TAMPEREEN TEKNILLINEN YLIOPISTO

ANTTI AHOLA

A PROGRAMMABLE LONG TERM ELECTRICAL STIMULATION SYSTEM FOR CELL CULTURES ON MICROELECTRODE ARRAYS

Master of Science Thesis

Examiners: Prof. Jari Hyttinen,
D.Sc. (Tech.) Jarno Tanskanen
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ABSTRACT

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Microelectrode arrays (MEAs) provide a means for measuring the electrical activity of cell cultures and their electrical stimulation locally, but their viability for long term stimulation of entire cultures is limited at best. For this purpose, a novel platform for electrically stimulating excitable cells on MEAs in long term cell culturing was devised. The designed and implemented system is a programmable electrical stimulation platform, capable of producing different kinds of electrical stimuli for several days on its own in a controlled environment, such as an incubator.

The system consists of three parts: a MEA container with stimulation electrodes, electronics for creating and amplifying the stimulation signal and a software designed for controlling the stimulus on a personal computer. Apart from the waveform generator and the personal computer, all aspects of the device were designed and built for this thesis. This work is a part of Stemfunc, an Academy of Finland funded project for developing novel methods to produce transplantable functional neuronal cells and cardiomyocytes from stem cells.

This thesis presents the research done for building the system, its capabilities, the results obtained by using it and gives viable plans and ideas for future research by using the device.

The designed system is capable of electrically stimulating cell cultures by user defined stimulus waveforms. When compared with the possibilities provided by existing commercial systems for cell culture stimulation, the designed system provides greater customization possibilities of several cell cultures with a low production cost. It is also among the few long term electrical stimulation platforms of cell cultures that utilize the microelectrode arrays, with the stimulation occurring in an incubator.

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Solujen sähköisen toiminnan mittauksen ja paikallisen sähköisen stimulaation mahdollistavat mikroelektrodijärjestelmät (MEA-levyt) tarjoavat tärkeän työkalun solujen viljelemiseen. Niiden käyttäminen kokonaisten soluviljelmien pitkäaikaiseen sähköiseen stimulaatioon on kuitenkin parhaimmassakin tapauksessa rajallista. Tätä tarvetta varten kehitettiin uusi järjestelmä stimuloituvien solujen pitkäaikaiseen sähköiseen stimulaatioon MEA-levyillä. Tässä työssä suunniteltu ja toteutettu järjestelmä on tietokoneohjattu ohjelmoitava stimulaatiojärjestelmä, jonka avulla voidaan tuottaa erilaisia sähköisiä stimuluksia koko soluviljelmälle useiden päivien ajan hallitussa ympäristössä, kuten esimerkiksi inkubaattorissa.

Luotu järjestelmä koostuu kolmesta osasta: kotelosta MEA-levylle elektrodeineen, elektroniikasta stimulaatiosignaalin luomiseksi ja vahvistamisesta sekä tietokoneohjelmasta, jolla voidaan hallita stimulaatiota. Aaltomuotogeneraattoria ja tietokonetta lukuunottamatta laite suunniteltiin ja toteutettiin itse tätä työtä varten. Tämä työ on osa Suomen Akatemian rahoitaamaa Stemfunc-projektia, jonka tarkoituksena on kehittää uusia menetelmiä toiminnallisten hermo- ja sydänsolusiirrännäisten tuottamiseksi kantasoluista.

Tässä työssä esitellään järjestelmän rakentamiseksi tehtyä tutkimusta, laitteen ominaisuuksia, laitteella saatuja tuloksia ja mahdollisia suunnitelmia ja ajatuksia laitteen jatkokäytöstä tutkimuksessa.

Suunnitellulla laitteella voidaan stimuloida sähköisesti soluviljelmiä käyttäjän määrittelemillä stimulusaaltomuodoilla. Verrattuna markkinoilla jo oleviin solujen sähköisen stimulaation laitteisiin, luotu järjestelmä tarjoaa paremmat mahdollisuudet erilaisten aaltomuotojen luomiseen useille soluviljelmille edullisin tuotantokustannuksin. Laite on myös yksi harvoista pitkäaikaisen sähköisen stimulaation mahdollistavista laitteista mikroelektrodijärjestelmille, jossa stimulaatio tapahtuu inkubaattorissa.

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CONTENTS

TERMS AND ABBREVIATIONS

ac	alternating current
ANF	Atrial natriuretic factor
AP	Action potential
ATP	Adenosine triphosphate
AV	Atrioventricular
CrP	Creatine phosphate
DAC	Digital to analog converter
DAQ	Data acquisition
DC	Direct current
EB	Embryoid body
ES	Embryonic stem
hESC	Human embryonic stem cell
MEA	Microelectrode array
MHC	Myosin heavy chain
Op-amp	Operational amplifier
PC	Personal Computer
PMMA	Polymethyl methacrylate, acrylic
ROS	Reactive oxygen species
Tn	Troponin

1. INTRODUCTION

The purpose of this Master of Science is to present the designing, building and development process of a programmable system for long term electrical stimulation of cell cultures. The design is based on a review of the literature of electrical stimulation of cardiac and neural cells.

The currently used stimulation systems are based on numerous methods, including both waveform generators capable of producing a limited number of waveforms and programmable systems. The device designed in this thesis makes it possible to simultaneously stimulate multiple cell cultures electrically by virtually any kind of waveform, as it allows the user to design them. As a basis for cell culturing, the system uses microelectrode arrays (MEAs), which are used for localized electrical cell measurements and stimulations. The system developed and built in this thesis is among the first to apply homogeneous electrical field stimulation on MEAs in long term cultures in an incubator. The advantage of stimulating cells in long term directly on the MEA is that measuring their electrical activity becomes easier and more reliable, as it is no longer needed to move the cells from one culturing chamber to another.

There have been numerous studies on the electrical stimulation, for varying purposes. It has been shown that the electrical stimulation induces positive effects in cell culturing, and lately with the development of stem cell technology, the need for developing the stimulation systems is emphasized. The mechanisms behind the positive effects for different cells have been studied, but they are not yet fully understood. There are various studying methods, for instance by measuring different marker proteins, with which the exact effects of the electrical stimulation become more known.

The process of culturing stem cells for regenerative medicine is a task that has numerous requirements in order for the cells to thrive. The production of cells outside of their natural environment – living organism – requires the surroundings to mimic the *in vivo* environment as well as possible. This means not only the temperature, humidity, pH and growth factors – to only mention a few – but also the electrical environment. Cardiac, neural and muscle cells all have electrical activity of their own and it contributes towards the electrical surroundings of other cells. In the cell culturing environment, this should be taken into account.

The stem cell culturing process takes a lot of time. In order to keep applying the electrical field to mimic the in-vivo electrical environment in culturing or to apply other desirable electrical stimulus, a setup for long term stimulation of cells is needed. For this purpose, a device for long term electrical stimulation of stem cells on a multi-electrode array platform was designed for Regea Institute for Regenerative Medicine in

Tampere. Stem cells had previously been electrically stimulated, but the advantages of this system are the possibility of keeping the cells on multi-electrode arrays for measurements, programming of electrical stimulation patterns, the homogeneity of the applied electrical field, the possibility to stimulate in an incubator and the simultaneous stimulation of six cell cultures on MEAs.

This work is a part of STEMFUNC, an Academy of Finland project, which aims to develop novel methods to produce transplantable functional neuronal cells and cardiomyocytes from stem cells. This is to be achieved by developing a biomimetic active environment for cell differentiation. The project is carried out by the Department of Biomedical Engineering, the Department of Automation Science and Engineering of Tampere University of Technology and Regea Institute for Regenerative Medicine of University of Tampere.

2. CELLULAR BACKGROUND

Understanding the electrical activity of the cells requires knowledge in both electricity and cell physiology. The two areas are well intertwined, as the cellular processes and interactions deal with ions and thus electricity. This is especially true when considering their electrical stimulation. While both electricity and its applications in physiology are relatively old areas of science – dating back to even prehistoric times – the recent developments in biological systems have allowed us to study cells much closer than what has ever been possible.

Electrical activity within the human body is well researched, due to its importance in understanding the physiology of cells and how different organ systems operate. The study dates back to the end of 18th century, when Galvani performed his famous experiments on frogs with electricity. The importance of his observations of electrical stimulation of muscles was realized only later when instrumentation had improved: in the end of 19th century, it was understood that the normal activity of these cells carried an electric current. In other words, the contraction of muscles is an intrinsically electromechanical process.

2.1. Basic physiology and electrical functions of cells

In order to understand the basis of cellular electrophysiology, one must be acquainted with the basics of cell anatomy. From the electrical point of view, in this work, the most interesting aspect is how the cells interact with their environment.

Eukaryotic cells, found in more complex forms of life, contain various organelles, such as the nucleus, ribosomes and mitochondria. Surrounding all this, the cells have a plasma membrane that separates the intracellular matrix from the extracellular matrix. This membrane is a complex structure of different lipids and proteins that serves an important purpose for instance in interaction with the outside world [1]. The physiology of cells differs greatly among different species. In general, the following chapters deal only with vertebrate physiology.

2.1.1. Membrane

The cell membrane is mostly composed of a phospholipid bilayer. This bilayer consists of phospholipids, which have a polar, hydrophilic head and two non-polar hydrophobic tails. When multiple phospholipids are immersed in liquid, the tails face each other – creating a hydrophobic core and the hydrophilic heads forming a barrier. This effectively creates a bilayer. The membrane consists also of different kinds of proteins, which can span the thickness of the whole layer - integral proteins - or peripheral proteins, which reside on the membrane surface.

The membrane and its proteins serve various functions. The membrane serves as a barrier between the intra- and extracellular compartments: it's not permeable to most water-soluble molecules and it can regulate the passing of different ions and particles. This allows the cell to keep a cytoplasmic composition different from the extracellular matrix [2].

2.1.2. Membrane potentials

In the scope of electricity, the different ionic concentrations between the intra- and extracellular matrices are of interest. Ions tend to diffuse from a higher concentration to a lower concentration. Similarly ions, being electrically charged particles, tend to flow towards electrical potentials of different polarity. These two effects form an electrochemical gradient, which governs the flow of ions through the membrane. Nernst Equation formulates the equilibrium between these two forces:

$$E_A - E_B = -\frac{RT}{zF}\ln\frac{[X]_B}{[X]_A},$$

where E_A and E_B are electrical potentials of compartments A and B, respectively, $[X]_A$ and $[X]_B$ concentrations of ion X in compartments A and B, respectively, R is the universal gas constant, T the absolute temperature, z the ion number and F the Faraday constant. Since there are multiple types of ions in both compartments, the overall voltage depends on all of them – the Nernst equation tells only the effect of one particular type of ion on the membrane potential. The equation can, however, be extended for multiple types of ions.

The embedded membrane proteins can move different ions either actively or passively through the membrane. This in turn makes changes in the electrical potential difference across the membrane [1].

Electric potentials across cell membranes can be found in every cell of the body, but some cells are capable of changing these potentials for different purposes – namely muscle (in this case heart muscle) and nerve cells. In muscle cells, a contraction is initiated by the change of membrane depolarization, whereas in nerve cells, a change in membrane potential may initiate an action potential (AP), which is used for communication when the activation spreads in the structure of the neural cells [1, 2].

2.1.3. Ionic concentrations

The resting membrane potential in cells is largely dictated by the concentrations of ions in both intra- and extracellular matrix. Depending on the cell type, the ions of most interest are potassium (K^+), sodium (Na^+), chloride (Cl^-) and calcium (Ca^{2+}). For neural cells, the intracellular space is rich in potassium ions, whereas the extracellular space has an abundance of sodium ions. For cardiac cells, the main ions are K^+, Na^+ and Ca^{2+}. Like with neural cells, intracellular space has potassium ions and extracellular space sodium, but also the extracellular concentration of calcium is much greater than the intracellular one. Chloride plays a smaller part in the equation due to its generally low concentrations [3, 4].

2.1.4. Ion transportation

As stated before, the cells can affect the ion concentrations. The membrane has numerous proteins that can transport ions through the membrane even against the electrochemical gradient, when certain requirements are met.

The methods with which ions can be transported through the membrane can be classified as five major types: bulk flow, diffusion and osmosis, exchange diffusion, co-transport and active transport. In the diffusion process, molecules move down their concentration gradient through a membrane, due to their random thermal movement. Osmosis on the other hand, is a process, in which water flows through the membrane, in order to lessen its chemical potential [1, 5]. Exchange diffusion and co-transport move two different substances through the membrane, with the former in opposite directions and the latter in the same direction. Depending on their gradients, moving one ion releases energy for the other to use - allowing it to pass through at the same time without directly using energy [1]. Active transport uses energy provided by adenosine triphosphate (ATP), to move ions against their gradient.

These methods are used by certain proteins, which are referred as ion channels and ion pumps. Ion channels when open, allow ions to flow through - thus increasing the permeability of the membrane. It is a passive mechanism, as it doesn't require work. Pumps, on the other hand, are active transporters and thus require energy [4].

Sodium-potassium pump (Na^+-K^+ pump) is one of the most researched ones. It is able to move the ions in opposite directions by moving 3 Na+ out of the cell and 2 K+ into the cytosol simultaneously – in the process using one molecule of ATP. Due to the pump moving an odd number of positive ions through the membrane in total, the cytoplasm becomes slightly more negative. Another example of an ion pump is the Na^+-Ca^{2+} pump, which can be found both in the cell membrane and endoplasmic reticulum.

The ion channels effectively set the permeability of the cell membrane by allowing ions to flow through. Depending on the type of the channel, the flow is regulated: some channels pass cations, some anions. Some channels can be selective and only allow specific ions to go through. Since the ion channels consist of multiple protein subunits that form a cylindrical structure, its geometry is a factor in defining its selectivity. Furthermore, there are multiple mechanisms which control whether the channels are open or not. Voltage-gated ion channels open or close depending on the magnitude and polarity of the voltage across the cell membrane. Ligand-gated channels require certain neurotransmitters or other ligands to bind to their receptors in order for the channel to open or close. Stretch-sensitive channels react to mechanical stretching force [6, 7].

For cardiac cells, gap junctions link the cell interiors of adjacent cells. They allow almost free diffusion of ions, which from a functional point of view makes it possible for the ions to move easily along the longitudal axes of the cardiac muscle fibers [8]. The cardiac AP propagation is covered in greater detail in chapter 2.2.6.

2.1.5. Action potentials

Electrical activation in nerve and cardiac cells occur with the same mechanism: rapid changes in membrane potential. This behavior is called an AP. A current pulse flows through the membrane which, depending on the direction of the current, either depolarizes or hyperpolarizes the membrane. When the voltage across the cell membrane reaches a certain depolarization limit called the threshold value, an AP occurs. In an AP, the transmembrane potential rises rapidly, reaching a positive peak, and then declining back to its resting state [1, 4].

2.2. Electrical properties of cardiac and neural cells

Even though the basic principles of excitable cells – channels, pumps, electrochemical gradient – are seemingly similar, cells have different kind of functions and mechanisms in handling the electrical information. Cardiac and neural cells differ very much from each other. In order to understand their stimulation needs, their physiological properties and electrical environment need to be examined first.

2.2.1. Nerve cells and their electrical properties

The basic structure of a neural cell consists of three elements: a soma; the central structure of the cell, dendrites; branched neural projections which transmit the signal from other neurons to the soma and an axon; a single elongated projection which passes signals from the soma to other neurons. The connection between an axon and a dendrite is called a synapse.

The larger axons have myelin sheats, which improve the electrical conduction. These myelin sheats, consisting of Schwann cell, are not continuous, but have gaps at regular intervals. These interrupts of the sheats are called nodes of Ranvier [5].

Neural cells and muscle cells are capable of creating electrical potentials spontaneously. The membrane potential of nerve cells goes through changes, as the neuron generates APs, or responds to sensory stimuli – as mentioned before in chapter 2.1.2.

As the neural cells serve as conductors in the human body, the geometry of the cell plays a crucial part. There are three important electrical characteristics: the resting membrane resistance, membrane capacitance and axial resistance along axons and dendrites. Essentially, a neuron is an electrical circuit, which determines the amplitude, timing and speed of signals passing through it [9].

A single nerve axon can be thought as long conductor, much like an electric wire. The main function remains the same – to provide a path for electric potentials to pass.

Based on the basic laws of electric physics, the longer the conductor, the more resistance the electric potential will experience.

$$R = \frac{\rho \cdot l}{A}$$

The resistance R is directly proportional to the resistivity ρ and length l of the conductor, and inversely proportional to the cross-sectional area A of the conductor. For nerves, this is a fundamental property: the APs decay as a function of distance from the stimulus site. The decay has drastic consequences: if an AP was to be transmitted across a long distance, its energy might not be sufficient to induce a synapse and induce further AP spreading.

Similarly as with regular electric wires, the longitudal voltage decay due is not the only affecting factor: the amount of current to one end of the conductor is not the same that comes out of the other end – a transverse, or a membrane resistance affects the flow of current as well [4].

2.2.2. Form and generation of neuronal action potentials

As explained in 2.1.5, APs occur, when the cell membrane reaches the threshold value. For neural cells, the resting potential is approximately -70 mV. The sodium and potassium channels open, when their opening conditions are met. The opening of the sodium ion channels causes the permeability of the cell membrane for that ion to rise very fast, creating an influx of sodium ions inside the cells. This effectively depolarizes the cell, due to the positive charge of the ions. The permeability of potassium increases much slower, therefore making the flux of potassium ions from inside the cell happen at a later time. Despite the slowness, the positive potassium ions repolarize the cell to its resting state. In the resting state, the Na-K pumps in the membrane restore the ion balance [1, 4].

As with other excitable cells, the neuronal APs obey the all-or-nothing –law. However, in nerve stimulation, larger stimulation amplitude can result in a greater population response. This is due to single axons having differing threshold voltages – a smaller response only indicates that a smaller number of neurons were recruited. A supramaximal stimulus occurs, when an increase in stimulation does not increase the response. Neuronal APs last typically about 2 ms [1]. A typical AP is illustrated in Figure 2.1.

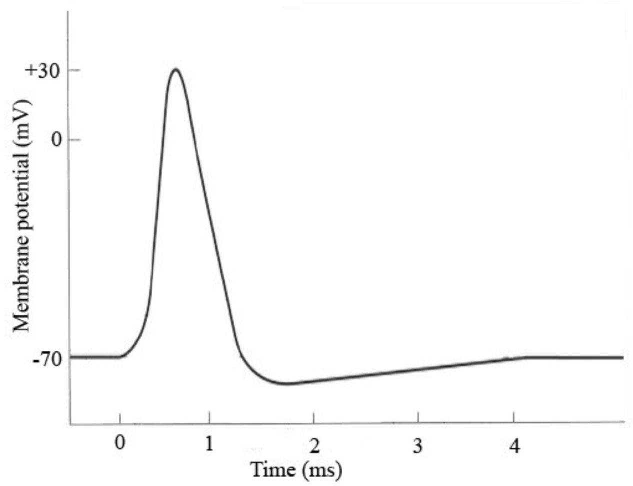

Figure 2.1: Neuronal membrane potential as a function of time.

2.2.3. Propagation of neuronal action potentials

When AP has been generated, it further depolarizes membrane area in the vicinity, making the AP travel along the axon due to the voltage-gated ion channels on the membrane reacting to the potential change.

The myelin sheats, mentioned before in 2.2.1, act as insulators. This allows the AP to cover long distances fast by jumping from one node of Ranvier to another via saltatory propagation. Essentially, the depolarization at a node of Ranvier is sufficiently large to change the potential of the next node in order to initiate an AP. This means that the electrical potential does not propagate as waves along the axon. The process is significantly faster than regular conduction, as can be seen from the illustration in Figure 2.2.

