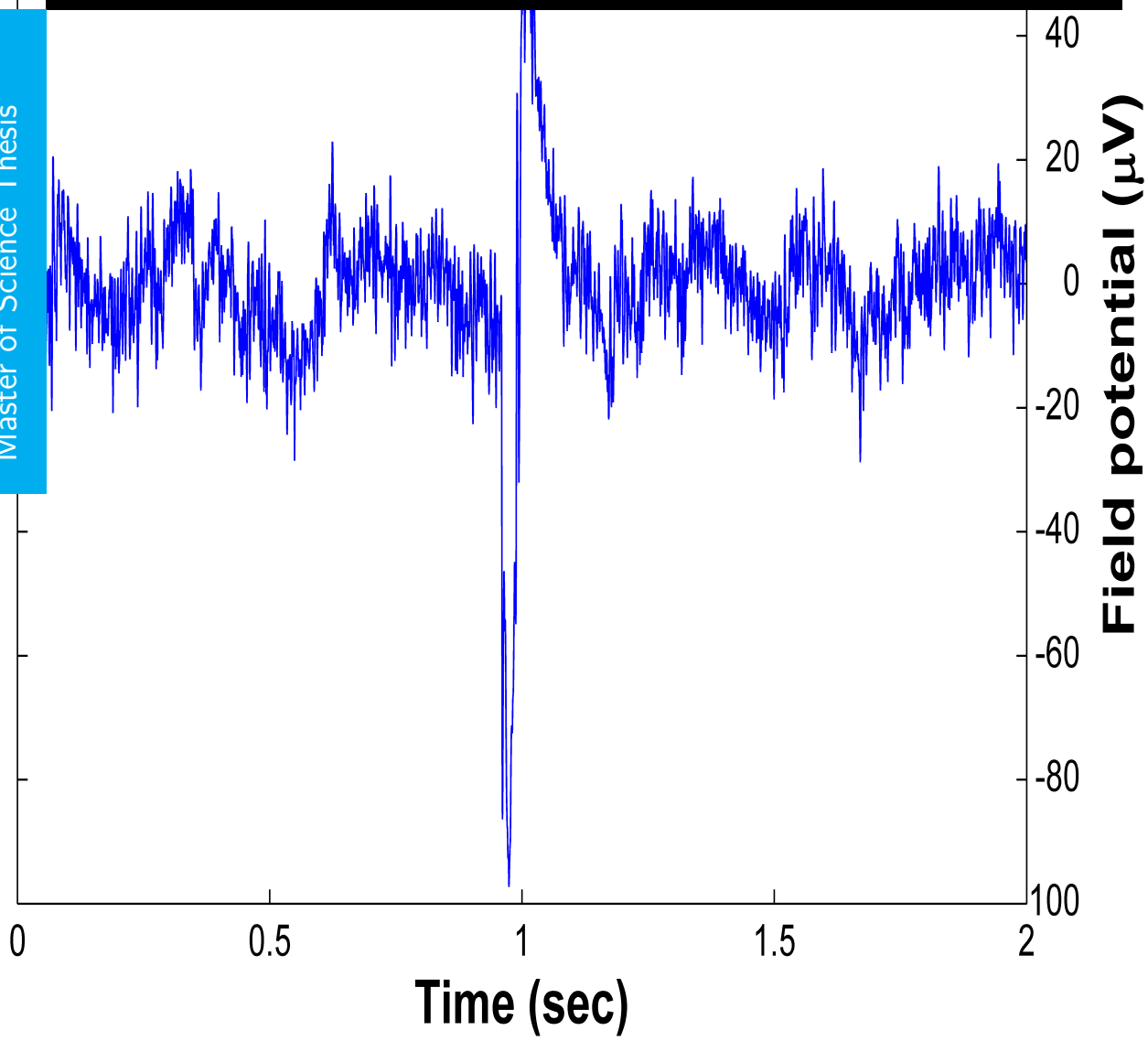
Assessment of Cytostretch - A Heart-On-Chip device

Experiments on Cardiomyocytes

Shruti Agrawal

Master of Science Thesis



TU Delft Delft University of Technology

Delft Institute for Microsystems and Nanoelectronicsl

Assessment of Cytostretch - A Heart-On-Chip device

Experiments on Cardiomyocytes

Master of Science Thesis

For the degree of Master of Science in Biomedical Engineering at Delft University of Technology

Shruti Agrawal

September 25, 2014

Faculty of Mechanical, Maritime and Materials Engineering (3mE) · Delft University of Technology

The work in this review was supported by TU Delft, UMC Utrecht and Philips Research. Their cooperation is hereby gratefully acknowledged.

Copyright © (Department of Microelectronics)
All rights reserved.

Delft University of Technology
Department of
(Department of Microelectronics)

The undersigned hereby certify that they have read and recommend to the Faculty of Mechanical, Maritime and Materials Engineering (3mE) for acceptance a thesis entitled

Assessment of Cytostretch - A Heart-On-Chip device

by

Shruti Agrawal

in partial fulfillment of the requirements for the degree of

Master of Science Biomedical Engineering

Dated: September 25, 2014

Supervisor(s):

Prof. Ronald Dekker

Dr. Teun P. de Boer

Reader(s):

Prof. R. Dekker

Prof. P. M. Sarro

Prof. W. A. Serdijn

Dr. T. P. de Boer

Abstract

An organ-on-chip device, called Cytostretch (Philips NV), that is capable of mechanically stimulating cultured cells, is the subject of this study. The device comprises a microthin PolyDiMethylSiloxane membrane that is highly elastic, on which titanium nitride electrodes have been embedded. Its utility is projected in cardiac research to emulate the mechanically beating on the muscle cells (cardiomyocytes) of the beating heart and record the extracellular potentials simultaneously. The device is in its nascent state and requires validation through suitable experiments, for which a pneumatic apparatus was built.

Following an overnight cyclic stretching, the membrane deforms permanently owing to hysteresis, which inhibits the formation of a uniform monolayer of cells on the membrane. There was a wide device-to-device variation in the noise levels at the electrodes in the MircoElectrode Array (MEA) recordings. Field potential recordings during spontaneous beating of cardiomyocytes cultured on the membrane were obtained successfully in the relaxed state of the membrane. The MEA recordings are however, accompanied by artefacts at the instance of stretching or relaxing the membrane which interfere with the field potential of the cardiomyocytes. The cardiomyocytes in the device died after their cyclic stretching, thought they were not detached from the membrane.

In conclusion, the permanent deformation challenges the utility and re-usability of the device when uniform monolayer of cells is desired. Therefore, the mechanical properties of the membrane and metallic interconnect should be revised. The interference from the stretching artefacts hampers the quality of MEA recordings. The method of stretching needs revision to prevent damage to the cells and high frequency stretching artefacts. The susceptibility of the silicon chip toward optical noise during MEA recording omits the microscopic investigation together with MEA recording.

Table of Contents

List of Figures

List of Tables

Preface & Acknowledgment

This thesis is based on the project which is a requirement to obtain the Master's degree in Biomedical Engineering at Delft University of Technology. The project was conducted in the Division of Heart & Lungs at the Department of Medical Physiology in the University Medical Centre of Utrecht. It was initiated together by UMC Utrecht, TU Delft and Philips Research with the aim of learning the effect of mechanical stretching on the maturation of stem cell derived cardiomyocytes, using Cytostretch device. However, it was soon realized that the device did not meet the standards required to perform meaningful experiments. Therefore, the project was re-directed to the assessment of the device and finding the issues with its design that affect its function.

I would like to thank my thesis advisor Dr. Teun P. de Boer at UMC Utrecht for giving me valuable insight and for the interesting ideas during the project. I am also thankful to him for teaching me the skills needed to conduct life science experiments, something that I had never imagined before. I thank him for his valuable feedback on writing the thesis. My supervisor Prof. Ronald Dekker at TU Delft is deeply thanked for giving me the opportunity to work on this interdisciplinary project, and providing me with necessary resources. I thank Saeed Pakazad for fabricating the devices and guiding me through its usage. I thank the PhD students and the laboratory technicians at UMC Utrecht for teaching me the skills and knowledge required to perform all the experiments. I regard Prof. Martin Rook (UMC Utrecht) for his involving discussions on electrophysiology and for leaving the laboratory late in the evening when I had extra work. I also acknowledge his guidance when I had to speedily design the electric circuit to drive the solenoid valve within 1 week. I regard the staff at the workshops at 3ME faculty of TU Delft for providing me with materials and machines to manufacture the holders. I cherish the company of Marlieke, Sanne, Ivar, Ian and Marieke with whom we cracked jokes in the laboratory. I would miss forever the affection and cookies of Tony (our laboratory secretary). I thank my family for making me so capable that I worked with this esteemed group. Last but not the least, I thank my friend Dhruv Mehta for his valuable time and support.

Delft, University of Technology — MSc. Biomedical Engineering

"To Dhruv"

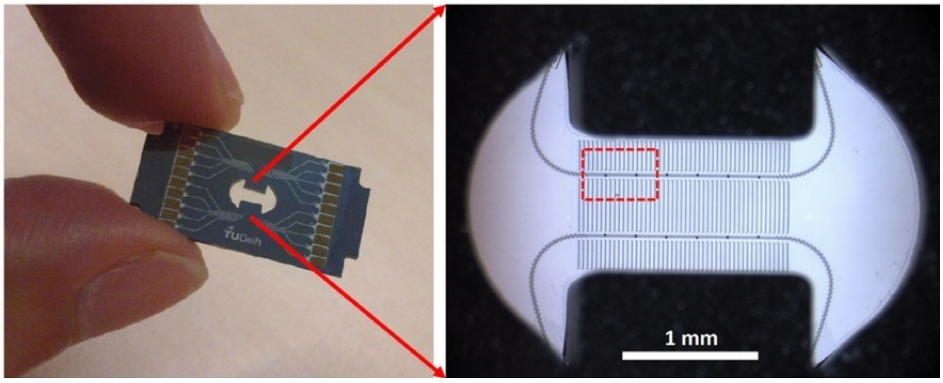
Chapter 1

Introduction

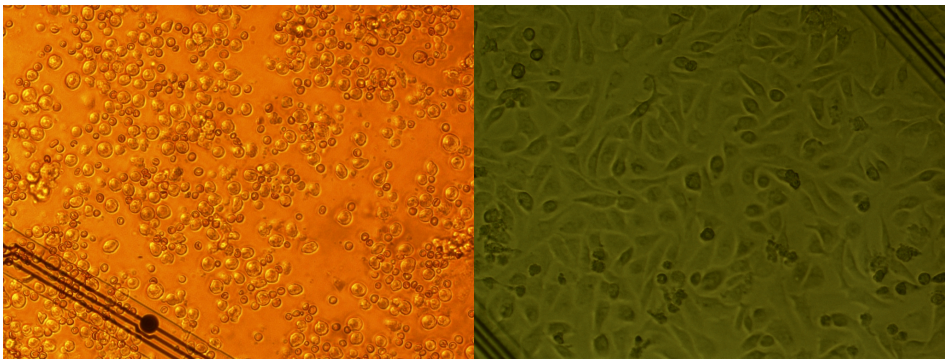
Animal testing is an integral part of health research because some of the organs mammals like mice, rats, rabbits and dogs, resemble those of humans. However, animal testing has received sharp criticism with regards to preventing cruelty to animals during experimentation, mainly in medicinal and cosmetics research. Typical experiments involve the induction of diseases in animals by altering their genes, surgical operations, the injection of toxins and life in captivity. On the other hand, a complete embargo on animal testing would certainly halt research on the much needed drugs and healthcare products. Thus, there is an imminent need to replace animals with high-fidelity experimental and theoretical models.

To promote research in medicine without animal testing, engineers have proposed a new range of micro-fabricated devices or biomimetic microsystems called *Organ-on-Chip*, which aim to emulate the micro-environment of living cells at tissue or even cellular level. The purpose of Organ-on-chip devices, is to precisely encompass the physical conditions of the cells present in the human body and thus, provide a more natural environment for culturing cells than the one provided by the conventional methods using Petri dishes. An Organ-on-chip may possess microchannels for the flow of biochemicals (OrganoPlate, MIMETAS [1]), stretchable PolyDiMethylSiloxane (PDMS) membranes to mimic muscle cells or myocytes (Lung-on-Chip, Wyss Institute at Harvard University [2]) etc. The present study uses Cytostretch, a Heart-on-Chip device, developed in 2013 through collaboration between Philips Research N.V. at Eindhoven and Delft University of Technology. It contains electrodes embedded in membranes to record extracellular electrical potential [3]).

The motivation behind Cytostrech are the rampant Cardiovascular diseases that have been a major cause of death amongst adults over the last few decades, resulting primarily from the death of cardiomyocytes (heart muscle cells) or *myocardial infarction.* A possible treatment involves replacing the dead cardiomyocytes with live ones, a fully functional copy of which, is yet to be produced *in vitro* (in the laboratory). However, such experiments rely completely on cardiomyocytes isolated from animals and reports on their success are rare [5, 6]. Thus, considering the disapproval of animal testing, scientists are exploring the possibility of culturing myocytes from precursor stem cells, hence, stem-cell derived cardiomyocytes. This task is



(a) An overview of the chip and the dog-bone shaped membrane. The electrical interconnects (each 2 μm thick) connect the circular electrodes (12 μm diameter) to the silicon chip. [4]



(b) Isolated cardiomyocytes, freshly-seeded on the membrane. The dark circle is one of the microelectrodes.

(c) Cardiomyocytes attached to the membrane.

made challenging by the fact that stem cells must be exposed to an environment that can provide the correct physiological environment conducive to their differentiation (specialisation) into cardiomyocytes.

This thesis seeks to explore whether Cytostretch can indeed provide such an environment. Unfortunately, the experiments in this thesis are based on neonatal rat cardiomyocytes (< 10 hours post-birth) because they are affordable and easier to handle than the stem-cell derived cardiomyocytes, while having similar electrophysiology.

1-1 Cytostretch - 'Heart-on-Chip'

Cytostretch, as a 'heart-on-chip' model, is a cell culture device that facilitates the application of mechanical stress on cultured cardiomyocytes, thus inducing their *in vitro* development.

Saeed et al. [4] describe Cytostretch as a Stretchable MicroElectrode Array (Stretchable Micro-Electrode Array (SMEA)) that consists of a silicon chip with a dog bone-shaped hole in the centre and covered with a transparent PDMS membrane containing the electrodes and micro-features intended to promote cell alignment and adhesion. The electrical interconnects traverse from the contact-pads on the silicon substrate to the electrodes on the PDMS membrane. There are in total 12 electrodes arranged linearly in 2 rows on the membrane. The PDMS membrane is only 25 μm thick and is the functional area where the living cells are cultured and stretched in coherence with the membrane. The functioning of the electrodes and the integrity of the structure has been tested by recording the field potential signals from cardiomyocytes with the aid of Multi Channel System (MCS) in both, relaxed and stretched condition of the membrane. Its biocompatibility has been verified with Caco-2 cancerous cells from a colon that are easy to culture.

Cytostretch is meant to serve as a more accurate *in vitro* model of cells for drug testing, differentiation of stem cells and studying the mechanosensitivity of cardiomyocytes.

1-2 Aim

An Organ-on-chip device must be robust and compatible with a multitude of experimental set-ups with regards to structural integrity, surface properties, transparency for microscopic examination and biocompatibility. In spite of the proven functioning of Cytostretch, there is a requirement to further develop it ergonomically and improve its compatibility with all kinds of cell-based experiments, before its market release. Its biocompatibility with cardiomyocytes must be verified specifically, which requires more stringent culture conditions than those of the cancerous colon cells.

The aim of this thesis is to evaluate and enhance the performance of Cytostretch as a 'Heart-on-Chip' device through experiments in cardiac electrophysiology at University Medical Centre (UMC) Utrecht.

1-2-1 Objectives

The objective for this study is to use the Cytostretch devices extensively and obtain the maximum amount of information about them through a combination of experiments, preparation of the apparatus, cell culture, field potential recording and processing of data. The details are as follows:

1. *Pneumatics* - To develop a device for controlling the flow of air delivered by a pneumatic pump to stretch the chip's membrane and hence, the cultured cells. The frequency and the extent of strain in the membrane must be adjustable by the user.

2. *Chip-holder*- To ensure an efficient transfer of energy from the pump to the membrane, some robust holders must be built that confine the Cytostretch device and direct the flow of pressurised air to the chip without leakage.

3. *Biofunctionality* - The PDMS membrane that is the functional area of the device must be made biofunctional, i.e. it must permit the attachment of cardiomyocytes. For culturing any cells, their attachment to the substrate is a primary requirement. PDMS being hydrophobic is non-adhesive toward organic compounds like protein solutions, and hence not a favourable culture ground for the cells. Thus, a chemical coating that can render the PDMS biofunctional must be found through suitable experiments.

4. After the validation of the above set-up, cardiomyocytes from neonatal rat hearts must be cultured on the Cytostretch and monitored to assess, firstly, the device's ability to promote *in vitro* sustainability of the cells and secondly, the interaction of the cardiomyocytes with the Cytostretch by stretching the membrane. Monitoring will involve microscopic investigation and calcium imaging to assess the electrophysiological and morphological properties of the cardiomyocytes.

5. Baseline measurements to detect the sources and levels of noise in the electrical signal from Cytostretch's Micro-Electrode Array (MEA) must be performed. This is important as noise and interference can hamper the quality of the electrical recordings with the MEA.

6. To formulate the protocols for the careful use of the Cytostretch primarily because of its delicate nature and its incompatibility with procedures like Immunohistochemistry and Patch-Clamping.

The supply of the devices was very limited (15 devices) and they are very susceptible to damage. Therefore, the test cases in the this experimental study were insufficient to calculate the statistics of the results, although the results indicated that the device in its present state is not reliable to for use in the life science experiments.

Chapter 2

Background

2-1 Cells of the heart

An adult human heart consists of about 10 billion cells of several types, whose location and function differ from each other, yet, they work in harmony. Less than one-thirds of the cells are cardiac muscle cells, which are excitable or electrically activated, while the remaining are non-excitable. The majority of non-excitable cells are fibroblasts, while a few excitable cells other than cardiomyocytes for example, stem cells, are also present in the heart.

2-1-1 Cardiomyocytes

Muscle cells are excitable, i.e., they are capable of responding to a depolarising stimulus (external), with a temporary reversal of the polarity of the electrical potential difference between their interior and the surrounding. In response to such an excitation, muscle cells develop a force through their contraction. *Cardiomycocytes* or *myocardiocytes*, are the muscle cells of the heart. In course of a cardiac cycle, each cardiomyocyte undergoes contraction and relaxation once, during which sodium, potassium and calcium ions flow across its cell membrane through ion channels.

2-1-2 Field potential

Field potential is the electric potential due to a cell's electrical activity (such as action potential) at any distance outside the cells. It is a significant characteristic of muscle and neuronal cells as it is governed and is indicative of the ionic currents that flow across their membranes whenever the cells are excited. In case of cultured cardiomyocytes, it is generally measured using an array of electrodes that are almost in contact with the cell membrane. The field potential of some distant cells may interfere with that of the cells directly in contact with the electrode [7], however, the amount of interference is negligible because the field potential

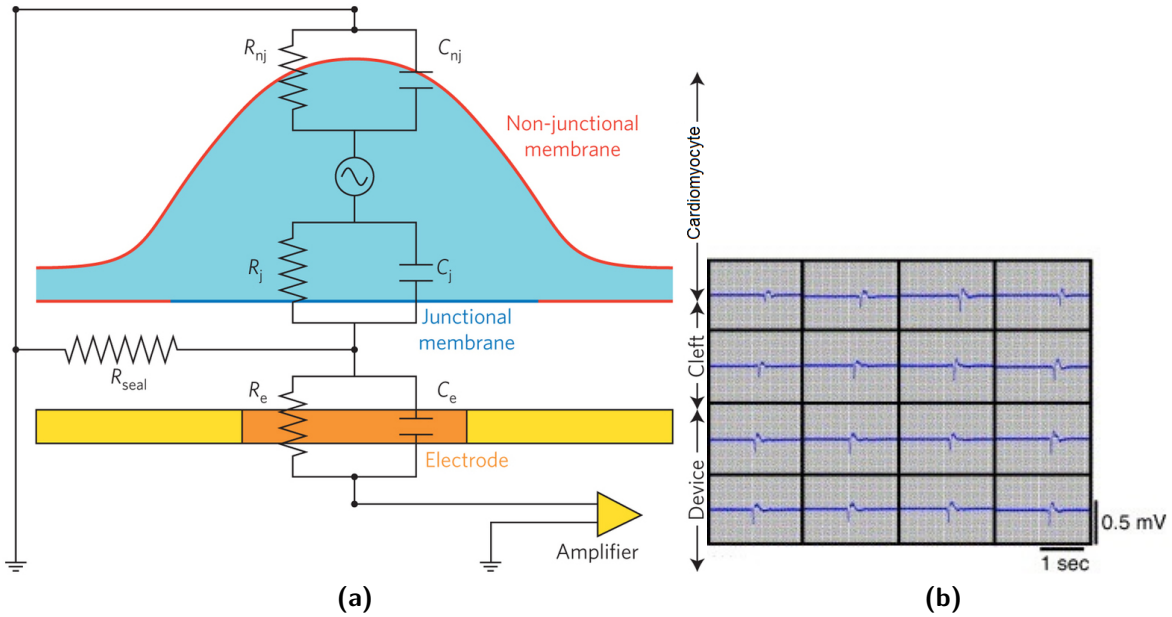


Figure 2-1: (a) One face of the cell interfaces with the electrode, generally present on the substrate, via a cleft filled with a physiological medium. The membranes are modelled with a resistor and capacitor (RC) in parallel. The ground of the circuit is set using another electrode, generally immersed in the ionic medium of the cell culture. Thus, the resistance between the membrane and ground is determined by the medium's conductivity and that of the cleft. The impedance of the electrode has been modelled as a resistor and capacitor in parallel. Thus, time-derivative of the extracellular potential of the cell is amplified and recorded. Image adapted from [8]. (b) Field potential recorded from a neonatal rat cardiomyocyte sheet using an array of 16 microelectrodes. Reproduced from [9]

follows a 1/R decay law. Figure 2-1a shows a model of an interface between a cell and an electrode outside the cell as described by Spira et al. [8].

A recording of a cells field potential with time may not always be the same, even for a particular type of cell. Changes in field potential, are in general, predictive of proarrhythmic properties of physical or chemical stimuli. Figure 2-1b shows an example of extracellular field potential recordings.

2-2 Measurement methods

Micro-Electrode Array (MEA) and calcium imaging have been used to assess the functioning of the device and the electrophysiology of the cultured cardiomyocytes, respectively.

2-2-1 Microelectrode Array recording

An array of Titanium Nitride microelectrodes are present have been embedded in the Cytostretch's membrane for the purpose of sensing the extracellular field potential of the cultured cardiomyocytes. This study encompasses the array's noise susceptibility and quality of the interface between the cardiomyocytes and electrode that affect the field potential recordings.

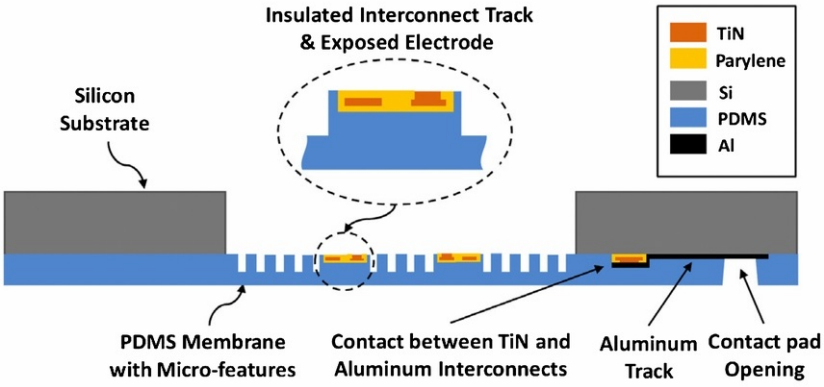


Figure 2-2: The schematic of the chip showing how titanium nitride electrodes are embedded in the PDMS membrane, that extends across the hole in the silicon substrate. Reproduced from [4]

An apparatus developed by Multi-Channel Systems GmbH (Reutlingen, Germany) has been used as the amplifier shown in Figure 2-1. It also helps process the acquired data on field potential.

2-2-2 Calcium Imaging

Every time that a cardiomyocyte beats, Calcium flows into the cell through the ion channels in the membrane, which is later released. The inward current produced by the flow of calcium ions, is another measure of the electrophysiological well-being of cardiomyocytes. One can observe the increase in intracellular calcium ions as fluorescence brought about by a fluorescent calcium dye.

Fluo-4 AM is one such dye that binds to calcium ions that enter the cytoplasm and exhibits green fluorescence upon absorption of blue light. If the cardiomyocytes are beating spontaneously, one can see the cells lighting up when the ions go in and turning dark when they flow out.

Chapter 3

Materials & Methods

This chapter describes the apparatus that was designed for stretching the membrane and the protocols of subsequent electrophysiological experiments adapted to the requirements of Cytostretch.

3-1 Structure of Cytostretch

The Cytostretch's chip must be mounted over a culturing chamber that comprises two parts: an acrylic plate and an acrylic ring, as shown in Figure 3-1a. Please refer to Appendix A for the details on the assembly of the device's components. The acrylic plate has two holes punched strategically at its ends to fix it to a pneumatic holder using nuts and bolts.

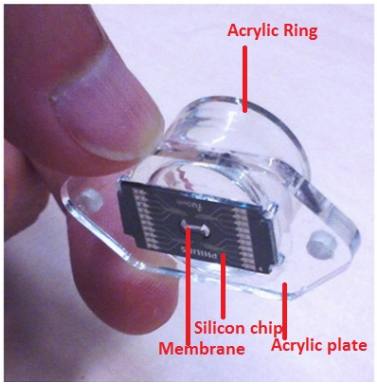
3-1-1 Membrane's mechanical properties

The average strain imparted to the rectangular region of the membrane along its transverse direction, is mathematically calculated assuming that the membrane will take up an arc's shape Figure 3-2a. The strain has a one-to-one relationship with the height, H, at the centre of the inflated membrane with respect to that of the relaxed membrane. Thus, the strain has a linear relationship with the pressure applied beneath the membrane (Figure 3-2b), assuming that the PDMS membrane is purely elastic [4].

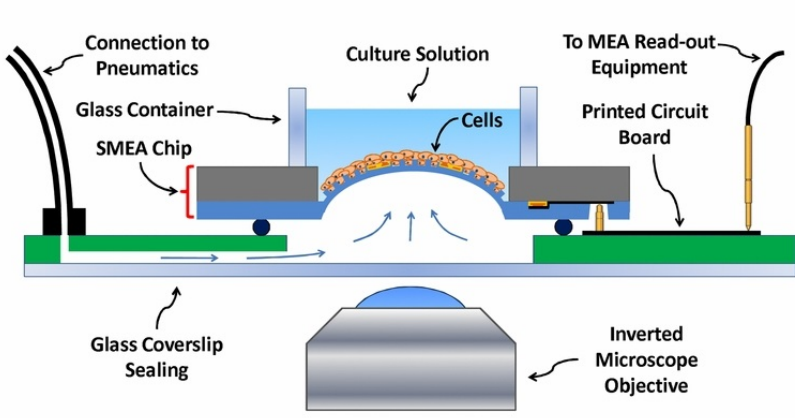
For stretching the cardiomyocytes in physiological range, one must only a strain of 10 to 20% strain. However, one may strain the membrane to 60% according to the article describing the technical specifications of the Cytostretch [4].

3-2 Pneumatic valves and instrumentation

To apply cyclic or constant pressure on the device's PolyDiMethylSiloxane (PDMS) membrane and to modulate the frequency of cyclic stretching, an electronic control system was designed,

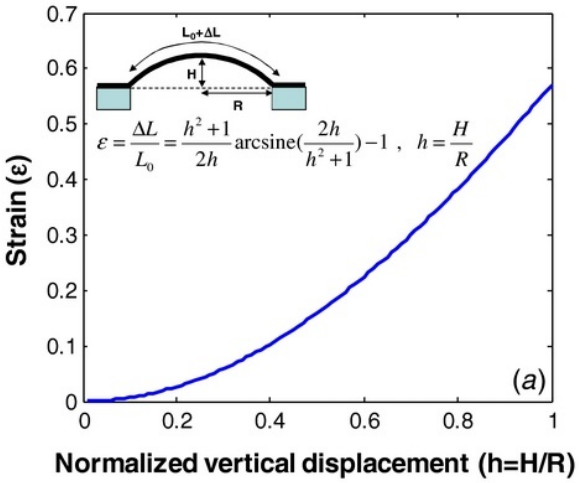


(a) The 3 distinct components of Cytostretch

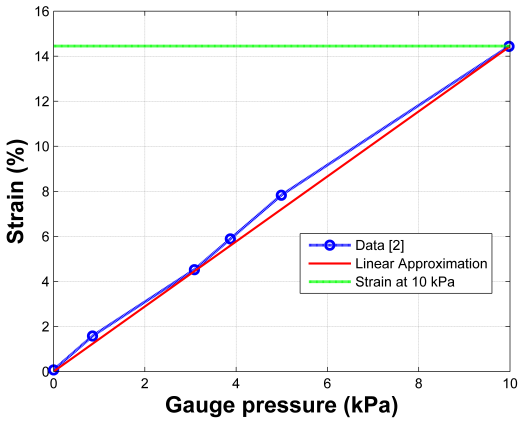


(b) A cross-sectional view of the chip holder system. A pneumatic connection is present, which leads to the membrane through a channel milled onto the rear side of the holder.

Figure 3-1: Components of the Cytostretch device and the chip holder pneumatic system. Reproduced from [4]



(a) The geometrical relation between the normalized vertical displacement of the centre of the membrane and the strain (L_0 denotes the original length of the membrane's cross-section, i.e. $L_0 = 2R = 1.2$ mm.)



(b) A graph relating the strain induced in the membrane with the applied air pressure. It is shown using the red line that the strain and gauge pressure are nearly linearly related from 0 to 15% strain with a slope = 1.5 %/kPa.

Figure 3-2: Components and the mechanical characteristics of the membrane in Cytostretch. Reproduced from [4]

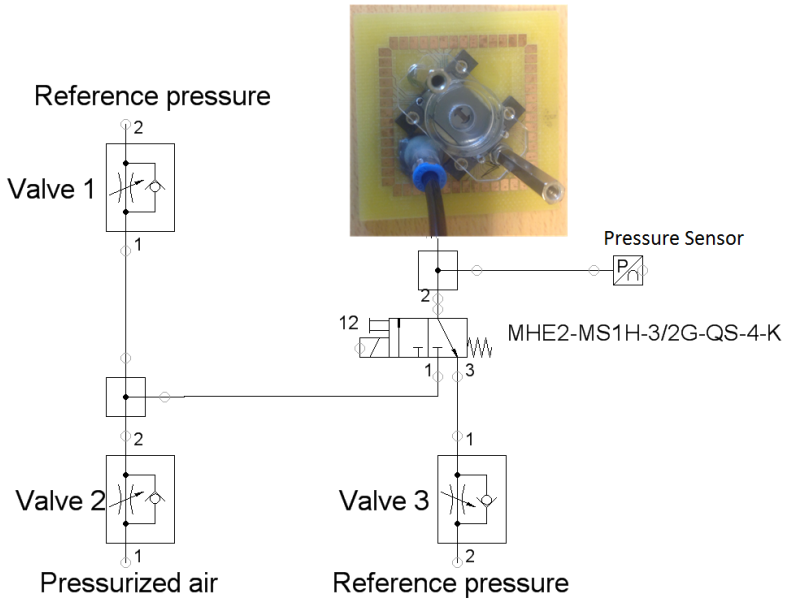


Figure 3-3: The arrangement of the valves for regulating the pressure being supplied to the chip membrane. The valves make the air flow viscous. Valve 1 and valve 2 are in series between the atmosphere and the pump outlet (gauge pressure 30kPa). By adjusting the 2 valves pressure valve between them can be adjusted to a value up to 30 kPa above atmospheric pressure. This pressure is directed to a 3/2 solenoid valve, which is a fast switching gate valve, i.e. the air entering through vent 1 in it passes through the vent 2 in one cycle and the air flowing from vent 2 passes through vent 3 in the next cycle. The solenoid valve does not damp the flow at all, but only directs it. Due to the presence of valve 2, the pressurised air is damped while flowing towards the membrane, which has an advantage that the inflation of the membrane is made gradual, like a viscoelastic material. Similarly, it is also crucial to have a viscous flow of the air that is exiting the membrane, therefore valve 3 has been added at the vent 3. It is necessary that there is not leakage in the chip holder, else the deflating flow of air would rather pass through the low resistance path causing an abrupt deflation of the membrane.

the construction of which, is described in Appendix B. The pneumatic apparatus to regulate the pressure is shown in Figure 3-3. To start and stop the flow of the air towards the membrane cyclically during the experiments, a solenoid valve is chosen as the *gate valve*. A combination of two needle valves is provided to control the pressure applied to the membrane and the pressure sensor. The needle valves also dampen the inflation and deflation of the membrane and smooth the transition between the two states.

The amount of applied pressure for an experiment, was measured using the pressure sensor and rendered using a digital multimeter, an oscilloscope or the analog input of Multi Channel Systems. Since the membrane is highly elastic, the pressure at any instant was a direct indicator of the strain in the membrane. The frequency of cyclic stretching can be set by the user using the digital clock.

3-3 Air-tight holders

The device has to be mounted on a holder that was provided to connect it with the ports of the Micro-Electrode Array (MEA) reader. This holder also directs the pressurised air towards the membrane as shown in Figure 3-1b. In addition to this holder (Figure D-2a), a separate holder is required while hold the chip while its membrane is only being stretched but without obtaining MEA recordings. This is because the former holder cannot be used for long duration stretching experiments inside an incubator due to its metal contacts that reactive to water and carbon dioxide, which are abundant inside the incubator.

An important requirement for the design of the holders is that they must have a thin base of not more than 3 mm, to minimise the gap between the objective and the membrane of the chip, while being observed under an inverted microscope. The proposed designs and their technical drawings have been manifested in Appendix D.

3-4 Method of stretching

The membrane was stretched using the air-tight holder and the instrument described above. Application of damped and free flow of pressurized air through the tubes to the membrane have different effects on the strain rate. The effective pressure sensed by the pressure sensor was measured using the Multi Channel Systems analog reader. The response of the membrane towards a step input of pressure is shown in Figure 3-4 and we see that the membrane pressure changes immediately when the excess pressure is released by the instantaneous opening of the solenoid valve. There are only short lasting oscillations and the pressure settles within 0.05 second. This proves that the membrane does not creep, unlike a viscoelastic flow. Therefore, it can be safely assumed that membrane strain can be directly determined from the pressure value at any instant, using the graph in Figure 3-2b.

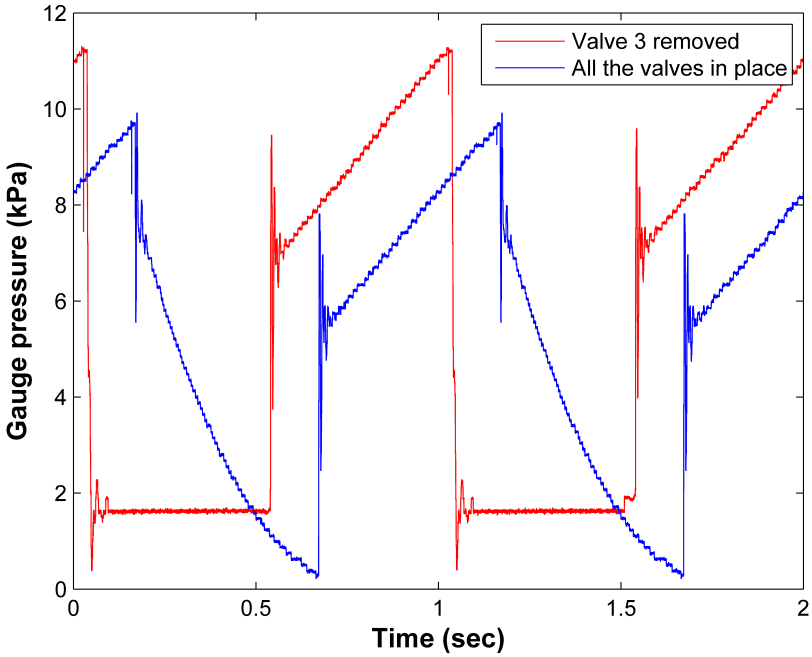
On the other hand, if the flow of the air is dampened using a valve, then the rate of the change in pressure decreases following an initial fast response. This is because of the added viscosity by the valve 3 in series with the membrane. The initial fast response cannot be reduced further by the adjustments of the valves. However, this is a more desired way of applying the pressure than the previous one, because it is more physiological for cardiomyocytes.