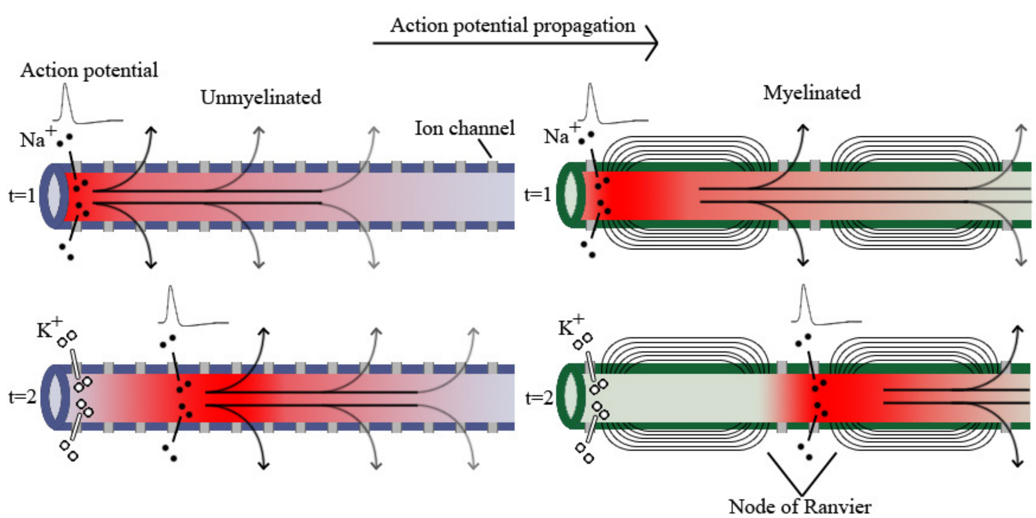

Figure 2.2: Neuronal AP propagation in unmyelinated and myelinated axons compared. Sodium ions are illustrated with solid dots and potassium ions with hollow dots. Adapted from [10]**.**

Whereas the neurons are able to transmit APs short distances by passive conduction, their main purpose is to release transmitters to convey the neuroimpulse further in a synapse. The synapse takes place in the synaptic cleft – the space between the terminals of the axon and the other cell. When the AP reaches the presynaptic terminal in the axon, chemical transmitter is released from synaptic vesicles. The transmitter then diffuses across the synaptic cleft and activates the postsynaptic membrane [4].

2.2.4. Cardiac cells and their electrical properties

The contraction of cardiac cells is mechanically similar to that of any other striated muscle cells found in the body. However, their electrical behavior is very different. Striated muscle activity is started, when an AP in motor neurons reaches the synaptic terminal, releasing acetylcholine, which in turn triggers the AP in the muscle cell. For cardiac cells, the APs are not triggered by neural activity, but by specific cells which start the AP independently – which then spreads to other muscle cells. Neural activity does, however, regulate the functioning of cardiac cells. A single AP results in a full cardiac contraction, whereas a single AP generates only a very minor force in other striated muscle cells. However, the contraction force can be modulated by cardiac nerves.

Cardiac cells can be roughly divided in two types: contractile and conductile cells. Contractile cells are responsible for the mechanical action, but can also spread activation to neighboring cells. Conductile cells have limited mechanical properties, but can spread activation fast and initiate APs. These cells serve various purposes in conduction – the most important regions where these cells can be found are the sinoatrial node, atrioventricular (AV) node, AV bundle – also known as bundle of His – and finally the Purkinje fibers [2].

2.2.5. Form and generation of cardiac action potentials

Even if some of the mechanisms producing APs in cardiac cells are the same as in neural cells, the result is very different. The membrane potential starts at its resting state, varying from -85 to -95mV. Like with neural cells, if the membrane voltage reaches certain threshold value, fast sodium channels open and the membrane quickly depolarizes. The process is fast due to the large resting potential and the abrupt change of conductance of the membrane to sodium ions. Also, the ionic concentration difference between intra- and extracellular spaces is considerable. When the membrane voltage reaches -35mV, slow and long lasting calcium ion channels open. The membrane potential keeps rising until it becomes slightly positive. After this, the sodium flow stops due to the depolarization of the membrane voltage and the inactivation of sodium channels. The membrane enters a phase of small repolarization not only because the channels close, but because specific potassium ion channels open briefly.

The cardiac AP goes through a plateau phase when the potassium ions flow out of the cell and an influx of calcium ions balance the membrane voltage. Ca^{2+} ions flow in, because of the increased conductance, low concentration inside the cell and because the membrane voltage is much lower than the equilibrium voltage. A typical cardiac AP is illustrated in Figure 2.3.

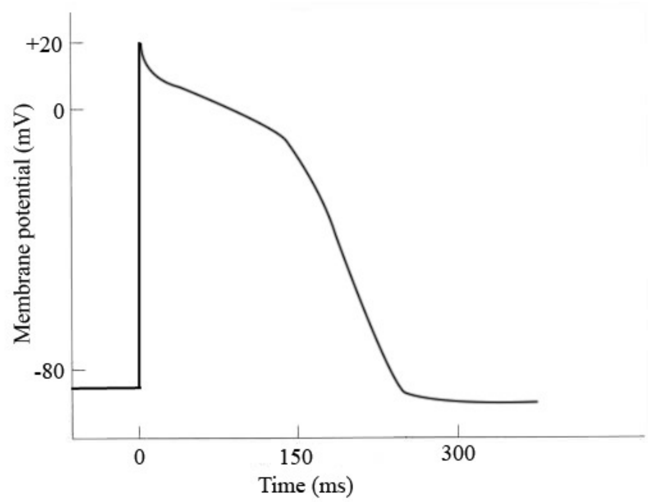

Figure 2.3: A ventricular cardiac AP as a function of time.

2.2.6. Propagation of cardiac action potentials

Passive conduction between cardiac cells has an important role in the depolarization. Adjacent cardiomyocytes in cardiac tissue are separated by intercalated discs which have an important role in transmitting the force generated during the contraction of the cell. Intercalated discs also support the AP spread and help with the synchronized contraction of the heart. For electrical impulses, the interesting junctions in intercalated discs are the gap junctions, which have a low resistance. The electrical impulses may spread through these connections, depolarizing the heart muscle as they proceed to the membranes of neighboring cells, as illustrated in Figure 2.4.

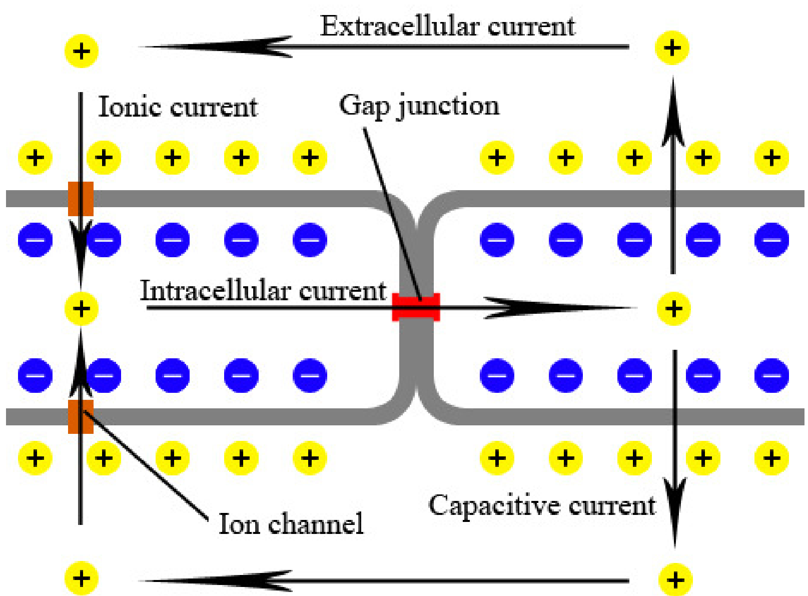

Figure 2.4 Conduction between cardiac cells. Adapted from [11]**.**

Various factors affect the velocity of depolarization: not only does the orientation of the cells and their position in regard to other cells matter, but also factors such as ischemia, electrolyte imbalance and drugs.

It has been suggested [12] that the gap junctions operate dynamically in each cardiac cycle. The APs would propagate by two main mechanisms, a free flow of current and an electric field. The theory suggests that gap junctions would be closed in the plateau phase of the AP and the extracellular electrical field would be the main route of propagation during that time.

2.3. Stem cells and culturing

Cells, which in theory have the capability of endless proliferation in vitro through self-renewal and of generating mature cells of different tissues through differentiation, are called stem cells [13]. These functions provide biomedical engineering with a very powerful tool, when such cells are produced: their therapeutic potential in regenerative medicine is large, along with their research potential in drug development and advances in human development biology [14]. However, their therapeutic use is currently inhibited by the unpredictability of the process: only a fraction of the cells develop to be useful for medicine. One of the driving forces behind developing the stimulation system was to improve this differentiation.

2.3.1. Stem cell differentiation

In general cell culturing, the process of culturing cells starts from isolating cells from a tissue. The cells extracted from an organism for culturing is called the primary culture. Some of the cells, in theory, are able to keep on dividing indefinitely. These cells are used to form cell lines, which are kept in cell banks [15].

In practice, cell culturing is a delicate process and requires specific equipment and manual work, all the while minimizing the risks for contaminations. All cultures used in the same research must have the same kind of environmental conditions, so that the results from the individual cultures are comparable and to achieve results that can be reproduced.

Human embryonic stem cells (hESC) were first derived by Thomson *et al.* in 1998. Embryonic stem (ES) cells are derived from inner cell masses of preimplantation stage embryos at blastocyst stage [16].

The spontaneous differentiation of hESC to cardiomyocytes was first demonstrated by Kehat *et al.* in 2001 [17]. In the study, ES cell differentiation to cardiac cells was induced by spreading the cells to small clumps. The cells were transferred to Petri dishes, where they were cultivated in suspension for 7-10 days and aggregated to form embryoid bodies (EBs). After the aggregating step, the EBs were plated on culture dishes, where they were observed for spontaneous contractions.

The differentiation of hESCs to neural precursors was shown by Reubinoff *et al.* in 2000 [18]. The stochastic nature of spontaneously differentiating hESCs makes it inefficient to generate neural cells. It is, however, possible to direct their differentiation towards neural lineage.

2.3.2. Incubator

A cell culture incubator is a device, which provides the laboratories with a means to upkeep ideal conditions for cell development. It is basically a container, in which the cells can be kept during their various stages of life.

Different cell lines require different kinds of environments. For most human cells, however, the usual conditions include a temperature of 37ºC, relative humidity of 95% to minimize media evaporation and condensation and 5% CO_2. Different conditions may be used for instance in cell stress studies. Opening the incubator door causes fluctuations in the temperature and other conditions inside, which the incubator should be able to compensate.

Another important aspect is the use of a container for the cell cultures in incubators. There are several possibilities, such as flasks, bottles and dishes, which reduce the possibility of contamination and environmental conditions changing while opening the incubator door. The incubators should be compatible with the types of containers used to keep the conditions ideal [19].

3. ELECTRICAL STIMULATION OF CELLS

In order to provide the cardiac and neural cells with the type of electrical environment that mimics their natural environment, external stimulation is needed. When mature enough, cardiac cells start their intrinsic beating, thus creating an electrical field, but in the early stages of development this behavior is not present. Nerve cells, on the other hand, relay APs when stimulated with appropriate stimuli, such as sensory stimuli.

3.1. The effects of stimulation

The electrical stimulation of cells has a lot of options – voltage, current, frequency, shape and strength of the electric field, waveform, delays and the total stimulation time all have their contribution. In order to elicit an AP, the pulse must be strong enough, However, weaker electric stimulation can also evoke desired effects in the cells. The stimulation should not be too large, however, to prevent damage caused by too high charge injection or charge densities [20]. Furthermore, the electrodes themselves may suffer from the stimulation, if electrical charge accumulates on one electrode due to an unbalanced stimulus pulse [21]. So far, there has been little research on their effects in long-term stimulation.

The application of electrical stimulation on cells has been shown to have positive effects. For instance, the application of electrical stimulation modulates the differentiation of ES cells. Mild electric stimulation strongly influences ES cells to develop into neurons [22]. For fibroblasts, electrical stimulation has been shown to induce cardiomyocyte predifferentiation. The exact mechanism of why the electrical stimulation affects the cells in this way remains still partly unclear [23].

3.1.1. Stimulation parameters

When the stimulation aims to cause an AP, the stimulation impulse must have sufficient strength with a sufficient duration. The relation of strength and duration can be illustrated as a curve, which shows for each stimulus duration the strength needed for invoking an AP. The shape of the curve varies, but as the duration grows, the strength starts approaching a certain value, rheobase. A stimulus pulse with the rheobase strength of theoretically infinite duration will induce an AP. The relationship of stimulation strength and duration has been formulated in detail in different empirical equations. A typical strength-duration curve is shown in Figure 3.1.

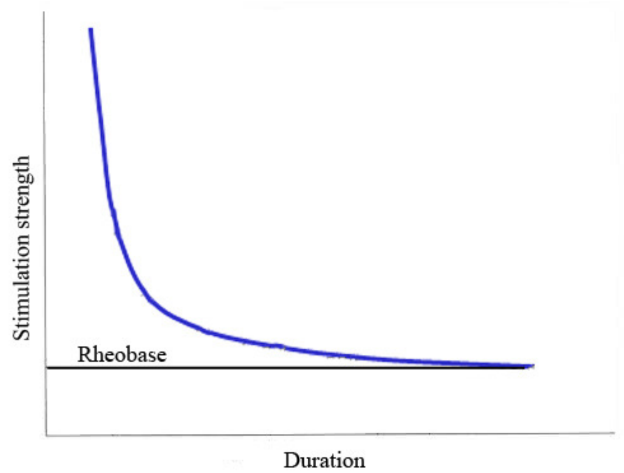

Figure 3.1: A typical strength-duration curve with rheobase

However, not all electrical stimulus deals with creating APs. The natural environment of cardiac and neural cells is intrinsically electric, so they experience electrical fields with lower strengths as well. In this work, the AP inducing electrical stimulation is mainly of concern, but the sub-threshold stimulation is not ignored.

Electrical fields with low strengths affect cells as well, as their electrical functioning is based strongly on the exchange of ions between the intra- and extracellular matrix. While applying an electric field might not cause an AP, it does open ion channels, which otherwise might be closed and cause electrochemical changes in the cell.

3.1.2. Cardiac cells

Electrical stimulation of cardiac cells is well-researched: cardiac pacing, defibrillation and resynchronization of the heart serve as the basis of treating several cardiac problems. Similarly as with the sinoatrial node and the other native pacemakers of the heart, an artificial pacemaker delivers a depolarizing electric impulse to the contractile cells.

For cardiomyocytes, the depolarization of the cell membrane exceeding the threshold voltage causes a contraction. This stimulation-contraction coupling is an essential factor when studying the stimulation of cardiac cells. The shape and form of the electric activity changes drastically, as it travels through the different specialized cells: the waveform in atrial muscle cells is different than that in ventricular cells [4].

3.1.3. Neural cells

Electrical stimulation for neural cells has been used in studying the nervous system and also attempting to restore function after a disease or an injury. It has also been used to study the connections between neurons and their projection patterns.

A nerve cell can be artificially stimulated by depolarizing the cell membrane. The exact effect on the cell depends on the nerve cell type, size and geometry – not to mention the stimulus itself. Both strength-duration relationship and the current-distance relationship also have an effect. It has been found out that for cathodic pulses, being negative pulses with current flowing out of the system, the threshold for activation of passing axons is less than the threshold for activation of local cells. With anodic pulses, being positive pulses with current flowing into the system, however, the threshold for local cells is less than that of passing axons. This means to say that the neuronal population can be targeted by simply choosing the waveform of the stimulation: in a cathodic stimulation, the site of excitation in axons is at the depolarized node of Ranvier while in local cells it is at the hyperpolarized node of Ranvier [24].

3.2. Research on electrical stimulation of cells

Stem cell culturing and cell products are a rather new market. Efficient production of cells requires a lot of basic research. Currently, the number of articles published is relatively low and they are not very detailed, especially when it comes to stimulation parameters. Even though there are various results on the effects, reproducing the results based on the given stimulation parameters may be difficult. Also, the studies have mainly concentrated on only a few aspects of the results: the stimulus impulses may have had effects that had gone unnoticed. If only orientation and elongation was studied, the effects on protein expression might not have been noted.

It is not well understood how exactly the electrical stimulation promotes cell differentiation. For cardiac cells, the generation of reactive oxygen species (ROS) in the cells has been suggested as one mechanism. It has been shown on mouse EBs that external electrical field increases ROS production in the cells. ROS, on the other hand, have been shown to activate transcription factors, which regulate for instance the transcription of genes for cytokines which may play a role in cardiac development. Although ROS can in large quantities be harmful to the cells, in small amounts they act as messengers within the cell and activate signaling cascades, which play a role in growth and differentiation of many cell types [25]. It is also not clear, whether the effects of electrical stimulation on cardiac cells are a result of the regular mechanical contraction due to electrical stimulation, or because of the electrical field stimulation *per se* [26].

3.2.1. Cardiac cells

Cardiac cells have been electrically stimulated in several studies, with different kinds of results. Most of the studies have been conducted on animal cells, such as rat or rabbit cells. For the purpose of gaining guidelines for both building and using the stimulation device, recently done publications were studied. The publications, used stimulation parameters and results can be seen in Table 1.

Table 1: Research on electrical stimulation of cardiomyocytes

Publication	Cell type	Pulse parameters	Purpose	Result
Au *et al.* 2007 [27]	2-day neonatal rat	Square monophasic waveform, 1 ms duration, 1 Hz frequency, 2.3 and 4.6 V/cm, 72 h	Determine the effects of electrical field stimulation on elongation and orientation of fibroblasts and cardiomyocytes	Electrical stimulation enhanced fibroblast and cardiomyocyte elongation, promoted orientation of fibroblasts
Chen *et al.* 2008 [28]	Murine ES-D3	Anodic-first biphasic waveform, 10 ms duration, 1 Hz frequency, 8.3, 24.9, 49.8 $\mu A/mm^2$, 96 hours	Develop a system allowing the use of point source electrical stimulation on ES cells for studying physiologically-relevant electrical stimulus	Later stages of ES have larger changes in cardiac and embryonic gene expression. At terminal stage, ES differentiating to cardiomyocytes showed positive correlation with stimulation amplitude
Genovese *et al.* 2008 [23]	HFF-1	Biphasic; 10, 20 and 40 V (C-Pace), 5 ms duration, 0.5 Hz, 72 and 96 hours	Evaluate the effectiveness of electrical stimulation to induce pre-commitment of fibroblasts into cardiomyocytes in vitro	Reduced cell population number, induced muscle-like cell morphology, protein expression and transcription factor expression, increased expression of cellular cardiac troponin I
Hedgepath *et al.* 1997 [29]	Left ventricular porcine	Waveform?, 7±1 V, 5 ms duration, 0.5-1.5 Hz	Measure myocyte orientation with respect to electrical field	Myocyte orientation with respect to stimulation electrodes can influence contractile behavior
Holt *et al.* 1997 [26]	Adult rat	Bipolar waveform, 5ms duration, 0,25 Hz-2 Hz frequencies, 5 V/cm, 24 hours	Investigate mechanical properties and calcium handling of cardiomyocytes after 24 hours of electrical stimulation	Regular, rhythmical electrical stimulation enhances mechanical properties and calcium transients.
Klauke *et al.* 2003 [30]	Adult rabbit	Various. Unipolar biphasic square waveform, 27 V/cm to 150 V/cm.	Develop a microchamber array allowing continuous field stimulation of adult cardiomyocytes	N/A
McDonough *et al.* 1992 [31]	neonatal rat	Pulsatile waveform, 80-150 V (Brevet *et al.* setup, 1976 [32]), 5-10 ms duration, 1-5 Hz, 72 hours	Study the relationship between cardiomyocyte contraction frequency, gene expression and cardiomyocyte growth	Increased cell size and myofibrillar organization, 5-10-fold increase of cardiac gene expression

Publication	Cell type	Pulse parameters	Purpose	Result
McDonough *et al.* 1994 [33]	Neonatal rat atrial myocytes	60-100 V (Brevet *et al.* setup, 1976 [32]), 10-20 ms duration, 1-8 Hz, ? hours	Characterize the effects of cellular events associated with contraction on atrial natriuretic factor	Pacing has various effects on the secretion of atrial natriuretic factor
Radisic *et al.* 2004 [34]	Neonatal rat ventricular myocytes	Rectangular waveform, 5 V/cm, 2 ms duration, 1 Hz frequency, 120 hours	Study the excitation-contraction coupling and whether it determines the development and function of engineered myocardium	Correlation with cell differentiation observed. After 8 days, alignment, elongation and central elongated nuclei
Radisic *et al.* 2007 [35]	Neonatal rat ventricular myocytes	Suprathreshold square biphasic waveform, 5 V/cm, 2 ms duration, 1 Hz frequency, 192 hours	Review biomimetic tissue engineering approach	Induced cell alignment and coupling, increased amplitude of contractions and improved organization
Sauer *et al.* 1999 [36]	Mouse embryonic fibroblasts	Square waveform, 1 V/cm to 5 V/cm, 90 ms duration, frequency?	Study the effects of electromagnetic fields on the differentiation of cardiomyocytes in EBs derived from pluripotent ES cells	Increased number of EB differentiating beating foci of cardiomyocytes and the size of beating foci
Serena *et al.* 2009 [25]	hESC, line H13	Square waveform, 10 V/cm, 1 and 90 s duration, 4 days	Study the effects of electrical field stimulation on ROS generation and cardiogenesis in EBs derived from hESC	Electrical stimulation plays a role in cardiac differentiation of hESCs, through mechanisms associated with the intracellular generation of ROS

As can be seen from Table 1, the pulse strengths vary and the results have been different based on what has been studied. Also, the stimulation setups have been very different. The results, however, are encouraging: the stimulation has had a positive effect on various aspects, such as cell alignment and orientation, gene and protein expression and from the stem cell point of view most interestingly, differentiation. The effects of the electrical stimulation had been assessed usually on functional and molecular level.