For more details on the usage of pneumatic apparatus, please refer to Appendix C.

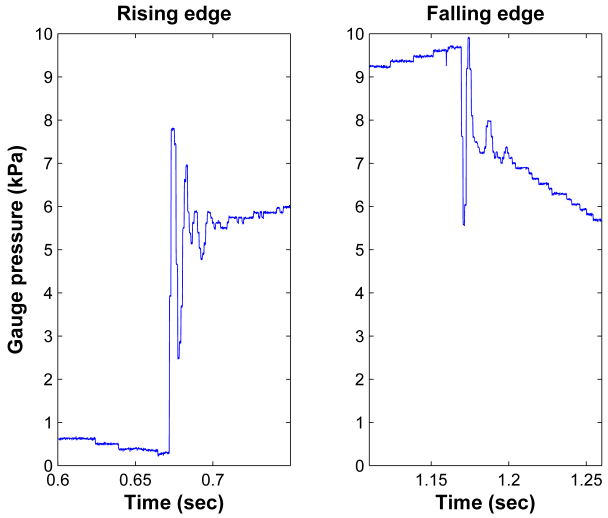
3-4-1 Effective strain on cells

The effective strain on the cells was calculated with the image processing software, ImageJ, using the micro-photographs of the cultured cells in both, relaxed and stretched states, as shown in Figure 3-5. This measurement helped in verifying the strain predicted from the strain - pressure relationship. Strain $= (L_2 - L1)/L1$. This method is applicable only to the cells attached to the central region of the membrane where the membrane can be assumed to be horizontal and flat.

In this case, it was difficult to locate the exact centre of the membrane because the electrodes are far apart from each other. Also, it is difficult to focus on the highest point of the membrane



(a) Pressure at the membrane, when the membrane is stretched and relaxed by an incoming and outgoing pressurized air at 10 kPa, at a frequency of 1 Hz. The initial steep change at positive and negative edges shows the elasticity of the membrane, and the following curvature is due to the valves. The negative pulse in the red plot appears appears to be rectangular because the valve for the exiting was fully open or removed.



(b) A closer look at the ringing at the rising and falling edges of pressure pulses at the

Figure 3-4: The pressure measured by the pressure sensor at the membrane while periodically stretching it using the designed pneumatic system containing a solenoid valve. The actual strain is directly related with the pressure, given the linear relationship in Figure 3-2b

because the centre point (which is the highest point while the membrane is inflated) is not known. It would be highly advantageous to the users if a small dot could be marked at the centre of the membrane during its microfabrication.

3-5 Materials

The chemicals and apparatus that have been used for the cell culture, membrane coating and the measurements of biomarkers in the cultured cells, are listed here. The protocols and the concentration of proteins and chemicals used in conventional practice have been optimised for use with the Cytostretch.

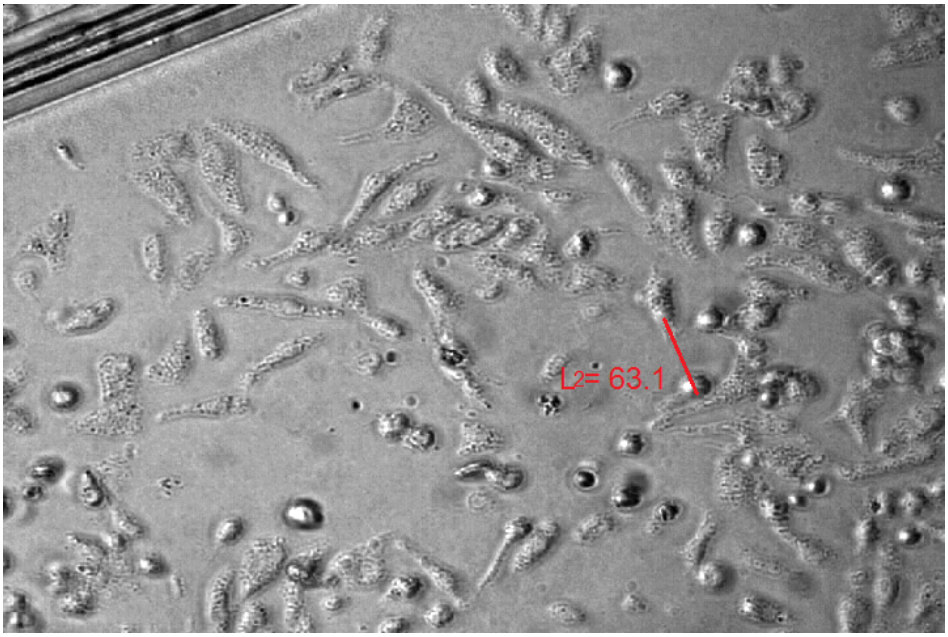
- Deionized water: Specific conductivity $\leq 0.04\mu$S/cm
- BiTy solution: An extracellular solution for *in vitro* experiments. $NaCl$ (8.1816g) + KCl (0.04026g) + $CaCl_2$ (1mL of 1M) + $MgCl_2$ (1mL of 1M) + $NaHCO_3$ (1.4702g) + 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (3.5745g) + Glucose (1.0810g) in 1L of deionized water. This solution is prepared and sterilised in the lab. The pH is corrected to 7.4 using $NaOH$. The presence of bicarbonate ions and HEPES ensures the pH of the cell culture medium is balanced at 7.4.
- APTES: (3-Aminopropyl)triethoxysilane (Sigma-Aldrich Co.)
- Pure EtOH: 99% Ethanol denatured with 5% methanol
- Fluo-4 AM dye
- Laminin: from EHS sarcoma (mouse) (Roche Diagnostics)
- Fibronectin: F0895 from Human Plasma (Sigma-Aldrich Co.)
- Ham's F10 Nutrient Mixture (Life Technologies): Supplemented with penicillin/streptomycin, Foetal Bovine Serum and L-Glut-amine to form the cardiomyocyte culture medium.

3-5-1 Machines

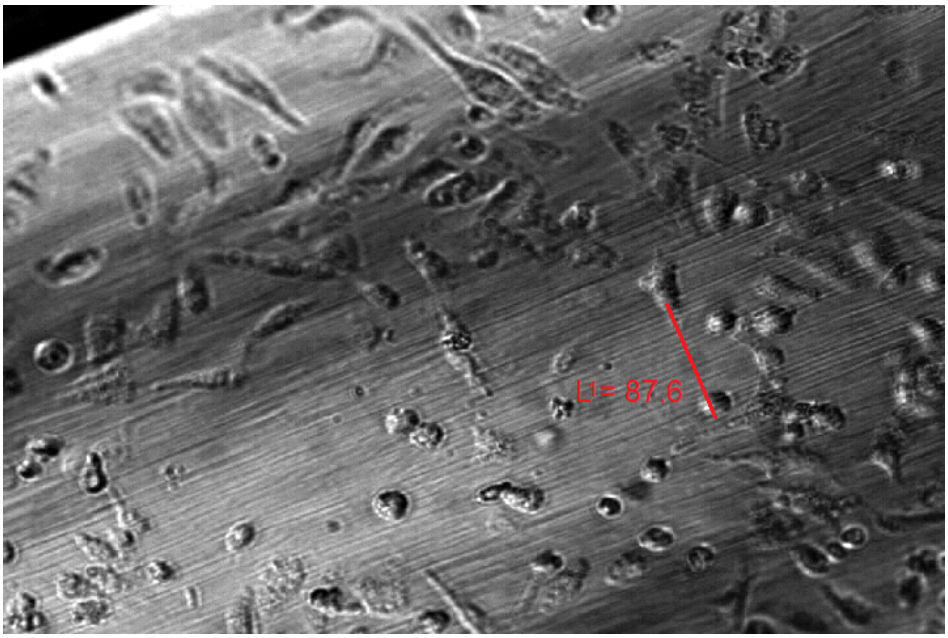
Edwards Auto306: available at the Cell Microscopy Centre in UMC Utrecht. For plasma cleaning.

3-6 Biofunctionality of PDMS membrane

Strong attachment between the cells and the surface via a layer of protein is essential for the further growth of cardiomyocytes in culture medium. Cells adhere to an *in vitro* substrate via a protein coating. The ability of a surface to interact with biological materials is called biofunctionality. In the case of Cytostretch, the PDMS membrane must be functional towards the proteins like laminin and fibronectin. This can be achieved by further subjecting the Cytostretch to air plasma and a silane primer.



(a) Relaxed membrane



(b) Stretched membrane

Figure 3-5: The photomicrographs of the relaxed and stretched membrane. 2 distinguishable points on the membrane are chosen in both the images, in the central area of the membrane and the length is determined by the tool.

3-6-1 Introducing -OH bonds at the surface

PDMS is highly hydrophobic which prevents its adhesion with protein and therefore, cardiomyocytes cannot adhere or flatten on the membrane. PDMS is often oxidised in Oxygen or Air plasma, which not only cleans the surface but also makes it hydrophilic by converting the $SiCH_3$ to SiOH at the surface[10]. Concentrated NaOH has also been recommended as a cheap substitute to plasma cleaning [11] because it forms Si-OH bonds on the surface owing to a high concentration of OH^- ions. But its contact with the Cytostretch is avoided because of the possible damage by a strong base NaOH at several points of failure on the chip mentioned in section 3-1.

Air Plasma cleaning protocol

The MEA and Cytostretch were cleaned with air plasma every time before culturing new cells to increase their hydrophilicity. The devices were exposed to air plasma under vacuum pressure of 0.015 mBar for 15 to 20 seconds.

The device is proposed to be reusable by Saeed Pakazad, following an thorough washing with detergent specified in Appendix B, which was performed every time before culturing new cells in them.

3-6-2 Silanization protocol

The effect of plasma cleaning alone was not found sufficient to make the PDMS hydrophilic, so a further application of silane [10] on plasma cleaned - PDMS was tested and found to increase its cellular adherence largely. This process of application of silane is referred to as *silanization* and is described below.

Organosilanes like (3-Aminopropyl)triethoxysilane (APTES) and (3-Aminopropyl)trimethoxysilane (APTMS) have been recommended in literature to enhance biofunctionality of PDMS [12]. For the presented experiments, APTES has been used because it was available in the laboratory. Pure ethanol, APTES, 70% ethanol and deionised water are required to perform silanization of Cytostretch, as given below. This method can be adapted for any culture dish with PDMS substrate.

- Prepare a 1% (v/v) solution of APTES in pure ethanol and pour 100μL of it in the inner well of the Cytostretch.
- Add 5% (of the volume of APTES solution poured) distilled water in it.
- Keep the Cytostretch at the room temperature for 10 min.
- Aspirate the Cytostretch and rinse it up to the brim once with 70% ethanol.
- Rinse 3 times with sterile deionised water.

3-6-3 Protein coating

The protein used for coating a substrate differs with respect the cell-type. To culture monolayer of cardiomyocytes, laminin or fibronectin are advised, amongst which laminin is more physiological. Following protocol was followed for protein coating of Cytostretch or any other culture dish:

- While using laminin, dissolve it in a pH neutral salt solution like Phosphate Buffered Solution (PBS) in the ratio of 1mg/100mL.
- If fibronectin is to be used, dissolve 60 - 70 μg per mL, which is 6 - 7 times the normally recommended ratio, because of extreme hydrophobicity of PDMS.
- Incubate the device or the culture dish into the incubator for at least 1 hr.
- Aspirate the protein solution and then let the substrate dry.
- Cells can be now immediately poured in the culture dish or the protein coated dish can be stored in 4°C for later use.

To compare the various methods specified above for the introduction of -OH bonds and protein coating, with regards attachment of cells, they were tested on PDMS covered wells in well plates. 8 test cases were prepared as shown in Table 3-1 and cardiomyocytes were cultured on them. Effect of air plasma and silanization over surface properties of the PDMS membrane of fresh was studied. It was found that air plasma followed by silane coating followed by laminin or fibronectin were the best ways to induce cardiomyocyte attachment on PDMS surface.

Method to induce hydrophilicity	Silanization	Protein	Cell adhesion
Air plasma	Yes	Fibronectin	Moderate
Air plasma	Yes	Laminin	Good
Air plasma	No	Fibronectin	No
Air plasma	No	Laminin	No
NaOH	Yes	Fibronectin	No
NaOH	Yes	Laminin	No
NaOH	No	Fibronectin	Little
NaOH	No	Laminin	No

Table 3-1: The extent of cardiomyocyte adhesion on PDMS surface as a result of various surface treatments and 1% laminin and 6% fibronectin coating.

3-7 Neonatal Rat cardiomyocyte isolation

The experiments have been conducted on neonatal (8-10 hour old) ventricular cardiomyocytes of Sprague-Dawley rats. This isolation process lasts 6 hours and is performed together by 2 to 3 members of the laboratory on 3 out of every 4 weeks. From the extracted neonatal hearts, atria are discarded and the ventricles are further disintegrated using forceps. The

disintegrated ventricles are treated with DNAase and Trypsin to break down their intercellular matrix and to acquire individual cardiomyocyte cells suspended in the medium.

The fibroblasts and cardiomyocyte are separated by differential adhesion selection process. This suspension is plated in Petri dishes for 2 hours because the fibroblasts settle down and adhere to the bottom of the dish first while the cardiomyocytes do not adhere that fast, then the cardiomyocytes are gently collected from the Petri-dishes. Eventually, at the end of the day, about 1 million cells are obtained from each heart. The cells were seeded on protein coated dishes of Cytostretch immediately.

3-8 Cardiomyocyte cell culture

After the isolation, the neonatal rat cardiomyocytes were cultured in the culture medium. To form a monolayer of cells after their attachment to the membrane, 15×10^3 cells/mm^2, thus 10^5 cells were poured into the protein coated inner well of Cytostretch device. 10 minutes later, when the cells had settled down, additional medium was added gently. The devices were left in the incubator undisturbed over night, after which they were examined under an inverted microscope to check the cell attachment. Following that, the medium was replaced thoroughly washing away the unattached, dead cells. A more exact sequence of steps is mentioned in Appendix F.

3-9 MEA recording

Prior to any MEA recording, the medium of the cells was replaced by the BiTy solution for pH maintenance. The Cytostretch fixed on the holder was mounted on the MEA reader. After the MEA recording, the cells were washed at least twice with culture medium before putting them back in the incubator, to wash away any possible contamination. The ground is set using the Ag/AgCl bath electrode. Baseline MEA recording of Cytostretch devices was performed by filling them with BiTy solution, but without culturing cells in them. All the measurements were taken at 37°C.
CAUTION: The ambient light was found to cause noise in the MEA recording so the set-up must be shielded from light using a cover.

3-10 Calcium imaging

Calcium imaging has been performed with fluo-4 AM dye in the following way:

- Add 1μM Fluo-4 AM dye to the medium in the cell culture dish.
- Label the cells for 15 minutes in the incubator.
- Replace the existing medium of the cells with fresh medium and incubate for 15 minutes.
- Change the medium to BiTy solution to perform Calcium imaging outside the incubator.

- Maintain the temperature of the culture at 37°C. For these experiments, the culture dish was placed over the heating plate provided by the Multi Channel Systems.

Calcium Imaging of the Cytostretch in fluorescent light has a better contrast when the device is placed in an acrylic Petri dish or one of the macro-machined pneumatic holders, rather than the PCB holder. Although, the desirable level of contrast is seen only with the Petri dish amongst all the possible holders due to the auto-fluorescence of materials of the holders.

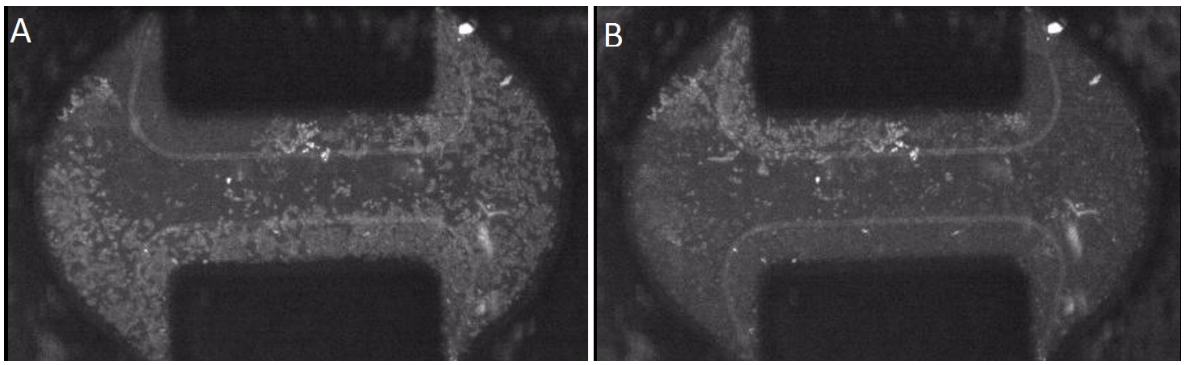


Figure 3-6: A.The bright cells containing calcium intracellularly. B.Now the other bright cells contain calcium intra-cellularly. The 2 sets of cells seem to be completely disjunct via gap junctions.

Chapter 4

Experiments & Results

Experimental tests were performed on the Cytostretch with or without culturing cardiomyocytes in the devices. First, the tests that were aimed at validating the Cytostretch as a suitable substrate for cardiomyocyte cell culture, in static conditions, are presented. Next, the repercussions of prolonged cyclic stretching on the membrane shape and on the cell culture is presented. The quality of Micro-Electrode Array (MEA) recordings with regards to noise and interference is presented.

4-1 Viability of the membrane

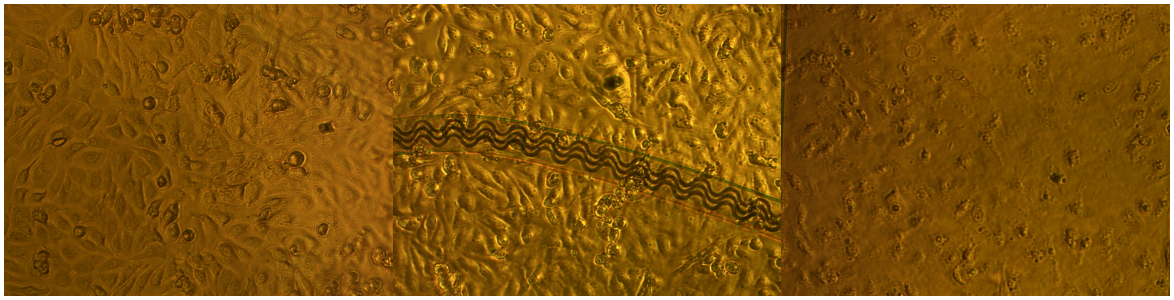
The viability of the devices was related to its usage history by examining the pattern in cell attachment and their survival.

4-1-1 Biofunctionality of fresh device

Through cell culture in Cytostretch devices, it was discovered that the percentage of cardiomyocytes that attached in fresh devices was only 20 to 30%, with which it is not possible to obtain a monolayer. A fresh device is referred to as the one in which neither, the cells were cultured previously, nor was it plasma cleaned more than once. The cardiomyocyte attachment on a relatively older device was 80% to 100% because the membrane turns more hydrophilic after repeated use in cell culture. Therefore, it is recommended to the users either, culture some cells in the device before making a final experimental model, or plasma clean them for a longer duration than 20 seconds, approximately 30 seconds.

4-1-2 Laminin vs Fibronectin

In the methods and materials section for protein coating, 2 types of protein coatings are mentioned. Although, the isolated cardiomyocytes attach to the PolyDiMethylSiloxane (PDMS) membrane coated with either of them, the sustenance of life in the 2 cases is not the same.



(a) Healthy cells on day 1 in the central region.

(b) Healthy cells on day 3 at an edge.

(c) Dead cells in the central region.

Figure 4-1: Photomicrograph of attached cardiomyocytes on the Cytostretch's membrane at different time points and in different regions, showing that the cells in the central region died earlier than those in the periphery.

3 samples each of laminin and fibronectin coated devices were prepared, and cardiomyocytes were cultured in them. The medium was also changed in them in parallel and regularly. A follow up after 3 days of the cell attachment revealed that the cells in the laminin coated devices died, whereas they survived in the fibronectin coated devices even till the fourth day. It was mysterious that the cells in the centre would die earlier than at the periphery, depicted through Figure 4-1. However, it proved that the PDMS membrane coated with fibronectin is more viable than laminin for cardiomyocytes.

To check whether there was contamination in the cell culture which could have caused the death of the cells in laminin coated devices, calcium imaging was performed using a wide angle and fast macroscope. An advantage of fluorescent imaging from the top of the device is that it makes it possible to observe the calcium activity, and hence the beating of the cells, of the cells present on the membrane as well as on the silicium substrate beside the membrane. It was not feasible otherwise with light microscopy because the objective can not be lowered in the present culture chamber.

There was a more coordinated beating activity in the cellular mass on the silicium than in the cells at membrane, as shown in Figure 4-2, implying that there was no contamination in the device. There were only cells in patches on the membrane, indicating that the membrane covered with laminin was not an optimal method of coating a PDMS substrate.

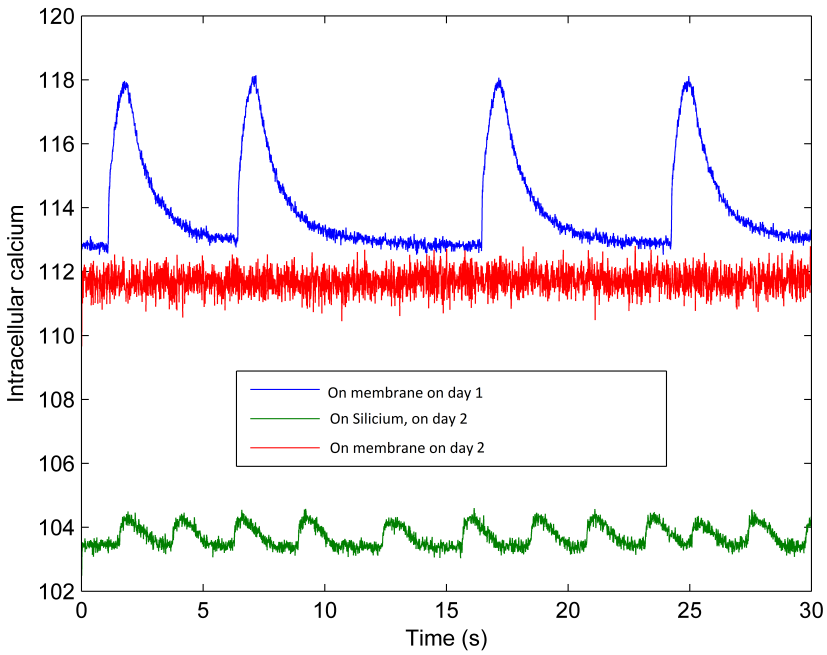


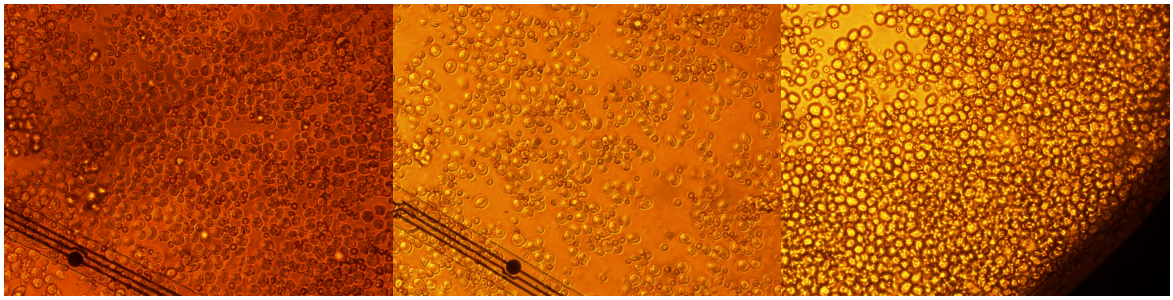
Figure 4-2: Calcium transients of cardiomyocytes

4-2 Permanent deformation in the membrane

The membranes of the devices were stretched occasionally, overnight, through the course of experiments. Permanent deformations were found in the membranes of the devices after using them extensively for stretching. The membrane was no more flat. A vertical height difference of 30 to 40 μm was measured between the edge and the middle point of the interconnect track, with the edge being at a lower level. Assuming a smooth curvature across the membrane, the centre point of the membrane would be higher than 40 μm. This hysteresis depicts a viscoelastic nature of the PDMS membrane, in contradiction to the presumed elastic nature by Pakazad et al. [4], or possibly a deformation in the metal interconnects.

This curvature in the substrate prevents a uniform spread of the cells on it, and thus is a hindrance towards the monolayer formation. When the freshly isolated cardiomyocytes are seeded on the membrane, they are spherical in shape with a diameter ≤ 12 μm, as it is visible in Figure 4-3. Therefore, most of the cells roll down towards the periphery. This phenomenon has been demonstrated in Figure 4-3 through the photomicrographs taken 10 minutes and 1 hour after seeding the freshly isolated ccardiomyocytes on the membrane.

Hence, the attached cardiomyocytes get localized to the edges of the dogbone-shaped membrane where no electrode is present and therefore, their electronic activity cannot be measured by the sMEA.



(a) Isolated cardiomyocytes 10 minutes after seeding them on the Cytostretch, uniformly spread from the periphery to the center.

(b) Isolated cardiomyocytes in the centre of the membrane 1 hour after seeding. They are less dense now.

(c) Isolated cardiomyocytes near the edge of the membrane. The cells are more dense over here.

Figure 4-3: Photomicrographs of isolated cardiomyocytes seeded on the Cytostretch membrane showing an increasing density towards the edges because of rolling down the slope. The cells are slightly smaller than the circular electrode, which is 12 μm in diameter.

4-3 MEA recording

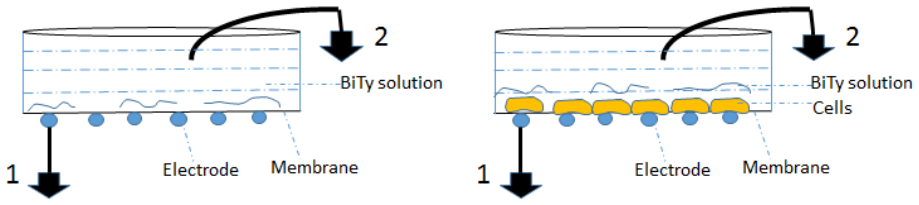
The reference or the ground electrode needs to be set by the user before starting a MEA measurement. Multi Channel Systems permits 3 methods of setting a voltage ground in a MEA, as shown in Figure 4-4.

1. MEA electrode - Only the microelectrodes present in the MEA are grounded in this case. The potential at the other microelectrodes in the array are measured with respect to the ground electrode.

   Thus, it would be possible to record the exact field potential wave at any other electrode only if there is sufficient time gap between the beats of the cells at the grounded electrode and the other measurement electrode. For this to be true, the electrode must be so far apart from each other that the travelling time of the depolarization pulse between them is greater than the field potential duration itself.

   Here is an approximate quantitative reasoning for this argument: The time duration of a field potential wavelet of cardiomyocytes in cell culture may last up to 1 sec (Figure 4-6). The size of a neonatal rat heart is roughly $3mm$ and if the beat rate is 1 beat/second, then the conduction velocity would be approximately $3mm$/sec. The minimum inter-electrode gap in Cytostretch is 240μm, therefore the minimum time difference between the field potentials at 2 electrodes would be $0.240mm/3mm = 0.08sec$ (if the cells are synchronized via gap junctions), which is much smaller than the field potential duration (= 0.5 sec). Thus, it is highly likely to miss some field potential wavelet information in the MEA recording. Therefore, MEA electrodes cannot be a reliable ground for measuring the cardiomyocytes.

2. Bath electrode - Only the bath electrode is grounded in this case. The bath electrode is immersed into the medium only by a short depth to maximise the ground resistance.



(a) Without a monolayer of cells. The electrodes are in direct contact with the medium.

(b) With a monolayer of cells. The electrodes are completely shielded from the liquid medium.

Figure 4-4: The 2 models of the MEA showing the environment of the electrodes with and without the cellular monolayer. 1 and 2 are the two independent ways of grounding the MEA electrode and the bath electrode, respectively. The third way of grounding is by grounding both of them together.

In this case, the field potential at all the electrodes can be recorded more accurately than in the first case, because now the reference is far from all the electrodes and hence has a greater resistance too. Therefore, this mode of setting the ground is the most optimum for recording cellular field potential.

3. MEA and bath electrode - In this case, some of the MEA electrodes are grounded along with the bath electrode. The voltage reference is similar to the case when the MEA electrodes alone are grounded.

The implication of different grounding methods on the MEA recording was verified through MEA recordings and has been further shown in Appendix E.

4-3-1 Noise and Baseline measurements in relaxed membrane

The 2 major sources of noise in the MEA recording using the Cytostretch are the mains hum and the ambient light. Light induced noise was eliminated by completely covering the MEA set-up. The mains hum was filtered in the Micro Electrode Array Rack using a bandstop filter for 50 Hz. The harmonics of 50 Hz were major constituent of noise, the signal was low pass filtered at 200 Hz. Occasionally, low frequency disturbance was seen, therefore a 2 Hz high pass filter was also applied. Finally, the peak to peak noise amplitude ranging from 20 μV to 30 μV was present in most devices, though the noise was very random and inconsistent in different devices (Figure 4-5). The field potential itself has frequency modes up to 200 Hz, therefore it was not filtered any further.

4-3-2 Field Potential

Field potential of neonatal rat cardiomyocytes were recorded using some of the devices in the static and relaxed state of the membrane and when the peak to peak noise voltage level was as low as 10μV. The amplitudes of the high frequency peaks were as high as 150μV, so the beat to beat time difference could be easily measured from theMEA recording, although

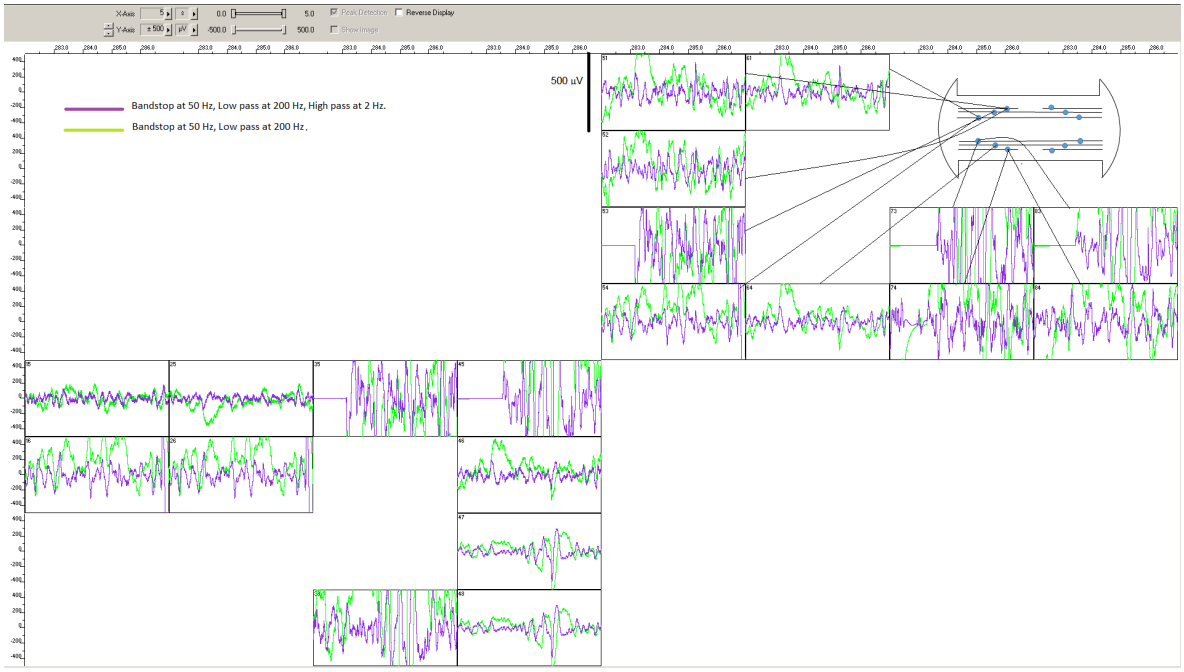


Figure 4-5: Noise measured during a MEA recording for a duration of 5 seconds on all the channels, in one of the Cytostretch devices not containing any cells. The optical noise was completely shielded during this measurement. The corresponding location of half of the electrodes on the membrane are shown. Some of the electrodes are connected with 2 channels while some have been shorted by the interconnects.

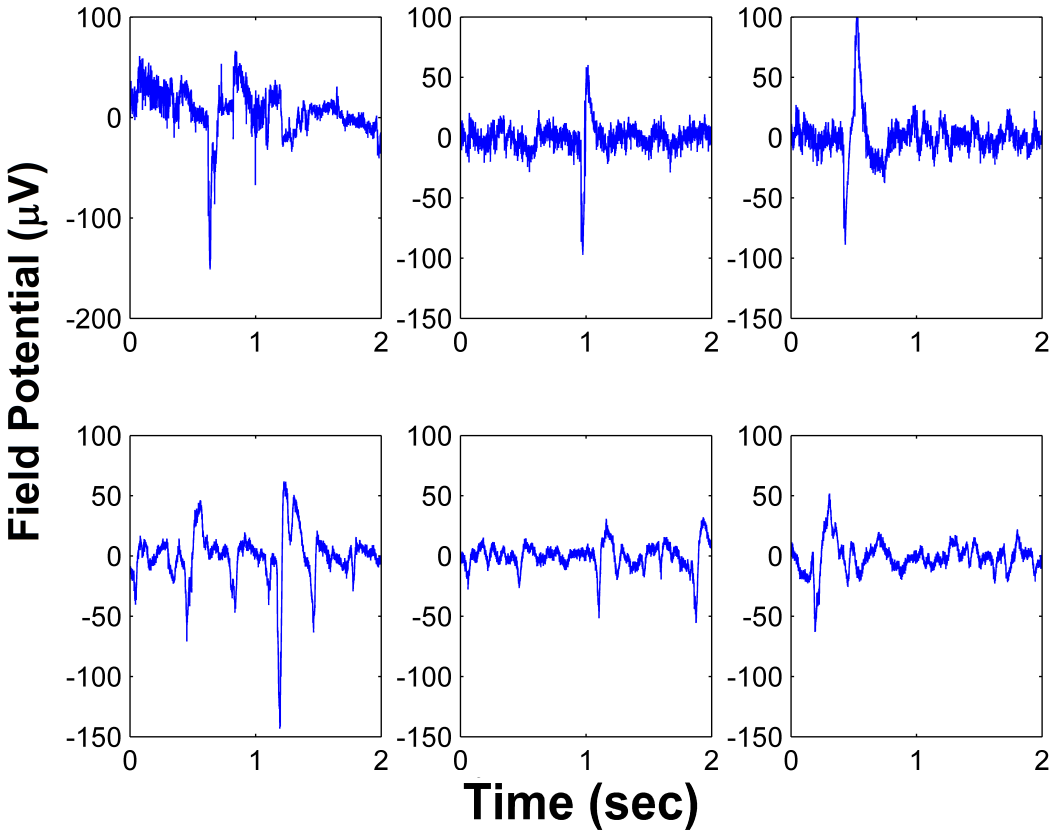


Figure 4-6: Field potential of cardiomyocytes recorded using Cytostretch. The signal in plot 2 to 6 was obtained after filtering the signal measured by MEA amplifier to remove 50 Hz hum, the frequencies above 200 Hz and below 2 Hz. The signal in the first plot was not high pass filtered.

the spontaneous beating was very occasional, so immediate effects of stretching on their field potential could not be concluded. Figure 4-6 shows some of the field potential wavelets in 2 different devices.

4-3-3 Stretching artefacts

The Cytostretch device has been designed to sense the field potential in the relaxed and stretched states as well as during transitions between the relaxed and the stretched states of the membrane. The resistance of the interconnects in the membrane, however, rises by 0.15% as the strain increases from 0 to 15% [4].

Interference

Baseline MEA recording, i.e. without any cultured cells, was taken while cyclically stretching the membrane at 1 Hz by 15 %. In case of any physical disturbance in the medium, such as perfusion or dropping some liquid gently into it, vigorous disturbance was seen in the MEA

recording. Similarly, sharp spikes are observed at the instant of stretching or relaxing the membrane. These are referred to as *stretching artefacts* in this text. The stretching artefacts are typically high frequency signal in the measured voltage and can be misunderstood as the extracellular or intracellular field potential of cardiomyocytes.

The spikes are faster when the stretching is abrupt and slower when the transition is damped by the use of a valve, as shown in Figure 4-7. These spikes interfere with the field potential of the cardiomyocytes because they lie in the high frequency region of the field potential.

As it is visible in the Figure 4-7, the stretching artefacts at the electrodes are very inconsistent with regards time and space is very high, so it is not straightforward to filter the interfering signal.