The most commonly used electrical fields vary from 1 V/cm to 10 V/cm, with 5 V/cm being used a lot. It should be noted, however, that the cell types vary and the results achieved by stimulating rat cardiomyocytes may not apply directly to stimulation of human cells. Instead, the field strengths have been slightly higher. The waveforms used have been almost exclusively square waves, mostly biphasic pulses. Some articles did not state the used waveforms, or the field strengths used, but instead mentioned voltages at which the cells had been stimulated.

3.2.2. Cardiomyocyte behavior

In 1997, the study by Hedgepath *et al.* [29] examined the cardiomyocyte function by electrical stimulation. Conclusive evidence on the relationship between changes in electrical field orientation and myocyte contractile function remained unclear, though.

Cardiomyocyte alignment and orientation was also studied by Au *et al.* in 2007 [27]. The exact mechanism of the causes of these effects also remained unclear, although it was noticed that the orientation effects were abolished by the inhibition of actin polymerization and partially by the inhibition of the PI3K pathway.

Contractile properties were studied by Holt *et al.* in 1997 [26] by measuring calcium transients of electrically stimulated cells. Systolic fura-2 ratio was measured to be 25.4 % higher in stimulated myocytes than in unstimulated ones along with developed fura-2 ratio. Diastolic fura-2 ratio was slightly lower in stimulated cultures when compared to unstimulated cultures. Also, ATP and creatine phosphate (CrP) levels were compared in both cultures. A moderate 11 % increase was observed in stimulated myocytes and a small 5.5 % increase in CrP. No significant difference of Ca^{2+}-ATPase content or [^{3}H]-ryanodine binding was observed.

McDonough *et al.* conducted studies considering the Atrial Natriuretic Factor (ANF) in 1992 [31] and 1994 [33]. It was found the electrical stimulation induced ANF expression. It was also noticed in the 1992 study that the ANF levels increased as a function of frequency and in the 1994 study that the ANF released reached a plateau at 8 Hz. When pacing was stopped, ANF secretion quickly returned to control values, indicating its close coupling with contractile behavior. MLC-2 expression was also dramatically increased. Phosphoinositide (PI) hydrolysis and cAMP formation were not increased, indicating that the protein kinases C and A are not involved with the contraction-induced growth response.

Radisic *et al.* [34] studied the role of excitation-contraction coupling in development and function of engineered myocardium. The electrical stimulation had enhanced the amplitude of contractions and the excitation threshold had decreased. Furthermore, the levels of cardiac proteins such as myosin heavy chain (MHC), connexin-43, creatine kinase-MM and cardiac troponin (Tn) I had increased. Polymerase chain reaction confirmed the expression of genes for sarcomeric α-actin, α-MHC, β-MHC, connexin-43 and β-integrin. With time in culture, the ratio of α-MHC (mature) and β-MHC isoforms increased in stimulated constructs whereas it decreased in nonstimulated ones, suggesting the maturation of cardiomyocytes to depend both on culture duration and electrical stimulation.

3.2.3. Stem cell specific cardiac differentiation

Stem cell differentiation has been studied for a relatively short period of time, only for a couple of years.

Serena *et al.* [25] showed that electrical stimulation plays a role in cardiac differentiation of hESCs. The differentiation was evidenced by spontaneous contractions, expression of Tn-T and sarcomeric organization [25]. Cardiac differentiation of ES cells was also studied by Chen *et al.* in 2008 [28] on murine cells. Gene expression analysis was done by measuring for cardiac markers β-MHC and Tn-T as well as for ES cell marker nanog. It was found that the stimulation caused a significant increase in both β-MHC and Tn-T, when compared with non-stimulated samples. The ES cell marker nanog increased significantly as well.

In 2008, Genovese *et al.* [23] showed the electrostimulation to induce cardiomyocyte predifferentiation of fibroblasts. In the study, it was concluded that due to electrical stimulation, an upregulation of Mef2C, muscle cell related transcription factor was detected. Furthermore, the stimulation increased the Tn-I expression, confirming the findings of Radisic *et al.* [34] for general cardiomyocyte stimulation.

Sauer *et al.* [36] used direct current (DC) electric fields lasting 90 s for studying the cardiomyocyte differentiation on mouse ES cells. Similarly as in the study by Serena *et al.* [25], intracellular ROS was increased due to electrical stimulation. The presence of ROS was verified by loading the EBs with DCFH-DA, a sensitive ROS indicator. It was also concluded that a single electric pulse of 90 s, rather than long-term DC exposure, was sufficient to enhance cardiomyocyte differentiation. Cytotoxic effects were observed with DC exposures longer than 2 hours.

3.2.4. Neural cells

Computer models of neural cells are often used in simulations and in studying the neurons. For the purpose of designing the device, literature reviewing was done also for neural cell stimulation. Publications found are listed in Table 2. Neural cell stimulation has been done for almost a century, but effective parameters for stimulating have been studied only lately. Wagenaar *et al.* [21] studied the effects of different kinds of stimuli on dissociated cultures and found the biphasic voltage-based electrical stimuli with negative-first pulses to be the most effective way of stimulation. In monophasic stimulation tests, it was found out that anodic pulses were significantly less effective than the cathodic pulses [21]. This behavior likely can be explained by the fact that the waveform shape has an effect on the areas of stimulation, as was explained in 3.1.3.

Table 2: Research on electrical stimulation of nerve cells.

Publication	Cell type	Pulse parameters	Purpose	Result
Fields *et al.* 1990 [37]	mouse dorsal root ganglion neurons	Various. Biphasic waveform, 1 and 5 V, 2.5 Hz, 5 pulses separated by 100 ms followed by 1.6 s rest, 5 Hz and 10 Hz	Determine the effects of patterned electrical activity on the morphology and motility of mammalian central nervous system growth cones	Phasic stimulation halted axonal outgrowth. Observed accommodation to electrical stimulus.
Hwang *et al.* 2009 [38]	Human bone marrow stromal cells	5 µA, 7.5 µA and 10 µA, 25 µs, 125 µs and 250 µs, frequency?, 72 hours	Investigate the effect of biphasic electric current stimulation on the differentiation of human bone marrow stromal cells into Schwann cell lineage	Higher and longer neurite formation, enhanced the functional capacity of differentiated Schwann cells
Kimura *et al.* 1997 [39]	PC12 (rat)	Square wave, 100 mV, 100 Hz, 30 minutes every 24 hours	Study gene expression in electrically stimulated differentiation of PC12 cells	Neurite growth
Patel *et al.* 1982 [40]	Embryonic Xenopus laevis	Various. 0.1 to 10 V/cm, 1 Hz and 0.1 Hz	Quantitative characterization of effects of the electric fields on neurite growth	Accelerated growth towards cathode, increased average neurite length
Wagenaar *et al.* 2004 [21]	rat embryo	100-1000 mV 100-800 µs duration, 1-20 µA, 10-1000 µs duration	Study the efficacy of pulse shapes and determine parameter ranges	Voltage control may be advantageous in stimulation scenarios due to control over electrochemistry
Yamada *et al.* 2007 [41]	R1 Mouse ES cell line	0, 5, 10 and 20 V, train of 5 pulses, 950 interpulse interval (C-Pace)	Examine the influence of inter- and intracellular ionic balance on differentiation	Electrical stimulation can bias the fate of differentiating ES cells toward neuronal lineages

Electrical stimulation has various positive effects in neural growth: guiding, formation and growth. The fields used by Patel *et al.* in 1992 [40] are somewhat in line with the cardiac stimulation field strengths. The study showed the electrical stimulus to induce growth towards the cathode and to increase the length. The publication by Wagenaar *et al.* [21] is especially interesting, as various waveforms, voltages and currents have been studied.

3.2.5. Neurite growth and guiding

The study by Patel *et al.* [40] showed the neurites to grow toward the cathode with the growth toward anode reduced. More neurites appeared to be initiated on the cathodal side of the soma and in general, the number of neurite-bearing neurons per culture and average neurite length increased. Possible cellular mechanisms were studied as well – it was found that the electrical field caused a "lateral electrophoresis" of the concanavalin A receptors on the surface of the neurons on the cathodal side of the cell. The immobilization of concanavalin A receptors removed the asymmetric growth response of the neurites, which would indicate the receptors having responsibility in the orientation of neurite growth.

3.2.6. Neuronal differentiation

Yamada *et al.* [41] showed stem cells to assume neuronal fate with the presence of mild electrical stimulation. Inter- and intracellular ionic densities were considered. Almost all cell colonies receiving electrical stimulus contained cells immunoreactive to a marker for early committed neuronal cells (TuJ1), whereas the control cultures contained only 10% of TuJ1-positive cells. The neuronal cells differentiated in a significantly shorter time than in other systems, which use growth factors for cell differentiation initiation. The role of Ca^{2+} for electrically induced neuronal differentiation was studied as well by using EGTA, a calcium chelator. EBs electrically stimulated with the presence of EGTA failed to assume a neuronal fate, whereas in absence of the chelator assumed a neuronal fate. A Ca^{2+} -influx can thus be seen as a necessary component of neuronal differentiation.

Similar results were obtained already in 1998, when PC12 cells were electrically stimulated by Kimura *et al.* [39] in order to study the gene expression in differentiating cells. The electrically stimulated cells hardly differentiated, but proliferated as well as normally cultured cells. The effect of calcium ion influx to differentiation was studied by blocking the calcium ion channels by lanthanum ion treatment. As a result, the treatment perfectly inhibited the induced differentiation. It was concluded that electrically induced differentiation may require calcium ion influx. Also, c-fos gene level, essential for PC12 cell differentiation, was investigated. The c-fos mRNA level was increased in stimulated cultures, indicating the electrical stimulation can induce not only morphological changes, but also cell differentiation.

3.3. Stimulus production

Before building an electrical stimulation system, already existing systems were considered. In the literature, several types of stimulus systems had been used. In Table 3, their characteristics have been listed.

Table 3: Stimulation hardware used in literature

Publication	Electrodes	Cell container	Stimulator	Additional info
Au *et al.* 2007 [27]	Carbon rod (Ladd Research Industries)	100x15 mm^2 petri dish	Grass s88x stimulator (Astro-Med)	Microabraded surface
Chen *et al.* 2008 [28]	MEA	MEA	MSP430 microcontroller driving a modified Howland voltage-controlled current source	
Genovese *et al.* 2008 [23]	?	4-well plates (NUNC)	C-Pace chronic stimulation unit (IonOptics)	
Hedgepath *et al.* 1997 [29]	Platinum	Thermostatically controlled chamber with a coverslip	Grass S11 stimulator (Astro-Med)	
Holt *et al.* 1997 [26]	25.4 cm x 0.64 cm carbon rod	175 cm^2 tissue culture flasks	?	
Klauke *et al.* 2003 [30]	MEA	MEA	Custom built stimulator	
McDonough *et al.* 1992 [31]	Ag:AgCl electrodes	Multiwell dishes	Grass stimulator (Astro-Med)	Based on Brevet *et al.* 1976 [32] setup
McDonough *et al.* 1994 [33]	Ag:AgCl electrodes	Multiwell dishes	?	Based on Brevet *et al.* 1976 [32] setup
Radisic *et al.* 2004 [34]	1/4-inch diameter carbon rod (Ladd Research Industries)	Glass chamber	Nihon Kohden cardiac stimulator	
Sauer *et al.* 1999 [36]	Stainless steel (V4A), 0.4 cm^2 area	24-well plates (Falcon)	Custom made electropulser	
Serena *et al.* 2009 [25]	Stainless steel, titanium-nitride coated titanium, titanium	Custom made bioreactor wells	Astro-Med Stimulator	
Wagenaar *et al.* 2004 [21]	MEA	MEA	Adlink digital to analog converter (DAC), custom DAC based on TLC-7628 (Texas Instruments)	
Xia *et al.* 1997 [42]	Ag:AgCl electrodes	12-well dishes	Grass stimulator (Astro-Med)	Based on Brevet *et al.* 1976 [32] setup

As can be seen, there is no one clear standard way of electrically stimulating cell cultures. In literature, a commonly referenced electrical stimulation setup is by Brevet *et al.* in 1976 [32], which included electrodes in culture dishes. A diagram of the Brevet et al. based system can be seen in Figure 3.2, by McDonough *et al* [33]. Based on this, similar kinds of solutions have been devised.

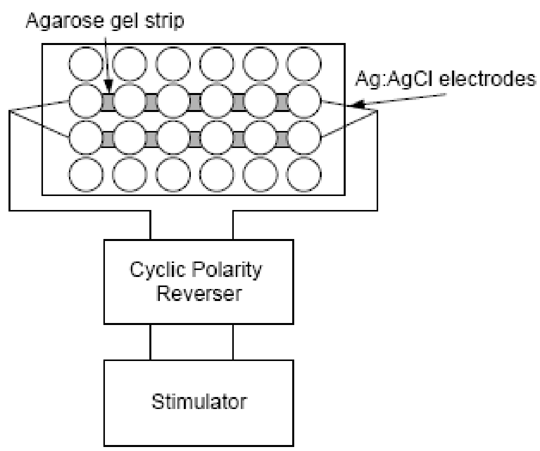

Figure 3.2: Diagram of a Brevet et al. type stimulation apparatus. Adapted from [33]**.**

The idea is simple – the wells are connected via agarose gel strips and electrical stimulus is applied to both ends of multiwall dish. The cyclic polarity reverser reverses the output from the stimulator periodically. The voltages used with the setup are high – the highest values being in the range of even 150 V. However, when the electrodes are placed on opposite sides of a large multiwall dish, the electric field will be in the range of below 10 V/cm.

For the electrodes, three studies [21, 28, 30] have used MEAs, which have the capability of localized electrical stimuli. In studies with more emphasis on creating a uniform field, carbon rod electrodes have been more widely used. Stainless steel as a material is also used.

The cell containers used are almost exclusively commercial products, consisting of mainly plates, wells and dishes. Even if they are not designed especially with electrical stimulation in mind, their status in cell culturing is well established and they provide a familiar platform for studying. Furthermore, a commonly referenced study by Brevet *et al.* [32] used the same method.

In waveform creation, Grass Instruments (a part of Astro-Med Inc.) stimulators are used in several studies. These stimulators use mostly square pulses for stimulation [43].

3.4. Cell stimulation technology

As can be seen in Table 3, the technology on cell stimulation is not very standardized. MEAs are used in several studies, but also more custom made devices have been made. Standard cell culture wells with external electrodes have been used before, along with different other containers.

3.4.1. Microelectrode arrays

The MEA, shown in Figure 3.3, is in principle two-dimensional electrode arrangement, consisting of miniscule probes. The electrodes are designed for both stimulation and recording of electrical activity of different cells.

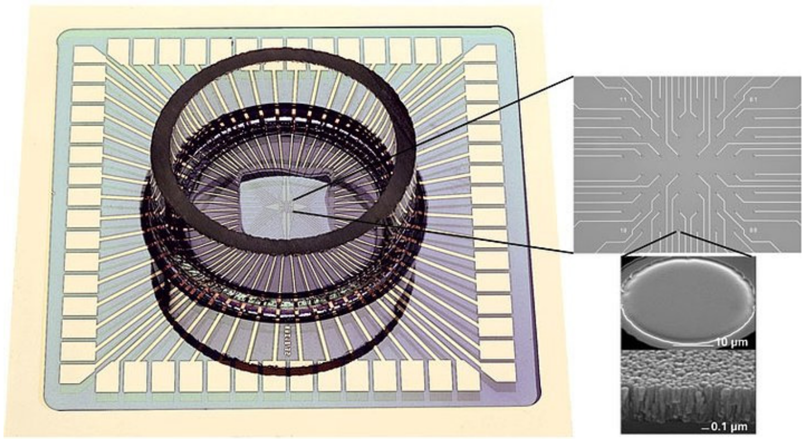

Figure 3.3: A microelectrode array by Multi Channel Systems MCS GmbH. With permission [44]

The basic structure of a MEA consists of typically 60 electrodes. Like shown in Figure 3.4, the micrometer scale electrodes are in a square formation.

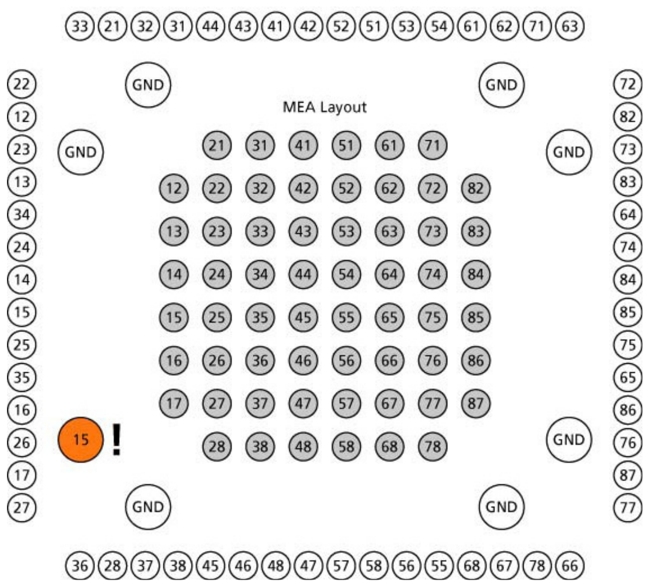

Figure 3.4: Pin and MEA layout. With permission [45]

This allows the recording and stimulation of targeted areas in both cell cultures and tissues. Micrometer-scale electrodes are accessible via wiring, with the electrode pads on the outer rim of the whole MEA plate, and the culturing area is surrounded by a glass ring, attached to the MEA surface, as in Figure 3.3. This allows the cells to be cultured directly on the MEA surface, while immersed in medium.

However, the downside of the intricate design lies in electrical stimulation. Cells placed on a MEA can be stimulated locally, by selecting the desired electrodes to which electrical stimulus can be applied. When dealing with large cell populations, stimulating only some parts of a culture does not have the same effects in other parts of the cell culture. The system is well suited for studying the responses of cells to electrical localized stimuli, but MEAs are of little to no use when stimulating whole cell cultures, since the fields generated are not homogeneous.

3.4.2. Commercial and scientific stimulation systems

It seems there are not a lot of commercial systems available for stimulating cells. IonOptix manufactures a cell culture pacing system, C-Pace, designed for stimulation of bulk quantities of cells in culture incubators. The cells are placed in culture dish systems, called C-Dish. C-Dishes consist of carbon electrodes in a standard culture dish, for 4-, 6- or 8-well culture dish. In effect, the stimulation system is similar to the system constructed, but the differences can be seen in the architecture: C-Pace uses the culture wells, whereas MEA dishes are used in this system. Also, as will be presented later on in 4.6, the waveform options implemented in this work are more extensive than in the IonOptix culturing system [46].

6 Individual 35mm Dishes

Figure 3.5: A C-Dish platform. With permission [46]

SciMedia manufactured ESTM-1 is an electrical stimulation system for cells cultured in 35 mm culture dishes. In contrast to the IonOptix system, the aim is not to a uniform rectangular field, but instead a circular one. When electrical stimulation is applied, the current spreads from the stimulation electrode in circular fashion and flows into the loop electrode. The ESTM-1 setup is illustrated in Figure 3.6.