4-4 Stretching the Cardiomyocytes

According to [13], isolated neonatatal rat cardiomyocytes are able to sustain strain immediately after their overnight attachment to the substrate. Therefore, the cardiomyocytes cultured on Cytostretch were stretched on day 1, i.e. after their overnight attachment on the membrane, by 15 % at a frequency of 1 Hz for 4-5 minutes, while taking the MEA recording.

4-4-1 Field potential

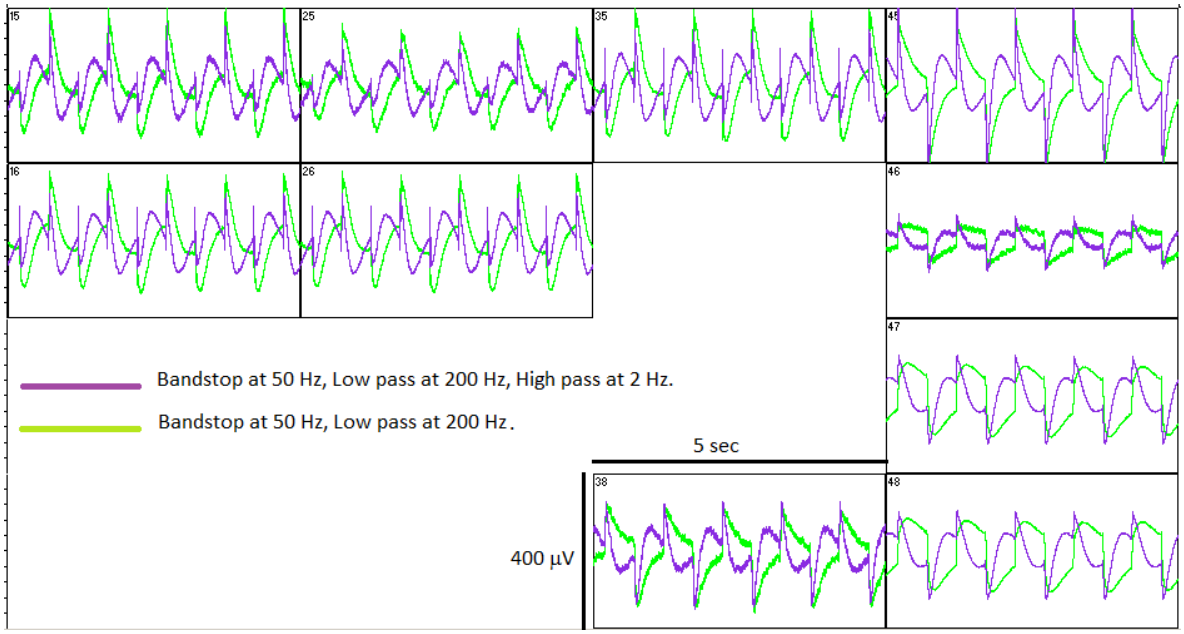
First, the field potential in the relaxed state of the cells was measured, which revealed an occasional spontaneous beating of the cardiomyocytes (Figure 4-8). The spontaneous beating declined gradually as shown in (Figure 4-9). After the MEA recording in relaxed state for 2 minutes, the periodic stretching was commenced, continuing the MEA recording (Figure 4-9). Figure 4-9 shows an enlarged version of the MEA recording before and after the start the stretching, to highlight the spikes accompanied with cyclic stretching. It is important to note that these spikes are monophasic unlike the field potential in the relaxed membrane, which is mostly biphasic.

As mentioned in section 4-3-3, the stretching artefacts have similar high frequency peaks as the field potential, so it cannot be distinguished whether the signal contains purely the cardiomyocyte filed potential, or the stretching artefacts or, a superposition of both.

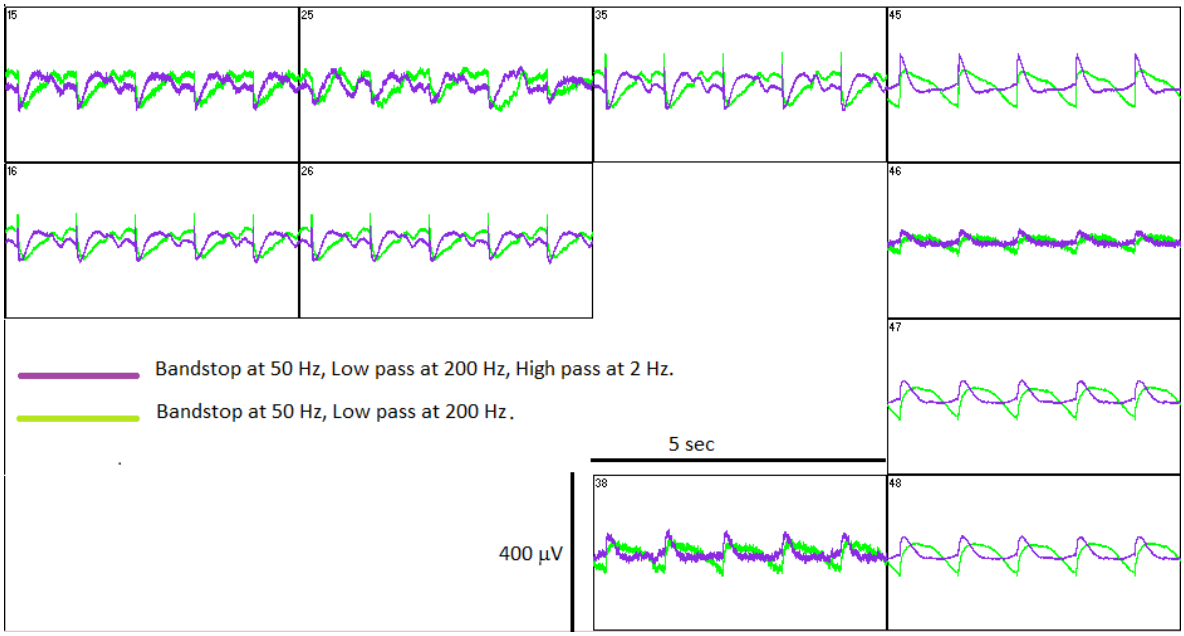
4-4-2 Impact of stretching on cardiomyocytes

The microscopic observation of the cardiomyocytes after 4-5 minutes of stretching (described above) showed that the cells had died, though the cells had not detached from the substrate. They looked very granular. The cells died, possibly, because they were overstretched at the edge of transition between the membrane's stretching and relaxation. Figure 4-10 explains that at the edges of pressure pulses, there is a very fast response of the membrane causing a high strain rate of 1400%/sec.

This method of stretching the membrane may not be physiological because most of the experiments in the past involving cyclic stretching used a constant strain rate approach, which was not fatal to the cells [14, 15, 16, 17, 18, 19, 20]. However, the solenoid valve is a gate



(a) No regulating valve is present at the exit of the air.



(b) A regulating valve is present at the exit of the air.

Figure 4-7: Artefacts measured in half of the channels from a device while cyclically stretching the membrane at 1 Hz by a pressure of 9.5 kPa (≡14 % strain). The amplitude of the artefacts increases upon completely opening valve 3 shown in Figure 3-3

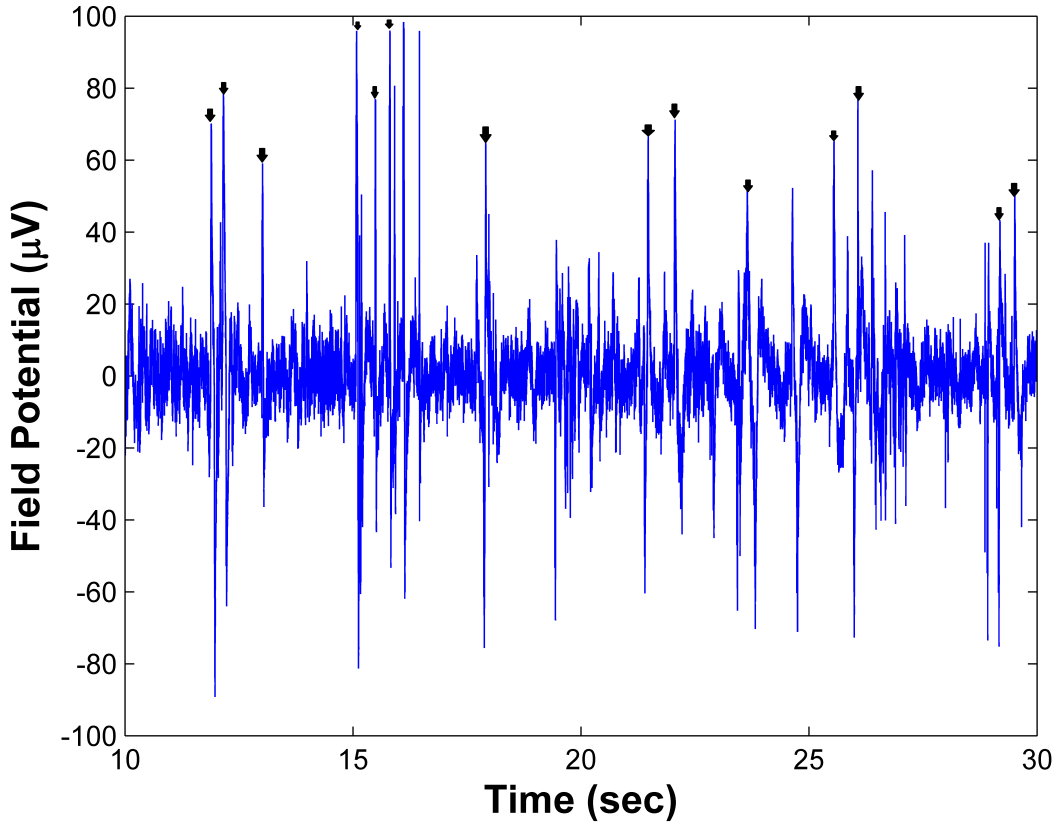
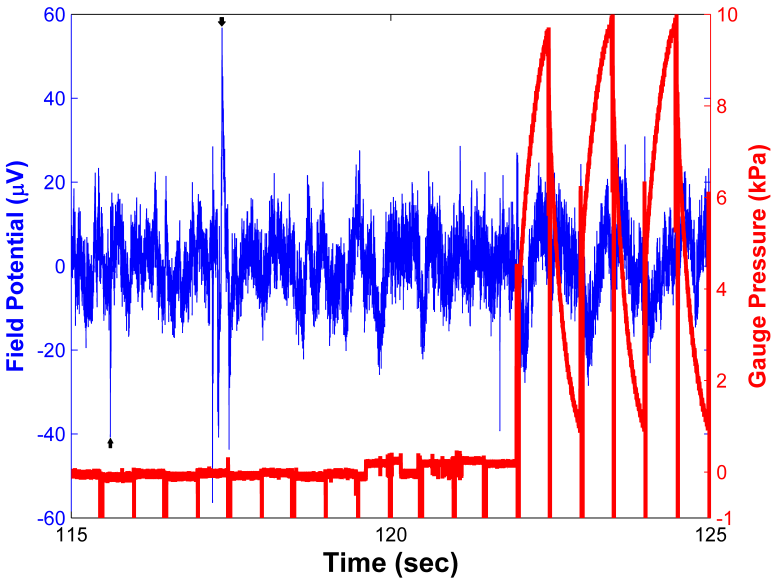
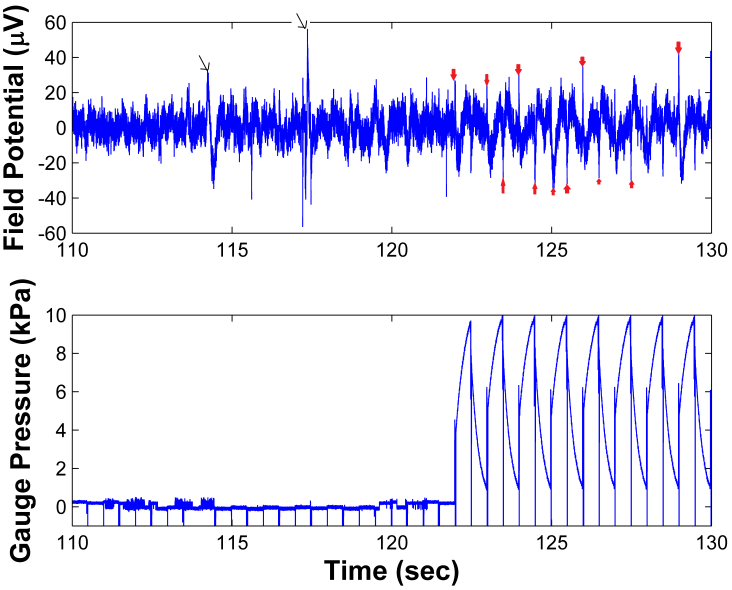


Figure 4-8: Field Potential recorded at one of the electrodes from a monolayer of cardiomoyocytes cultured in a Cytostretch device, in the relaxed state of the membrane, from 10th to 30th second after the commencement of MEA recording. The arrows point out some of the field potential instances.



(a) The frequency of occurrence of field potential has declined over time, comparing with Figure 4-8. The pressure graph shows when the cyclic stretching was started.



(b) The MEA recording while stretching is typically monophasic, rather than biphasic.

Figure 4-9: Field Potential recorded in a monolayer of cardiomoyocytes cultured in a Cytostretch device, first in the relaxed state of the membrane and a subsequent cyclic stretching. The black arrows point out some of the field potential instances prior to stretching and the red arrows indicate the spikes in the MEA signal during cyclic stretching. Note: The steep fall in the pressure curve is present because of a loose connection in the circuit because of which the sensor disconnected periodically at the switching of the solenoid valve. It does not indicate a steep fall in the pressure applied at the membrane.

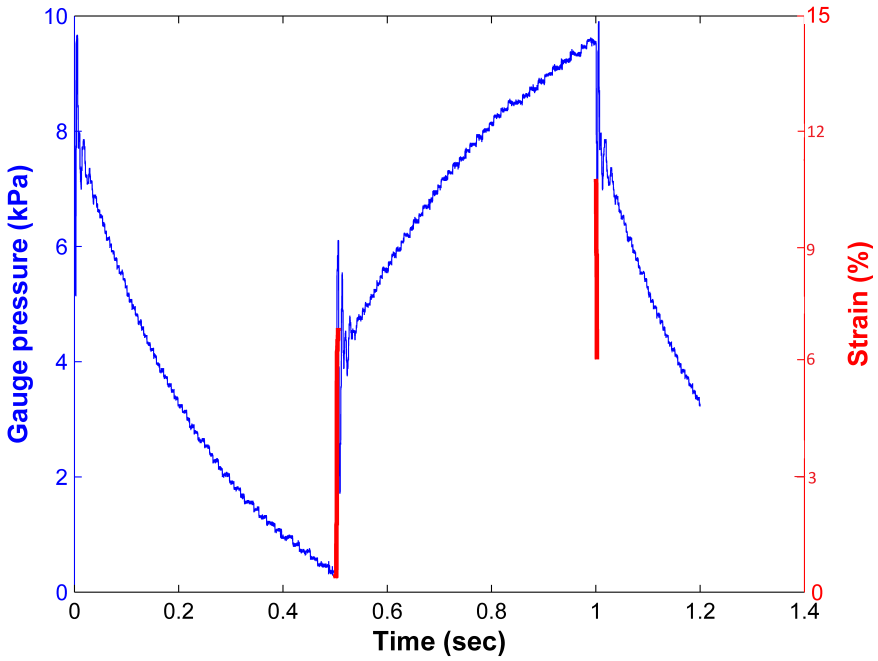


Figure 4-10: Sudden straining of the membrane during cyclic stretching using the solenoid valve aided pneumatic apparatus.

valve and hence, accompanied with a fast response. Therefore, a method of stretching that forces the pressurised air into the membrane a slower rate is required.

4-5 Stretching using a syringe

To verify the hyposthesis that slow stretching would exclude the stretching artefacts, a syringe was used instead of the combination of a pneumatic pump and solenoid valve. This test was performed on 2 devices. The outlet of the syringe was connected to the membrane and the pressure sensor. The piston of the syringe was forced back and forth manually and periodically. The gauge pressure developed in the membrane and the corresponding MEA recording is shown in Figure 4-11. This baseline recording reveals that there are consistent low frequency component in the artefact and also high frequency spikes that resemble the cardiac field potential can appear in the MEA recording, even without any cultured cardiomyocytes.

To study the effect of such stretching on the cardiac MEA recording, neonatal rat cardiomyocytes were cultured. First, the extracellular field potential due to the spontaneous activity of cardiomyocytes was observed, which was occasional, rather than frequent or regular (Figure 4-12). Then, the cyclic stretching was commenced. The pressure that was sensed by the pressure sensor was observed using the analog input of MEA reader, and the field potential of the cardiomyocytes was recorded simultaneously, as shown in Figure 4-13. The maximum strain in the membrane ranged from 9 to 18%, while the average frequency was only 0.35 Hz (because it was performed manually).

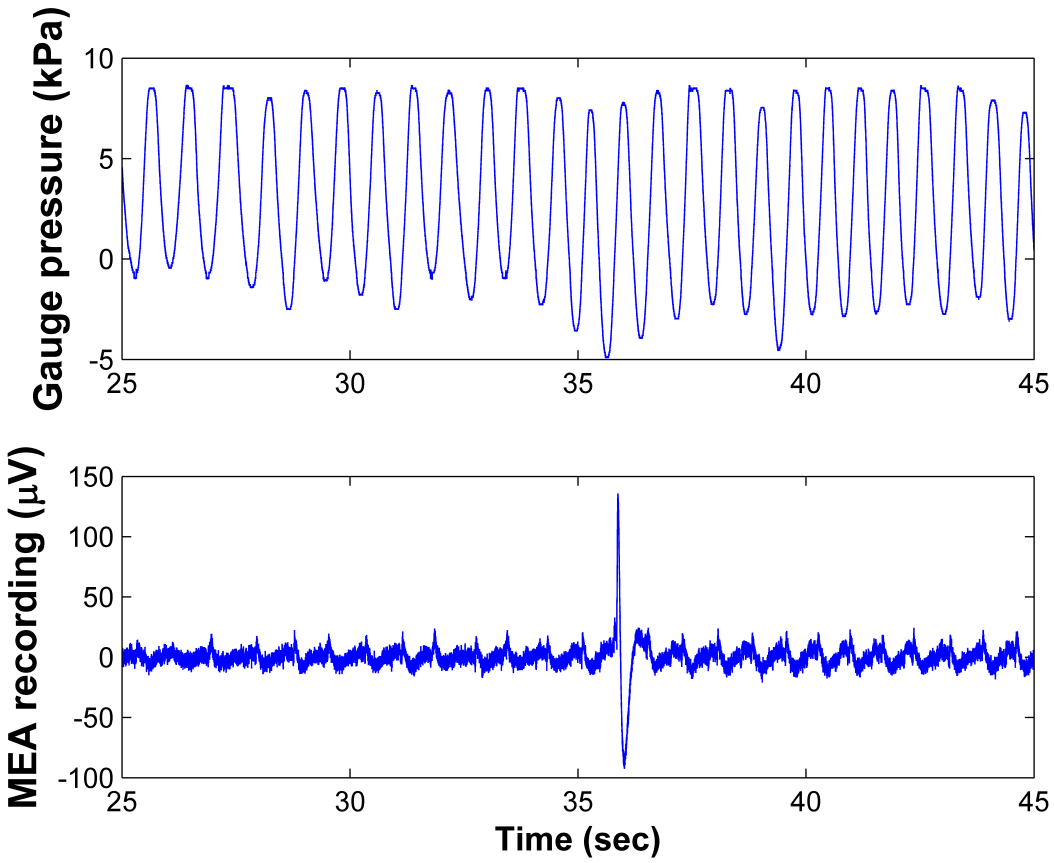


Figure 4-11: Applied pressure and MEA recording of a Cytostretch device (no cultured cells) being manually stretched and relaxed using a syringe. The pressure plot shows that the frequency was approximately 1 Hz, while the wave is not a perfect sinusoidal wave. The MEA recording reveals that there are some sharp artefacts with an amplitude of approximately 10 μV and there is a sudden large biphasic spike too. This means that stretching artefacts can occur even with a smooth cyclic stretching.

Like the case of solenoid valve, in this case too, high frequency peaks were present in the MEA signal that were synchronized with the inflation and deflation of the membrane. The peaks were however, higher and sharper in this case, comparing the Figure 4-14 with Figure 4-9. A microscopic observatio of these gently stretched cardiomyocytes revealed that the cells had died, although the cause of their death cannot be explained. However, the sample set is also not suficient to conclude much.

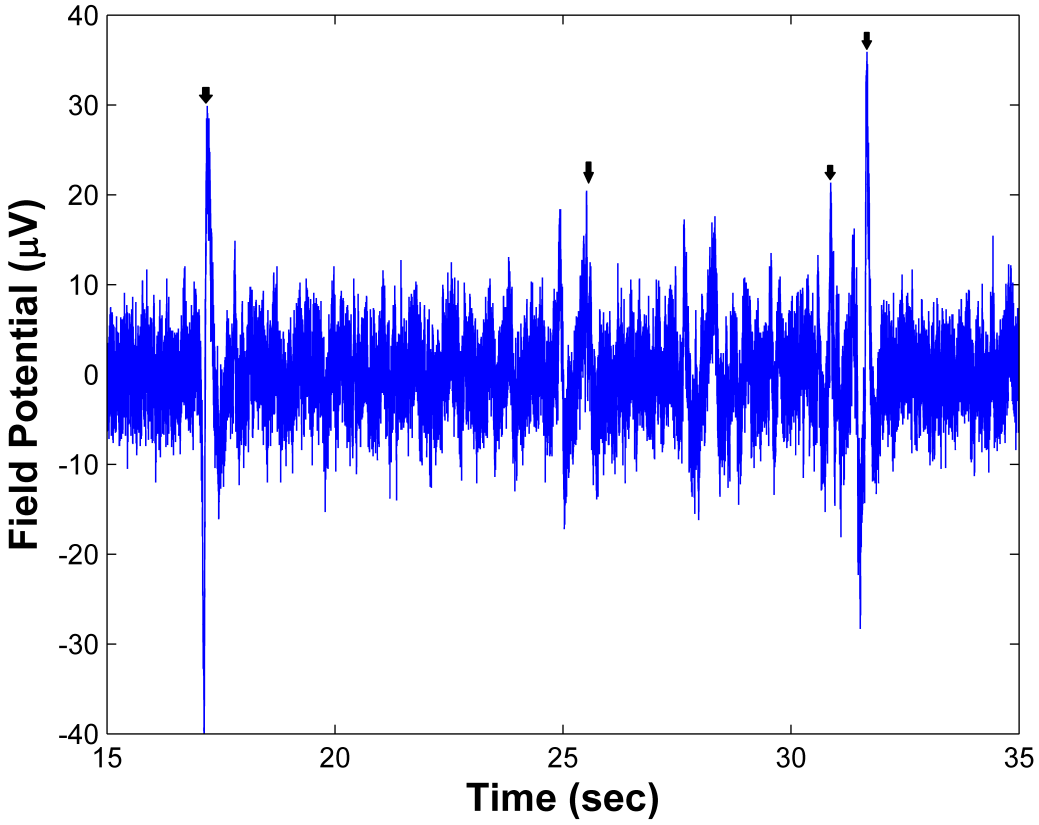


Figure 4-12: Field Potential recorded at one of the electrodes in a monolayer of cardiomoyocytes cultured in a Cytostretch device, in the relaxed state of the membrane, from 15th to 35th second after the commencement of MEA recording. The arrows point out some of the field potential instances. This device was later manually stretched using a syringe.

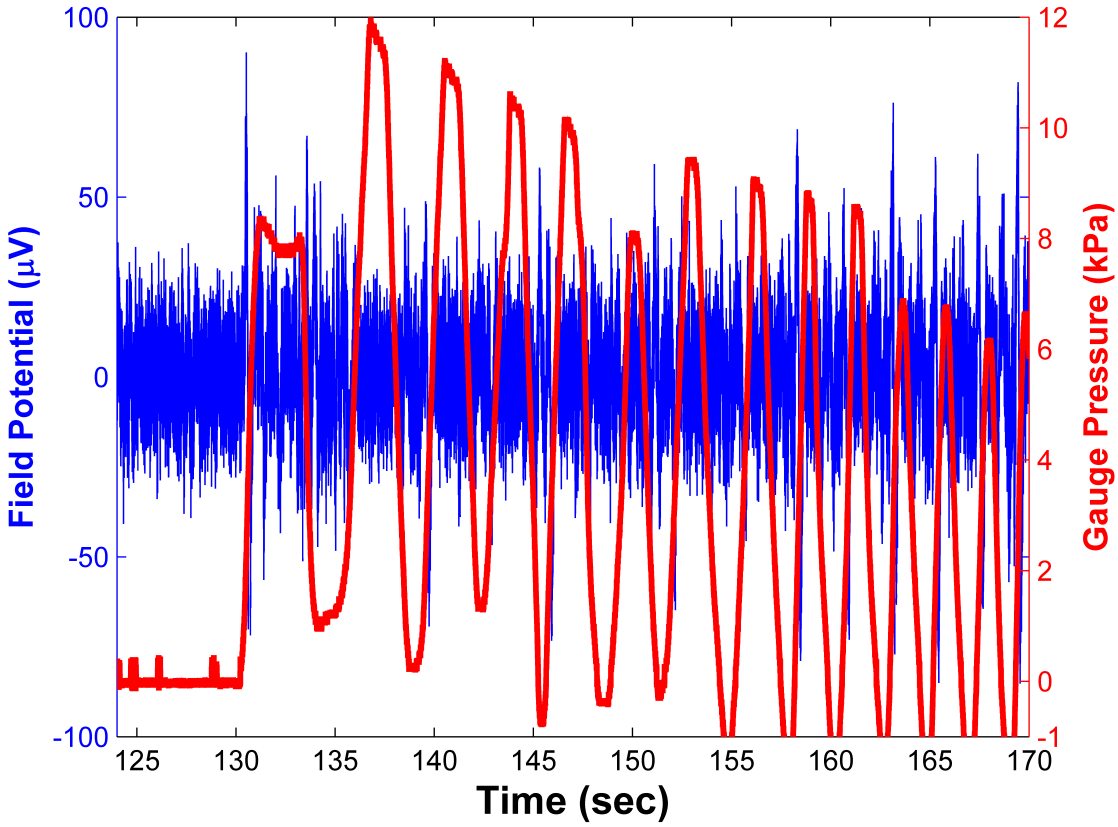


Figure 4-13: Field Potential and pressure at the membrane recorded in a monolayer of cardiomoyocytes cultured in a Cytostretch device, later during the MEA recording. The arrows point out some of the field potential instances. The pressure graph shows the instant when the cyclic stretching was started. The amplitude of the field potential peaks during stretching vary from 30 to 80 μV.

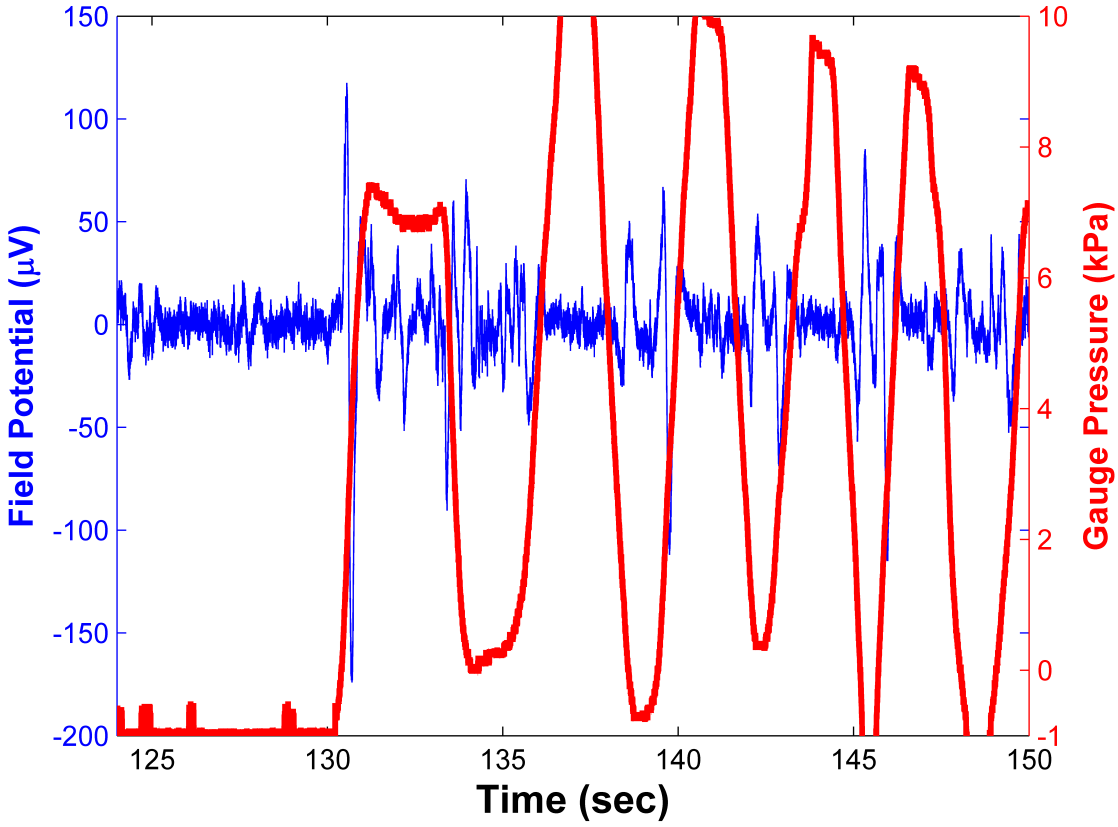


Figure 4-14: Field Potential recorded in a monolayer of cardiomoyocytes cultured in a Cytostretch device, first in the relaxed state of the membrane and a subsequent cyclic stretching. The red arrows indicate the spikes in the MEA signal during cyclic stretching. The MEA recording while stretching is typically monophasic, rather than biphasic.

Chapter 5

Conclusion

The primary purpose of the Cytostretch device is to sustain living cells in culture, stretch them and record their field potential in all the states of the membrane. Therefore, its performance was assessed directly through experiments using neonatal rat cardiomyocytes. In conclusion, there is a wide scope of improvement in the design of the device, the mechanical properties of the membrane and the pneumatic apparatus. A major challenge is to record clearly the filed potential of cardiomyocytes on a stretching membrane using the MEA.

5-1 Membrane viability

Inducing the attachment of cardiomyocytes on PDMS membrane was a challenge because laminin and fibronectin do not bind directly with the PDMS surface. Therefore the hydrophilicity of the PDMS was increased by application of (3-Aminopropyl)triethoxysilane (APTES), a silane primer. Finally, the cardiomyoyctes could attach overnight after seeding them in the coated membrane, indicating that the adhesion of PDMS coated with a silane primer was higher than that of PDMS with laminin.

Following the attachment of cardiomyocytes, it was discovered that cardiomyocyte culture does not sustain with laminin coating, which is otherwise a conventional method for culturing on glass or plastic substrates. However, this problem was solved by replacing laminin with fibronectin.

5-2 PDMS hysteresis

A huge drawback of the membrane is that it exhibits hysteresis, opposed to the assumption that the membrane is elastic [4]. After repetitive cyclic stretching of the membrane, it was permanenetly curved. Although the permanent curvature is not very high in absolute terms ($\sim$ 0%), the height of the curvature 2-3 times the diameter of the freshly isolated round cardiomyocytes. Therefore, the slope dislocates the freshly isolated cardiomyocytes from

the centre to the edges of the membrane and thus, preventing the formation of a uniform monolayer. It was observed in most of the devices because of they were periodically stretched overnight and it was the major reason, after the shortage of devices, why more experiments could not be performed. On the other hand, if there was a greater supply of new devices, then the permanently distorted devices could have been discarded.

This observation challenges the claim of Saeed Pakazad (personal communication) that it is a reusable culture device. The device can be cleaned using detergent, therefore it can be reused for cell culture. However, cyclic stretching for long duration would cause a permanent deformation of the membrane and hence, rendering it useless.

5-3 Quality of MEA recording

The Micro-Electrode array recording of the cardiomyocytes cultured on the membrane of Cytostretch, in its relaxed state, show that the Cytostretch device can capture the cardiac field potentials. Since the cells were only occasionally beating during the experiments, the field potential was also irregular and less frequent than *in vivo*. Although, a high level of noise (400 mu V ptop) was measured in some of the devices, most of them had a fairly low noise level (10 mu V peak-to-peak) making it easy to distinguish the field potential visually, which ranged from 100 to 150 μV (peak-to-peak).

The silicon in the chip is highly sensitive to light which produces noise as high as 1 V (peak-to-peak), but it can be easily shielded from ambient light to produce a noise free signal from its MEA. However, the optical noise poses a great restriction to its compatibility with experimentation because the cultured cells cannot be observed through a microscope simultaneously with MEA recording. For a device whose functionality is under question, it is desirable to validate its measurements using other methods like microscopy or calcium imaging, simultaneously.

It can be seen in Appendix E, that many microelectrodes in the Cytostretch are shorted with each other, which imposes restrictions on the field potential recording of a cardiomyocytes in a monolayer. When 2 distinct cells are attached on 2 different electrodes present on an interconnect, the interconnect would force their extracellular potential to be equal; although the cells may be not be naturally connected with each other via gap junctions. Thus, there would be a loss of information about their spontaneous beating. In the standard MEA, designed by MultiChannel Systems GmbH, none of the electrodes are shorted.

5-4 Pneumatic apparatus

The existing method of stretching, using a pneumatic pump and a solenoid valve, forces the membrane to stretch instantaneously by almost 10%, which does not comply with their stretching pattern under physiological conditions. This is owing to a highly elastic nature of the PDMS membrane that responds fast to the closing and opening of the solenoid valve. The applied strain rate and the pattern of stretching does not match with any other studies involving cyclic stretching of cardiomyocytes [14, 15, 16, 17, 18, 19, 20]. It is suggested that the absolute strain rate be controlled to be uniformly low throughout the cycle.

The high frequency stretching artefacts interfere largely with the MEA recording too. Similar artefacts occur when the fluid is stirred gently during the MEA recording, and also while stretching slowly using a syringe. The MEA measurements made from the cardiomyocytes during manual stretching also exhibit spikes that resemble cardiac field potential. But it cannot be explained what do they represent - the intracellular or extracellular field potential, the artefacts or both. Additionally, the death of the cells following slow stretching also could not be explained. Further experiments must be performed to substantiate this observation.

A plausible explanation behind the artefacts is the movement of the ions present in the medium that cause local currents and hence, the artefacts in MEA recording. However, it poses a question- is it possible to record a clear MEA signal while the medium is not at rest with respect to the microelectrodes, when the signal amplitude is only about 100 μV?

To automatize a physiological cyclic stretching, a ventilation pump used for ventilating rat hearts may be used. The frequency and the volume of pumped air can adjusted with this instrument and the pattern is rather physiological than jerky (Figure 5-1).

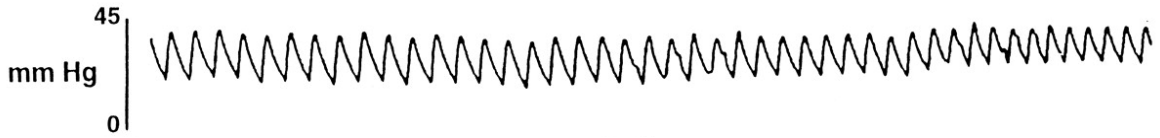


Figure 5-1: Rhythmic gauge pressure vs time generated by a mechanical ventilator [21]. This pattern does not have fast pressure change, which is more physiological than the present pneumatic apparatus.

5-5 Compatibility with other experiments

Other experiments that a user is likely to perform on cultured cells are immunohistochemistry, fluorescence imaging, patch clamping using mico-pipettes or intracellular measurements using sharp micro-pipettes. It is possible to do fluorescence imaging of the cells cultured on the Cytostretch, but only when the device is placed in a non-fluorescent dish. All the developed holders exhibit auto-fluorescent which interferes with the image of the cells and spoils the image quality. Therefore, it is required to design a holder that allows stretching of the membrane and does not add to fluorescence. One of the solutions would be a holder made of PDMS because it shows low fluorescence [22, 23]. To improve the calcium imaging it is also better to increase the size of the central hole and O-ring in the holder, to prevent any interference from the O-ring that flattens upon compression. For details on the O-ring and central hole, please refer to Appendix D.

Immunohistochemistry is an invasive method to find the location and abundance of protein in the cultured cells. It is of great importance in studies involving stretching of muscle cells because the protein ultrastructure is known to be altered by strain [16, 24]. In the process of immunohistochemistry, the cells are fixated to the substrate in a way that it is not possible to wash them away. Therefore, one would avoid performing immunohistochemistry on the the cells cultured in Cytostretch for the fear that it renders a device useless afterward.