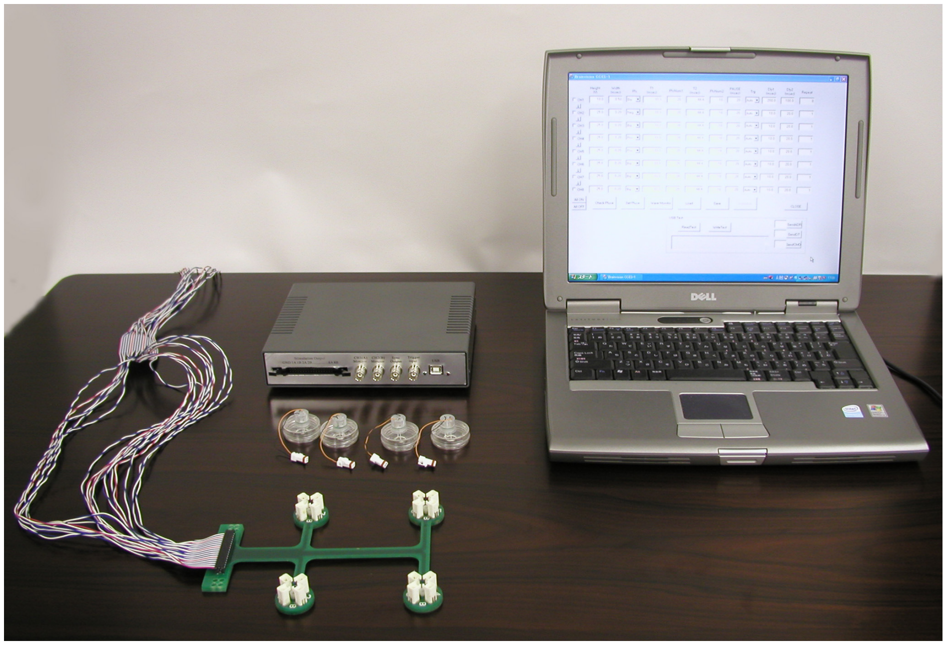

Figure 3.6: ESTM-1 Electrical stimulator. With permission [47]

The scientific setups differ greatly, as there is no one standard in performing electrical stimulation. Cell culture wells, petri dishes and MEAs are often used in custom systems. Systems may be as simple as signal generators connected to rod electrodes in culture flasks, or plate electrodes in multiwall dishes. Rod electrodes tend to generate circular fields around them, whereas plate electrodes generate fields more rectangular in form. The common reasoning behind the systems lies in striving to create a homogeneous electrical field for the cell culturing. Due to the small size of the cells, large electrodes are able to generate fields that are almost the same throughout the cell culturing area. A system done by Brevet *et al.* in 1976 [32] has been used as a reference for procedures and guidelines of culturing in studies, such as Xia *et al.* in 1997 [42] and McDonough *et al.* in 1992 and 1994 [31, 33].

4. MATERIALS AND METHODS

In order to study the effects of long-term electrical stimulation on cells, a device along with software was designed and implemented for the purpose in this thesis project. The stimulation device consists of three parts: a petri dish -like container for a MEA dish - nicknamed “Antti-Box”, the stimulation electronics and stimulation software installed on a laptop personal computer (PC). A MEA within the container is to be placed in an incubator, in order to provide the cells with the temperature, humidity and other conditions necessary for their well-being. The computer along with the stimulation control electronics supplies the electricity to the cells. This setup in its various forms was then used in several tests at Regea Institute.

4.1. Requirements for the device

As explained in chapter 2.3, the cell cultures require a very specific type of environment and conditions for living. This poses also certain requirements for the stimulation device, as it needs to be present in the same environment as the cells.

The place of use for the stimulator is in a MEA laboratory at Regea – more specifically to be used in an incubator concurrently with other cell cultures. Due to the need for sterilizing the components, only the container was to be placed inside the incubator. This allowed the rest of the stimulator equipment to be placed outside the incubator and thus be constructed from less expensive components than what would be needed for operating the electronics in warm, humid, and sterile conditions. Within the incubator, this also saved room.

As a passive component, the container needed to be like a petri dish: the lid would need to sit on top of the bottom so that air would flow between them, while still preventing dust and other particles from entering. Even though the container isn’t directly in contact with the cells, the biocompatibility of the container needed to be considered. During evaporation of the medium, the liquid may condense on the lid and drop back into the MEA well with the cells. If material particles are dissolved into the condensed liquid and the material itself is hazardous to the cells, the cells may die and in any case cell experiment results would be modified and not trustworthy.

Biocompatibility of the electrodes is a more important factor, as they are immersed in the medium with the cells. They need to be as inert as possible and well machined, so that foreign particles will not be worn off the electrode material in use. In long term use it is possible the electrodes will degrade – especially with the constant feeding of electric currents through them – so it should be possible to replace the electrodes relatively easily.

The laboratory room in which the device was to be used is relatively small. This fact needed to be taken into account in the device design: there are numerous devices and other equipment in the laboratory, so a large stimulation system would also be in the way of other research done in the same laboratory.

4.2. System specification

In its entirety, the setup was designed to consist of a container, stimulation electronics, a waveform generator, a personal computer, stimulation software, and wiring. The container was specified to have three parts: the bottom part, the lid and the electrodes. The bottom part would firmly house the MEA dish in a shallow recess, the lid would be placed on top as a cover and the electrodes would be attached through the lid so that they descent into the MEA well inside the container. Apart from the electrodes, this setup would resemble functionally a petri dish. The air exchange would be conducted with a small space between the bottom and the lid.

The electronics needed to be able to create various kinds of alternating current (ac) waveforms for long periods of time, since several types of different waveforms were going to be tested and the stimulation periods may last for several days. For this purpose, the electronics would consist of a waveform generator, which would create the general waveform used in stimulation, and the stimulation electronics, which would buffer and amplify the created signal so that it could be used in electrical stimulation. The computer USB was decided to be used as a supply voltage for electronics. The electronics would need to be able to deliver both voltage- and current-controlled pulses.

Software was specified to be MATLAB based. The user interface would need to be sufficient for allowing the user to select the desired stimuli easily by specifying the waveform, strength, duration and possible other parameters. The stimuli would need to be able to be queued so that stimulation with different stimulation profiles could be used, without changing the stimulation parameters while the test is running.

4.3. Container design

As specified, the stimulation platform was designed to be passively an equivalent of a petri dish, capable of housing a MEA dish and the cells, plated on the MEA electrodes. In order to electrically stimulate the cells, two electrodes were designed to be inserted through the container lid. The design was done using Solidworks 2006 SP4.1. Figure 4.1 shows the platform in use.

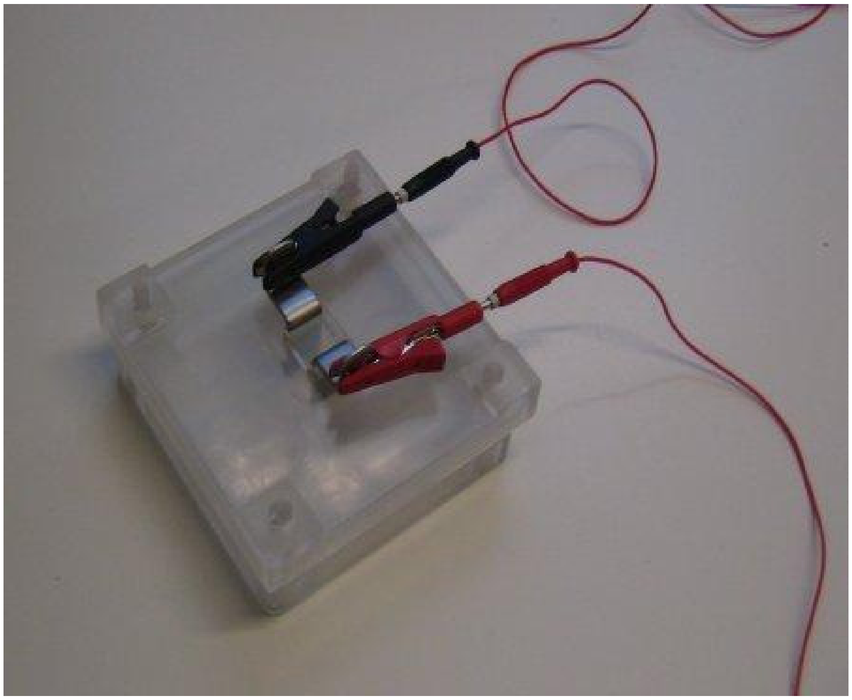

Figure 4.1: Stimulation platform in use

The single MEA container and the stimulator lid were built by the heavy laboratory of the Department of Biomedical Engineering at Tampere University of Technology.

4.3.1. Single MEA container

In order to counteract medium evaporation, the platform needed to have pools of extra medium. Laboratory personnel would still have to add more medium from time to time, but the time interval between refills was made longer by adding the pools. The volume required was estimated to be about 6 ml - the pools have 3.3 ml in each, so the requirement is fulfilled. The pools can be seen in the upper and lower parts of the container shown in Figure 4.2.

The round holes seen on both sides of the MEA dish are designed to make lifting the MEA dish from the platform easy. The grooves below the MEA dish are intended to decrease adhesion between the MEA dish and the platform.

The lid was relatively light and the clips and wires were deemed somewhat heavy, so the lid was designed to be secured in place by nuts. The bottom had four bolts with a helical ridge, so that one could fix the lid in place with nuts.

For the sake of research consistency, the lid can only be placed one way on top of the platform. This has been ensured by making one of the four guiding bolts for the lid slightly thicker than the rest – and similarly increasing the width of one of the coinciding holes on the lid. Since the difference is small, it is hard to notice and thus the thicker bolt has been marked with red, as seen in Figure 4.2.

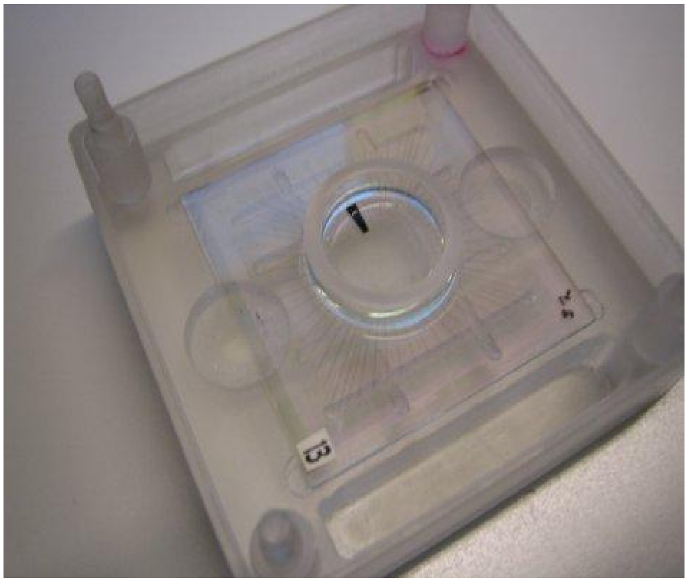

Figure 4.2: A single MEA platform without the lid.

When the lid is placed on the container, the electrodes are positioned on both sides of the center of the MEA. The electrodes are positioned as illustrated in Figure 4.3.

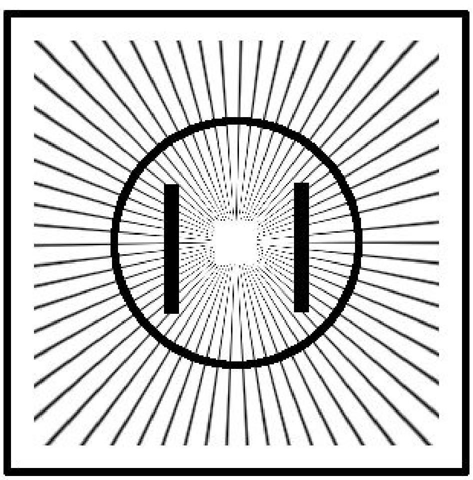

Figure 4.3: Drawing illustrating the positioning of electrodes on a MEA with a 9 mm electrode distance.

The schematic for the single MEA container can be seen in Appendix A: Single MEA container schematic.

4.3.2. MEA amplifier stimulation platform

After creating the stimulation system, the possibility of measuring the field produced by the system using a MEA amplifier while recording a video of the culture arose. A custom lid for the stimulation platform was designed. This lid allowed the stimulation of cells using external electrodes while on a Multichannel Systems MEA amplifier in short term. Some improvements were done on the design, based on the experience gained from using the single MEA platform. In comparison with the regular stimulation platform, the custom lid has a glass window on the top, which allows microscope imaging of the cells while stimulating. Also, it has a coverable hole for gas exchange. A concept drawing of the lid can be seen in Figure 4.4.

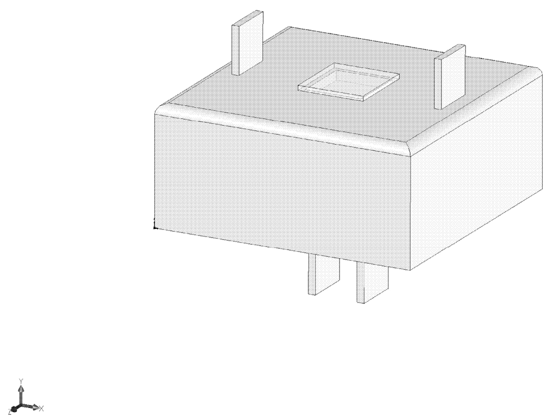

Figure 4.4: MEA stimulation lid concept drawing.

The stimulator lid was designed to be placed on top of the MEA amplifier. This created a small problem, as there was no way of ensuring the lid would stay in position during stimulation. For this reason, small raised corners were attached to the MEA amplifier to match the corners of the stimulation lid. The MEA stimulation lid on the MEA preamplifier can be seen in Figure 4.5. In other functional aspects, the lid is comparable to the single MEA platform.

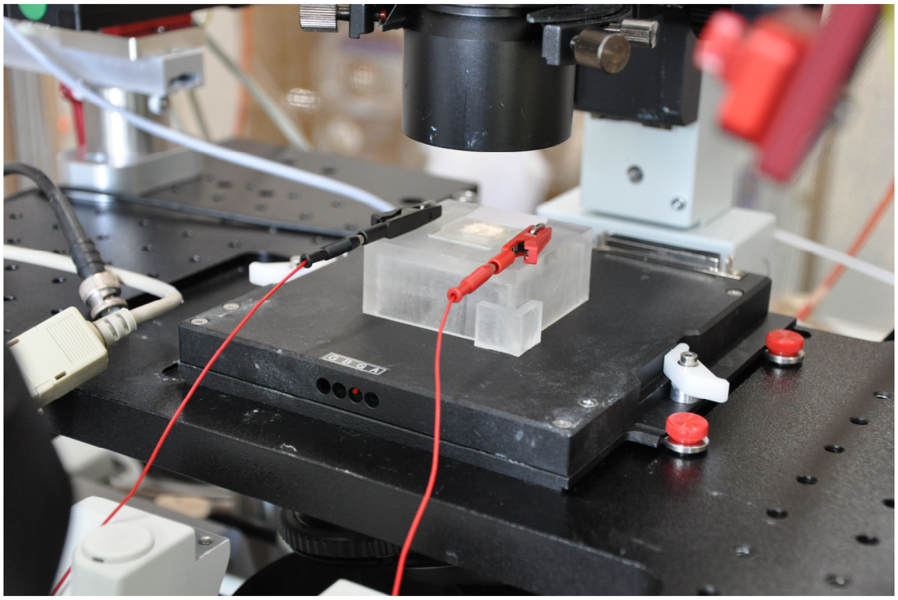

Figure 4.5: MEA stimulation lid on a MEA amplifier.

The schematics for the lid are given in Appendix D: Stimulation lid schematic.

4.3.3. Platform for six MEAs

With the successful creation of the single MEA container, a need for creating a six MEA platform quickly became evident. Cell culturing is not a fast process, and a large part of the already conducted studies on electrical stimulation of cells have taken 72 hours, or more. The volume of cells stimulated per month is thus rather small. Even more importantly, getting scientific results based on measurements on only a single MEA is not viable, as it is very difficult – if not impossible – to have exactly comparable stimulation instances. With the six MEA platform, it is possible to have for instance three instances of stimulated cultures and three instances left unstimulated as a control group in exactly the same environmental conditions. Figure 4.6 illustrates the six MEA stimulation platform. It uses the same principles as the single MEA platform, but is able to house six MEAs and it incorporates design improvements. This includes for instance glass windows for better visibility, which make it possible to observe cell cultures with a microscope without actually opening the container and thus ensuring sterility of the culture. The transparency of the container had been considered an advantage when choosing the materials in order to confirm the amount of medium still left in the container without opening it, but the visibility was not good enough for microscope use.

In this design, the electrode distance was decreased to 5 mm. This decrease almost doubled the highest possible electric field to be achieved because of the shorter distance between the electrodes. Alternatively, the shorter distance allowed the stimulus amplitudes to be halved, as the electric field is directly proportional to the voltage applied, but inversely proportional to the distance of the electrodes.

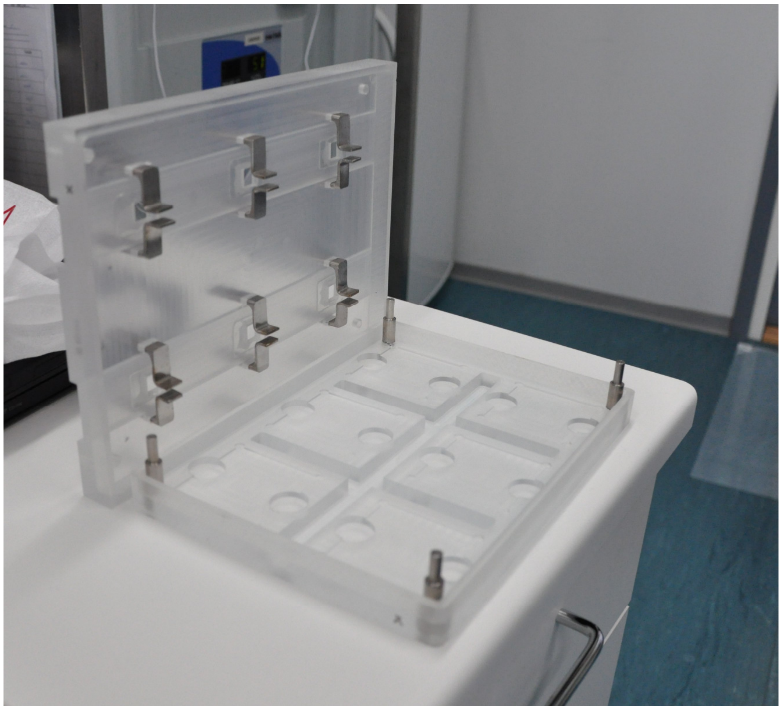

Figure 4.6: A six MEA platform

The six MEA platform was manufactured from acrylic (polymethyl methacrylate - PMMA), whereas the single MEA platform was created using polycarbonate. The decision was done due to the higher costs involved in manufacturing multiple units of the platform. Special care needs to be taken when sterilizing the PMMA platform, as the material is more susceptible alcohol than polycarbonate [48]. Platform schematics are given in appendices: Appendix B: Six MEA container bottom schematic and Appendix C: Six MEA container lid schematic. The six MEA platform was manufactured by Työkalu Kostamo Oy at Tampere.

4.4. Electrodes

Electrodes have a crucial part in the stimulator design. Not only do they serve to deliver the electrical stimulus to the cells, but they are also in the medium with the cells. The biocompatibility issues had to be taken well into consideration.

The electrodes were first simulated using Comsol Multiphysics 3.5a with simple 2D models. Simulation made it possible to estimate the electric field caused by the electrodes before creating them.

4.4.1. Electrode simulation

The models simulated with Comsol Multiphysics 3.5a consisted of three elements: two stainless steel electrodes and the medium. In real use, the mediums used at Regea are D:MEM+F-12 supplement and Neurobasal+B27 in 1:1 ratio. However, in the simulation the medium was approximated as water. The error caused by this was considered to be small, since the exact placement of the electrodes would not be precise either and the simulation would only serve as a guideline.

Using the models, the placement of electrodes could be tested and the intensity of the electric field verified. The first model considered the electrode placement seen from above, whereas the second model considered the view from one side. The first model shows the electrical field between the electrodes and the second model the electrical field under the electrodes, giving more insight into what the field is like for the cells at the bottom of the MEA well.

In both models, one electrode was set to 5 V potential, with the other remaining at 0 V. Based on the results obtained from the simulation, the physical electrodes were designed and built.

4.4.2. Physical electrodes

Conventional, 1 mm thick austenitic stainless steel 316L was chosen as the electrode material. It is easy to machine and most importantly – it has been found to have good biocompatibility [49]. In medicine, stainless steel has been used widely in surgical instruments and as implant material in screws, plates, prostheses and sutures, to only name a few. The material was inexpensive and widely available, so more expensive materials were not considered for long.

In the first container design, the distance between the electrodes was set to 9 mm. It was possible to vary the voltage between -2.3 to +2.3 V in the first generation device, and between 0 V and 4.8 V in the second generation device (as will be presented in 4.5). This meant that it was possible to reach field strengths of 5 V/cm peak-to-peak, 2.5 V/cm amplitude. Similar field strengths had been used previously [36].

It was important to achieve an electrical field as uniform as possible for the cells. That would ensure all of the cells experienced the same stimulus and the possibility to interpret results obtained by stimulation, when the field is well known in the cell culture area. The exact placement of cells on the MEA requires work, so the area on which they could be placed for stimulation needed to be large enough. Since the distance of the electrodes could not be increased without decreasing the strength of the electric field, the width of the electrodes was chosen to be large enough to increase the area. The final electrode design can be seen in Figure 4.7.

Figure 4.7: Stimulation electrode.

One of the crucial factors of maintaining a uniform electrical field was to have the electrodes sit as close as possible to the bottom of the MEA dish well. The electrodes should not touch the bottom in order not to scratch its dielectric layer on the microelectrode wiring and possibly causing measurement and stimulation errors. On the other hand, having them too far from the bottom would make the electric field vary on the surface. In practice, it is difficult to have the electrodes very close to the bottom. The manufacturers estimated the distance of the electrodes from the bottom to be fractions of a millimeter, but the exact values were not confirmed.

The electrodes were connected to the electronics using 2 mm clips and wiring, as can be seen in Figure 4.1. The wiring was chosen based on the wire thickness. With the cells in the incubator, the wires could not be too thick to prevent the incubator door from closing properly. The cheap thin copper wires available during the construction contained only few individual copper threads, so a more expensive one was chosen. Not only is a wire with more threads more reliable, but also the current density is lower.

4.5. Electronics

Throughout the designing process, various parts were considered and tested. The first version of the container design proved to be sufficient for the setup, but due to a change in the way waveforms were generated, the stimulation electronics and parts of the software had to be replaced for a second generation stimulus platform. At first, a sound card based setup was considered for the waveform generator, but later on the change to a data acquisition (DAQ) card was made.

4.5.1. Sound card based setup

The first version of the stimulation platform had a sound card as a waveform generator. The reasoning behind it was that sound cards can produce very different kind of waveforms, they are inexpensive and having them output data is relatively simple. They have, however, certain weaknesses – as they are designed for producing sounds audible by humans, their frequency response is optimized for the 20 Hz - 20 kHz frequency range. Due to this, forming square waves and other waveforms that require low frequencies, becomes essentially impossible.

Nevertheless, a setup was built around this system. The idea was to set the sound card output amplitude to maximum, amplify it to a desired maximum level for stimulation and afterwards control the voltage by software. This idea had the in-built assumption that the frequency response was stable, or at least not very steep in the desired frequency range. Because of this, the sound card quality could not be neglected.

Creative E-MU 0404 USB 2.0 Audio/MIDI –interface was selected for testing, due to being a higher quality product than regular consumer products. The frequency response seemed to stay stable enough for this purpose on low frequencies, even if the voltage was far smaller at 1Hz than it was at higher frequencies. This was deemed good enough for sound cards, and the system entered testing phase.

The amplifier electronics were designed to use the 5 V DC supply voltage from PC USB. E-MU 0404 output voltage was at its peak about +/- 3 V. Since the USB provides only a positive supply voltage, the operational amplifiers (op-amps) used only single-sided supply voltage. A DC bias was thus implemented in the system, so that the single-supply op-amps could amplify the whole waveform and not just the positive side. The DC bias was removed later on in the signal path with a high capacitance. This removed the DC bias, but allowed the ac signal to pass with only little decrease in amplitude, albeit with slight changes in phase. Due to the prototype nature of the device, no attention was paid to the general looks of the device. It was built in a simple connection box.

4.5.2. Data acquisition card setup

The disadvantages in the sound card setup and the discovery of an inexpensive DAQ card by National Instruments (NI USB-6008) led to further testing. The card seemed to function relatively well at low frequencies and it was easily programmable with the aid of MATLAB DAQ Toolbox.

Like with the audio card, there are two analog outputs. However, the card is a lot smaller than the audio card and has multiple digital outputs and also numerous digital inputs, which may prove to be useful when the system is developed further.

The data output method is slightly different from the sound card setup: with the sound card, the whole data sequence was fed to the sound card, which then produced the output. The NI USB-6008 DAQ card, however, accepted inputs of only one sample at a time. A whole data sequence input method would have been more preferable, but the inexpensive USB-6008 card did not support this.

This time, the amplifier electronics was designed with more precision – as the data output from the DAQ card was 0-5V, the electronics were designed for an input of +/-2.5 V with a 2.5 V bias. As the same bias existed in both output voltage electrodes, the effective output voltage was +/-2.5 V. USB-6008 data sheet provided some further information on its performance: the resolution of 12 bits along with the absolute accuracy of 7 mV were more than necessary for the stimulation use, as the there was no need for that great precision. The software-timed maximum update rate of 150 Hz seemed to be low for the stimulation use: it would have allowed changes in stimulation amplitude in 6.7 ms intervals. The preliminary tests on the device, however, showed the device to be capable of changing states at a higher rate and this was not considered a setback. Output current was limited at 5 mA. This was not a problem either, as a buffer circuit was needed between the DAQ card and the electrodes, in any case [50]. Also, the appearance of the device was improved. Proper connectors were used and the device was built in a more sophisticated junction box.

4.5.3. Electronics design

The electrical circuits in the stimulation platform consist of mainly amplifiers. As the DAQ card provides 0-5 V output voltage and USB provides a 5 V supply voltage, the op-amps are used as buffer circuits in order to provide sufficient current for the stimulation. The circuitry provides a voltage buffer for both channels and in addition, a current supply circuit for them. The supply voltage of 5 V means that the output amplitude of the electronics is 2.5 V at most. A 9 mm electrode distance thus means a 2.3 V/cm field and a 5mm electrode distance a 5 V/cm field.

For current stimulation, a sufficient output current was deemed to be 200 μA. The current density of stimulation depends on the amount of medium added into the MEA culturing chamber, as it is defined as current per area. As the current flows only in the medium, the level of medium regulates the current density. Typically, 1 ml of medium is added. With a 10 mm radius of the MEA well, the medium level will be about 3.5 mm. This means the maximum possible current density is almost 570 $\mu A/cm^2$.

The circuitry can be divided into two sections, one for each channel. Both sections contain a voltage buffer using TS921 op-amps and current supply using AD8391 op-amps. A double-sided board was used, with the electric components being surface mounted on one side. This allowed the other side to be left as a ground potential and thus to make it more stable.

Schematic and design was done using PSpice 9.1 circuit simulation software. The circuit board design was done using Mentor Graphics PADS software. The schematics for the design can be seen in Appendix E: Circuit schematic for stimulation electronics and Appendix F: Stimulation electronics circuit design and parts listing. After designing the circuit, an etching mask was created. The mask was printed with a laser printer on an overhead film and the board was etched by photolithography. The components were manually soldered on the circuit board.

4.6. Software

Due to the need for processing, combining and creating signals, the StemStim-software was coded with MATLAB. In general, the software allows the creation and production of electrical stimuli of pre-determined and user created waveforms. Terminology for the software was developed: a stimulation sequence consists of electrical stimuli with the same stimulus parameters. A stimulation session may consist of several stimulation sequences.

As a regular procedure, the user selects the waveform and its duration in hours, minutes and seconds. When using pre-determined waveforms – sine and square waves – the user selects also the amplitude in volts. For square waves, the user may decide to change the duty cycle – although it may cause electrode degradation in a long run and is thus not recommended. After pressing the add-button when the necessary parameters have been set, the sequence is listed in the list for sequences in the session, as shown in Figure 4.8.

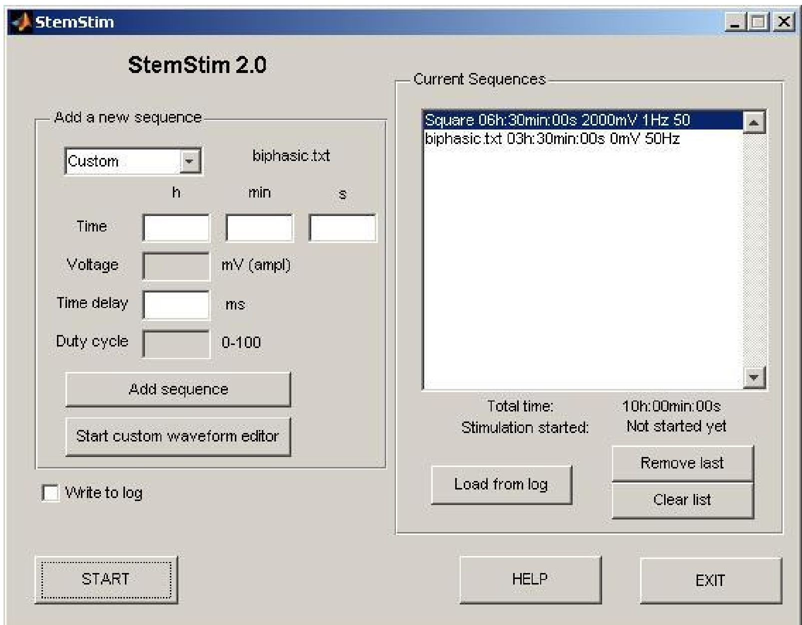

Figure 4.8: StemStim software.

The user may also create arbitrary waveforms using a custom wave editor, also supplied with the software. In the editor, the user creates the signal in small pieces: by determining start and end voltages of direct lines, and the duration of this voltage change. For instance, to create a 1 V ramp signal lasting 1000 ms, the user would need to determine 0V as the starting voltage, 1000 mV as the end voltage and 1000 ms as the duration. These small pieces can be added as many times as necessary to the signal, in order to achieve even complex waveforms. The designed waveform is saved on the computer hard drive as sample values. Previously created waveforms can be loaded from hard disk for viewing. Removing components from previously created waveforms is not possible, but new components can be added. Figure 4.9 illustrates the variety of waveforms possible to be created.

When creating custom waveforms, it's possible to create signals that may not be balanced and thus may cause damage to the electrodes. To prevent this, the software calculates the total charge transferred by the electrical stimulation between electrodes for the custom stimulation sequence. Charge Q can be calculated from current I and time t using a differential equation:

$$dQ = I \cdot dt\ .$$

Since the interface uses voltage stimulation, current I is substituted by Ohm's law and then integrated with respect to time in order to formulate the following equation:

$$Q = \frac{1}{Z}\int U dt\ ,$$

where Z is the impedance of the medium. With this knowledge, the user may attempt to balance the waveforms so that there no net charge is accumulated on either electrode and the electrodes stay in condition longer. Also, the charge accumulation per hour is calculated.

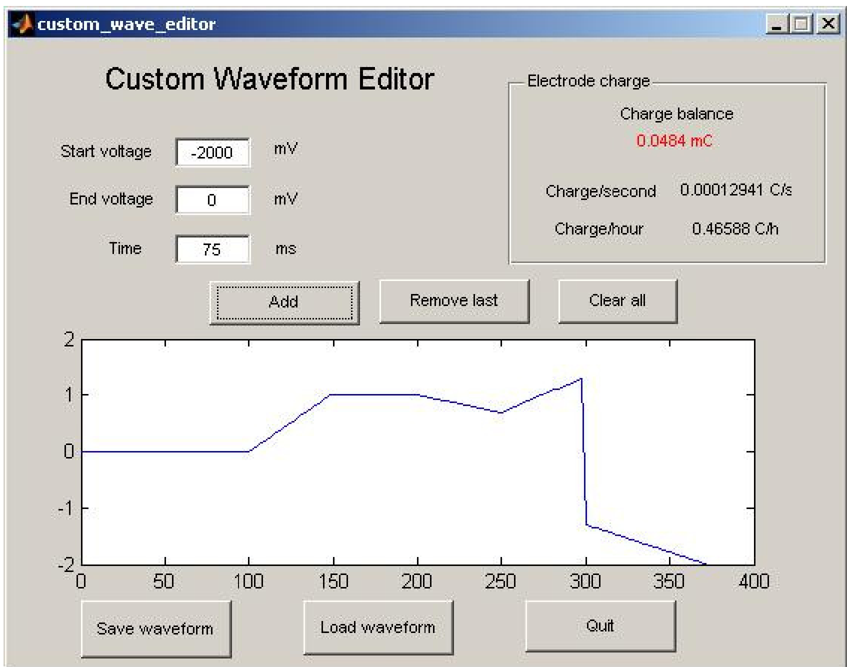

Figure 4.9: StemStim Custom Wave Editor.

Saved waveforms from the custom wave editor can be used in the software by selecting the created signal from the hard drive, determining the overall duration of the stimulation and the delay between each waveform. This allows the user for instance to create a 100 ms waveform with a 900 ms delay before the next waveform – one waveform per second. If the user decided to choose a 400 ms delay instead, the same waveform would be repeated twice in one second and the frequency would thus be 2 Hz.

There can be multiple stimulation sequences in one stimulation session, and the stimulation sessions can be logged. This allows the user to replicate the stimulation sessions easily and confirm the parameters that were used in stimulation afterwards.

When the user starts the stimulation, the program notifies the user of the stimulation starting time, and should the user wish to stop the stimulation before it is scheduled to end, an abort button is provided.

The code in MATLAB was written largely from scratch. A large part of the code deals with processing of the user input to the software, the constructing of signals based on the given parameters and the arrangement of variables for output. Interfacing the DAQ card with MATLAB was not as easy as was imagined – each sample had to be sent to the card individually. More expensive versions of the device would accept whole vectors of input, but with the USB-6008, it had to be done one at a time. This was not a big issue per se – in theory a single for-loop going through the vector, setting the output one sample at a time would have been sufficient. However, the difficulties became apparent in synchronization. The heavier the for-loop, the slower it fed the samples to the DAQ card, thus interfering with the timing of the stimulation. Furthermore, the for-loop had to be possible to be stopped while running. Due to the way MATLAB handles loops, the only way to break out of a loop while it was being processed was to check for a stop-variable. This variable could be set for instance by pressing a button. However, doing this check in every loop slowed down the processing time drastically, resulting in desynchronization. This is why optimization of code was necessary, both in checking for the stop-variable and outputting data for the DAQ card.

Some freely available code routines were used in achieving the desired result. STOPLOOP [51] was used to create a user interface button, which would stop the ongoing loop effectively without consuming too much processor time. ex_func.m and ex_func2.m [52] were used in processing the data in matrices to cell array form in order to save and load waveforms. Both external code components were modified before use for better suitability. Program 1 illustrates the way data output to the DAQ card was eventually done. The user interface was done by using the GUIDE application in MATLAB.

```
while(~FS.Stop() && i<=cols-2)
%The loop is kept running as long as the stop button has not
%been pressed and the loop has not reached the last cell
   while(j <= size(channel1,1) && ~FS.Stop())
   %Go through each sequence in the channel1 data matrix
   k=0;
         while (k <= samples_to_play(j) && ~FS.Stop())
         %Repeats the waveform template as many times as
         %needed
               for i = 3:channel1(j,2)
                     %Goes through the template and sets the
                     %output
                     putsample(ao,channel1(j,i));
               end
               k=k+template_sizes(j);
         end
   j=j+1;
   end
   break;
end
```

Program 1: Data output to DAQ

4.7. System evaluation methods

Consulting previously ran stimulation tests on cardiac and neural cells – not necessarily stem cells, or even human cells – testing parameters for the completed device were designated and new methods of testing developed. The tests were conducted at Regea MEA laboratory, which has the necessary equipment for measuring and observing cells.

4.7.1. Waveform verification

The waveforms created by the device were tested by two methods, both using a GW Instek GDS-1022 oscilloscope in the measurement. With the first method, the generated voltage pulses were measured directly from the output of the electronics. This method removed the noise caused by the electrodes and served the purpose of verifying whether the signal was in fact what the system had been programmed to generate.

With the second method, the stimulus actually experienced by the cells within the stimulation volume was measured by using the MEA Amplifier. The test was done without cells, by measuring voltages directly from the MEA electrodes. Because of the DC bias voltage, it was not possible to measure the voltage with the MEA electrodes when the amplifier was connected to other electrical equipment and large currents would have occurred between the two devices. Furthermore, the MEA amplifier has a constant amplification which would saturate the voltage sensor input easily, when using external stimulation of above microvolt scale. In any case, it would not have been viable to measure the voltages at the same time as the stimulation, since the stimulation pulses would be far greater than the potentials caused by the cells. Any measurements would have had to be carried out between stimulus pulses, thus defeating the purpose of the measurement.

However, the MEA amplifier pins stay in contact with the MEA dish with the amplifier disconnected and the waveforms could be verified by attaching oscilloscope probes to different MEA amplifier pins. The pins were chosen based on the electrode configuration of the MEA dish.

4.7.2. Video recordings

As the custom lid had a glass window on top, a microscope was able to be used when observing the reaction of electrical stimulation to cardiac cells. The tests were run on rat cells and human matured cardiomyocytes grown from Regea 08/017p46 stem cell line. The cultures had intrinsic beating frequencies, although the rate seemed to slow down after moving them from the environment they were used to. The microscope was attached to a secondary computer, which captured the data sent by the microscope on video files. Videos with several stimulation parameters were recorded.

The aim of the testing was to evaluate the response of the heart cells to different kind of stimuli, test the stimulation platform and to produce a valid testing concept for future tests.

The test on the human cardiomyocytes was done using rectangular biphasic 1 Hz square wave. Two parameters, field strength and pulse width, were changed to observe their effects on the stimulation.

For the rat cell culture, monophasic square waves were used. The reason for this test to differ from the human cell culture tests was that the stimulation parameters were studied to see how the cells would be stimulated using values found in literature. Therefore 2 ms, 5 ms and 10 ms pulses were used with different field strengths.

4.7.3. Long term stimulation

The long term stimulation was started first by using the sound bard based setup. In the first few tests, there was no systematical research plan and the process was considered more or less as figuring out the practical procedures required for a long term stimulus. Also, it revealed problems both with the hardware and software that would occur in long term – mostly related to the memory usage of the software but also to the overall performance of the hardware in low frequencies.

Testing with the second generation device (DAQ card based system) was done with a more focused goal and documentation. Several tests had been conducted, with the target being medium with H7 cardiac cells, END-2 cells, NRC heart cells and H7 differentiating cells. Each stimulation test was performed with a control sample in the same environment, to be able to pinpoint the effects of the stimulation.

5. RESULTS

This chapter lists the results of the work: the simulation results, the device created, the measurements done with the device and the practical experiences from using the device in real cell culturing. Estimating the performance of the device is not a simple task: comparable scientific results can be obtained only after extensive testing on real cells. However, based on the requirements set for the device in previous research and the observations done, one can determine how well the device fulfills the expectations.

Using the methods for system evaluation described in chapter 4.7, results were obtained both on the capability of the system for stimulation, as well as on the viability of the stimulation on cells.

5.1. Electrode simulation results

Using the simulation models described in 4.4.1, results on the electric field strength dependence of the electrode placement were obtained. One electrode was set to 2.5 V with the other remaining at 0 V, in order to demonstrate the maximum field strengths possible to be created. The first model considered the placement of electrodes in the MEA well as seen from above. A resulting image can be seen in Figure 5.1.

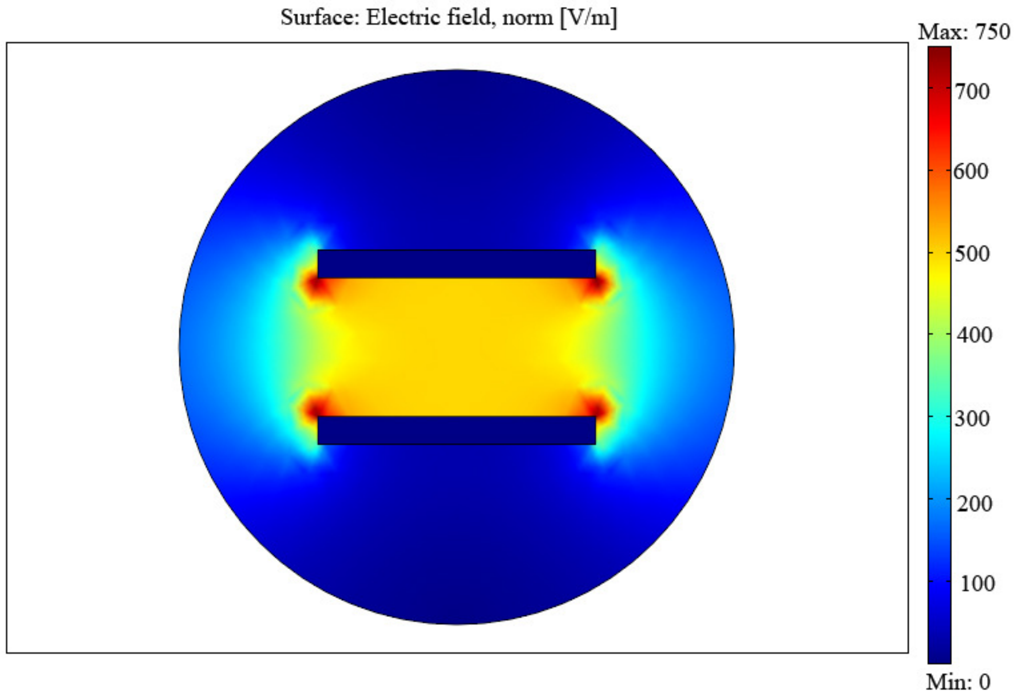

Figure 5.1: Simulated electric field with a 5 mm electrode distance in a MEA well.

As can be seen from Figure 5.1, the electric field between the electrodes is not constant everywhere between the electrodes. The further the cells are from the center of the MEA dish (and the electrodes), the less uniform field they experience. A cross-sectional plot from between the electrodes shown in Figure 5.2 indicates that the field strength decreases a maximum of 2%, from 500 V/cm to 490 V/cm, in the area of ±3 mm from the center point. It is thus relatively constant in the area, but beyond the 3 mm distance the field strength starts to strongly decrease. The 6 mm wide area in the center, however, had relatively uniform field strength. If cells were to be placed outside of this area, the results would not be comparable with the cells in the middle of this area.

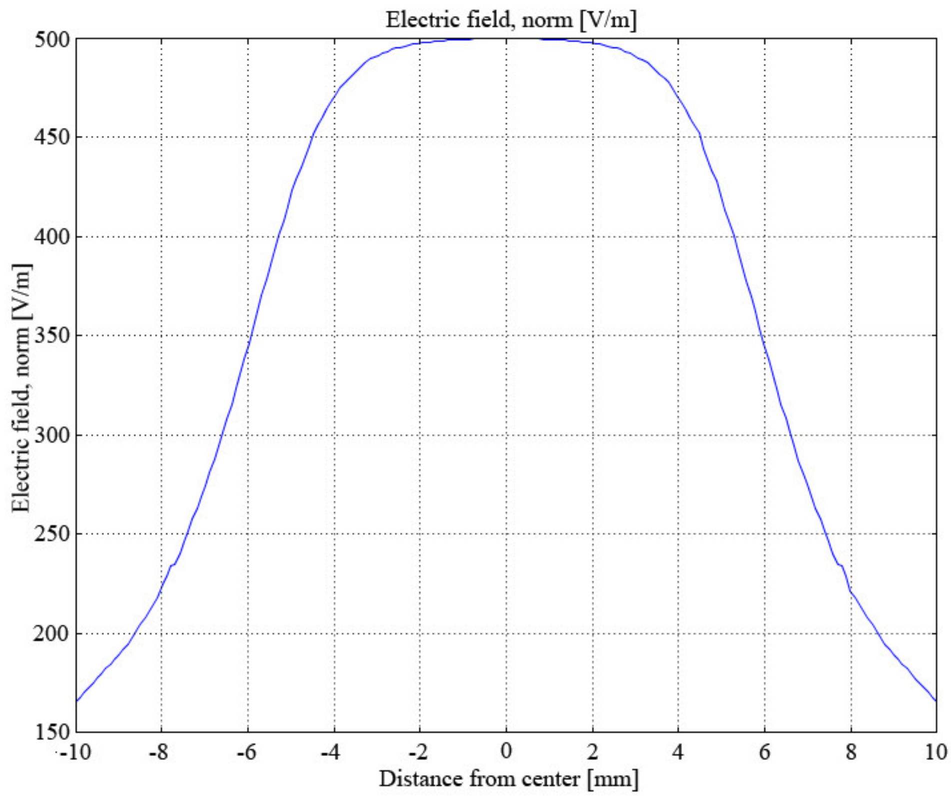

Figure 5.2: A cross-sectional plot of the electric field between the electrodes as a function of distance from the center line between the electrodes.

In the second model, it was approximated that the electrodes would be 0.5 mm from the bottom of the MEA well. The resulting image of the second model can be seen in Figure 5.3.

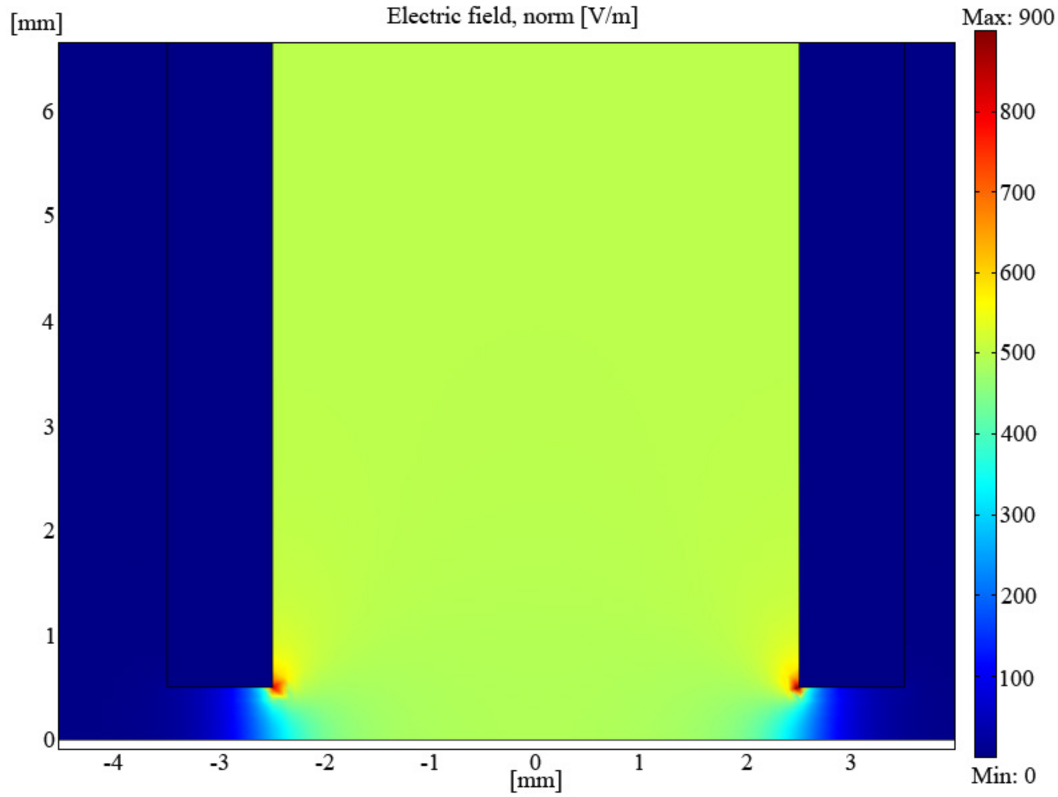

Figure 5.3: A cross-sectional image of the electric field under and between the electrodes with 0.5 mm electrode distance from the bottom. The color indicates the intensity of the electric field.

The model shows that at the bottom, the field strength decreases a maximum of 3%, from 490 V/cm to 475 V/cm, in the area ±1.5 mm from the center. It is thus relatively uniform even at the bottom, where field is weakest in the vertical direction. In practice this means that the cells should not be placed closer than 1 mm to either electrode. Figure 5.4 further shows the electric field to decrease strongly after ±1.5 mm from the center.

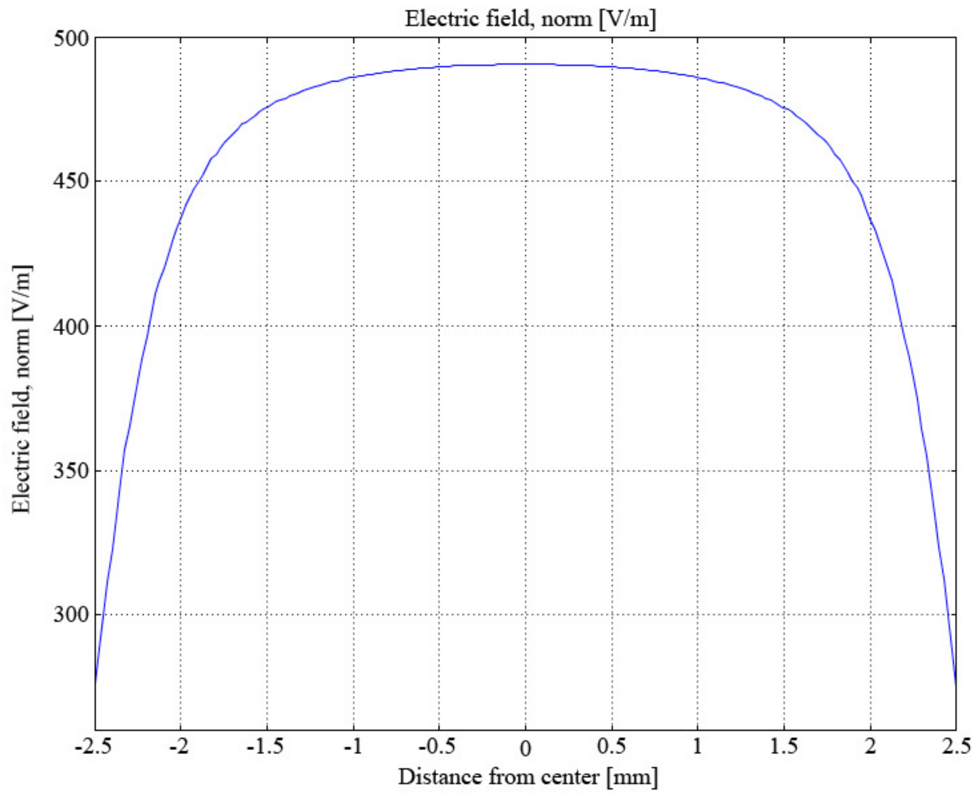

Figure 5.4: A cross-sectional plot of the electric field at the bottom of the MEA well, with the electrodes 0.5 mm from the bottom, as a function of distance from center.

Having the electrodes as close to the bottom as possible is important, since the further the electrodes are from the bottom, the less uniform the electric field becomes. The electric field also becomes weaker. If the electrodes were placed at 1 mm height, the field would change drastically, as can be seen in Figure 5.5.

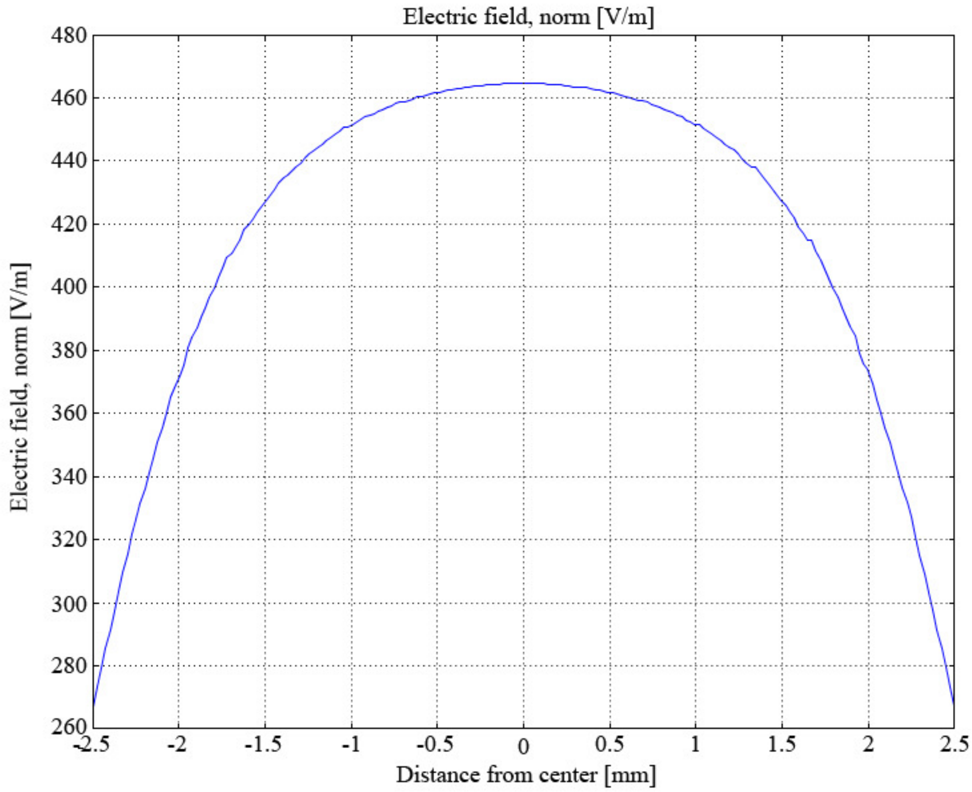

Figure 5.5: A cross-sectional plot of the electric field at the bottom of the MEA well, with the electrodes 1.0 mm from the bottom, as a function of distance from the center.

It can be seen from Figure 5.5 that if the electrodes are placed 0.5 mm higher, the electrical field does not even reach 4.75 V/cm – a value, which was possible in the area ±1.5 mm from the center in the model with the electrodes 0.5 mm from the bottom.

If the electrodes are close enough to the bottom of the MEA, the electric field does not change drastically when moving upwards from the bottom of the MEA well. Figure 5.6 illustrates how the electric field decreases as a function of distance in the center between the electrodes from the bottom of the MEA well, when the electrodes are placed 0.5 mm from the bottom.

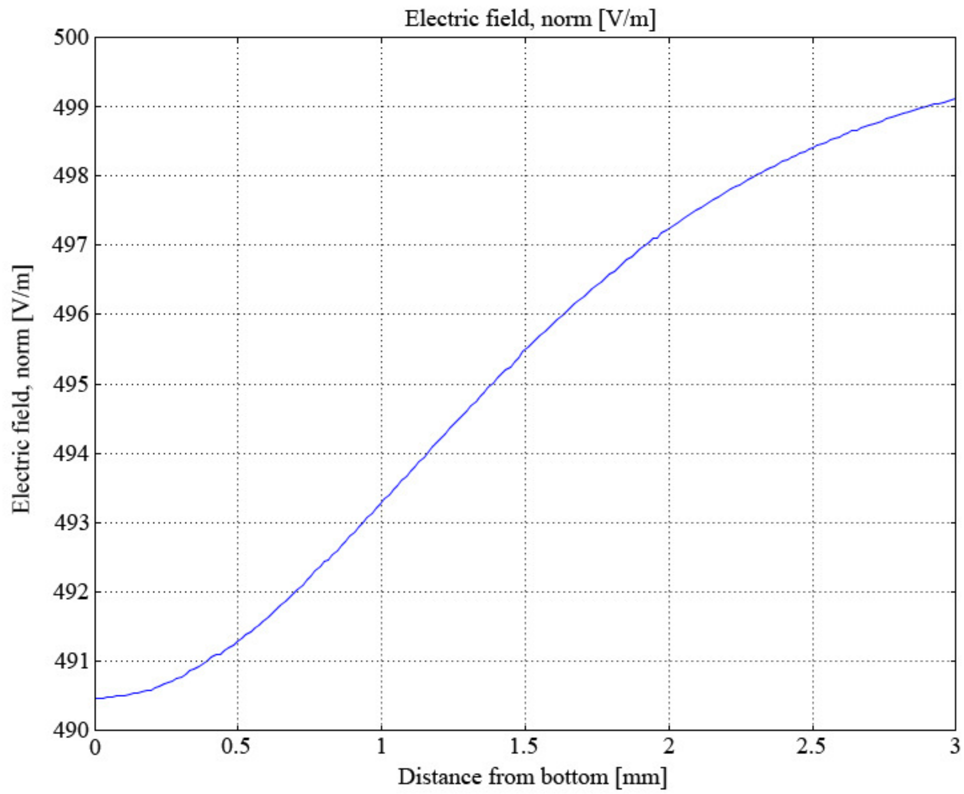

Figure 5.6: A cross-sectional plot showing the electric field in the center between the electrodes as a function of distance from the bottom of the MEA well. The electrodes are placed 0.5mm from the bottom.

The cells, located at the bottom of the MEA, experience a roughly 4.9 V/cm field, when the electrodes are placed at 0.5 mm distance from the bottom. Even if the cells were located higher, the electric field would not change a lot.

5.2. System general results

In this thesis, a fully functional programmable long term electrical stimulation system for cell cultures was developed. The designed device consists of a MEA container, the stimulation electronics and software on a laptop computer, which controls the stimulation electronics.

The system fulfilled the requirements set for it in laboratory use – it can be sterilized easily, by using alcohol or by autoclaving. The container design is comparable to that of a petri dish, which makes it easy to use in an incubator. The materials used in the container are biocompatible. Replacing the electrodes is easy, as they are firmly placed but not permanently attached. As the system does not take a lot of space, it can be used in a very limited laboratory space. It is also safe for the user, since the voltages used are low.

It also functions according to the specifications: the system is capable of creating almost arbitrary waveforms. The amplitude of the waveform is limited by the 5 V USB supply voltage and the shape by the DAQ card, which has allows the resolution of 2 ms.

Stimulating for long periods of time poses no problems. The electronic circuits amplify the signal created by the waveform generator. The MATLAB-based graphical user interface makes it possible for the user to select different kinds of waveforms, stimulus strength and duration, along with waveform-dependent parameters such as frequency, delay between pulses and pulse ratio. Waveforms can be queued, meaning it is possible to have different stimulation sequences in one stimulation session. In addition, the system was expanded with the addition of a six MEA platform as well as with a stimulator lid for MEA amplifiers.

5.3. Notes on the use of MEA amplifier

Using the MEA amplifier at the same time as electrical stimulation was being applied proved to be difficult due to the 2.5V bias at the stimulation electrodes. When the stimulation electrodes with their bias potential are submerged in the medium, the electrodes on the MEA itself are connected to the ground potential via the amplifier. This means that even the presence of the stimulation electrodes would cause a large current to flow from the stimulation electrodes to the MEA electrodes, due to the short distance between them. To avoid this, the MEA amplifier is disconnected from the mains and computer while the stimulation is taking place.

5.4. Waveform verification results

Images of the waveforms were taken using the waveform verification method described in chapter 4.7.1. The oscilloscope image in Figure 5.7 was taken directly from the output of the electronics. The image shows the output voltage with light blue color and the reference voltage with yellow color. A 2.0 V amplitude 1 Hz square wave was used. The figure shows the output to be stable and the waveform closely matches the specified square wave. The amplitude of the signal is 0.08 V higher than specified. The frequency is precisely 1 Hz.

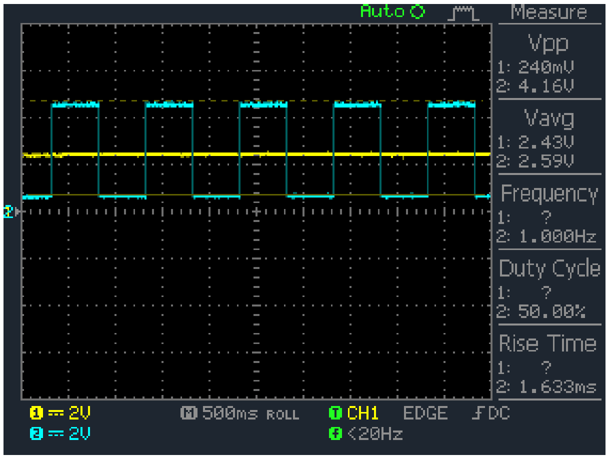

Figure 5.7: The waveform measured from electronics output. The curve with light blue color shows a 1 Hz square wave with the amplitude of 2.0 V and the yellow line shows the voltage of the reference electrode. The oscilloscope measured numerical values of the stimulation waveform, which indicate for instance the peak-to-peak voltage of the square wave to be 0.08 V greater than specified.

In order to test the effect of the stimulation electrodes to the waveform, Figure 5.8 was taken. A 1 V peak-to-peak square wave was used.

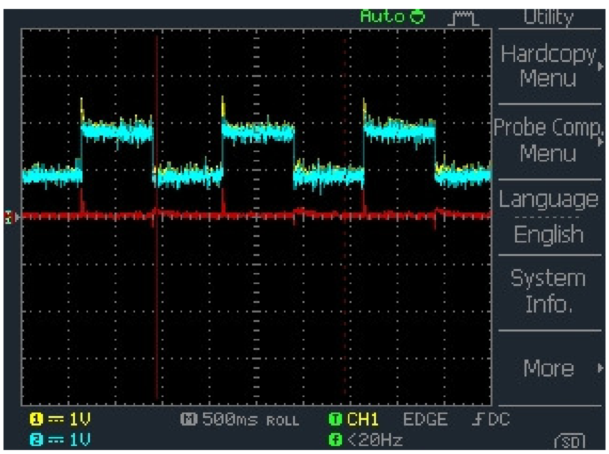

Figure 5.8: A square wave measured from MEA electrodes. The almost overlapping yellow and light blue curves show the vertically displaced voltages of individual electrodes with regard to the embedded reference electrode of the MEA. The red curve shows the potential difference between the electrodes.

In the image, the red curve indicates the voltage measured between the MEA electrodes 14 and 84 [45]. The electrodes were chosen to be nearest to the stimulation electrodes, in the center of the electrode grid. The yellow and light blue curves show the voltage measured from both electrodes individually with regard to the embedded reference electrode of the MEA. The two curves were together vertically displaced to be able to more clearly show also the red curve, which is the potential difference between the two microelectrodes, meaning the difference between the yellow and light blue curves. In practice, the red curve indicates the change of electrical potential actually experienced by the cells between these electrodes, showing spikes at the sites when the voltage is changed. The noise of the electrodes seems to be cancelled out from the perspective of the cells.

The waveforms were not without anomalies, however, due to the sampling frequency of the DAQ card. According to measurements with the oscilloscope, the device was not capable of creating pulses with 1 ms duration or less – each such pulse was widened to 2 ms duration. This was not unforeseeable, as the device data sheet did specify the output rate to be 150 Hz. When testing with a pulse consisting of a 2 V spike lasting 2 ms followed immediately by a -2 V spike lasting 2 ms, repeated at 1 Hz frequency with 996 ms between each pulse, the oscilloscope showed some of the pulses to be missing. In a minute-long stimulation sequence, on average 5 bipolar pulses were missing either the positive or the negative pulse. However, when the same pulses were tested with live cardiac cell cultures with video recording on, contractions did occur regularly even when the stimulus pulse was seemingly missing.

5.5. Video recording results

The specific custom-made lid for the MEA amplifier, along with the microscope, provided a view on how cardiac cell cultures react to the stimuli given by the system. The results indicated that the electrical stimulus given to the cells was indeed causing a reaction. When stimulated, the cell culture contracted according to the stimulus pulses. Apart from the stimulus pulses, the cells also contracted on their own occasionally, according to their intrinsic beating rhythm. However, this was not clearly visible in the recordings – only a few pulses without stimulation were observed. This behavior could be explained by the changing environment causing stress to the cells, as they were removed from their standard culturing platform.

For the human cardiomyocytes, the stimulation showed the effect of the stimulus frequency along with the stimulus energy and pulse width: the cell culture was not able to beat uniformly with all settings. If the interval between voltage pulses was too short, the cell cluster started beating in an unsynchronized fashion – some cells had a different rhythm than others. It was commonly seen that the cells would start contracting first on one side of the cluster, with the contraction continuing later on the other side of the cluster. When the interval between the stimulation pulses was increased, the cells started beating with more synchronization.

Defining the field strength or the pulse width at which the cells had been stimulated was not easy. When either the field strength or the pulse width was increased, more and more cells started to beat at the stimulation rhythm. In order to determine the required settings for stimulating different cultures, the methods of observing the results need to be developed.

Judged by eye, stimulation induced beating started to occur when using a 2.7 V/cm field strength with 100 ms pulse width – 50 ms positive followed by a 50 ms negative pulse. Similarly, using a 500 ms positive pulse followed by a 500 ms negative pulse, cell stimulation was deemed to occur at the field strength of 0.5 V/cm.

As expected by the strength-duration curve, when the stimulus pulse width was decreased, some pulses reached levels below the stimulation threshold and the beating caused by stimulation was decreased and with even shorter pulses, it stopped. However, with an increased stimulation voltage, the energy was also higher and thus the stimulation induced beating could be observed.

The rat heart cell culture reacted at lower field strength, as was expected. Similarly as in the tests with the human cells, it was difficult to determine the threshold for stimulation. However, it was clearly seen that the cells reacted at far lower pulse strengths as pulses with durations even as short as 2 ms did induce beating in the culture with low field strengths. In order to state the exact field strengths for inducing beating, the stimulation threshold would need to be defined for cultures first. However, at field strengths in the magnitude of 1 V/cm some reactions were seen.

5.6. Long term stimulation results

The results varied in the long term testing. Partly this was due to the changes in stimulation parameters, duration and pulse ratio between the tests, but also due to materials and coating. Most tests were done using fetal bovine serum+gelatene coating, but also poly-D-lysine coating was used. One of the problems in achieving consistent results on the performance of the device was to have the cells attach properly even in the control samples. In several tests, the cells had died in both stimulated and control samples due to this. Also, the evaporation of the medium had been a problem, but the control samples would indicate this has more to do with the placement in the incubator than with the actual stimulation. These kinds of problems are natural with cell cultures, especially when the cell culture protocol has not yet been fully optimized.

In general, it was found that the pH of the samples remained OK and the stimulation did not affect it. When the attachment of the cells had succeeded, beating was observed in some instances in stimulated samples. Cell orientation was not seen to take place because of the stimulation. The effects of long term stimulation on the growth of stem cell cultures are still being researched using the developed platform.

5.7. Notes on general use

In laboratory use, the system was found to serve well in its intended use. The containers were comparable to a petri dish and the design felt sturdy, while still being easy to handle. Sterilization of the container did not pose problems material-wise. Both polycarbonate and acrylic proved to withstand alcohol well enough.

Using the software user interface in laboratory environment was handled with a mouse. As the laptop had a touchpad for control, using it with laboratory latex gloves was not easy.

6. DISCUSSION

The produced system is fully functional and it is being used for the purpose it has been designed. The results obtained from the measurements are encouraging, as the device is capable of serving as a platform for future studies. Even as the device is finished, improving the device further will unlock more areas of research. In this chapter, the overall performance of the device along with advantages and future improvements is considered.

6.1. Overall performance

The scope of this work included designing mechanical parts of the device, the electronic circuitry combining commercial products with the system being developed and the software to use it all. The developing has been a continuous process. Several functions and properties of the system were devised after the first versions of the device had been in use.

The system fulfills its requirements in cell stimulation: it is able to generate and deliver electrical impulses to cells during a long lasting culturing phase. The device has not been found to be unsafe to the stimulated cells, neither by cytotoxicity nor by harming the cells by the electrical stimulation. The device is also safe for the user: the voltages used are low, when compared to the Brevet *et al.* system, which requires voltages to be in the range of 100 volts [33].

Usability of the device and the program can still be improved, as well as adding more functionality and possible new modules. These functions are best developed according to user feedback, as there are no strictly similar systems available – and even compared to the systems commercially available, it has more features.

From a strictly engineering point of view, the stimulation system is not a very complex device and with future work, it can be improved. The software is MATLAB-based, which makes further development easy. More advanced functionality may, however, require changing to other programming languages. Fortunately, the code can be implemented on other programming languages with relatively little effort. The stimulator circuit along with the waveform generation may be a future issue – while the system works well now, improving the circuitry will provide waveforms with better resolution, less noise and a possibility to reach lower frequencies.

6.1.1. Hardware

The container for the MEA is one of the strong points of the stimulation device. The experiences of the cell culture and research personnel using the device have been positive, as the device has a professional feel to it. It is easy to use: the electrode lid could only be placed one way, the MEA dishes stayed in their places, the amount of medium could be easily seen and the overall design felt sturdy.

The design was aimed to mimic that of petri dishes. When the results on growing cultures on MEAs placed in petri dishes and the container designed in this work were compared, no differences were observed. Thus it can be concluded that the container works well as a petri dish substitute. When using MEAs, the designed system has the advantages of keeping the MEA better in one place and the in-built pools of medium counteract evaporation.

6.1.2. Electronics

The electronics in the device are fairly simple, as the waveform generation itself is done using an external device. The advantages are clear: programming is simple, design is robust and the DAQ card itself is inexpensive. However, the disadvantage of the DAQ was seen when verifying the waveforms, as it was not capable of creating pulses lasting for 1 ms.

Basing the stimulation system on the DAQ card instead of a computer sound card enables the use of low frequencies, a stable frequency response and the possibility of having outputs of single samples. These advantages make it vastly superior to the sound card in electrical stimulation use, since it provides much better waveform customizability.

6.1.3. Software

The software went through multiple changes and development cycles during the project, and it is still being developed further. The key aspect in the software when considering other stimulation systems is the customizing aspect of the waveforms possible to be generated. The user is able to create arbitrary waveforms, which can then be used in stimulation. The software allows the queuing of different stimulation sequences and is capable of controlling the waveform generator in long term.

Current stimulation was omitted when creating the software. This was done due to the research interests at Regea, and the inherent control of voltage – with current stimulation, larger voltage spikes might have occurred. The advantages of voltage control had also been documented by Wagenaar *et al.* [21]. The hardware and electronics, however, support the current stimulation so adding it would be relatively trivial.

The users found the system easy and intuitive to use without intensive training. However, since the software was not developed for a large number of users, the user help documentation was not completed and the user training was done in person. Documentation and help files will be written in the future, as they are important aspects which require attention and development, even if the software is intuitive to use. The source code is, however, commented for future use.

6.1.4. Field strength limitations

One of the important factors of the device is the electric field strength and waveforms it is capable of creating. The DAQ card output had the output voltage capability from 0 V to 5 V, which combined with the 9 mm electrode distance ensured only the maximum theoretical electric field strength of 2.78 V/cm. In practice, the maximum field strength was closer to 2.3 V/cm because of the op-amps. As the field strength of 5 V/cm was commonly used in several studies, the six MEA system had a reduced electrode distance of 5 mm in order to be able to reach 5 V/cm with a one sided 5 V USB power supply.

When it comes to technical performance, perhaps the greatest error in development was the electrode distance in the single MEA container. With higher field strengths, the tests could have been run with settings largely similar than in other studies. This is, however, easy to state in hindsight, as several studies had also used lower field strengths, for instance Sauer *et al.* [36]. Furthermore, the naturally occurring DC fields in animal tissues are in the range of 0.1-2 V/cm [36], so stimulating with similar field strengths would not be out of question. In addition, cardiac cell beating was observed even with the stimulation lid, which had an identical electrode distance with the single MEA container. This indicates the single MEA platform does serve its purpose, even if the field strengths possible to be reached are lower than the most commonly used fields in the literature.

Also, a more powerful power supply might have been a good idea, even if it had added an additional power cord to the laboratory. It would have allowed greater headroom in stimulation and would have provided a powerful tool in studying the cell apoptosis by too large electric stimulus.

6.1.5. Noise removal

The noise in the system originates mainly from the electrodes. The output waveform measured directly from electrodes is relatively clean, although some noise can be noticed. Since the DAQ has relatively low noise levels and the amplifier does not introduce a lot of noisy components, some of the noise may simply be conducted to the system via the laptop.

The USB supply voltage does fluctuate in use and some of its fluctuation may be transferred to the electronics output. This was not considered to be an issue, as the cells experience the stimulus only by the current induced by the potential difference and not by specific voltage levels applied to the electrodes. Introducing filtering capacitors to the supply voltage may be helpful in future designs.

6.2. Choosing the stimulation parameters

Although there is still a lot of testing to be done, when it comes to stimulation parameters – especially in long term experiments – some preliminary guidelines can be seen from the results.

When choosing stimulation parameters, all the parameters should be well thought out. Due to the research interests of the collaborators at Regea, the testing of the stimulation system concentrated mainly on the cardiac cells. In the literature, cardiac cells have been stimulated typically with the field strength of 5 V/cm. The beating observed in short term tests showed the cells to beat at lower field strengths, even with 2 V/cm biphasic pulses, albeit not as strongly as with higher field strengths. However, the durations of the stimulation pulses and the time between them had not been thoroughly documented in literature. Using the six MEA system with the decreased electrode distance, the maximum field strength was effectively doubled. This allowed the system to reach the most commonly used field strengths in the area of 5 V/cm.

In our tests, too short times between stimulation pulses did not allow the cells to return to their pre-stimulation state and uniform beating was not observed. Also, the differences between cells need to be considered, as their rheobase values are different. With a 1 Hz frequency, a short pulse width allowed a longer time interval between pulses – for instance 100 ms pulse followed by a 900 ms resting period. This in turn allowed all the cells to return to their resting state and thus created a more stable beating rhythm. On the other hand, this decreased the amount of energy in the stimulus pulses and not all stimulation pulses exceeded the stimulation threshold. This could, of course, be corrected by increasing the field strength. With very short stimulation pulses, the single MEA system was unable to provide the cells with high enough field strengths, as the peak-to-peak voltage value is restricted to the maximum of 5 V. However, the field strength was easily doubled by decreasing the distance between the electrodes in the six MEA system, so this was not seen as a severe problem.

6.3. System development

Even though the platform succeeds in fulfilling its requirements, further developing of the stimulation system will provide more possibilities and advantages. Developing the system further does not require decisive amounts of time or work, as some parts of the ideas have already been partly implemented in the device.

6.3.1. Six MEA platform development

For the future, the platform is going to be developed by adding a glass window under the MEA dish. This allows the use of a microscope without lifting the stimulation cover – which in turn decreases the stress to the cells caused by the change of the atmosphere. In the very first tests with the device, the evaporation of medium was seen as a larger issue than in the single MEA system. This is understandable, as the pools of medium support a relatively smaller amount of medium per area of evaporation. In future versions, this could be improved by increasing the depth of the pools.

6.3.2. Two channel stimulation

Currently the software supports stimulation using only one stimulation sequence at a time. However, the electronics have been designed with double capacity, so that it is possible to have two ongoing stimulations simultaneously. The DAQ card has two analog output channels and the stimulation electronics have been designed with double capacity, so that it is possible to have two simultaneous stimulations. Implementing the second channel in the software provides a logical step for further research: for multiple MEA platforms, different electrical stimulus parameters may be used.

Combined with the six MEA platform presented in chapter 4.3.2, a powerful cell stimulation research tool is achieved. For example, four MEAs can be stimulated in pairs with two different stimuli and two MEAs can be left unstimulated as controls. Not only does this drastically shorten the time required for stimulus research when compared to the single MEA stimulation platform, but it also provides more reliable data due to the possibility of having control samples for the stimulated cells in exactly the same environment conditions.

6.3.3. Current stimulation

During the early development of the device, both voltage and current stimulation methods were considered for the device. The possibility for current stimulation is included in the stimulation circuit, but it has not been implemented in the software so far. Also, the output current of the device is still to be verified.

The development of the current stimulation was postponed, because sudden changes in the stimulation current were estimated to cause high voltage spikes to the cells. Voltage stimulation was thus assessed to be a safer alternative. Furthermore, more papers have been published dealing with voltage stimulation than with current stimulation.

6.4. Future research possibilities and applications

The growth environment of the cells is not a passive one. It has, in fact, a lot of active components, in addition to the electrical stimulation. Merely taking care of the temperature, gases and humidity is not at all everything that can be done to mimic their native environment. Taking care of the other needs of the cells, such as adding growth factors, can be done manually by laboratory personnel, but can also be automated.

6.4.1. Electromechanical stimulation of cardiomyocytes

Adding the second channel as explained in section 6.3.2 provides a very interesting field of research. The contraction of the cardiac muscle is an inherently an electromechanical process, so a logical step from stimulating the cells electrically would be to stimulate them mechanically as well. While the mechanical stimulation platform would be an entirely new area of research, such a device could rather easily be combined with the electrical stimulation platform. One channel could be used to stimulate the cells, while having the other channel would provide the control signal for the mechanical stimulus. In fact, a mechanical simulation is currently being developed and tested by the Department of Automation Science and Engineering at TUT and after brief assessments, the electrical stimulation system may be used in parallel with the mechanical stimulation system. There are still some problems concerning the actual implementation of electrodes in the mechanical stimulation system, but all the technical prerequisites exist. The software itself would not require modification after adding the second channel, but an additional hardware module may be needed for further signal amplification. While the container itself would have to be redesigned for mechanical stimulation, the modifications will most probably be minor, and the electronics and the software could be used almost directly.

6.4.2. Long term microelectrode array stimulation

The electrical stimulation done using the created platform lacks the aspect of spatial localization of the electric pulses. As it is, all the cells and their parts experience roughly the same electric field, as was designed. Using the MEA amplifier, cells on a MEA dish may also be stimulated in a more controlled fashion, but only in short term. By connecting the designed stimulation electronics to the embedded MEA microelectrodes, it would be possible to stimulate the cells longer periods, while having the cells in the incubator.

While the electrical stimulus per se could be used in the microelectrode stimulation system, additional electronics would be required for being able to choose the electrodes for stimulation. In effect, demultiplexers would be needed, as a single input signal would be fed to electrodes of choice. Also, the stimulation amplitude would need to be decreased because of the small size of the microelectrodes, which could otherwise lead to current densities harmful to the microelectrodes, and because of the short distances between electrodes on MEAs. This would, however, require drastic changes and even redesigning of certain aspects of the device, but the overall framework would stay the same and ease further design.

6.4.3. Observing the effects

Not only can the electrical stimulation of cells be improved, but also the ways its effect is observed. It can be as important as the stimulation itself: a cluster of cardiomyocytes may appear to be beating uniformly with cells activating throughout the culture, but the observed motion may be caused only by a fraction of cardiomyocytes, with the rest of cells moving due to attachment to the beating cells. For the verification of actual beating mass, new methods need to be developed. Electrical measurement of the cells is not often feasible, because the stimulation pulse exceeds the potentials generated by the cells and usually also cause severe measurement artifacts. The voltage signals measurable from the cell cultures need to be amplified greatly by the measurement equipment in order to observe them properly, and because the stimulation pulse cannot be isolated from the measurement, the voltages can only be measured after the stimulation pulse. This calls for other methods, such as video analysis on the movement of cells.

6.4.4. Commercialization

As shown previously in glances to the literature in chapter 3.2.1, different custom stimulation platforms have been used. However, as was seen in Section 3.4.2, to my best knowledge only few commercially available systems are available for stimulating cells electrically in an incubator. The system built in this thesis work fulfills the expectations set on the system and is able to produce the scientific results achievable with similar setups, but due to its prototype nature, test type design and MATLAB based functionality it does not compete directly with commercial setups. With more emphasis on productization, however, the possibility would exist.

7. SUMMARY

The objective of this thesis was to design, build and develop a programmable long term electrical stimulation system for cell cultures on microelectrode arrays. The project was multidisciplinary, as it involved cellular electrophysiology, modeling, electronics, programming and hardware design. The scope was extremely wide: each of these aspects would have warranted individual research, if the project had not been outlined properly. Despite the outlining, additional components for the stimulation system were designed, such as the stimulation lid and the six MEA platform, which were produced in response to new needs of the cell research initiated by the first design realized in this work.

The device built is a functional, self-contained system, which can be used for its intended purpose – electrical stimulation of cell cultures in long term. Its functioning was verified by measuring the resulting waveforms with an oscilloscope, the interaction with cell cultures was tested by video recordings with the aid of a microscope, and its viability for long term stimulation by regular cell culturing at Regea. This system to my best knowledge among the first systems using MEAs placed in an incubator in long term electrical stimulation by uniform electrical ac fields, while allowing the user to design the stimulation waveform profiles.

Literature research done for designing the device revealed a relatively small number of systematical research articles on electrical stimulation of cells. Various articles had considered only some of the effects of the electrical stimulation, such as in the case of cardiomyocytes, elongation and orientation [27]. Different studies had shown, however, that electrical stimulation also affects their protein and gene expression [23, 28], mechanical properties, calcium transients and other aspects [26]. Also, the effect of different stimulation parameters had only been considered for neural cells and even there, the waveforms had been based on the square wave [21].

In the future, using the stimulation platform can provide interesting venues of research. Exploring the various stimulation parameters made possible by the customizable waveforms can result in better cell response to electrical stimulation and enable the research on the effects of long term stimulation on stem cell differentiation and tissue production. Due to the way the waveforms are generated, the system can be used in parallel with other stimulation methods, such as mechanical stimulation, in order to stimulate cardiac cells electromechanically.

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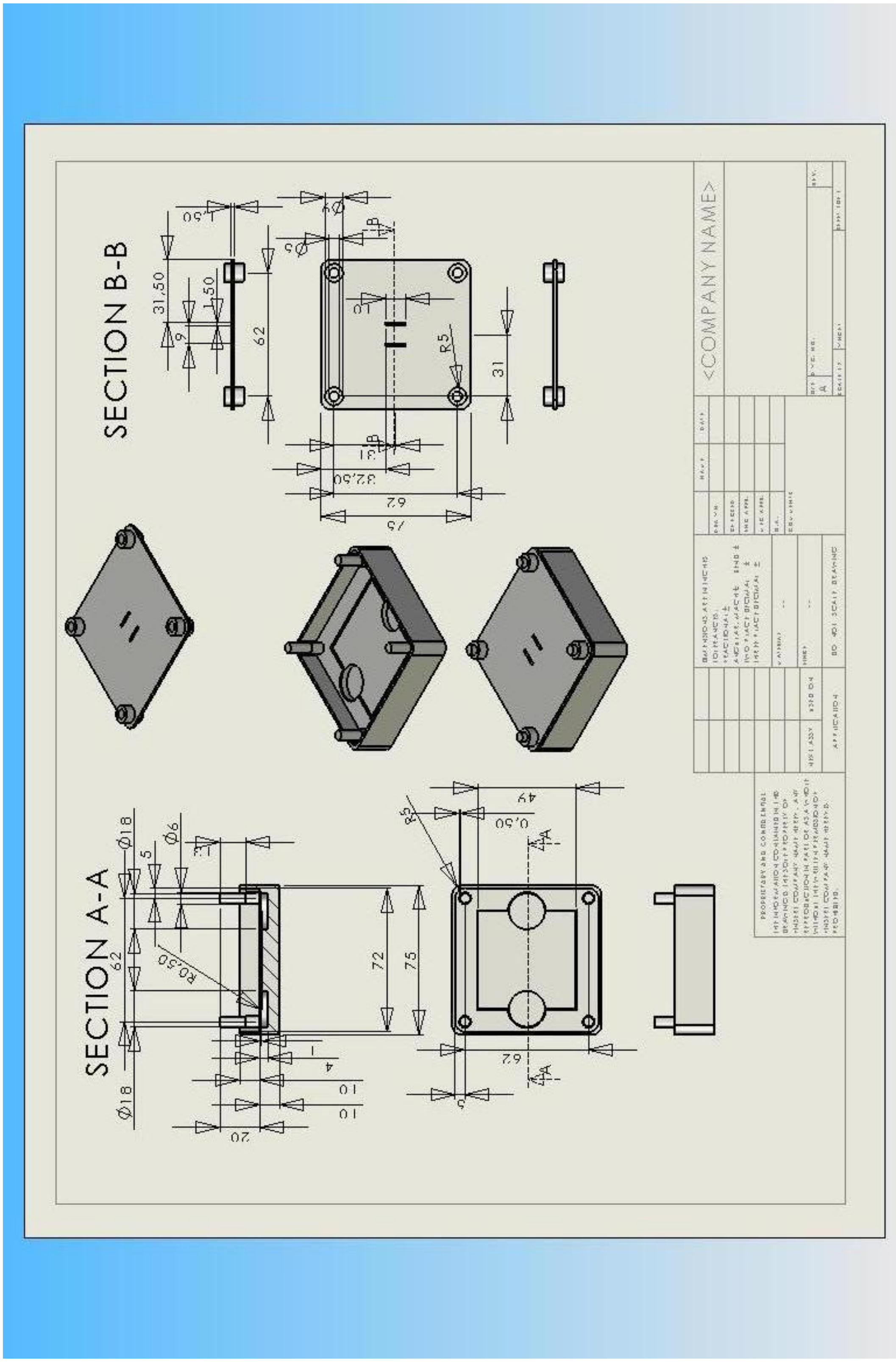
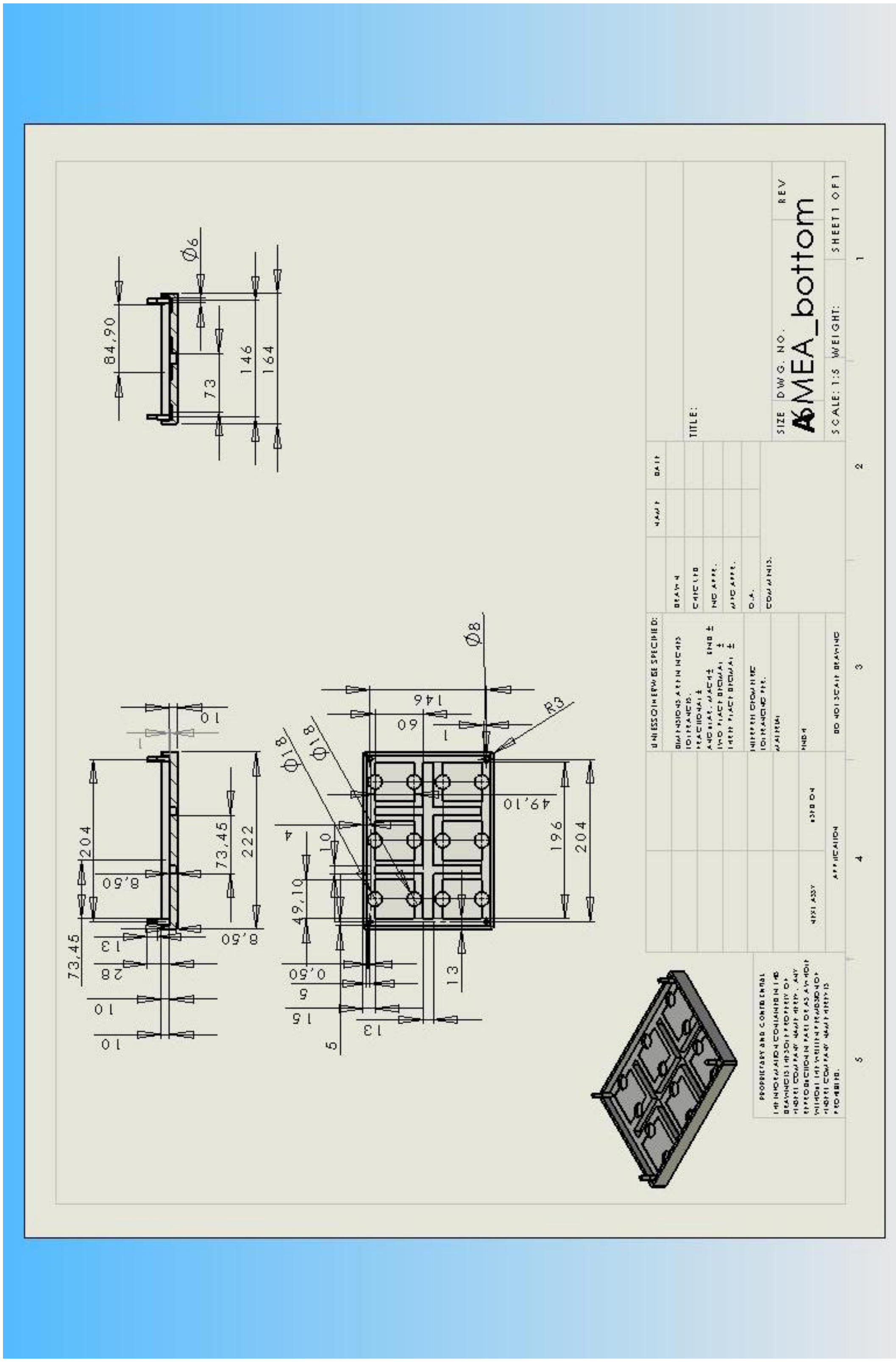
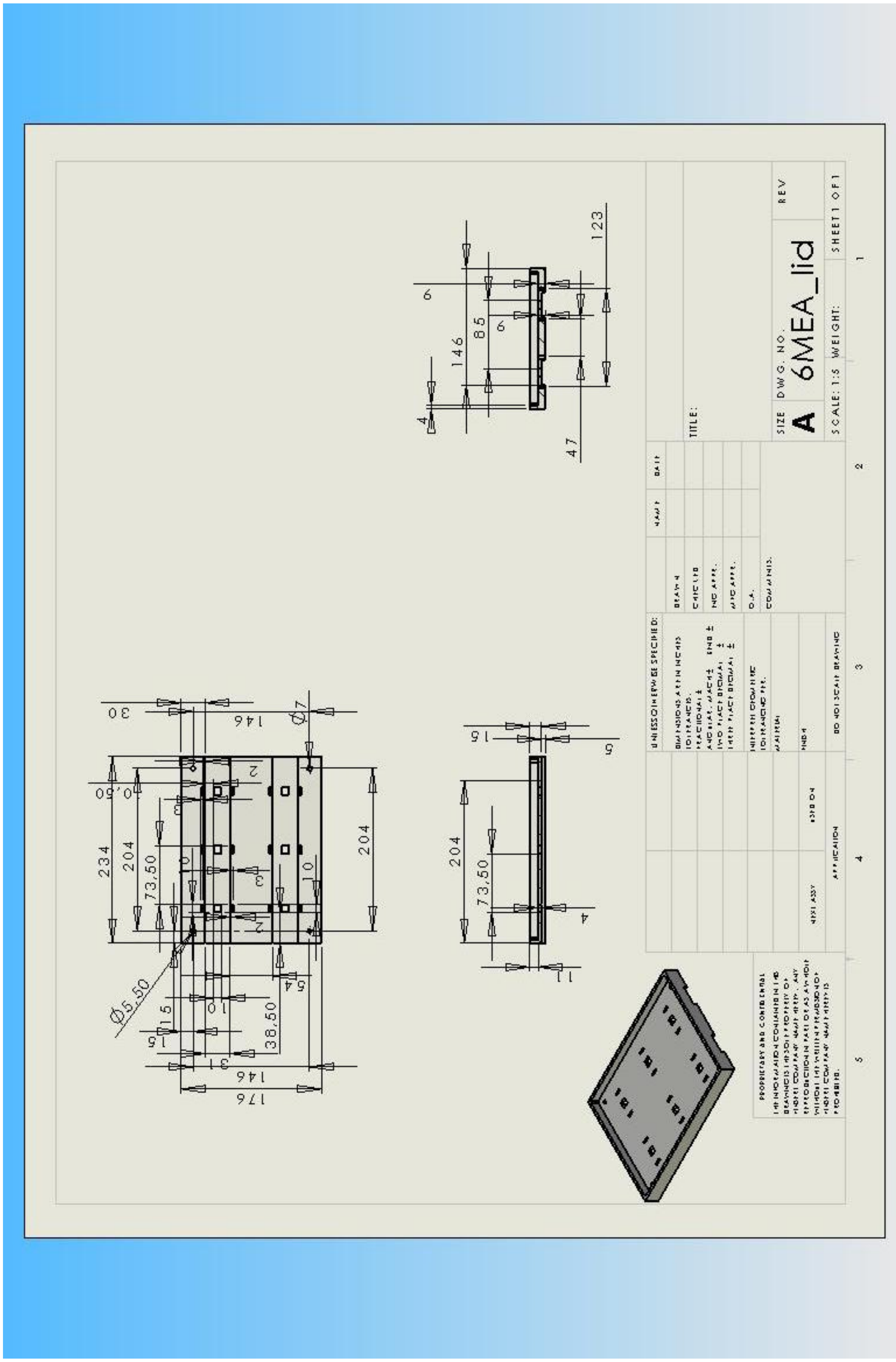
APPENDIX A: SINGLE MEA CONTAINER SCHEMATIC



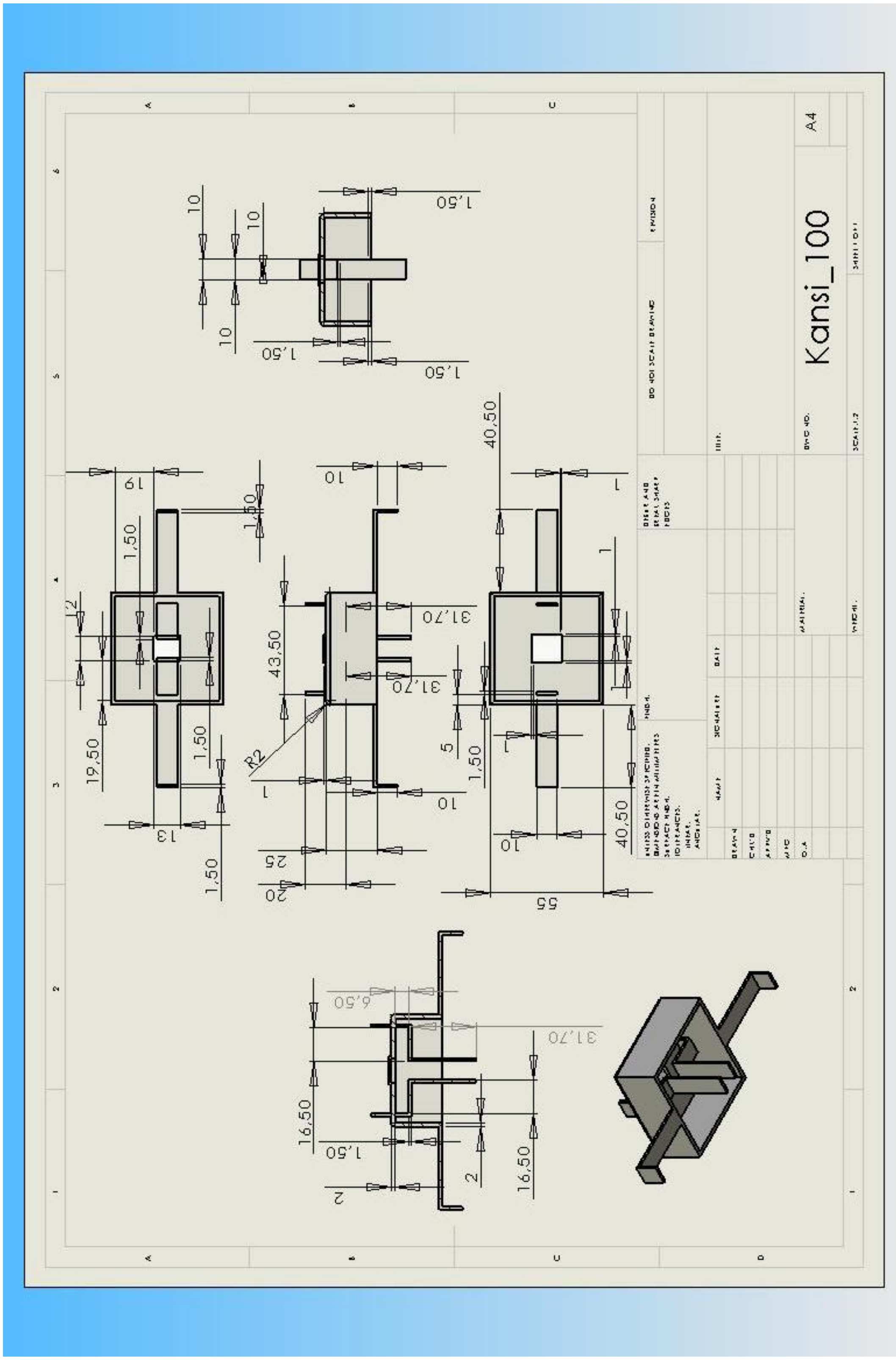
APPENDIX B: SIX MEA CONTAINER BOTTOM SCHEMATIC



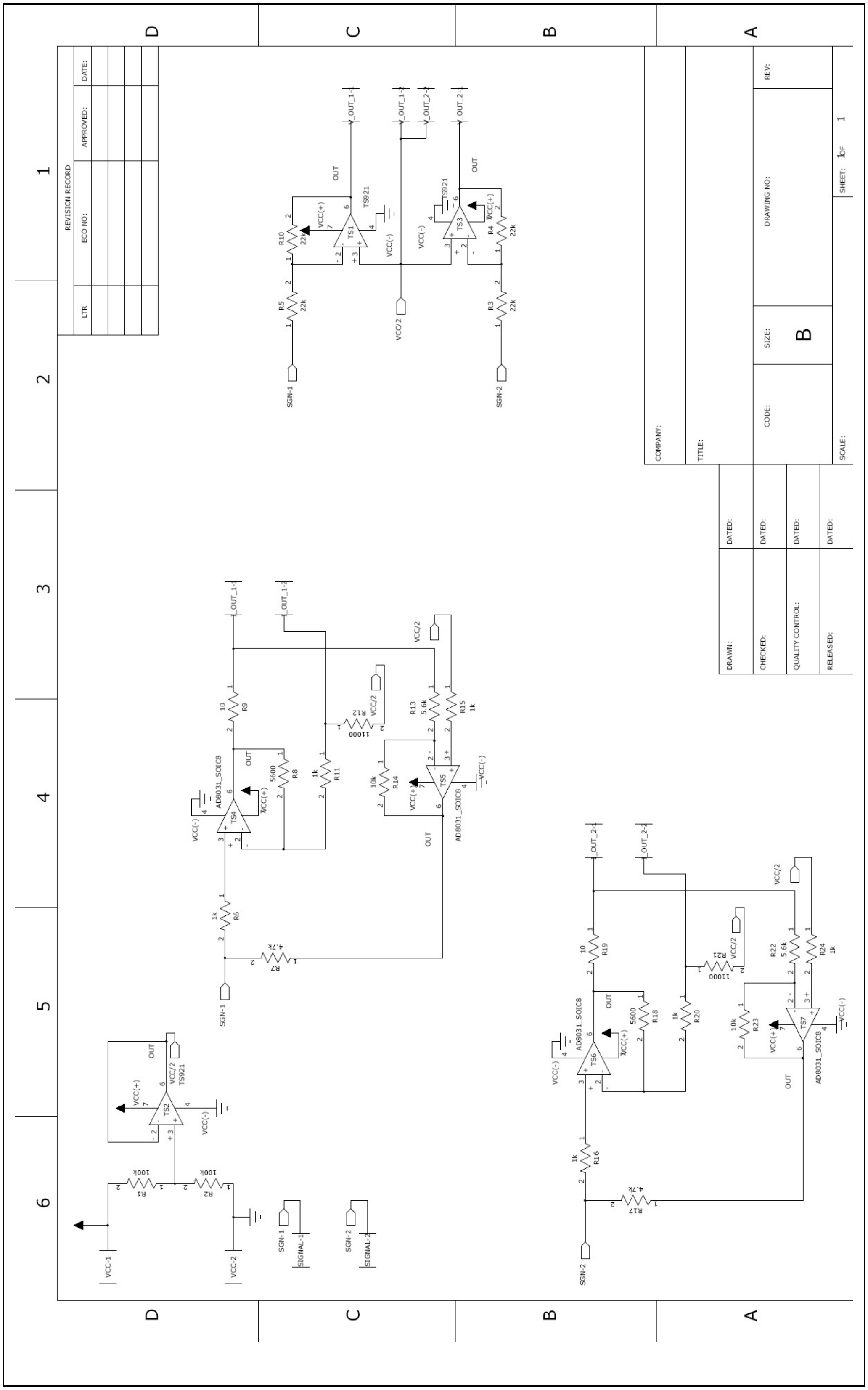
APPENDIX C: SIX MEA CONTAINER LID SCHEMATIC



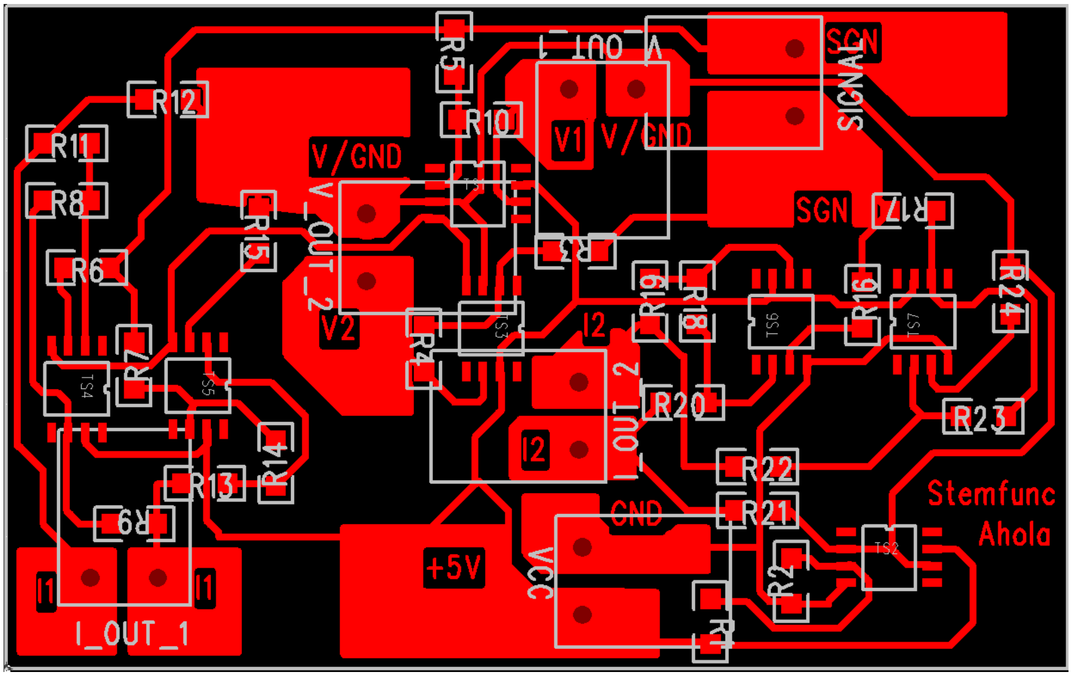
APPENDIX D: STIMULATION LID SCHEMATIC



APPENDIX E: CIRCUIT SCHEMATIC FOR STIMULATION ELECTRONICS



APPENDIX F: STIMULATION ELECTRONICS CIRCUIT DESIGN AND PARTS LISTING



Part Name	Reference	Quantity	Description
AD8031_SOIC8	TS4-7	4	Op-amp for current amplification
RES1206,10	R9, R19	2	Surface mount resistor, 1/8W
RES1206,100k	R1-2	2	Surface mount resistor, 1/8W
RES1206,10k	R14, R23	2	Surface mount resistor, 1/8W
RES1206,11000	R12, R21	2	Surface mount resistor, 1/8W
RES1206, 1k	R6, R11, R15-16, R20, R24	6	Surface mount resistor, 1/8W
RES1206, 22k	R3-5, R10	4	Surface mount resistor, 1/8W
RES1206, 4.7k	R7, R17	2	Surface mount resistor, 1/8W
RES1206, 5.6k	R8, R13, R18, R22	4	Surface mount resistor, 1/8W
TS921	TS1-3	3	Op-amp for voltage amplification