Microscopic observation of the membrane inflation is required by the user to accurately measure the strain in the cells and assess the deformation in the membrane from the vertical

increase in the height at the centre of the membrane. For that, it is required to focus on the centre point. Therefore, it is suggested that the centre be marked by a dot on the PDMS membrane. The dot could be placed by embedding the electrode or interconnect material.

Appendix A

Device handling

A-1 Assembling the components of the Cytostretch

All the three parts were supplied to me individually and were then joined together to build up the whole device using silicone gel. The chip was attached to a 2 mm thick acrylic sheet forming a hole around the PDMS membrane. This inner well that is now formed around the membrane has a volume of 100 μL. Since this amount of liquid medium is not sufficient for the survival of cells for more than a day, the plastic sheet has been augmented with an acrylic ring. The acrylic ring, bottomed with the combination of acrylic plate and silicone chip, can hold up to 600 μL. Both the above joints are made with PolyDiMethylSiloxane (PDMS) mixed with a curing agent in the ratio 10:1, because PDMS is biocompatible while the epoxy glues are not.

A-2 Careful handling of the device

The membrane, which is 20μm thick, is the most fragile part of the device and is susceptible to breaking, thereofre upon touching with anything rigid. The silicon chip itself is exposed to the environment and is prone to corrosion and cracking because it is brittle and has aluminium connections.

A-3 Cleaning

The device is reusable, i.e. cells can be repeatedly cultured in it provided that all the cells, protein layer and any bacterial growth have been washed away. Washing can be performed and has been performed between successive culture of cells, using 1% solution of Terg-a-zyme®enzyme detergent (Sigma-Aldrich) in distilled water. It is advisable to fill the device with the detergent solution and shake it over night to completely eradicate the deposited proteins. A fresh device also must be washed with the detergent to wash away any possible

contamination acquired during the fabrication or transportation. Here, a fresh device is that which has not been used to culture any cells, nor has been subjected to plasma.
CAUTION: Avoid spilling detergent on the ventral surface of the chip because the deposited detergent would alter the conductance between the device and the MEA holder.

Appendix B

Pneumatic valve controller

To apply cyclic or constant pressure beneath the PDMS membrane in the Cytostretch device and be able to change the frequency of the switching, an electronic control system has been instrumented. Pressurised air with at least 10 kPa pressure is necessary to stretch the membrane of a Cytostretch up to 15%. The pressure can be further tuned to any value less than or equal to the maximum value with needle valves. To stretch and relax the membrane cyclically during the experiments, a solenoid valve is chosen for it can switch between the open and closed state fast. A digital clock with a relay at its output has been used to switch the solenoid valve. The digital clock has a user interface with which the frequency and the duty cycle can be set as and when required.

A pressure sensor allows us to read the pressure delivered to the membrane. The analog output of the pressure sensor ranges from 0 to 5 V and is very well read using the analog input channel of the Multi Channel System.

The circuit schematic has been presented in Figure B-2. All the components were soldered and arranged in a box, named as pneumatic box to make a convenient interface with the users. The pneumatic box is a robust and portable electronic object which can be plugged in and out so it can be used in different settings, such as incubator, patch clamping, calcium imaging, microscope and Multi Channel System (MCS), placed in different laboratory rooms.

B-1 Circuit design

- Pneumatic valves: These are spiral valves and let the air flow through them when open.
- Solenoid valve *(Festo MHE2-M1H-3/2O-Q5-4-K)*: A solenoid 3/2 fast switching valve is used [25]. It is driven electronically on the principle of electric induction. So a snubber diode is connected to prevent any current passing because of the back EMF generated at the time of switching its power supply off. It is being operated on 24 V DC, 120 mA current. Its functioning is explained further in Appendix C.

- Pneumatic pump: A vacuum pump that generates up to 33.3 kPa of air pressure is used. A more powerful pump could be used because the other components namely, the solenoid valve and the pressure sensor can withstand 1 MPa and 400 kPa, respectively.

- Clock generator *(Omron H5CX-L)*: It is a user-friendly digital timer which offers many timer settings to generate pulses from the accuracy ranging from 10^{-4} seconds to 10^{4} hours [26]. Its output consists of a relay switching between 2 pins. It is being operated on 24 V DC power supply.

- Pressure sensor *(Honeywell 030PDAA5)*: This pressure sensor works on 5 V power supply and is biased at 2.52 V, i.e. at the atmospheric pressure its output voltage is 2.52 V [27]. The sensitivity when operated with 5V power supply is given by:

$$\frac{(0.9 - 0.1) \times 5V}{206.5kPa} = 0.01V/kPa$$

- Relay and transistor: A Bipolar Junction transistor is gated by the output of the timer and it drives the relay which drives the solenoid valve. The relay is being used in addition to the transistor for the safe operation of the clock. About 5 A current passes through the solenoid immediately when it is switched on which can damage the clock if driven by it directly.

- 24 V DC power supply

- 5 V regulator: It is a 7805 chip that converts 24 V DC to 5 V at its output. It is required here to drive the pressure sensor.

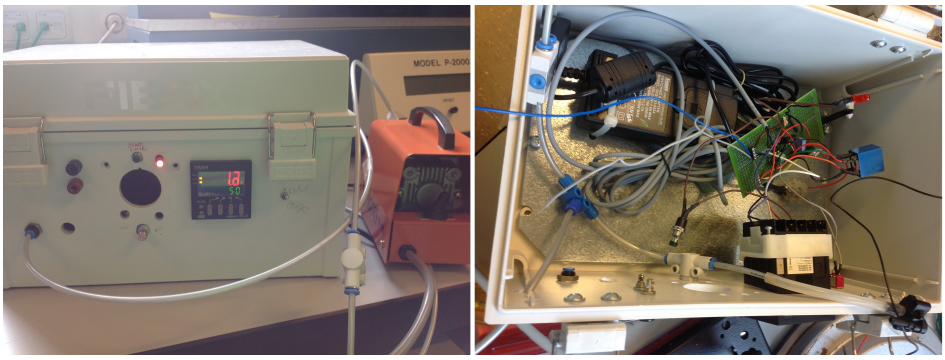


Figure B-1: The instruments in the pneumatic box that drive the valve.

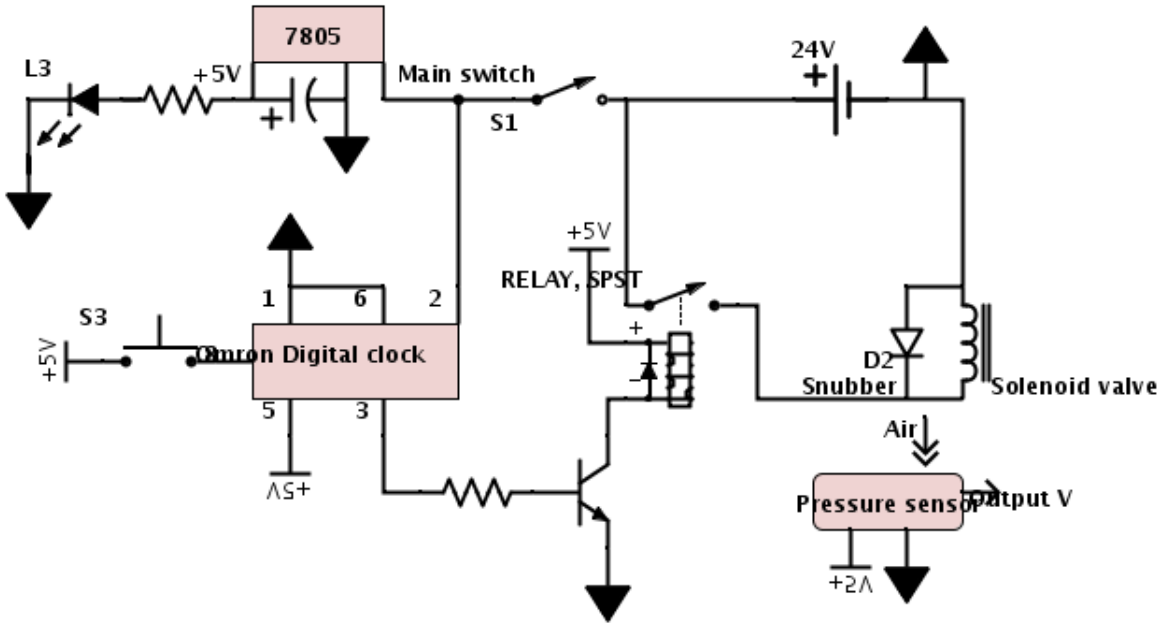


Figure B-2: The electric circuit that drives the solenoid valve.

Appendix C

Usage of pneumatic apparatus

1. It is important to ensure that there is no leakage to the atmosphere in the pneumatic apparatus. Leakage generally occurs when the chip is not tightly fastened, or the glass cover-slip at the reverse side of holder is broken, or the pneumatic fitting to the holder itself is not properly fixed. On must extra care to check all these possible points of leakage, lest the pressure should not attain the desired pressure levels and the sudden jerk in the membrane would traumatize the cultured cells.

2. The valve 3 must not be too tight, otherwise the minimum pressure may not equal the atmospheric pressure.

3. Actual applied pressure between valve 1 and 2 should be set to a much higher valuse than the pressure to be attained during the cyclic stretching, because the saturation time of this viscoelastic model is far greater than 0.5 seconds which corresponds to the normally used beat rate of 1 Hz.

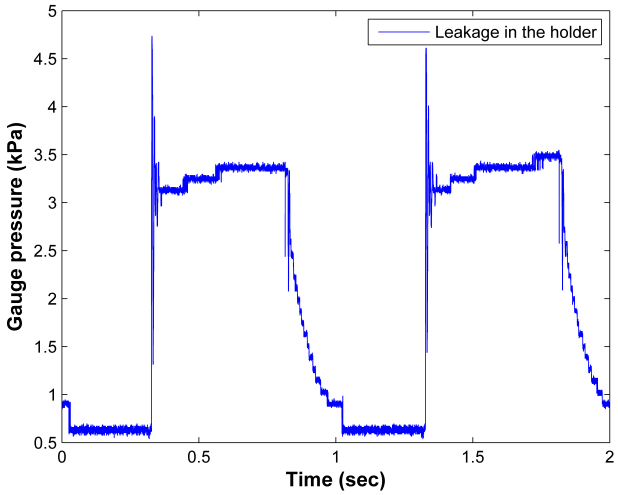


Figure C-1: Pressure at the membrane when there is some leakage in the pneumatic apparatus.

Appendix D

Device holders

1. 3D printed holder: Using SolidWorks (Dassault Systems SolidWords Corp.), I designed a plastic holder with horizontal pneumatic inlet that bifurcates into 2 branches to upply air to the membrane of 2 chips. I printed it in the Walk-in Workshop for Students in the 3ME faculty of TU Delft. However, this printer could print with a minimum resolution of 0.1 mm, because of which the surface of the produced device was neither smooth nor flat, but rathers porous. Nuts and bolts were fixed to hold the device in place and O-rings were attached to form a tight seal between the device and the chip around the dog-bone shaped membrane. A 3 mm wide tube was fixed permanently to the inlet using an epoxy glue.

   The set-up was tested for leakage using the pneumatic apparatus. There was a leakage at the interface between the O-ring and the holder because the surfaces were neither smooth nor flat.

(a) A schematic of the 3D printed holder.

(b) A cross section of the 3D printed holder showing the transverse pneumatic inlet.

Figure D-1: Schematic of the 3D printed holder.

2. Mechanically machined acrylic holder: This holder was manufactured with a 2 to 3 mm thick acrylic sheet so it could withstand the force of the , with horizontal grooves and a

vertical inlet drilled and milled through it, respectively. The machining was performed at the workshop in the 3ME faculty of TU Delft. It is shown in Figure D-2.
This holder can hold 3 chips at the same time to connect with the pneumatic inlet and thus, makes multiple samples. However, this is not a stand alone piece because it requires fixing of a highly transparent glass cover-slip to seal the air flow channels. There was only thin cover slip presen in the lab, which cracked by the air pressure. Also an air tight seal could not be fiormed in this case because the acrylic sheet was not very flat and fixing the chips with screws lead to further bending and hence, cracking the glass cover-slip.

3. 1 cm thick holder: Finally, I produced holders that are made from a 1 cm thick acrylic sheet to keep up the rigidity of the structure. A hole for the pneumatic inlet was drilled transversely, thus, eliminating the need of an extra cover slip to cover the groove. The goal of air-tightness was very well met in this case, however the thickness of the does not allow them to be used on an inverted microscope. Also, calcium imaging was not possible due to the autofluorescence of the material.

4. Single holder: Eventually a single-chip holder was manufactured, similar to the PCB based holder. Instead of a thin glass cover slips, microscope slide was fixed to cover the grooves at the back side. However, the thickness of the assembly was too big to view it over an inverted microscope.

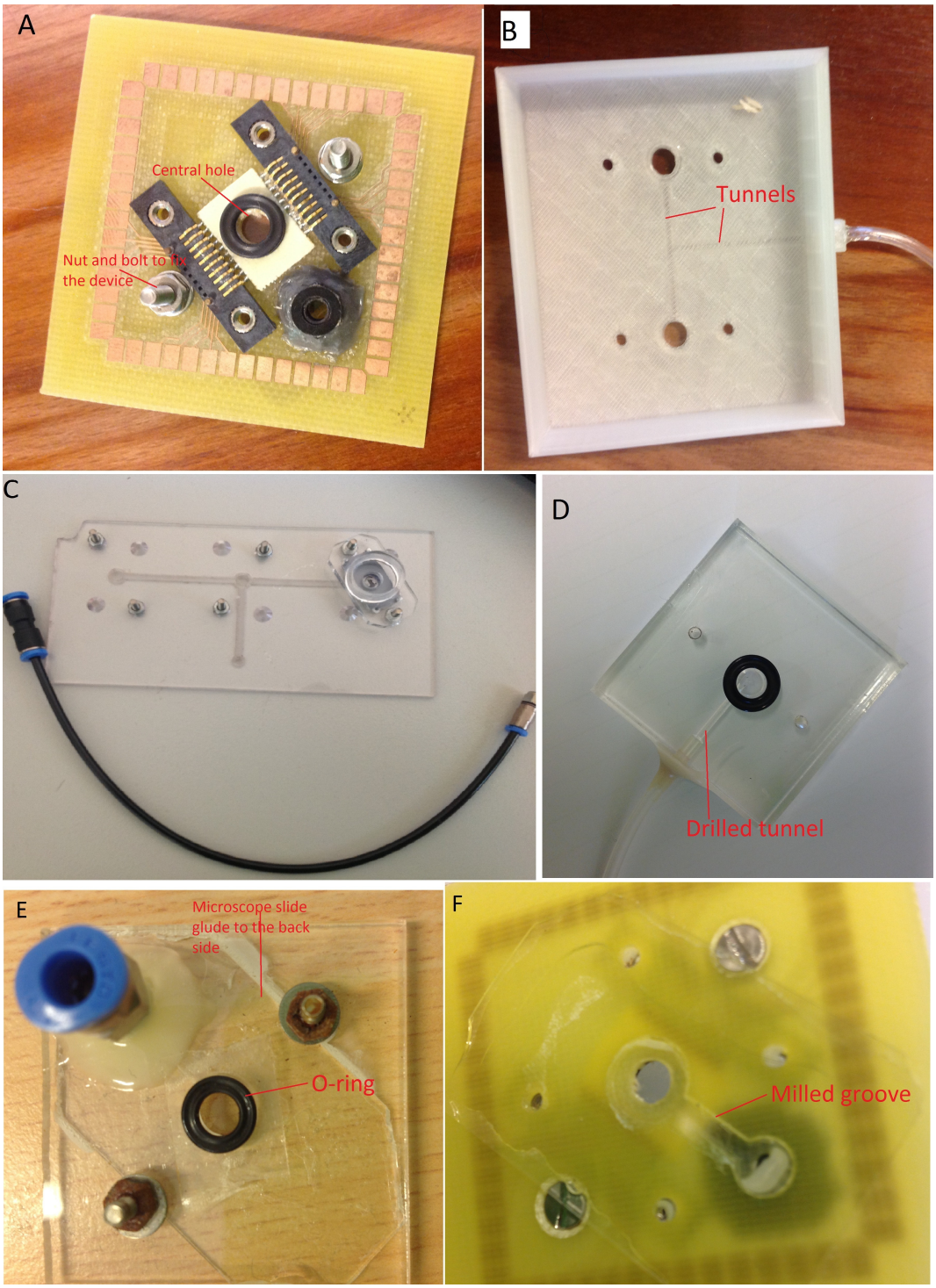


Figure D-2: (A) The PCB based holder with 60 metal contacts. (B) 3D printed holder with transverse channels for air contains slots for holding 2 chips simultaneously. (C) The acrylic holder for 3 chips. (D) 1 cm thick acrylic holder. (E) Single holder similar to the PCB based holder with a microscope slide. F. Groove in the PCB based holder.

Appendix E

MEA recording with different grounds

The layout of the microelectrodes has been shown in this appendix, emphasizing that the electrodes are interconnected with each other and there are 2 axes of symmetry in the their placement. The MEA recordrings in devices, containing only the medium, are presented to show the effect of the different grounding methods on the recording itself.

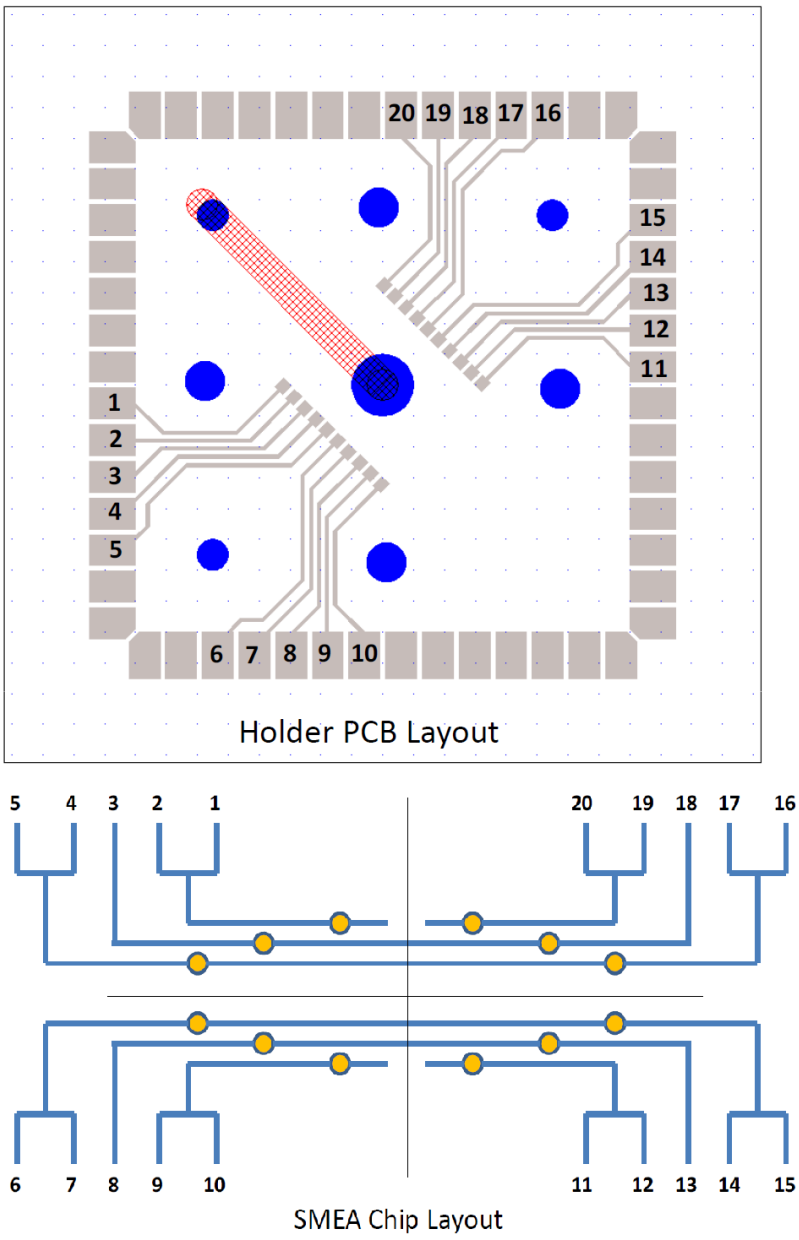


Figure E-1: The layout of electrodes on the membrane and their corresponding ports on the PCB holder. The 2 axes of symmetry amongst the positions of the electrodes on the membrane are shown.

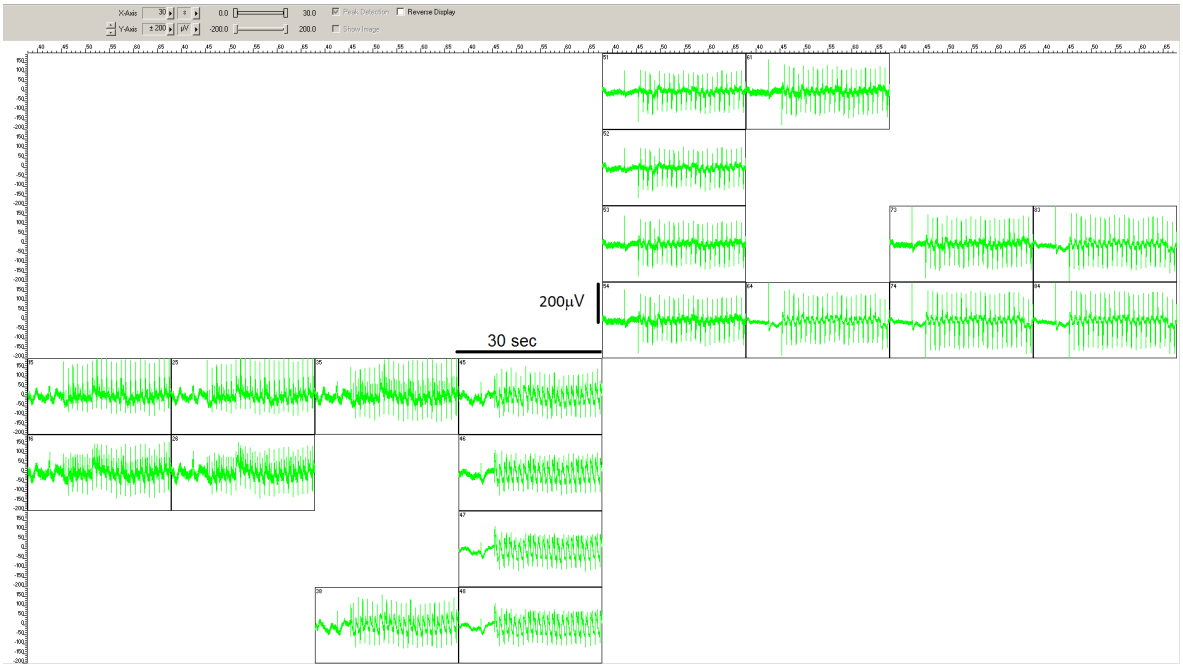


Figure E-2: The MEA recording on all the electrodes of a Cytorstretch device keeping only the bath electrode grounded, while cyclically stretching it at the frequency of 1 Hz. It can be noticed that there are sharp spikes on all the electrodes because the bath electrode is located far from the moving membrane.

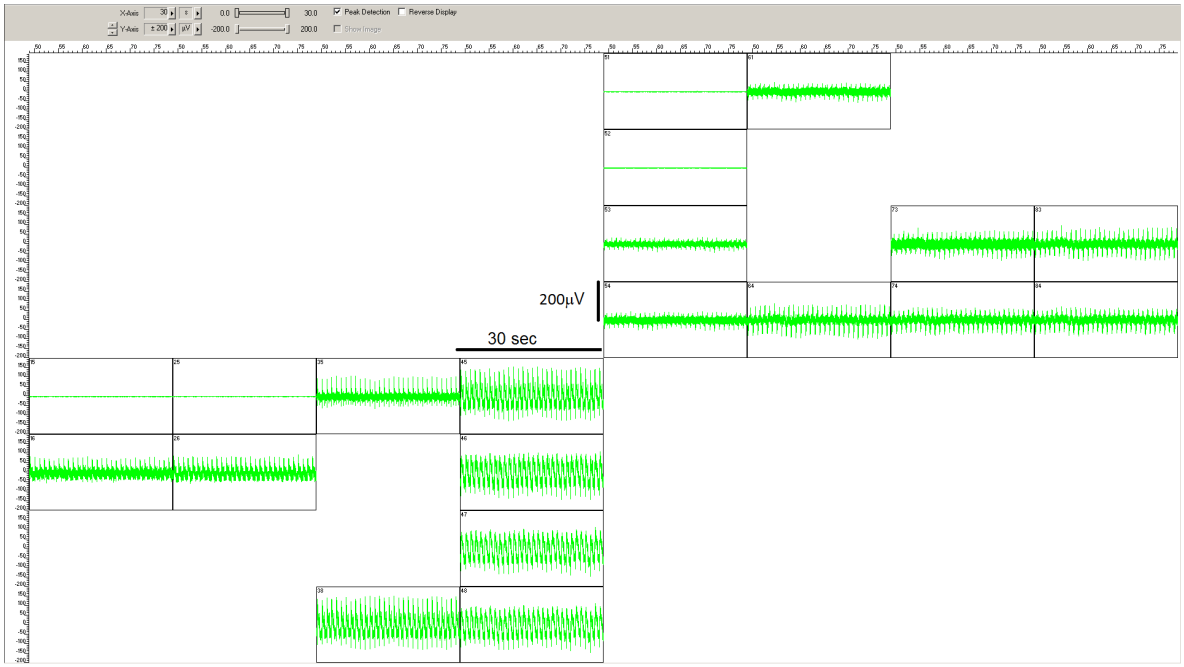


Figure E-3: The MEA recording on all the electrodes of a Cytorstretch device keeping only the electrodes 15 and 25 grounded, while cyclically stretching it at the frequency of 1 Hz. Electrodes 51 and 52 are shorted with 15 and 25, respectively. It can be noticed that there are sharp spikes on the electrodes that are spatially far from the grounded electrodes, whereas the electrodes 53, 54 and 61 that are spatially near them have lower spikes. This because they experience similar amount of displacement while stretching.

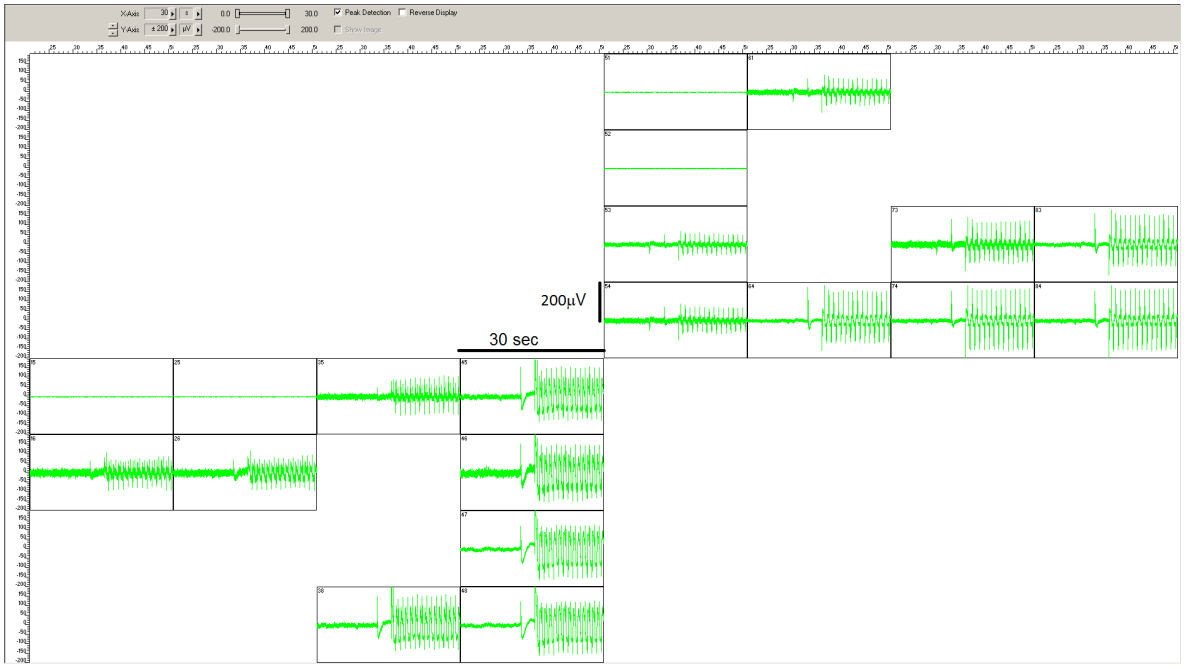


Figure E-4: The MEA recording on all the electrodes of a Cytorstretch device keeping the bath electrode as well as microelectrode 15 and 25 ground ground, while cyclically stretching at the frequency of 1 Hz. It can be noticed that there are sharp spikes on all the electrodes but some of the electrodes that are located near the grounded microelectrodes. This is because the bath electrode is far from the membrane, so it is not feel any disturbance, whereas the microelectrodes on the membrane do sense the artefacts.

Appendix F

Cell culture

Time point	Operations
Day 0 (Monday), afternoon	Devices cleaned and coated for new culture
Day 0, 5PM	Isolated cells seeded in the device
Day 1, 10AM	Microscopic observation of the cell attachment
Day 1, 11AM	The medium changed to wash out the floating dead cells

Table F-1: The steps followed to culture cardiomyocytes in Cytostretch chips or any other culture dish.

Bibliography

[1] S. J. Trietsch, G. D. Israels, J. Joore, T. Hankemeier, and P. Vulto, "Microfluidic titer plate for stratified 3d cell culture," *Lab Chip*, vol. 13, pp. 3548–3554, 2013.

[2] D. Huh, B. Matthews, A. Mammoto, M. Montoya-Zavala, H. Yuan Hsin, and D. Ingber, "Reconstituting organ-level lung functions on a chip," *Science*, vol. 328, pp. 1662–1668, 2010.

[3] S. Khoshfetrat Pakazad, A. Savov, A. Van De Stolpe, S. Braam, B. Van Meer, and R. Dekker, "A stretchable micro-electrode array for in vitro electrophysiology," *Proceedings of the IEEE International Conference on Micro Electro Mechanical Systems (MEMS)*, pp. 829–832, 2011.

[4] S. K. Pakazad, A. Savov, A. van de Stolpe, and R. Dekker, "A novel stretchable microelectrode array (smea) design for directional stretching of cells," *Journal of Micromechanics and Microengineering*, vol. 24, no. 3, 2014.

[5] I. Kehat, D. Kenyagin-Karsenti, M. Snir, H. Segev, M. Amit, A. Gepstein, E. Livne, O. Binah, J. Itskovitz-Eldor, L. Gepstein, *et al.*, "Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes," *Journal of clinical investigation*, vol. 108, pp. 407–414, 2001.

[6] S.-J. Gwak, S. Bhang, I.-K. Kim, S.-S. Kim, S.-W. Cho, O. Jeon, K. Yoo, A. Putnam, and B.-S. Kim, "The effect of cyclic strain on embryonic stem cell-derived cardiomyocytes," *Biomaterials*, vol. 29, pp. 844–856, 2008.

[7] Y. Kajikawa and C. Schroeder, "How local is the local field potential?," *Neuron*, vol. 72, pp. 847–858, 2011.

[8] M. Spira and A. Hai, "Multi-electrode array technologies for neuroscience and cardiology," *Nature Nanotechnology*, vol. 8, pp. 83–94, 2013.

[9] Y. Haraguchi, T. Shimizu, M. Yamato, A. Kikuchi, and T. Okano, "Electrical coupling of cardiomyocyte sheets occurs rapidly via functional gap junction formation," *Biomaterials*, vol. 27, pp. 4765–4774, 2006.

[10] J. Dunkers, H.-J. Lee, M. Matos, L. Pakstis, J. Taboas, S. Hudson, and M. Cicerone, "Effect of surface modification on protein retention and cell proliferation under strain," *Acta biomaterialia*, vol. 7, pp. 2902–2909, 2011.

[11] I. Hoek, F. Tho, and W. Arnold, "Sodium hydroxide treatment of pdms based microfluidic devices," *Lab on a Chip - Miniaturisation for Chemistry and Biology*, vol. 10, pp. 2283–2285, 2010.

[12] F. Sarvi, Z. Yue, K. Hourigan, M. Thompson, and P. Chan, "Surface-functionalization of pdms for potential micro-bioreactor and embryonic stem cell culture applications," *Journal of Materials Chemistry B*, vol. 1, pp. 987–996, 2013.

[13] H. Vandenburgh, R. Solerssi, J. Shansky, J. Adams, S. Henderson, and J. Lemaire, "Response of neonatal rat cardiomyocytes to repetitive mechanical stimulation in vitro," *Annals of the New York Academy of Sciences*, vol. 752, pp. 19–29, 1995.

[14] I. Komuro, T. Kaida, Y. Shibazaki, M. Kurabayashi, Y. Katoh, E. Hoh, F. Takaku, and Y. Yazaki, "Stretching cardiac myocytes stimulates protooncogene expression," *Journal of Biological Chemistry*, vol. 265, pp. 3595–3598, 1990.

[15] J.-I. Sadoshima, L. Jahn, T. Takahashi, T. Kulik, and S. Izumo, "Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. an in vitro model of load-induced cardiac hypertrophy," *Journal of Biological Chemistry*, vol. 267, pp. 10551–10560, 1992.

[16] W.-H. Zimmermann, K. Schneiderbanger, P. Schubert, M. DidiÃl, F. MÃijnzel, J. Heubach, S. Kostin, W. Neuhuber, and T. Eschenhagen, "Tissue engineering of a differentiated cardiac muscle construct," *Circulation Research*, vol. 90, pp. 223–230, 2002.

[17] J. Boublik, H. Park, M. Radisic, E. Tognana, F. Chen, M. Pei, G. Vunjak-Novakovic, and L. Freed, "Mechanical properties and remodeling of hybrid cardiac constructs made from heart cells, fibrin, and biodegradable, elastomeric knitted fabric," *Tissue Engineering*, vol. 11, pp. 1122–1132, 2005.

[18] D. Frank, C. Kuhn, B. Brors, C. Hanselmann, M. LÃijdde, H. Katus, and N. Frey, "Gene expression pattern in biomechanically stretched cardiomyocytes: Evidence for a stretch-specific gene program," *Hypertension*, vol. 51, pp. 309–318, 2008.

[19] M. Shachar, N. Benishti, and S. Cohen, "Effects of mechanical stimulation induced by compression and medium perfusion on cardiac tissue engineering," *Biotechnology Progress*, vol. 28, pp. 1551–1559, 2012.

[20] V. F. Shimko and W. C. Claycomb, "Effect of mechanical loading on three-dimensional cultures of embryonic stem cell-derived cardiomyocytes," *Tissue Engineering Part A*, vol. 14, pp. 49–58, 2008.

[21] E. W. Taylor, D. Jordan, and J. H. Coote, "Central control of the cardiovascular and respiratory systems and their interactions in vertebrates," vol. 79, pp. 855–916, 1999.

[22] A. Piruska, I. Nikcevic, S. H. Lee, C. Ahn, W. R. Heineman, P. A. Limbach, and C. J. Seliskar, "The autofluorescence of plastic materials and chips measured under laser irradiation," *Lab on a Chip*, vol. 5, pp. 1348–1354, 2005.

[23] S. Cesaro-Tadic, G. Dernick, D. Juncker, G. Buurman, H. Kropshofer, B. Michel, C. Fattinger, and E. Delamarche, "High-sensitivity miniaturized immunoassays for tumor necrosis factor α using microfluidic systems," *Lab on a Chip*, vol. 4, pp. 563–569, 2004.

[24] A. Salameh and S. Dhein, "Effects of mechanical forces and stretch on intercellular gap junction coupling," *Biochimica et Biophysica Acta - Biomembranes*, vol. 1828, pp. 147–156, 2013.

[25] Festo, *Solenoid valves MH2/MH3/MH4, fast-switching valves*, 6 2011.

[26] OMRON Industrial Automation, *Digital Timer*, 10 2012.

[27] Honeywell International Inc., *TruStability®Silicon Pressure Sensors: SSC Series-Standard Accuracy*, 3 2011.

Glossary

List of Acronyms

SMEA	Stretchable Micro-Electrode Array
PDMS	PolyDiMethylSiloxane
MCS	Multi Channel System
APTES	(3-Aminopropyl)triethoxysilane
APTMS	(3-Aminopropyl)trimethoxysilane
PBS	Phosphate Buffered Solution
MEA	Micro-Electrode Array
UMC	University Medical Centre
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid