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# Cell Printing: An Effective Advancement for the Creation of um Size Patterns for Integration into Microfluidic BioMEMs Devices

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# CELL PRINTING: AN EFFECTIVE ADVANCEMENT FOR THE CREATION OF µm SIZE PATTERNS FOR INTEGRATION INTO MICROFLUIDIC BIOMEMS DEVICES

by

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# ABSTRACT

The Body-on-a-Chip (BoaC) is a microfluidic BioMEMs project that aims to replicate major organs of the human body on a chip, providing an in vitro drug testing platform without the need to resort to animal model testing. Using a human model also provides significantly more accurate drug response data, and may even open the door to personalized drug treatments. Microelectrode arrays integrated with human neuronal or human cardiac cells that are positioned on the electrodes are essential components for BoaC systems. Fabricating these substrates relies heavily on chemically patterned surfaces to control the orientation and growth of the cells. Currently, cells are plated by hand onto the surface of the chemically patterned microelectrode arrays. The cells that land on the cytophobic 2-[Methoxy(Polyethyleneoxy)$_{6-9}$Propyl]trimethoxysilane (PEG) coating die and detach from the surface, while the cells that land on the cytophilic diethylenetriamine (DETA) coating survive and attach to the surface exhibiting normal physiology and function. The current technique wastes a significant amount of cells, some of which are extremely expensive, and is labor intensive. Cell printing, the process of dispensing cells through a 3D printer, makes it possible to pinpoint the placement of cells onto the microelectrodes, drastically reducing the number of cells utilized. Scaled-up manufacturing is also possible due to the automation capabilities provided by printing. In this work, the specific conditions for printing each cell type is unique, the printing of human motoneurons, human sensory neurons and human cardiac cells was investigated. The viability and functionality of the printed cells are demonstrated by phase images, immunostaining and electrical signal recordings. The superior resolution of cell printing was then taken one step further by successfully printing two different cell types in close proximity to encourage controlled innervation and communication.

Dedicated to my friends and family

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| BoaC | - | Body-on-a-Chip |
| BSA | - | Bovine Serum Albumin |
| CDI | - | Cellular Dynamics International (cell company) |
| cMEA | - | custom Microelectrode Array |
| DIS | - | Days in System |
| DETA | - | $N^1$-(3-Trimethoxysilylpropyl)diethylenetriamine |
| DMEM | - | Dulbecco's Modified Eagle Medium |
| DOD | - | Drop On Demand |
| EGTA | - | Egtazic acid |
| HEPES | - | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hMN | - | Human MotoNeuron |
| hNP1 | - | Human Neural Progenitor 1 |
| HSL | - | Hybrid Systems Lab |
| hSN | - | Human Sensory Neuron |
| KSR | - | Knock-out Serum Replacement |
| LDH | - | Lactate Dehydrogenase |
| NGF | - | Nerve Growth Factor |
| PBS | - | Phosphate-Buffered Saline |
| PEG | - | 2-[Methoxy(Polyethyleneoxy)$_{6\text{-}9}$Propyl]trimethoxysilane |
| PFA | - | Paraformaldehyde |
| Pt | - | Platinum |
| SCSC | - | Spinal Cord Stem Cell |
| UV | - | Ultraviolet |

# CHAPTER 1 INTRODUCTION

## 1.1 Problem Statement

The goal of this thesis is to show that cell printing is an effective method for cell placement onto a patterned substrate to reduce resource utilization (cost of cells) as well as increase repeatability and uniformity, while preserving cell viability and functionality.

The current methods used by the Hybrid Systems Lab (HSL) for cell plating are hand plating and printing cells. The advantages of printing cells include a reduction in time, resources and cost as well as an increase in repeatability and uniformity of cell plating, while maintaining viability of the cells similar to hand plating. This is achieved through precise cellular placement allowing for fewer cells used per surface and a higher throughput possibility through automation. The functionality and viability of the printed cells were compared to hand plated cells to determine feasibility using phase imaging, immunostaining, patch clamp, and electrical recordings.

## 1.2 Objectives and Overview

The objectives of this thesis are to demonstrate the feasibility of printing cells on chemically modified, blanket coated and patterned surfaces through comparisons between printing and hand plating utilizing morpholgical, functionality recordings, patch clamp (electrophysiology), and immunostaining analysis. The instrument used to print the cells is a RegenHu 3D Discovery printer. The cell types discussed will be human motoneurons (hMN), human cardiomyocytes, and a co-culture of hMN and human sensory neurons (hSN).

## 1.3 Application

One of the main objectives of the HSL is to create BoaC devices. The goal is to represent major organs of the human body on a chip, which can then be used as a platform to test drugs. The BoaC acts as a human model, which can reduce or eliminate the need for animal testing, providing a more accurate result of a human organ's response to drugs. When assembly for the BoaC systems scales-up, an automated cell plating technique utilizing as few cells as possible will be desired. Also surfaces representing a multi-cellular system, such as the reflex arc, may need to be fabricated at a resolution too fine to achieve via hand plating. Cell printing can address both of these objectives.

## 1.4 Organization of the Thesis

The first section of this thesis details the current printing techniques and applications found in the literature. The next section discusses the techniques utilized in this research study. The adaptation of the 3D printer will also be discussed. The main body of the paper includes printing hMNs on micropatterned surfaces to show no detriment to the cells when compared to hand plating, and demonstration of successful printing of cardiac cells as well as implementation in microfluidic systems to obtain electrical signals. Furthermore, printing of two cell types, hMNs and hSNs, showing intended innervation of hSNs into hMNs will be discussed. The thesis will then summarize the results and offer conclusions with a glimpse into future applications.

# CHAPTER 2 LITERATURE REVIEW

## 2.1 Introduction

This chapter explores the current state of bioprinting, providing a brief introduction into the benefits and difficulties of printing living cells. Discussion of the different methods of bioprinting will be addressed as well as a more in depth look into microvalve printing, the technique used in this paper.

## 2.2 Bioprinting Overview

Bioprinting is the process of using a printing machine or instrument to deliver biological material or cellular support matrices to a substrate. There are many advantages to the bioprinting technique, some of which include high and fast sample throughput, relatively inexpensive equipment, and adaptable programs that are controlled via desktop computer [1]. One of the difficulties of bioprinting is making sure the cells survive the printing process. Cells that experience too high of a shear stress will exhibit higher levels of lactate dehydrogenase (LDH) in the media, a sign of cell death [2]. The process of liquid flowing out of the microvalve hole creates shear in the flow of the liquid due to the increase of friction around the microvalve edge. Factors that determine the amount of shear stress imposed upon the cells are a function of the hole diameter that the fluid is flowing out of, the viscosity of the cell fluid, the speed of the liquid flow, and the size of the cells present in the liquid [3]. It is possible for the cells to survive the printing process without noticeable harm. Tao Xu et al. [4] studied the health of printed embryonic hippocampal and cortical neurons and compared them to hand plated controls via immunostaining and patch clamp. No statistical differences between the health of

the printed and hand plated cells were observed, and they stated that the health of both sets of cells was comparable to literature standards [4].

## 2.3 Types of Bioprinting

There are many different bioprinting techniques, including thermal drop on demand (DOD), piezoelectric DOD, electrostatic DOD and electrohydrodynamic jetting, acoustic droplet ejection, and micro-valve printing [5]. Many different types of proteins, hydrogels and cell types have been bioprinted, as listed below. For proteins: Bovine Serum Albumin (BSA), Biotin-BSA, Streptavidin, and Y-Biotin have been printed [1]. For hydrogels, fibrin gels are used for the creation of a matrix in 3D printing [4]. Cell types printed include Bovine aortal endothelial cells, and a smooth muscle cell line (ATTC) [6], rat Primary embryonic hippocampal and cortical neurons for 2D applications, as well as NT2 cells for 3D applications [4].

## 2.4 Benefits of Microvalve Printing

Microvalve printing is a low-cost method for printing cells. Many types of bioinks can be used, ranging from high to low viscosity cell solutions in media. All parts can be easily cleaned and the microvalves are interchangeable, allowing for a large variety of microvalve sizes. Cells printed with microvalve printing experienced greater than 90% viability, even though the cells experience higher shear stress than some of the other methods listed above [5].

## 2.5 Summary

As stated above, there are many different approaches for bioprinting and each method has its pros and cons. Microvalve printing allows for a variety of printing strategies to deliver

cells in a highly repeatable manner with high cell viability despite higher shear stress than other printing methods.

# CHAPTER 3 METHODS AND TECHNIQUES

## 3.1 Introduction

This chapter covers the methods for the creation of the substrates and printing tools used in this project, as well as the biological preparation and analysis, surface chemistry modification and printing techniques.

## 3.2 Fabrication of cMEA Surfaces

The custom microelectrode array (cMEA) surfaces used in this project were created at the Cornell NanoScale Science and Technology Facility, Ithica, NY. Fabrication of these cMEA surfaces was achieved by taking a fused silica wafer and cleaning it with a piranha solution. Photoresist was then added and the surface was exposed to UV light. The wafer was then placed in an oven with ammonia. The desired mask was then added and the exposed areas were developed away. Titanium thermal evaporation was used to create the metal traces. An oxygen, nitrogen, oxygen stack consisting of 150 nm of each per layer was added. The photoresist was then added, the desired mask put on, and the pattern developed. A plasma etcher was then used to drill through the SiO SiN SiO layers of insulation. The photoresist was then stripped, exposing platinum (Pt) in desired locations. The wafer was then cut into rectangles to create the separate cMEA chips. Figure 3-1 is an image of a fabricated cMEA chip.

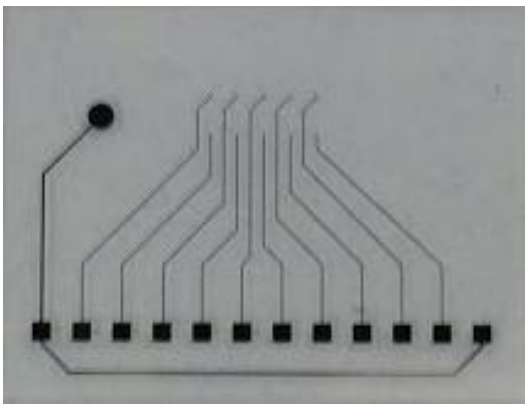


Figure 3-1: cMEA chip

## 3.3 Chemical Modification of Surfaces

The surfaces, which were used to hold cells, underwent a chemical modification before cell plating. Different surface modifications were performed depending upon the cell type and the desired use of the patterned device. The cytophilic surface that was utilized is DETA, while the cytophobic surface was PEG.

### 3.3.1 DETA Modification

The DETA-toluene solution was prepared in a nitrogen rich environment with very low humidity. The cMEA chips to be modified were plasma cleaned for 20 minutes and then added to the DETA-toluene solution in a fume hood in the laboratory atmosphere. The mixture was heated to 100°C for about 30 minutes. The mixture was then taken off the heat and cooled to near room temperature. The cMEA surfaces were then rinsed three times in distilled toluene and placed in a new container of distilled toluene. This fourth toluene rinse with the cMEA chips inside was heated to 100°C for about 30 minutes. The cMEAs were removed from the toluene solution and placed in a 110°C oven for approximately 12 hours, and then stored in a

desiccator until use. Quality control was performed on the surfaces using an XPS with an aluminum anode and a quartz monochromator with a spectrometer calibrated against a clean Ag sample. Si 2p, C 1s, N 1s and O spectra were recorded to quantitatively qualify the surfaces.

### 3.3.2 PEG Modification

The cMEA chips to be modified were plasma cleaned for 20 minutes and then added to a 3mM PEG solution with 37% HCl in toluene for 45 minutes at room temperature. The rinsing steps were as follows: toluene, ethanol (x2), and deionized water (x2). The surfaces were then dried and placed in a desiccator overnight [7].

## 3.4 Laser Ablation of Surfaces

The surfaces were patterned using a quartz photomask with a designated pattern, such as 500 μm circles around an electrode. The laser used was a 193 nm deep UV eximer laser from Lambda Physik. The surfaces were ablated for 45 seconds at a frequency of 10 Hz and a pulse power of 230 mW [7].

## 3.5 Fabrication of Cell Printer Plates

The acrylic printer plates used for alignment of the cMEAs and coverslips were scaled to the dimension of standard cell culture plates to fit the RegenHU printer. The plates were designed in either CAD or Autofusion360 and exported into a PDF for laser cutting. Small metal alignment pins were pressed into tiny laser etched holes to facilitate the alignment of the cMEA chips. The top and bottom parts of the plate were then adhered via a watertight seal using dichloromethane and leak tested. The plates were then cleaned with a 1% tergazyme solution in tap water, rinsed with Milli-Q water and sterilized with 200 proof alcohol before use.

## 3.6 Cell Printing Process

Methods describing the cell printing process, which includes gathering hMN, hSN and cardiac cells, the cell printer program, and the outlined printing process are listed below.

### 3.6.1 Cell Culture

The cells used for printing included hMN cells, cardiac cells, and sensory hMN cells. The following subsections describe the process necessary to take cells stored in liquid nitrogen and prepare them for use in cell printing. Due to their inherent differences, each cell type has a different preparation procedure.

#### *3.6.1.1 SC-hMN Cells*

The first step was to create a laminin coated surface. A solution of 10 µL of laminin to 1ml of sterile water was added to a small petri dish. Special care was taken to ensure enough solution was present to cover the entire bottom of the dish. The petri dish containing laminin solution was then placed in a fridge overnight. If coating was required same day, the petri dish was left out at room temperature for six hours before plating cells onto the laminin coating.

When the surface was ready to have cells plated onto it, the surface was removed from the fridge and warmed in an incubator. SC-hMN cells were then removed from liquid nitrogen storage and thawed, and then removed and placed in warmed neurobasal medium containing 4M NaCl. The cells were then spun down into a pellet and resuspended in hMN medium. The petri dish containing the laminin solution was then removed from the incubator and the laminin solution removed. The cells were then counted and plated in the laminin-coated petri dish.

Three days later, on the day of cell printing, the hMN media was removed from the petri dish and the surface rinsed with warmed phosphate-buffered saline (PBS). After the PBS sat on the surface for two minutes, the PBS was removed and accutase added for three to five minutes. When most of the cells detached, they were transferred into a conical tube. The cells were then spun down and re-suspended to create a cell density of approximately 5 million cells/ml. The specific cell density was recorded and the cells were ready for printing.

#### *3.6.1.2 Cardiac Cells*

A 6-well plate was obtained to culture the cardiomyocytes. A 10µg/ml solution of fibronectin was placed in each well and the plate was then incubated at 37°C for 30 minutes. The fibronectin was then aspirated and the wells rinsed with PBS. A vial of cardiomyocytes was removed from storage in liquid nitrogen and thawed, then added to thawing medium and centrifuged at 250g for five minutes. The cells were then resuspended to approximately 4-5 million cells /ml. The cells were then added to the fibronectin coated 6-well plate at a plating density of about 500,000 cells/well. After a week, the cells were disassociated using accutase and pelleted at 250g for five minutes. The cells were then re-suspended in cardiac medium at a cell density of approximately 5 million cells/ml. The specific cell density was recorded and the cells were ready for printing.

#### *3.6.1.3 Sensory Neuron Cells*

The first step was to coat a dish with Matrigel gelatanous matrix for cell adherence and growth using a 1:200 dilution in Dulbecco's Modified Eagle Medium (DMEM) for two hours. Human neural progenitor cells were thawed from liquid nitrogen storage, spun down at 300g for 5 minutes, resuspended and plated at 400 cells/mm$^2$ onto the Matrigel surface. The cells were fed every other day with human neural progenitor 1 (hNP1) growth medium until cells

reached confluency. The cells were then replated onto a polyornathine laminin coated surface prepared a few days in advance. The polyornathine laminin surface was prepared by adding 15 mg/ml of polyornithine and left to sit overnight at room temperature. The next day the surface was rinsed with PBS and then 1ug/ml of laminin and 10 ug/ml of fibronectin were added and left at room temperature for two hours to overnight. The laminin and fibronectin solution was then removed and ready for the cells to be immediately plated. Tripsin and tripsin inhibitor were used to remove the cells from the Matrigel surface and the cells were then plated onto the polyornathine laminin surface. The cells were fed every other day with hNP1 growth medium until confluency and differentiated using the specific media feeding protocol. Cells were fed every other day for ten days. Every feeding day, the media contained 500 ng/ml of Noggin and 10uM of SB431542, and a percentage of knock-out serum replacement (kSR) and N2B supplement that changed in concentration percentages every feeding day. The percentages of kSR and N2B supplements corresponding to each feeding day are listed below:

1. 100% kSR, 0% N2B
2. 75% kSR, 25% N2B
3. 50% kSR, 50% N2B
4. 25% kSR, 75% N2B
5. 0% kSR, 100% N2B

On day eleven, the cells were fed with hSN media. At that point, the cells were fed with hSN media every other day until harvested with trypsin for cell printing [8].

### 3.6.2 Cell Printer Program

The program to write the G-code for the printer is BioCAD. The main designs for the cell printer were drawn up in BioCAD, then special modifications were made to the G-code when minor changes were necessary.

### 3.6.3 Materials for Cell Printing

The materials for printing include the surfaces to be printed on, such as glass coverslips, cMEAs and cantilever chips, alignment printer plates to hold the surfaces in place, and all the necessary parts used in cell printing, including microvalves, screws, rubber O-rings, plastic adapters, and 10cc cartridges. The cell printer plates were designed in Autodesk Inventer Pro, and their fabrication is discussed in depth in section 3.5.

### 3.6.4 Printing Process

All materials were sterilized with 200 proof ethanol. When cardiac cells were to be printed, the surfaces were first coated with a 10µg/ml solution of fibronectin and then incubated at 37°C for 30 minutes. The fibronectin solution was then aspirated and the surfaces rinsed with PBS and allowed to dry.

A RegenHu 3D Discovery printer with an added printing chamber was used to print the cells, as seen in Figure 3-2 [9]. Surfaces to be printed on were placed on printer plates for alignment purposes. These plates also served as culture dishes for up to 24 hours after the print, and are shown in Figure 3-2C. The printing cartridges containing cell media or cell solution are stationed above the printer plate, shown as Figure 3-2A and Figure 3-2B. The cells were printed out of a microvalve using a jetting process. It is necessary to add enough cell suspension to the cell printer to ensure that the material reaches the print nozzle, about 125-150 µL. Settings such as microvalve opening time, dosing distance and air pressure were adjusted to find the ideal printing conditions. The microvalve opening time controls the time that the valve remains open when printing a spot. A larger microvalve opening time correlates to a larger dispensed volume. The air pressure controls the force that the liquid is transferred to the printing nozzle. A higher air pressure increases the amount of liquid that flows through the

printing nozzle. 0.25 bar is the air pressure used to print the cells. It is the lowest setting on the printer, and thus likely to cause the least shear stress on the cells.

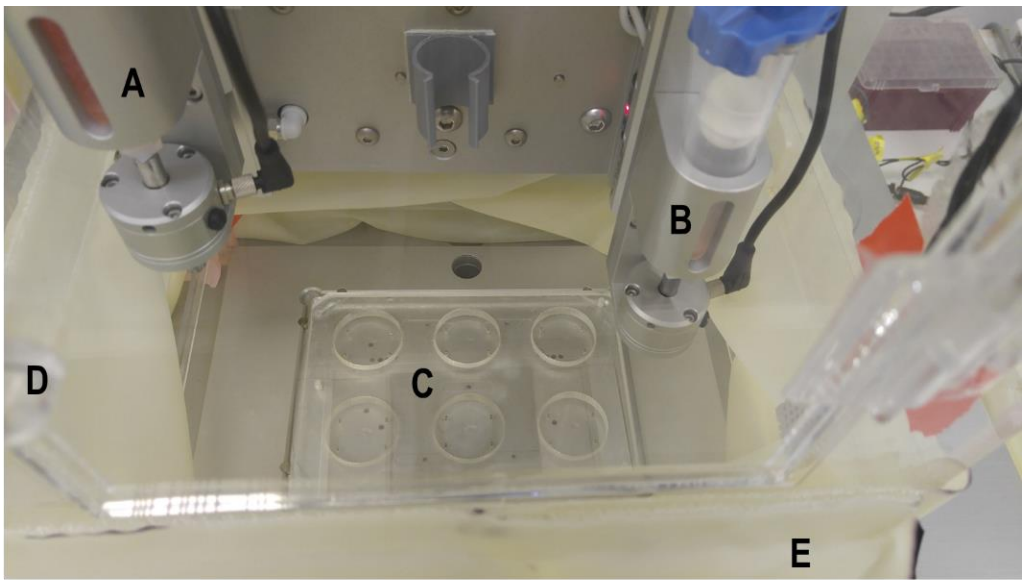



Figure 3-2: Image of printing chamber. A. Print cartridge holding media or first cell suspension. B. Print cartridge holding second cell suspension. C. Plate holding surfaces. D. Plastic encasement surrounding the print area to keep the environment sterile and humid. E. Latex material to complete the print area enclosure while allowing for movement of the stage and frame during printing.

Dosing distance defines the distance between spots in a line, therefore increasing the dosing distance decreases the amount of liquid printed in a line. The cell printer plates containing surfaces with printed cells were parafilmed and then placed in an incubator for half an hour. After half an hour, the necessary cell culture media was added to the wells and the plates were placed back into the incubator overnight. The surfaces were transferred the next day to 6 well cell culture plates and new media was added. Cells were maintained following the general cell culture protocol for the specific cell type.

## 3.7 Immunostaining

Immunostaining images were taken utilizing the Zeiss Axioskop 2 mot plus confocal microscope. The cells were fixed in place on the coverslip by adding PFA for 15 minutes, then removing the PFA and rinsing with PBS twice for 5 minutes each. The plates containing the coverslips were then wrapped in parafilm and placed in the fridge until they were immunostained.

For immunostaining, the cells were removed from the fridge and 0.1% Triton X-100 in a 1x PBS solution was added to the coverslips for 15 minutes to allow for the permeabilization of the cells. This permeabilization solution was then removed and 5% donkey serum with 1% BSA in PBS solution was added and let sit for an hour at room temperature. This is the blocking solution, or blocking buffer, used to prevent nonspecific binding of the secondary antibodies. The blocking buffer was then removed and the primary antibodies were added to the cells, and diluted in blocking buffer overnight at 4°C. The primary antibodies used for the hMN cells were mouse SMI32 (1:200 dilution) and rabbit MAP2 (1:400 dilution). The coverslips were then washed 3 times with PBS for 10 minutes each as a rinsing step. The secondary antibody was then added and let sit for 2 hours at room temperature in the dark. Diluted in blocking buffer, the secondary antibodies used for the hMN cells were a 1:250 dilution of donkey anti mouse fluorescing at 488 nm and a 1:250 dilution of donkey anti rabbit fluorescing at 568 nm. The secondary antibodies were then removed and the coverslips washed 3 times with PBS for 10 minutes each. The coverslips were then mounted to glass slides using Prolog Gold with DAPI mounting media and ready for imaging on the confocal microscope.

## 3.8 Patch Clamp

Patch clamp is the act of measuring single ionic currents in a cell to determine their function and degree of maturity. Patching of cells occurred on a Zeiss Axioscope 2 FS Plus upright microscope. The extracellular solution was neurobasal medium at 35°C with the pH adjusted to 7.3 using a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution. The intracellular solution contained K-gluconate, egtazic acid (EGTA), $MgCl_2$, $Na_2ATP$, phosphocreatine, phosphocreatine kinase, and was adjusted with HEPES to a pH of 7.2. Three mL of the extracellular solution was used and changed every ten minutes. A Multiclamp 700A amplifier was used to take voltage clamp and current clamp measurements, with an electrode resistance of 6 to 8 MΩ. Cells with an access resistance less than 22MΩ were analyzed [10].

## 3.9 cMEA hMN Recording

cMEAs were assembled into systems that allowed for recording analysis, as seen in Figure 3-3. The system to be recorded was removed from the incubator and transferred to the recording rig. The recording software used to play and record the electrical signals was MC_Rack from Multichannel Systems. Once properly attached to the recording instrumentation, the electrodes started responding and indicated spontaneous activity of the hMNs. The spontaneous electrode firing was recorded for five to ten minutes. Once the recording had been completed, the software was stopped, and the system was removed from the rig and placed back in the incubator.

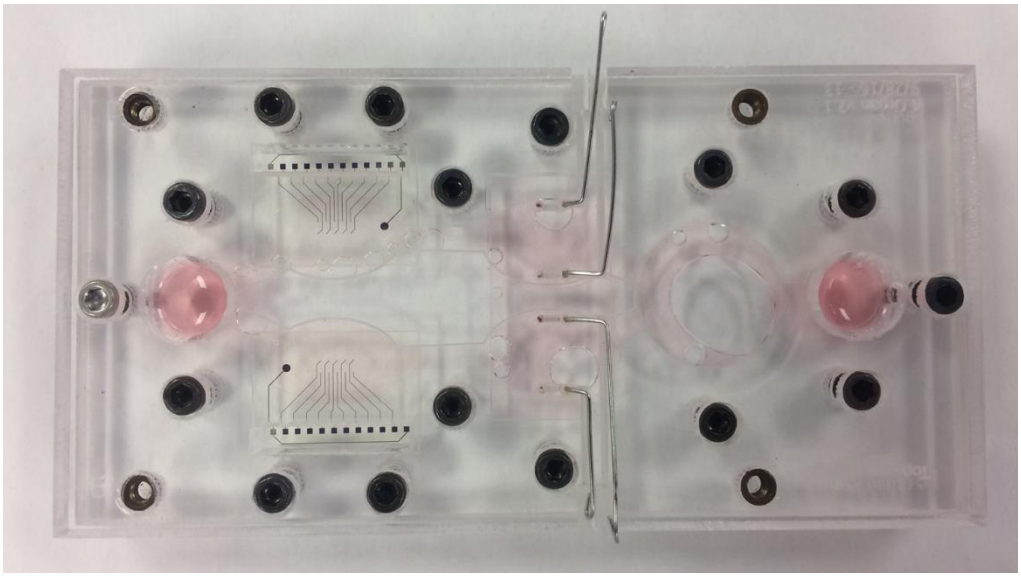


Figure 3-3: Image of assembled system containing two cMEAs.

### 3.10 cMEA Cardiac Recording

The system to be recorded was removed from the incubator and transferred to the recording rig. The recording software used to play and record the electrical signals was MC_Rack. Once properly attached to the recording rig, the electrodes should start responding and show spontaneous activity. The software MC-Stimulus was then opened to operate the stimulation of the cells. The usual beat frequency was 1 Hz starting at -800mV and stepping up until it was too fast for the cells. Once the stimulated recording was completed, the software was stopped, and the system was removed from the rig and placed back in the incubator.

# CHAPTER 4 ADAPTATIONS OF THE REGENHU 3D BIOPRINTER

## 4.1 General Introduction

Some modifications were necessary before the regenHU 3D printer was ready to print cells, such as the addition of the encasement material to ensure a sterile and properly humidified environment. Alignment methods were developed to ensure accurate placement of cells, chemical modification of the surfaces was performed to ensure the cells remained in the desired location on the chip and remained healthy, and proper pressure settings to ensure the shear stress on the cells was not too high during the print were taken into consideration.

## 4.2 Printer Modifications

In order to ensure a sterile, humid environment, the printheads containing the cells and the surfaces printed on were separated from the outside lab environment. Figure 4-1 shows the addition of an acrylic cube encasing the printhead area. Also shown is a latex skirt that connects the sample stage and print cartridge holder, allowing them to freely move both horizontally and vertically during printing. In order for the printing chamber to maintain the humidity required by the cells, a humidifier was attached via a hose, and an Arduino board was attached with a sensor displaying the temperature and humidity inside of the chamber.

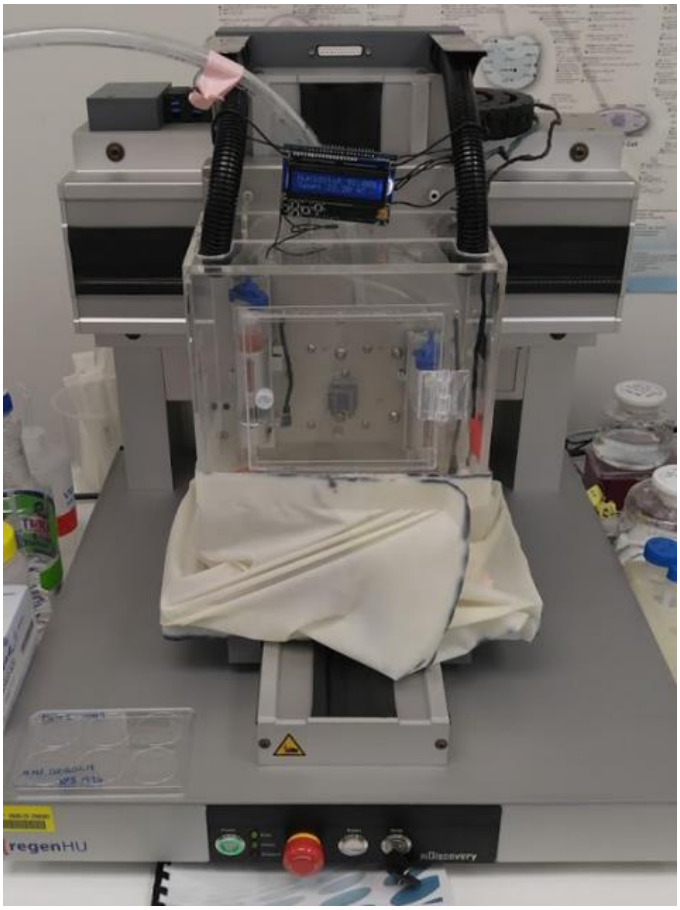



Figure 4-1: Image of printer with latex skirt and acrylic housing piece.

## 4.3 Printer Plate Alignments

Figure 4-2 shows the different printer plate models designed over the course of this project. A printer plate refers to the acrylic plate that holds either the cMEA or coverslip surface to be printed on. The purpose of the plate is to act as an alignment tool and a sterile humid environment for the first twelve to twenty-four hours after printing. The first design of the plates included a six well plate that holds six 18 mm diameter glass coverslips (Figure 4-2A). It was difficult to remove the coverslips from the printer plate with this design, so (Figure 4-2B) was designed with pins for alignment and a larger well that allowed for the tweezers to more

easily grab the glass coverslip. The next design was a six well plate that holds three cMEAs and three 18 mm diameter glass coverslips (Figure 4-2C). This design worked well when printing cMEAs and coverslips in the same print run was desired. This layout was less ideal when the demand for printing cMEAs increased. It was also necessary to wait ten to fifteen minutes after each print, with the plate sitting in the sterile and humid printer environment, for the cells to adhere to the surface before media was added to the cMEAs. To avoid undesired wait time, a new plate was designed (Figure 4-2D) that included a water reservoir between the wells, and a raised platform for the cMEA to sit on, creating a media reservoir in the well below the chip. Media was placed into this reservoir, while keeping the top surface of the chip dry, and water was added to the water reservoir. The surface was then printed on, and a lid was added while still in the humid printer environment. Parafilm was then added and the plate was placed in the cell incubator for twenty minutes. The printer was then ready for another plate to be printed during this twenty-minute wait time. After the twenty-minute cell settling time, the printer plate was removed from the incubator and the required amount of media was added to cover the cMEA.

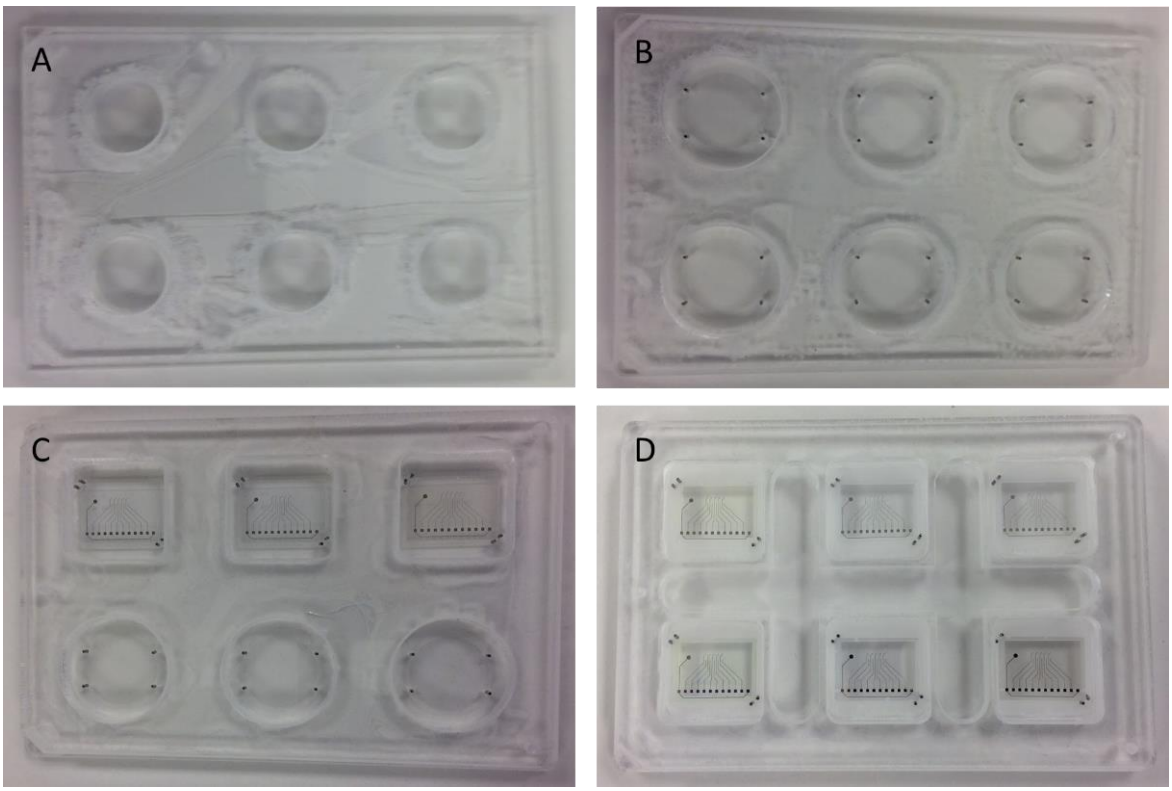



Figure 4-2: Different cell printer plates. A. Initial and B. final design used to hold six 18mm diameter glass coverslips. C. Design of plate to hold three cMEAs and three 18mm diameter glass coverslips. D. Humidity plate designed to hold six cMEAs.

## 4.4 Pattern on cMEA chips

To encourage the cells to grow in a desired location, different patterns were created on the surface of the substrate. The two organosilanes used to modify the surface were $N^1$-(3-Trimethoxysilylpropyl)diethylenetriamine (DETA) and 2-[Methoxy(Polyethyleneoxy)$_{6-9}$Propyl]trimethoxysilane (PEG), shown in Figure 4-3 [9]. DETA acted as a cytophilic surface while PEG acted as a cytophobic surface [10]. Their different patterns on the cMEA is shown in Figure 4-4. Figure 4-4A is coated with DETA over the entire surface of the chip. Once cells were placed on this chip's surface, they were free to migrate. In order to control cell migration, surface patterns shown in Figure 4-4B, C and D were used. Figure 4-4B consists of DETA

circles 500 µm in diameter centered over the electrodes, and works well for generating patterns ideal for single cell printing to gain spontaneous cell recordings. Figure 4-4C and D represent the "feed forward patterns." The goal of these patterns is to control the growth of one population of cells towards innervation into a second population of cells. All electrodes have the 500 µm diameter circle centered over the electrodes, with 5 µm width lines between the electrodes. The cells, whose axons are desired to grow along the lines and innervate cells on the other electrode, are deposited on the electrode that connects to the lines. The cells to be innervated are deposited on the electrode that has a gap between the lines and the electrode pattern. This gap is to discourage the growth of the cells axons, since these cells are to be innervated. Two different directions of line patterns were designed, a "top to bottom" pattern, Figure 4-4C, and a "side to side" pattern, Figure 4-4D. Figure 4-4E and Figure 4-4F are a magnified view of Figure 4-4C and Figure 4-4D, respectively.

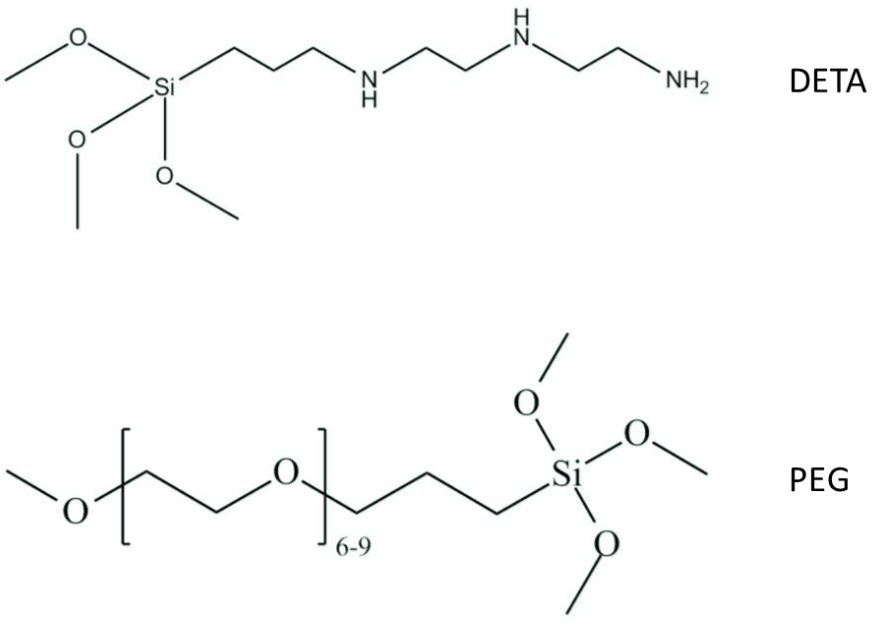



Figure 4-3: Molecular structure for DETA [10] and PEG [11].

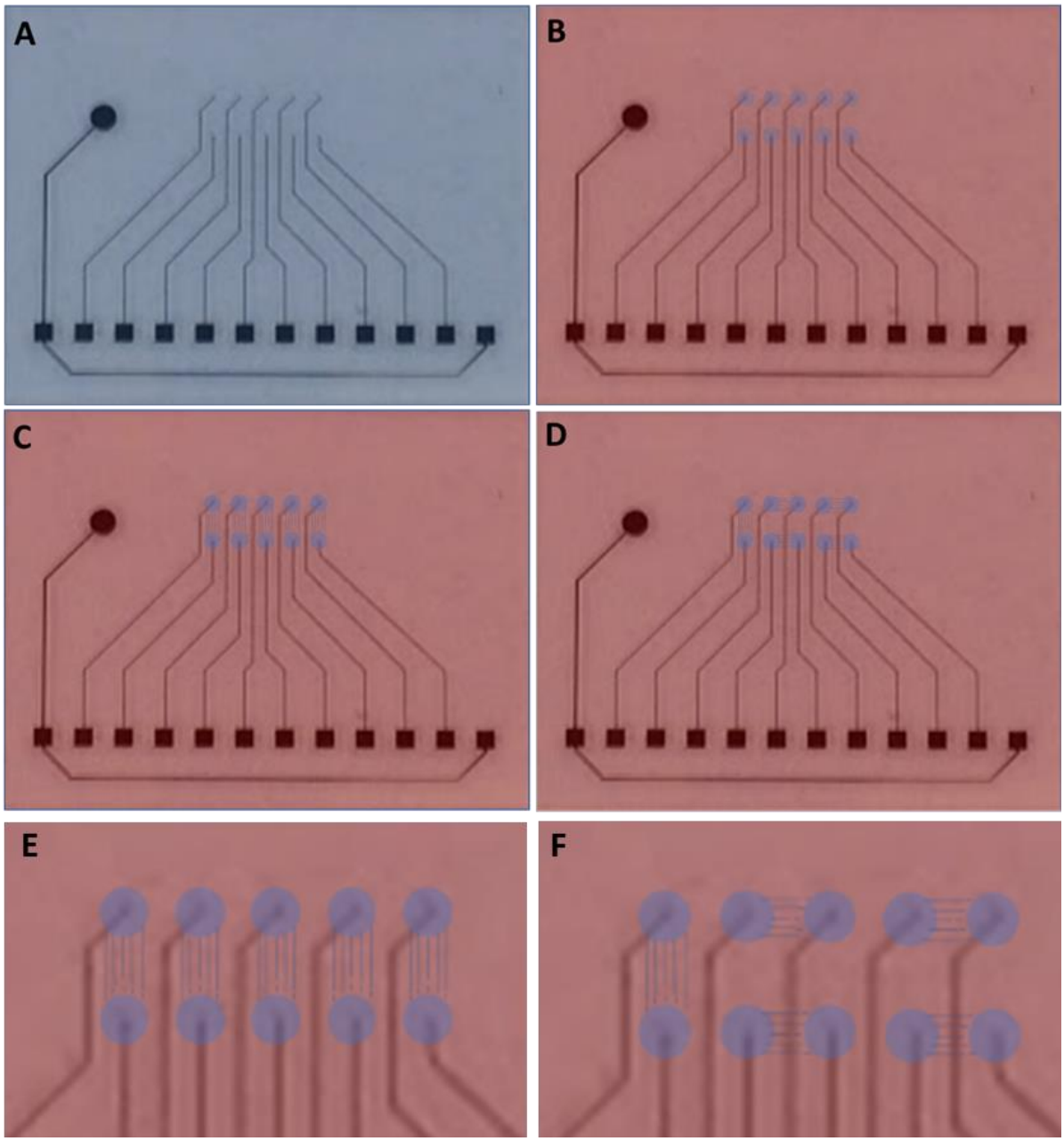



Figure 4-4: Four surface patterning designs for cMEAs. Blue represents DETA while red represents PEG surface modifications. A. Blanket DETA coated. B. 500 µm diameter circles over electrodes. C and D. Feed forward pattern with 500 µm diameter circles over electrodes. C. All top to bottom pattern. D. Side to side pattern with one top to bottom pattern. E. Zoomed in view of image C electrodes. F. zoomed in view of image D electrodes.

## 4.5 Microvalve Calibration

In order to determine the amount of liquid coming out of the nozzle, an experiment was performed where spots of water were printed using different spot sizes, and the mass of the different spots was measured. A calibration curve was obtained and is shown in Figure 4-5.

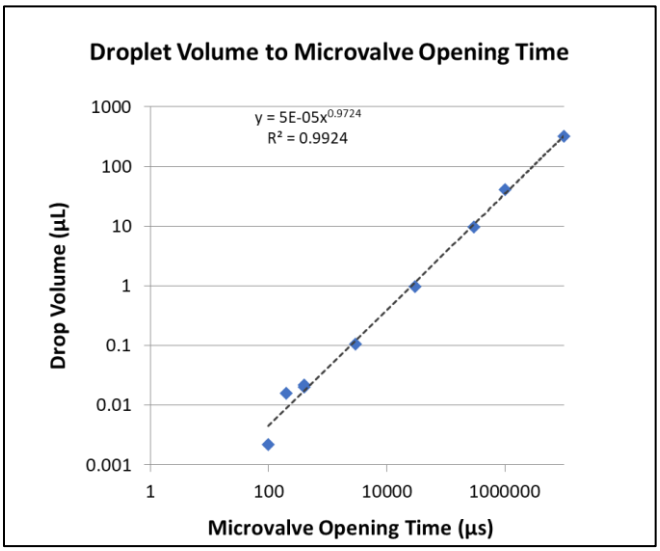



Figure 4-5: Calibration curve for droplet size with respect to microvalve opening time in µs.

## 4.6 Shear Stress on Cells

To reduce the shear stress that the cells experience, the cells were printed at the lowest measurable pressure of 0.25 bar.

## 4.7 Summary

From these preliminary experiments and optimization modifications, the general procedure for printing cells was produced. Cells were printed at a humidity of 90-99% and a pressure of 0.25 bar, unless otherwise stated.

# CHAPTER 5 GOAL 1: PRINTING SINGLE CELL TYPES ONTO BLANKET DETA AND PATTERNED DETA SURFACES - SCSC DERIVED HMN

## 5.1 Introduction

This chapter reveals one of the main purposes for cell printing, placing cells in precise cytophilic locations on surfaces. The current methods for plating cells in the HSL are hand plating and printing cells. The advantages of printing, as discussed in the introduction, include precise cellular placement allowing for fewer cells used per surface and the possibility of higher throughput via automation. The purpose of goal one was to show that printing a single cell type, spinal cord stem cell (SCSC) hMNs, onto glass substrates modified by covalently bonding DETA to the surface, maintain morphological and physiological characteristics similar to cells placed on the same type of surface by hand plating techniques.

The surfaces used in this experiment were glass cMEA chips and glass coverslips. The surface was either blanket coated with DETA or had DETA-filled circles in desired locations on the chip with a PEG background. The methods section covers the process of the chemical modification of the surface coating procedures in detail.

Once the cells were printed, the functionality and viability of the printed cells were compared with hand plated controls and analyzed using the following metrics: morphology, immunocytochemistry, electrical functionality recordings and patch clamp electrophysiology.

## 5.2 SCSC hMN Cells

The hMN cells were obtained from the spinal cord of human fetal tissue without any identifying markers and were fully differentiated from SCSCs to hMNs before freezing down in liquid nitrogen for storage [12]. Three to four days before printing, the cells were removed

from liquid nitrogen and plated on a petri dish to recover from the thawing process. On the day of printing, the cells were disassociated and added to the printer at a cell density between 5 to 7 million cells/ml. Section 3.6.1.1 in the methods chapter covers this process in more detail.

## 5.3 Printing hMN Cells vs. Hand Plating hMN Cells

Due to inherent differences between printing and hand plating that will be described in the following subsections, the cell density necessary for successful printing is significantly higher than the cell density for hand plating. The procedures and specific requirements are discussed in the following subsections.

### 5.3.1 Printing hMN Cells

The optimal cell density for printing is approximately 5 to 7 million cells per ml to obtain cell spots with enough cells to generate the desired patterns for functional electrical measurement recordings [14]. As shown in Figure 5-1 and Figure 5-2, cells can be placed in precise locations by the cell printer to form the desired cell containing patterns, such as spots or lines, and remain in that location for at least a few days.


Figure 5-1: Phase microscope images of hMN cells printed on DETA coated cMEAs the day of print showing good printer alignment at 25x magnification.


Figure 5-2: Phase microscope images of hMN cells printed on DETA coated cMEAs one day after printing. The top row of electrodes was printed using a spot pattern, while the bottom row of electrodes was printed using a line pattern. The image is at 25x magnification.

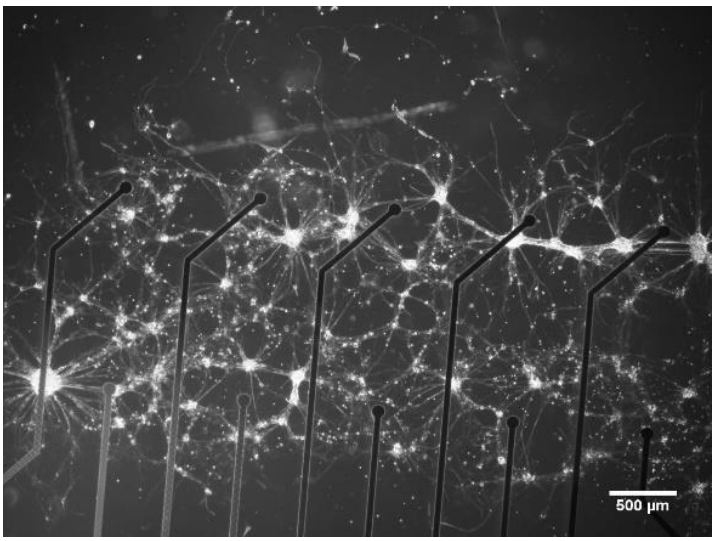



Figure 5-3: Phase microscope images of hMN cells printed on DETA coated cMEAs twenty-two days after printing indicating cell movement across the surface, away from their initial placement. The image is at 25x magnification.

Printing cells in precisely the correct location is important, but it is also imperative that they stay where they were placed. As seen in Figure 5-3, over time cells will move around on the surface of the chip. To prevent such movement, surfaces were printed on that contained not only the hydrophilic DETA coating, but also the PEG coating in locations cells were not wanted. Without the repulsive cytophobic PEG surface, cells would migrate freely, usually away from the electrodes. Printing the cells onto the DETA-coated electrodes, surrounded by the PEG coating, created a surface that maintained the desired pattern that was initially plated. This was shown over the course of sixteen days in Figure 5-4.

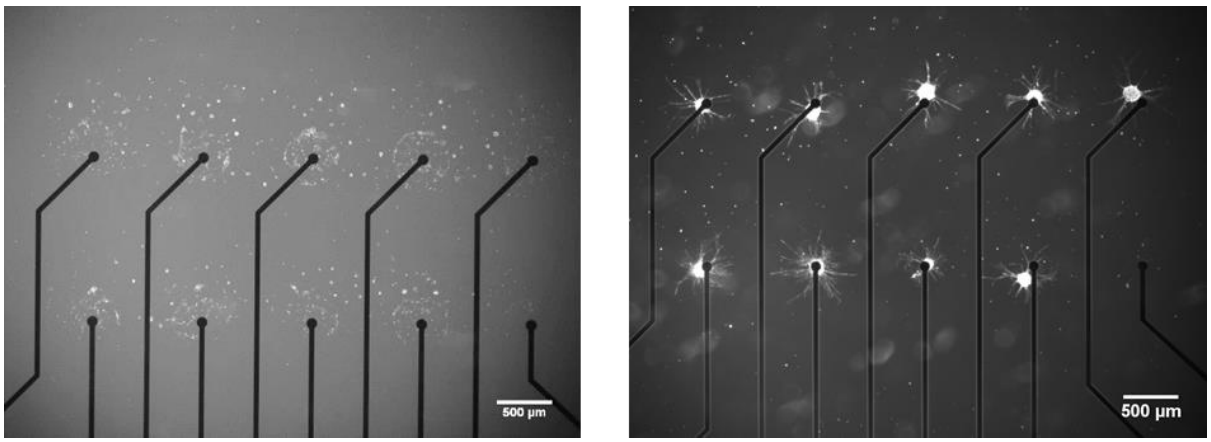



Figure 5-4: Images of hMNs printed onto a cMEA with 500 µm diameter DETA filled circles around the electrodes on a background of PEG coating. The left image was taken the day after print and the right image was taken sixteen days after print.

As indicated in Figure 5-2, cell spot size uniformity was not consistent throughout the print. Since one goal of printing cells was to create uniformity between all printed cMEAs in a given printing session, this variance was not preferred. Experiments were performed with OptiPrep, a density gradient medium usually used for cell isolation [15], to decrease the effects of cell settling during printing, which was the predicted cause of the variability in the cell density of printed spots throughout the experiment. An experiment was performed where an array of spots was printed on coverslips, and the number of cells in the first three printed spots and the last three printed spots were counted by hand using images from a phase microscope. This experiment was performed for three concentrations of OptiPrep, 0%, 5% and 10%, added to the cell solution, and two droplet volume sizes, 8 nL and 20 nL. Figure 5-5 plots the average standard deviation of cellular uniformity for the described experiment. Each dot in the graph represents the average standard deviation for the corresponding drop volume at the given OptiPrep concentration, with error bars representing the standard error of the mean for each data point. No error bars are indicated for the 8 nL droplet in the 5% OptiPrep dataset due to

an n of one coverslip. It was concluded that adding 5% OptiPrep to the cell solution provided the optimum increase in the density gradient of the media, allowing the cells to remain evenly dispersed throughout the duration of the print. When 0% OptiPrep was added to the cell solution, printing at the larger volume of 20 nL gave a very large standard deviation of cell uniformity. This suggested a large amount of cell settling occurred in the printer cartridge. At the higher percentage of OptiPrep, the larger drop volume also gave a higher standard deviation than the 5% OptiPrep. This suggested that the density of the print media became too high, again preventing the cells from being evenly dispersed throughout the media. After printing with 5% OptiPrep in the cell solution, Figure 5-6 shows improved uniformity compared to Figure 5-2. Both Figure 5-2 and Figure 5-6 cMEAs were patterned with the same mask design, 500 μm diameter DETA filled circles around the electrodes on a background of PEG coating.

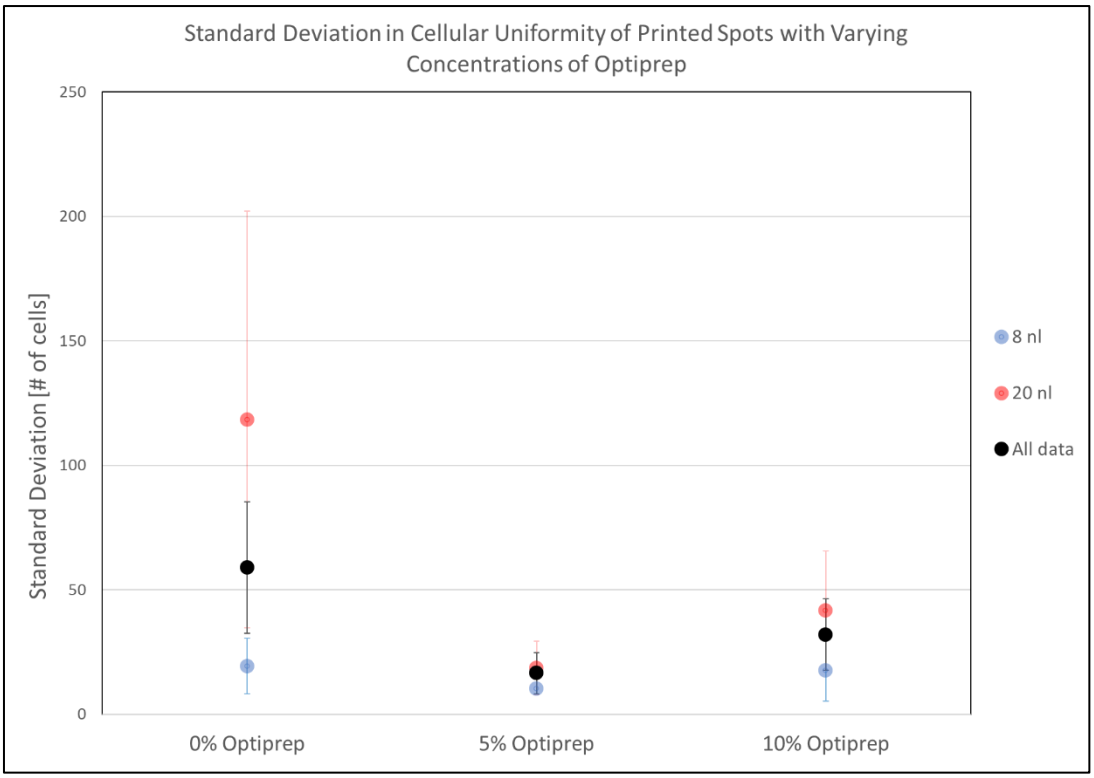



Figure 5-5: OptiPrep measurements testing the uniformity of cell spots from beginning to end on the coverslip print with respect to percentage of OptiPrep in cell solution. All data have multiple n's except the 8 nL point for 5% OptiPrep, resulting in no error bars for that data.

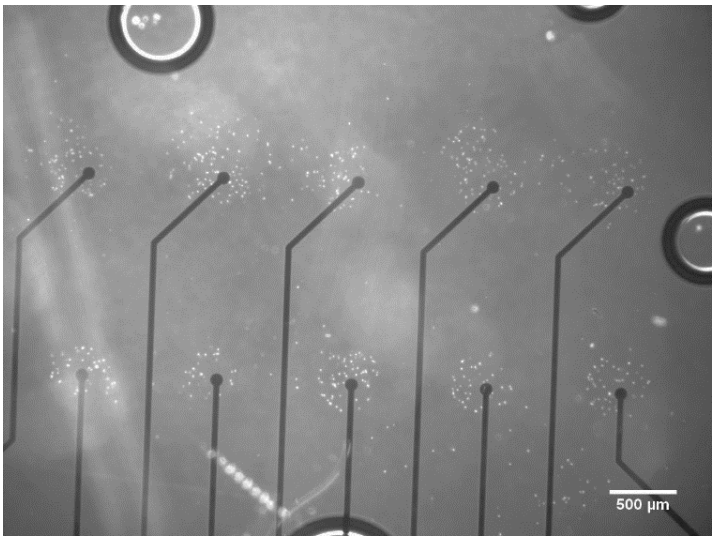



Figure 5-6: Day after print image of hMNs printed onto a cMEA with 500 µm diameter DETA filled circles around the electrodes on a background of PEG coating. Printed cell solution contained 5% Optiprep for uniformity.

### 5.3.2 Hand Plating hMN Cells

The typical hand plating procedure included thawing an aliquot of cells from liquid nitrogen and plating the cells at a surface density of 200 cells/mm$^2$ onto a 50mm$^2$ area of the cMEA surface that completely encompassed the electrodes. The density of the cell solution was 100,000 cells/ml. The surface pattern emerged over time as the cells migrated to the cytophilic surfaces around the electrodes. The hand plating controls performed during a cell print followed a different procedure. The technique described in section 5.2 was used to prep the cells. Before the cells were added to the printer, some of them were used for the hand plate controls by pipetting droplets of cells onto a cMEA or coverslip surface. Therefore, the density of the cells added to the printer was the same density as the controls that were hand plated, which was approximately 5 million cells per ml, significantly higher than the typical hand plating density. The volume deposited on the surface was larger than the printed volume due to the smallest pipettor volume available in the lab, 1 µL.

## 5.4 Morphology Comparisons Between Printed and Hand Plated hMNs

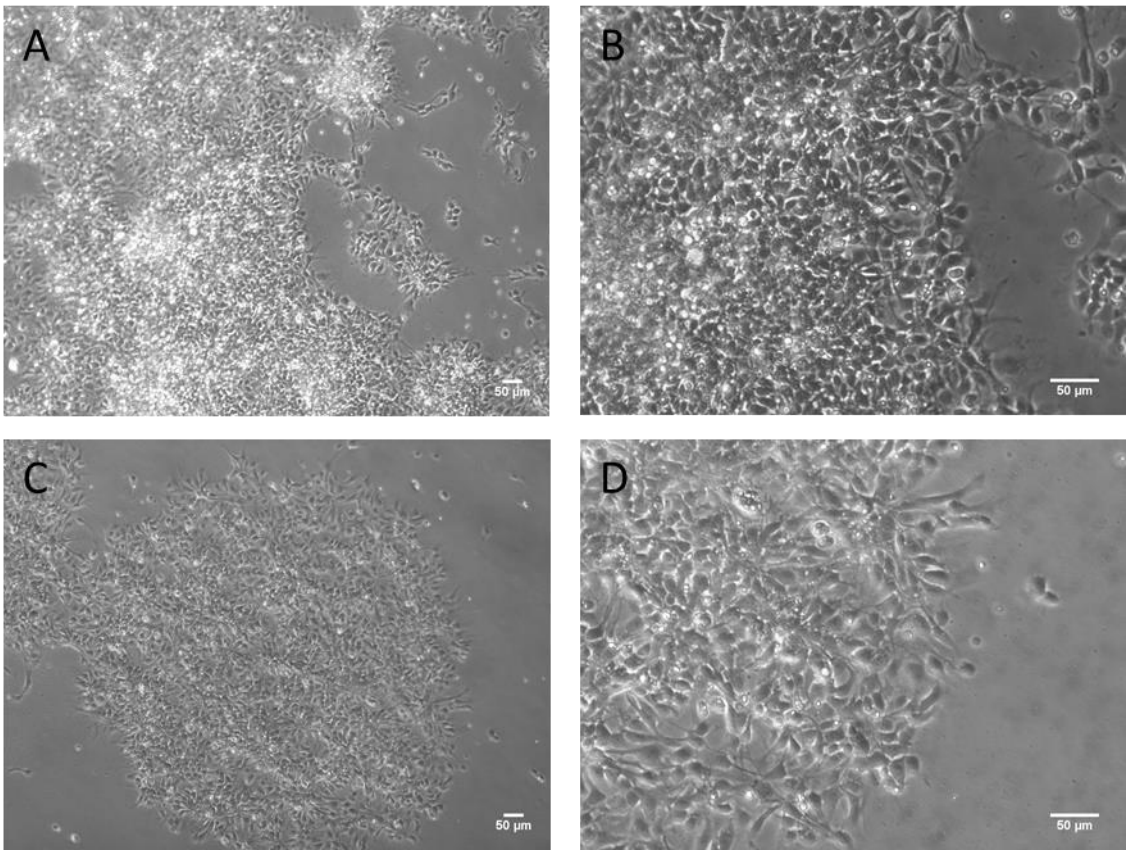



Figure 5-7: Images containing printed and hand plated cells at two different magnifications. A. Hand plated 100x. B. Hand plated 250x. C. Printed 100x. D. Printed 250x.

As seen in Figure 5-7, there are no morphological differences between the hand plated and printed cells. Both sets of cells are very similar looking the day after plating.

## 5.5 Immunostaining Comparisons Between Printed and Hand Plated hMNs

Figure 5-8 shows stained cells at specific timepoints after printing. Immunostaining was performed 1, 3, 7, 10, 20 and 28 days after printing. The markers used were SMI32, Map2 and Dapi. SMI32 is a specific immunostaining marker for motoneurons that indicates the maturity of the cells, and is noted in green in the figure. The axons increased in length over

time, indicative of growing, maturing neurons. Map2 is a general neuron marker. The soma and dendrites of the neuron, as well as some axons, are red in the figure. Taken together, the presence of these molecules justifies the conclusion that the printed neurons matured over the course of twenty-eight days in vitro. The last marker, Dapi, stains the nucleus of cells, and helps to discern the number of cells present and their location on the surface. As indicated Figure 5-8, after day seven, the cells started to migrate towards each other to form groups of cells. The first image for each of the days is a superimposed image of the three stained images.

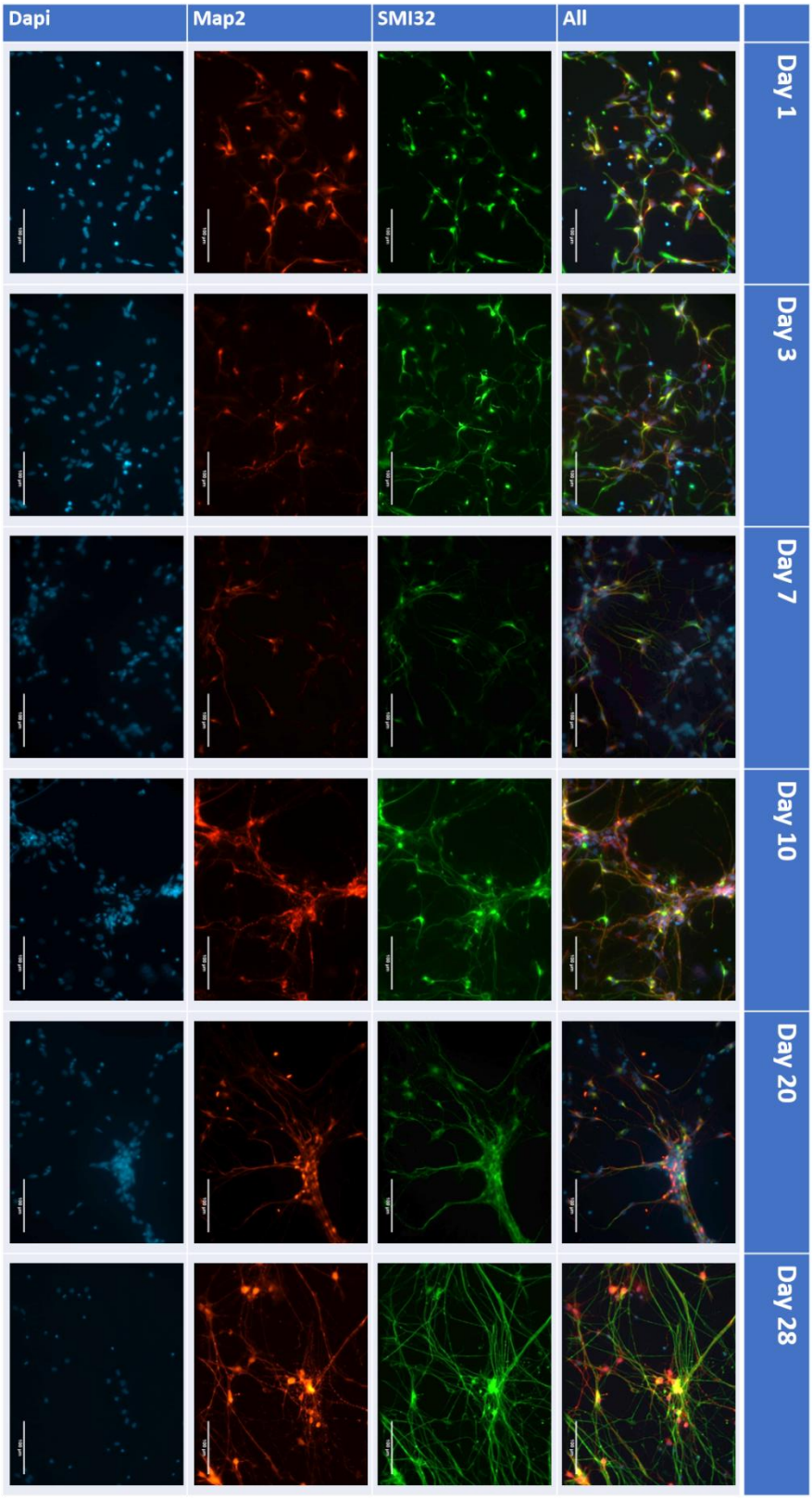



Figure 5-8: Immunostaining images containing SMI32, Map2 and Dapi markers of hMNs stained at 1, 3, 7, 10, 20 and 28 days after printing.

## 5.6 Functional Comparisons Between Printed and Hand Plated hMNs

Printed surfaces were assembled in acrylic systems seven days after printing, while hand-plated cMEAs were assembled four days after plating. Spontaneous electrical cellular activity was recorded over a period of twenty-eight days after system assembly, indicating functional neuronal maturation [12]. To make the printing and hand plating data more comparable, the averaged weekly plots shown in Figure 5-9 only contain data for the first four weeks, the day 65 and 66 printing data was not included in the figure. The Methods section contains the procedure followed for recording the cells in the microfluidic four organ systems. Plots of the percent of active electrodes, amplitude of the spikes and spike rate are shown in Figure 5-9 for systems containing printed and hand plated cells. The phase images of each cMEA on each day of recording is shown in the appendix, along with individual plots for the three sets of data for each cMEA over the recording period. The printed cMEAs took a few more days to have a higher percentage of active electrodes, but after week two, the printed cMEAs had a higher percentage of active electrodes. The amplitude of the printed cells was higher than the amplitude of the signals for the hand plated cells, as seen in Figure 5-9 B. The spike rate for the first three weeks of the hand plated cMEAs was higher than the printed spike rate. The spike rates either increased or remained the same for the printed cells, and decreased over time for the hand plated cells.

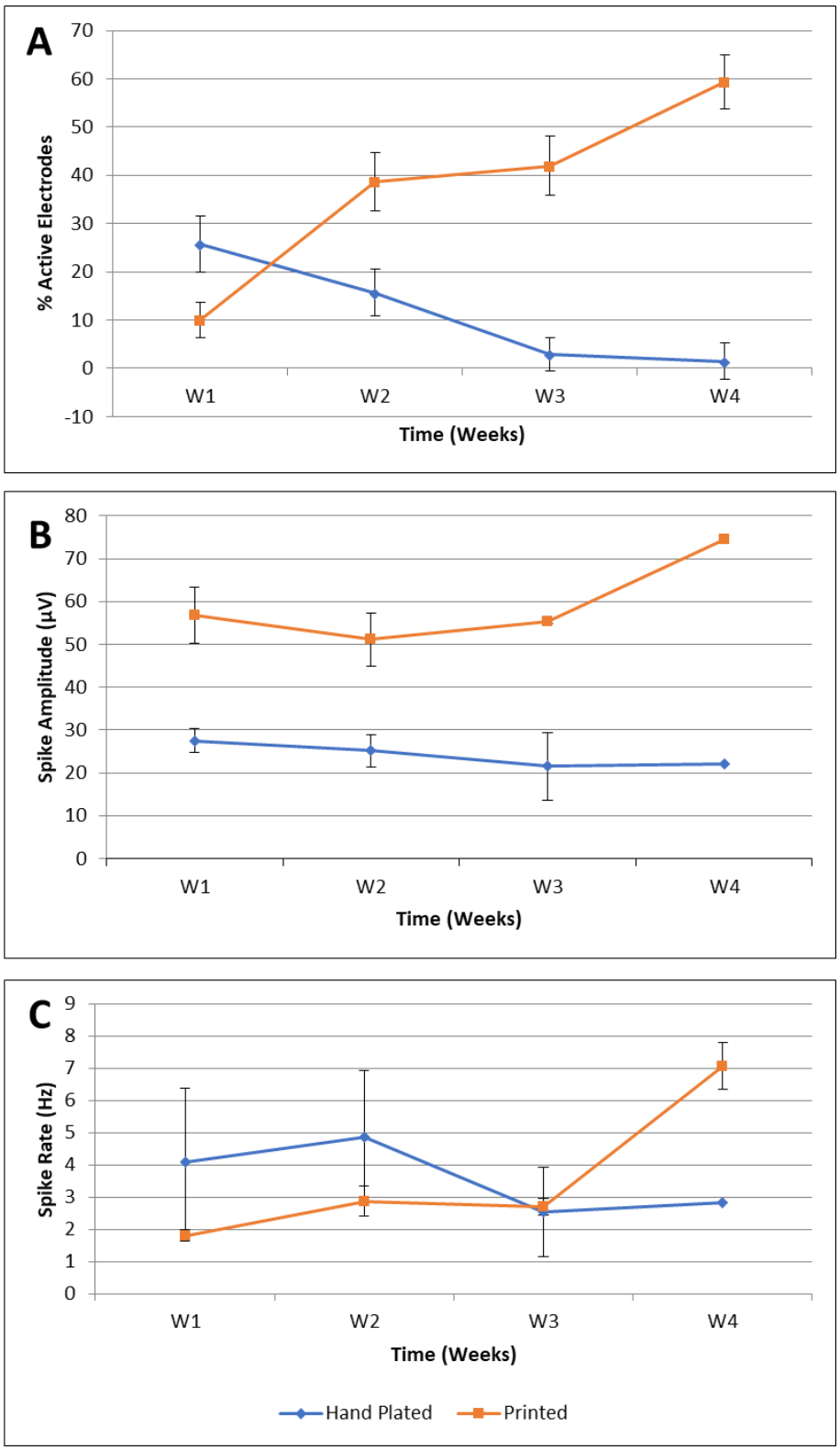



Figure 5-9: Recorded cMEAs in systems, blue diamonds represent hand plated system data while orange squares represent printed data. A. Percent of active electrodes. B. Spike amplitude C. Spike rate.

## 5.7 Patch Clamp Comparison Between Printed and Hand Plated hMNs

The results of the non-invasive recordings over twenty-eight days were supported by data previously obtained from patch clamp, as shown in Figure 5-10, confirming good cell viability and functionality [13].

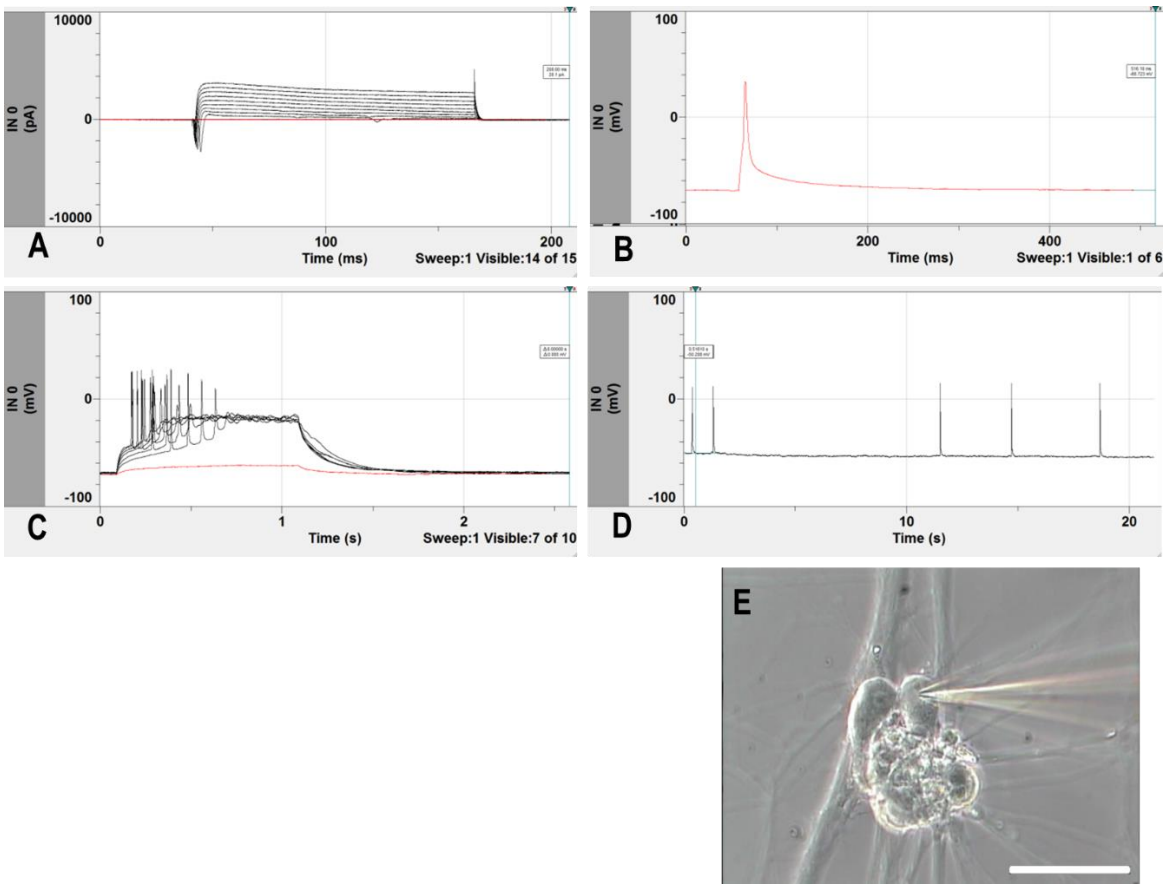



Figure 5-10: Patch clamp data for printed hMN cells on DETA coated glass coverslip twenty-eight days after print. A. Voltage clamp showing inward and outward current through cells. B. Single action potential from cell. C. Current clamp showing repetitive firing. D. Spontaneous activity of cell. E. Image of patching single hMN cell, scale bar 25 µm.

# CHAPTER 6 GOAL 2: PRINTING SINGLE CELL TYPES ONTO PATTERNED DETA SURFACES- CARDIOMYOCYTES

## 6.1 Introduction

The purpose of this chapter is to show printing of the cardiac cell type. Plating these cells with the cell printer is desired due to the extremely high cost of cardiomyocytes. With cell printing it is possible to fabricate more cardiac plated surfaces with the same amount of cells compared to hand plating.

Once the cells were printed, the functionality and viability of the printed cells were compared with hand plated controls and analyzed using morphological phase images and electrical functionality recordings.

## 6.2 Cardiac Cells

The type of human cardiac muscle used was obtained from Cellular Dynamics International (CDI), and is a highly purified iPSC derived cardiomyocyte line. A vial of cardiac cells contains about four million cells and costs $1,000 per vial. The procedure for prepping the cells prior to printing is covered in section 3.6.1.2.

## 6.3 Details of printing vs. hand plating

This section covers the technique differences between printing cardiac cells and hand plating cardiac cells. Also shown are the cost saving calculations when printing vs hand plating.

### 6.3.1 Printing Cardiac Cells

The process of printing cardiac cells involves the procedure outlined in section 3.6.1.2 to obtain the cells. The recommended printing density for cardiac cells is 5 million cells per

ml. After the cells were added to the printer, the printing process outlined in section 3.6.4 was followed. The ideal cost savings are shown below in Table 6-1, assuming complete cell survivability during the entire hand plating and printing process.

Table 6-1: Savings comparison between printing and hand plating cardiac cells

| Metric | Printing | Hand Plating |
|---|---|---|
| Cells used per cMEA surface | 6,000 | 50,000 |
| Surfaces plated per vial | 666 | 80 |
| Cost per chip | $1.50 | $12.50 |
| Cost to plate 100 surfaces | $150 | $1250 |

### 6.3.2 Hand Plating Cardiac Cells

Obtaining the cells for hand plating is described in section 3.6.1.2. The process of hand plating cells onto cMEAs involves PDMS barriers that create a small well around the electrodes, causing the cell solution to remain over the electrode region. Each hand plated cMEA surface utilized 50,000 cardiac cells. The procedure for hand plating the controls for the cell printer was to take the cells collected for printing and pipette 1 µL droplets onto fibronectin coated DETA coverslips. cMEA controls were also created by pipetting a few 1 µL droplets onto the PEG modified, DETA backfilled fibronectin coated cMEA surfaces in the U shape pattern. Immediately after hand plating, the surfaces were filled with media and placed into the incubator.

The benefit to printing cardiac cells instead of hand plating is seen in Table 6-1. Printing used significantly fewer cells per surface, allowing for the patterning of more printed cMEAs

than hand plated cMEAs. Even though 100% cell survivability is not experimentally obtainable, printing can be 13% effective and still be the preferred approach when only considering the cost of cells. At 13% efficiency, 86 surfaces can be obtained, which is 6 more surfaces than hand plating can produce. When personnel time and cost of other supplies are considered, the printing method must be more than 13% effective to be the preferred method for plating the cells.

## 6.4 Morphological Comparison

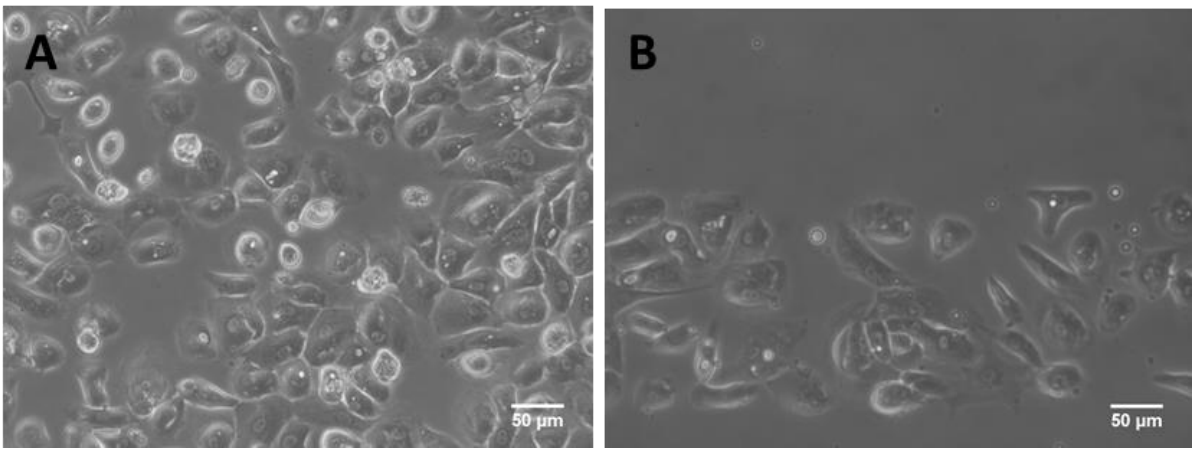



Figure 6-1: Cardiac cells one day after plating at 2,500x. A. Hand plate. B. Printed line.

As seen in Figure 6-1, the morphology of the cardiac cells the day after plating was similar, showing that printing cardiac cells does not harm them morphologically. Figure 6-2 and Figure 6-3 show the assembled printed and hand plated cMEAs, respectively. Both cMEAs have beating cells in a U pattern. Since more cells were used in the hand plating control, there are no breaks in the U pattern as noted in Figure 6-3. Even though the U pattern was not complete in Figure 6-2, there were still a few connected electrodes to show proof of principle for using the printer to print cardiac cells and allowed for the functional electrical

measurements to be tested in the next section. Fine-tuning the printing parameters in future experiments should overcome the breaks in the printed cMEA, leading to desired density of cells in the U pattern.

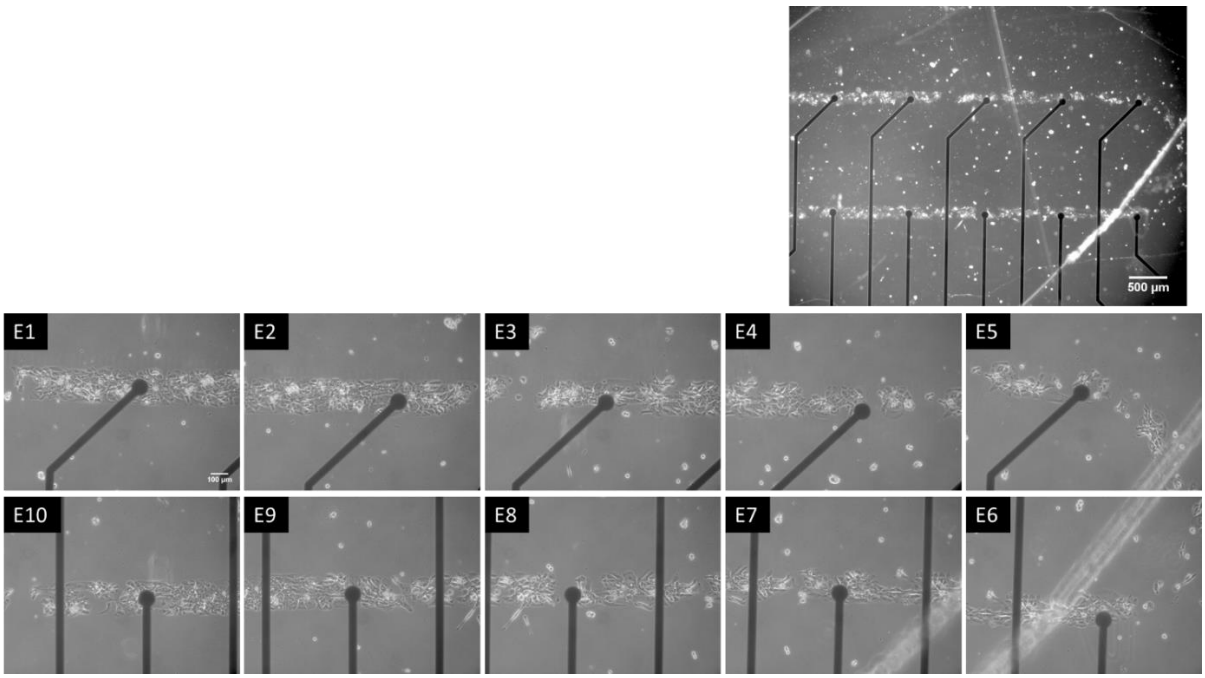



Figure 6-2: Image of assembled printed cMEA.

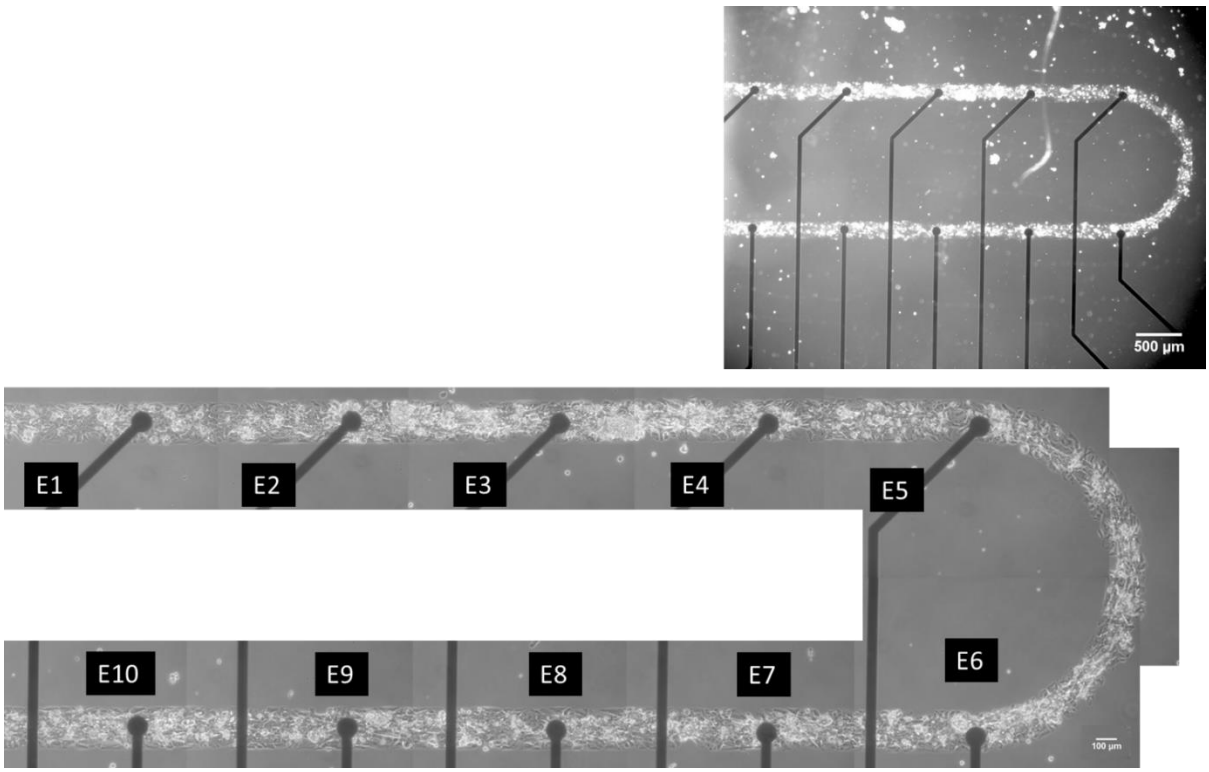



Figure 6-3: Image of assembled hand plated cMEA.

## 6.5 Recording Data

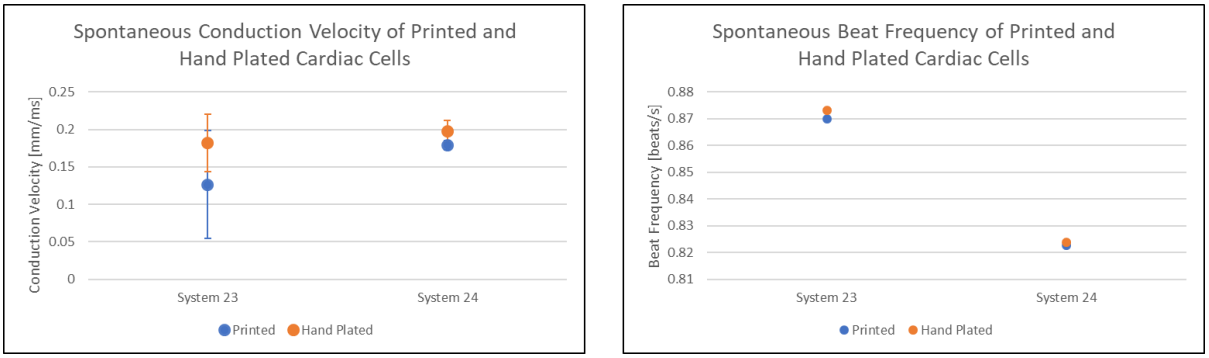



Figure 6-4: Plots comparing spontaneous conduction velocity and beat frequency for printed and hand plated cardiac cells. The standard deviation of the averaged conduction velocity values provided the error bars for the plot of conduction velocities. There was not enough variation in the beat frequency data for visible error bars.

Systems containing both the printed and hand plated cardiac patterned cMEAs, including those shown in Figure 6-2 and Figure 6-3, were assembled and recorded for spontaneous signals. The conduction velocity and beat frequency were also obtained for both systems. Conduction velocity was found by taking the distance between the electrodes connected with a continuous path of cardiac cells and dividing it by the time it took for an electrical signal to travel between the electrodes [15]. The error bars were calculated by taking the standard deviation of the averaged conduction velocities for each cMEA. The fact that the error bars for the printed and hand plated cMEAs in both systems overlapped shows the comparability of function for printed and hand plated cardiac cells. The beat frequency was obtained by counting the number of spontaneous beats that appeared within a known timeframe on a single electrode to obtain beats per second. The beat frequency variation within each cMEA was so miniscule that the error bars were not visible. The difference between hand plated and printed for each system was very small, reiterating that cardiac cells were not negatively affected by printing. The difference in the beat frequency between systems could be attributed to many variables in the lab, such as the temperature of the system before and during recording, and therefore should not be considered significant.

# CHAPTER 7 GOAL 3: PRINTING TWO DIFFERENT CELL TYPES SHOWING INTENDED INNERVATION-HMN AND HSN

## 7.1 Introduction

It has been successfully shown that a single cell type can be placed onto a cMEA surface using a cell printer to achieve similar or better results compared to plating the cells by hand. Now two different populations of cells were printed onto the same cMEA surface near each other. As shown in this chapter, the resolution necessary to place different populations of cells onto electrodes right next to each other, without undesired mixing, cannot be achieved via hand plating. This is where the cell printer excels and performs functions that hand plating cannot achieve.

This chapter will first discuss a proof of concept procedure where two populations of SCSC hMN cells were printed to show that the resolution of the printer was capable for the desired task of printing two different cell types on one cMEA surface. After it was shown that the printer was capable of printing two different cell types, it was decided that SCSC hMNs and hSN would be printed, with the goal that the hSN would innervate the hMN [19]. This occurs in the human body, so an in vitro model could be useful for future BoaC projects. The first item that needed to be considered was the type of media that both cell types could survive and function in, since there was not a media commonly used for both cell types. After a media formulation suitable for both cell types was developed, a printing procedure was developed and the cells were printed. Images were taken the day after printing and after axons had grown toward the opposite cell type which suggested innervation.

## 7.2 Fluorescent Dye Proof of Concept images

Two populations of SCSC hMN cells were loaded into the cell printer, each in their own cartridge. One population was labeled with a red fluorescent tracker dye, while the other population was labeled with a green fluorescent tracker dye [16]. Figure 7-1 shows the best printed and hand plated images for two different types of patterns. The first pattern was printing every other electrode with red or green. The second pattern was printing on all of the top electrodes with red labeled cells and printing on all of the bottom electrodes with green labeled cells. It was very apparent in Figure 7-1 that plating with the printer gave superior results. Since this proof-of-concept was shown, planning continued towards dual cell printing.

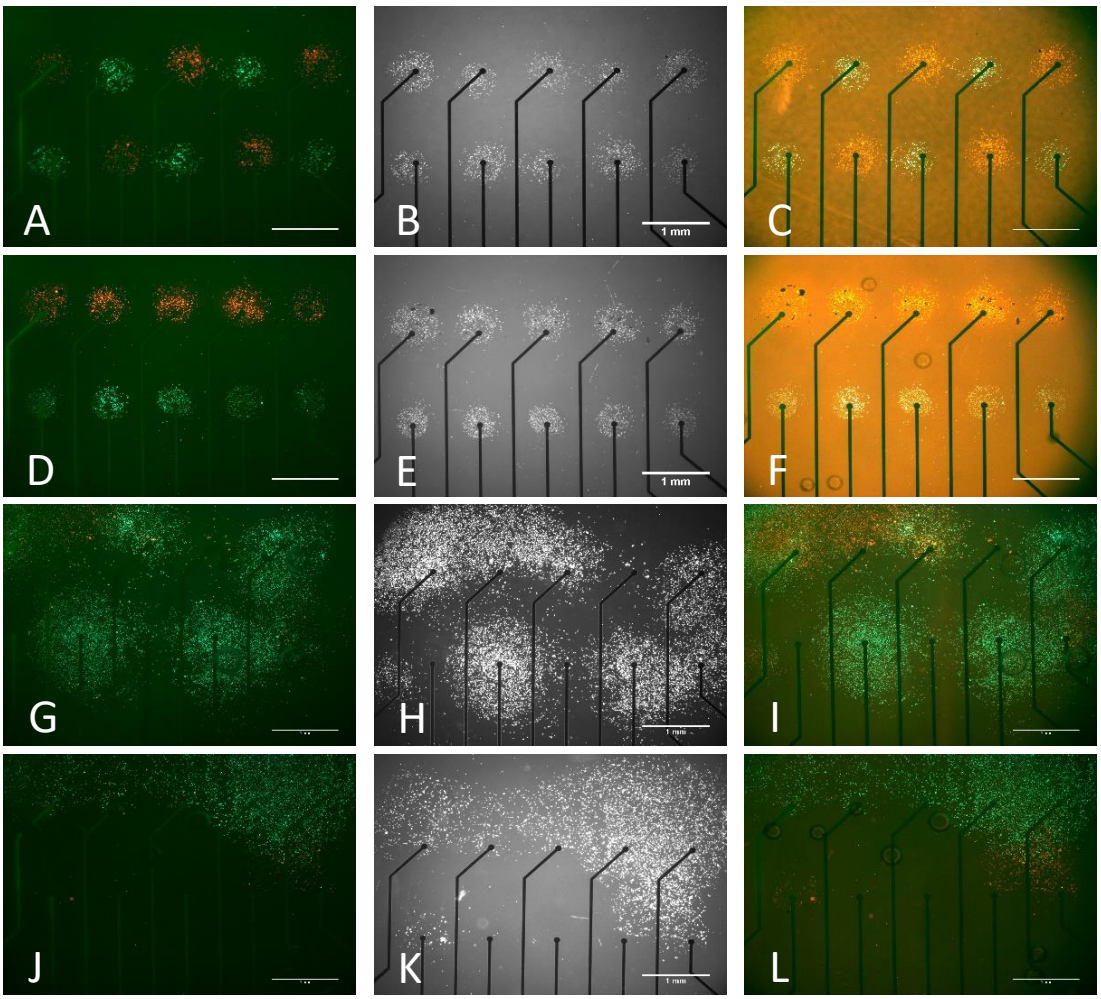



Figure 7-1: Images for hand plating and printing cell tracker dye cMEAs. All scale bars are 1 mm. The top row of electrodes were printed using a valve opening time of 400 µs, while the second row was printed using a valve opening time of 250 µs. Top row images: Printing red and green every other electrode. Second row images: Printing all top electrodes red and all the bottom electrodes green. Third row images: Hand plating attempting red and green every other electrode. Bottom row images: Hand plating attempting all top electrodes green and all bottom electrodes red. A, D, G, J. Fluorescent image. B,E,H,K. Phase image. C,F,I,L. Combined phase and fluorescent image.

## 7.3 Design of New Patterns

To direct the hSN to innervate the hMNs, a new mask for surface patterning was necessary. Figure 4-4 E and F show the mask pattern that encouraged the axons of the hSN to grow towards the hMN cells. The presence of the break in the line pattern on the hMN side was

designed so the hMNs would stay on their electrode, while lines connecting to the hSN were to encourage the hSN's axons to grow onto the line pattern.

## 7.4 Media Considerations

Sensory neurons and motoneurons have different recommended media formulations, so it was important to find a formulation of media in which both cells would survive. A hand plating experiment was set up to test how hSN and hMN cells survived in different types of media formulation. Table 7-1 and Table 7-2 show the details for the two-cell media experiment. Each media and cell combination listed were plated on both DETA coated coverslips and DETA coated coverslips covered with laminin.

Table 7-1: Controls for two cell media experiment

| | **Cell** | **Media** |
|---|---|---|
| Positive Controls | hSN | hSN |
| | hMN | hMN |
| Negative Controls | hSN | hMN |
| | hMN | hSN |

Table 7-2: Experimental formulations for two cell media experiment

| **Cell** | **Media** |
|---|---|
| hSN | 50/50<br>MN + NGF |
| hMN | 50/50<br>MN + NGF |
| hSN and hMN | 50/50<br>MN + NGF |

Table 7-3: Description of the images for Figure 7-2 and Figure 7-3

| Name | Cell | Media |
|---|---|---|
| A | hMN | hMN |
| B | hSN | hMN |
| C | hMN | hSN |
| D | hSN | hSN |
| E | hMN | 50/50 hMN and hSN |
| F | hSN | 50/50 hMN and hSN |
| G | hMN and hSN | 50/50 hMN and hSN |
| H | hMN | hMN + NGF |
| I | hSN | hMN + NGF |
| J | hMN and hSN | hMN + NGF |

Figure 7-2 and Figure 7-3 show how the cells looked the day after hand plating onto coverslips. Looking at the morphology from day 1, it was apparent that the hSNs were able to survive in hMN media better than the hMN in hSN media. The hSNs did not look good in the hSN media on DETA, but the cells had time to dry out before the media was added to the well during plating. It was predicted that the best media formulation for both cells would be the hMN + 0.01% nerve growth factor (NGF), since NGF is the main factor missing from the hMN media when compared to the hSN media. hMN media is rich in necessary neuronal trophic factors, making it an ideal base medium [12]. Two concerns to this hypothesis were raised, whether adding NGF to the hMN media would cause a proliferative growth in contaminant cells, and whether hSNs would survive well in a hMN base media. Looking at the phase image results in Figure 7-2 and Figure 7-3, neither concern occurred. In fact, the hSNs and hMNs in H, I and J in both Figure 7-2 and Figure 7-3 exhibit expected neuronal morphology. It is

predicted that in the co-cultures, the hSNs and hMNs produce factors beneficial to the opposite cell type. The cells survived well on both DETA and Laminin coated DETA, so DETA was the surface chosen to print on. It was necessary to print on a dry surface, and easier to create a reliable dry DETA surface, as the laminin surface should be plated on while still wet.

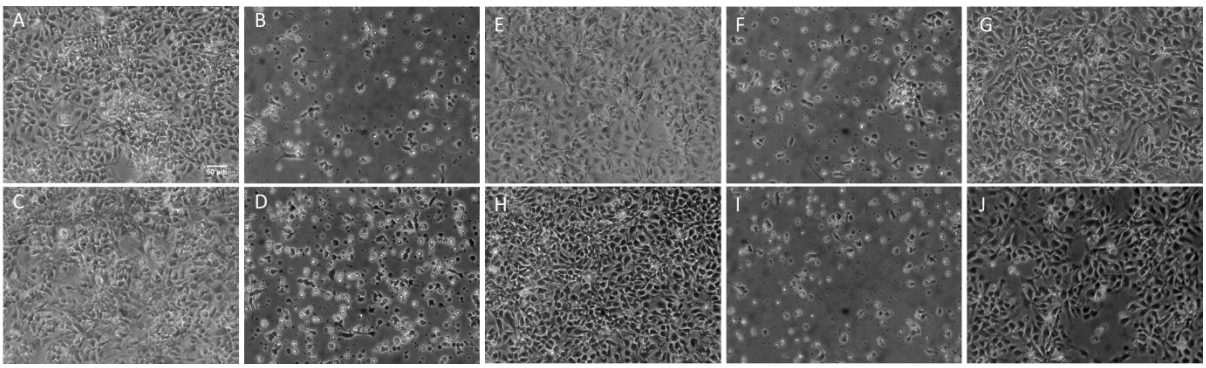



Figure 7-2: DETA coated coverslips, refer to Table 7-3 for image descriptions. Scale bar in image A, all images the same magnification of 100x.

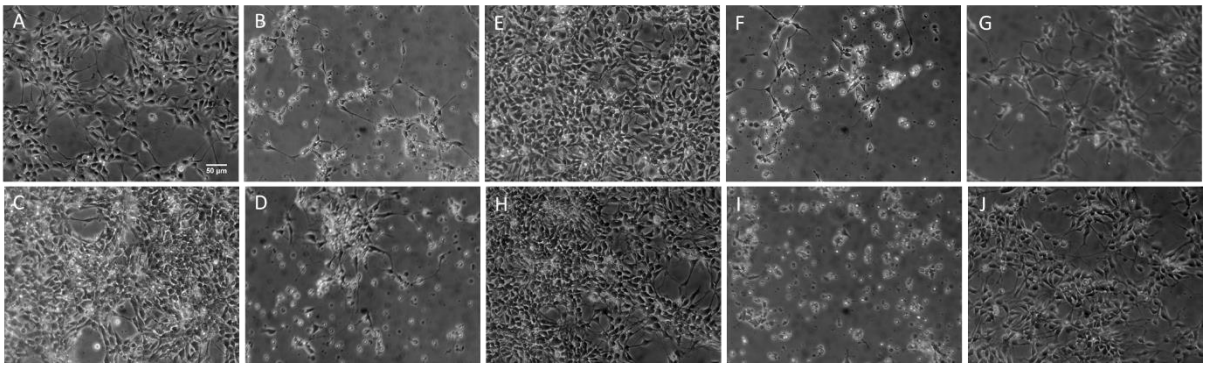



Figure 7-3: DETA and laminin covered coverslips, refer to Table 7-3 for image descriptions. Scale bar in image A, all images the same magnification of 100x.

## 7.5 Printing Procedure

Printing with two different cell types posed the same challenges as printing with two differently fluorescent populations noted earlier in this chapter. An important consideration to remember is to keep the printing spot size small enough to ensure that the cell populations stay separated. In order to do this while ensuring enough cells are on the electrodes for significant recording measurements, the cell density that was normally printed must be increased. It was shown that the hSNs exhibit less survivability when printed, so the printing density for the hSNs must be significantly higher than for the hMNs, about five to ten times higher. Further printing experiments would provide optimized printing conditions for hSNs.

## 7.6 Images of Innervation

After printing both cell types, the surfaces were imaged the day after print, four days after print, twelve days after print, and fifteen days after print. Twelve days after printing, via phase images a few of the electrode pairs showed signs of innervations between the two cell pairs on the electrodes, as seen in Figure 7-4, and Figure 7-5. Figure 7-6 shows signs of innervation fifteen days after print on a different cMEA surface, with improved alignment of cells on electrodes.

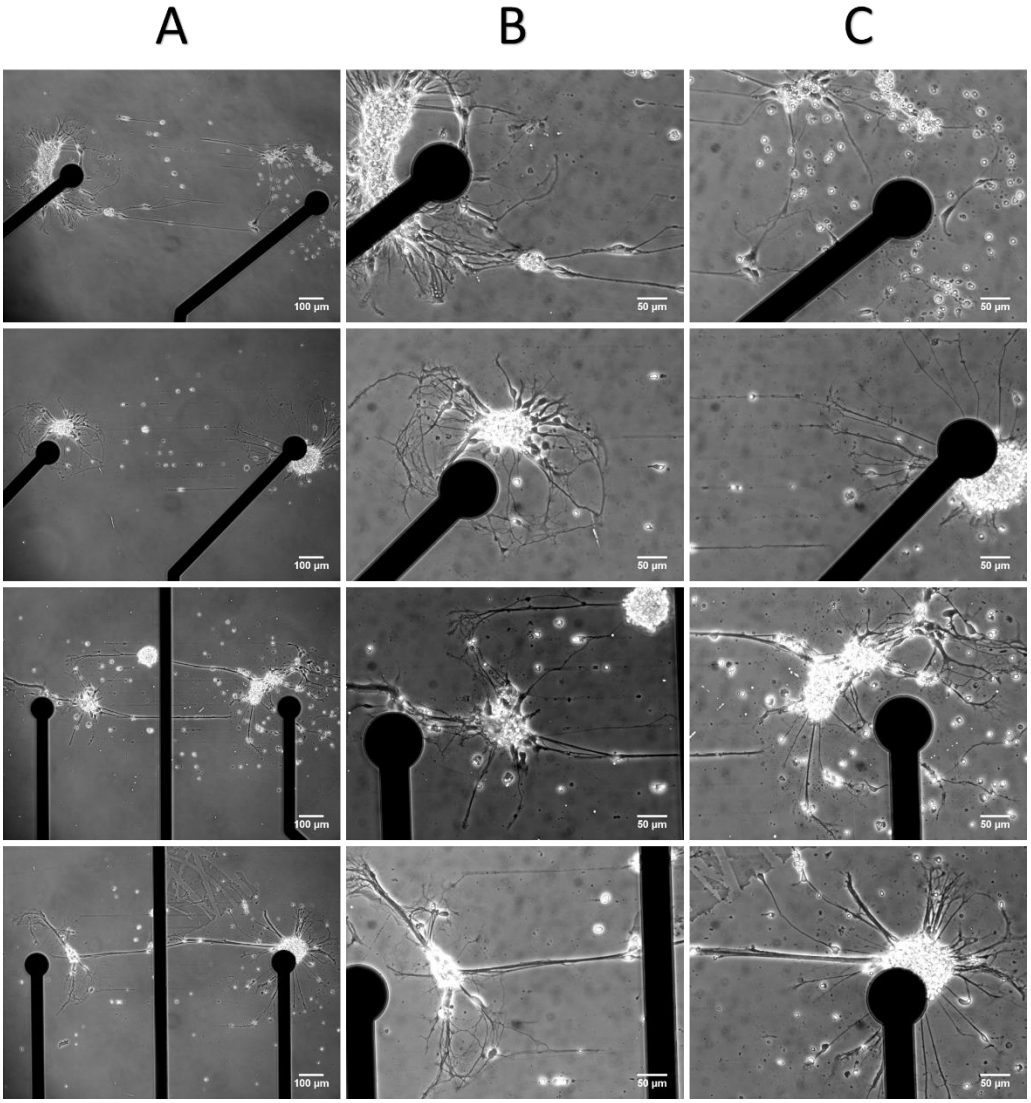



Figure 7-4: Images of innervated printed hMNs and hSNs twelve days after printing. Column A shows the two cells connected on the electrodes at 25x. The left electrode contains hMN cells, while the right electrode contains hSN cells. Column B shows a close up of the hMN electrode. Column C shows a close-up of the hSN electrode. Columns B and C images are at 250x.

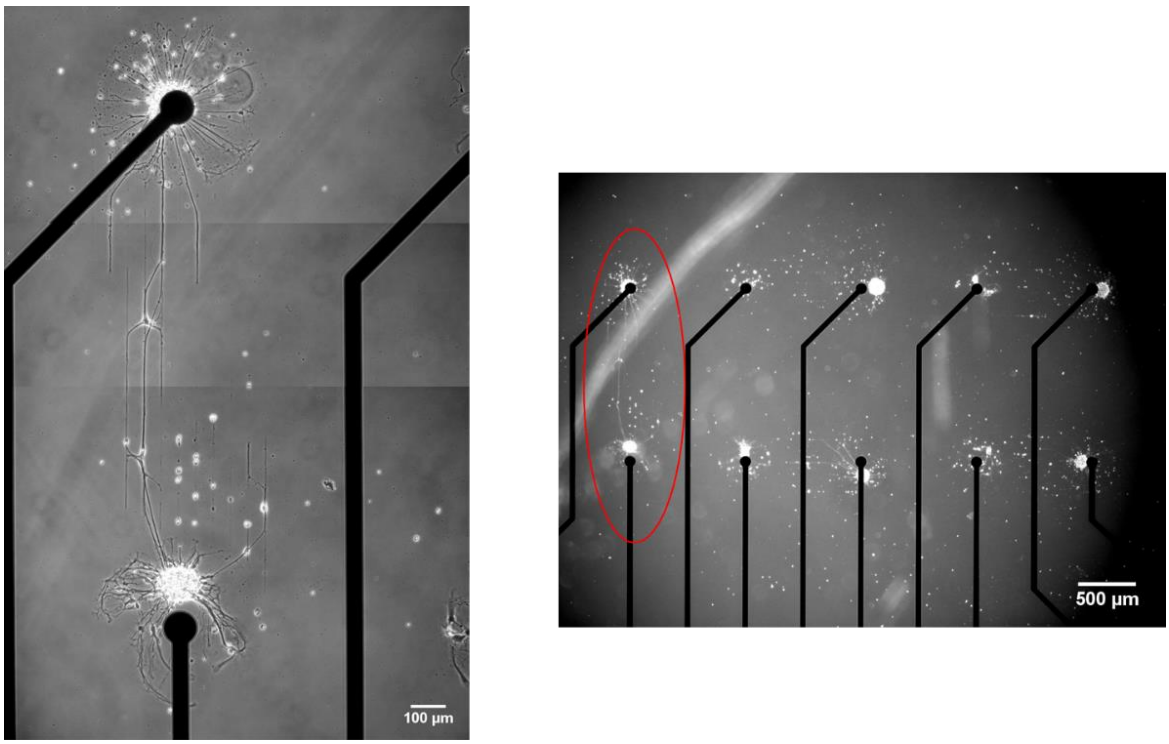



Figure 7-5: L: Image of innervated printed hMNs and hSNs twelve days after printing at 100x. The top electrode contains hSN cells, while the bottom electrode contains hMN cells. R: Location of left image on its cMEA chip at 25x.

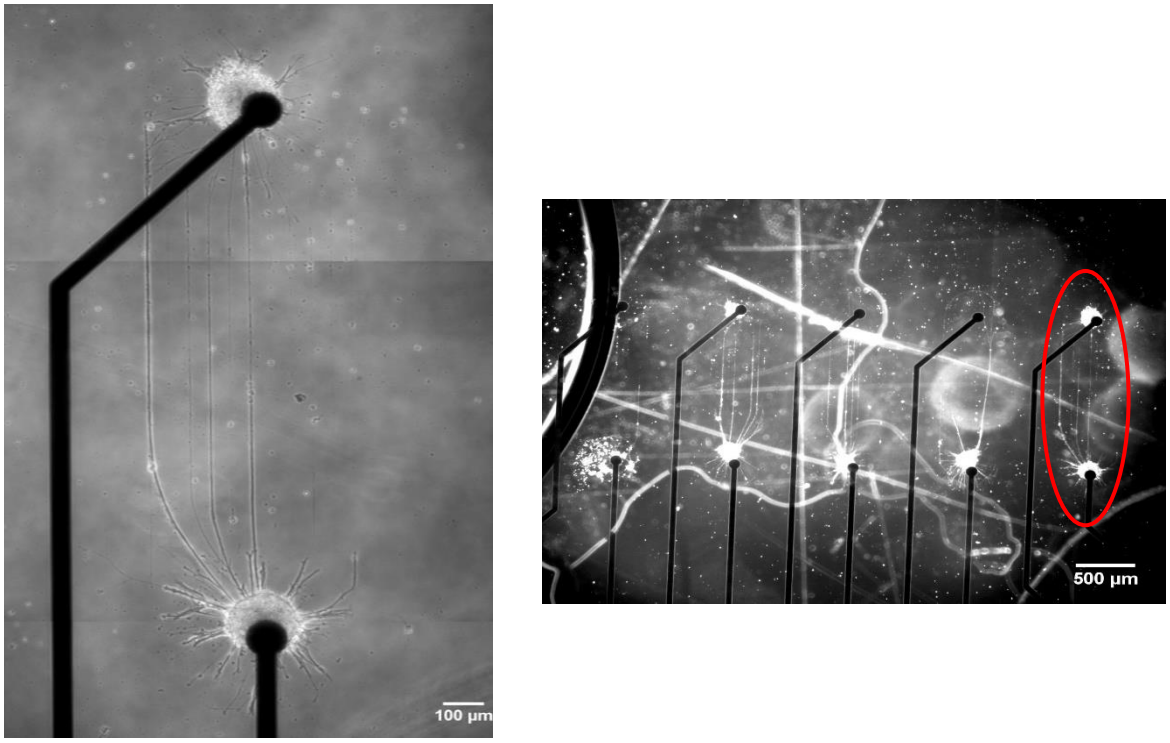



Figure 7-6: L: Image of innervated printed hMNs and hSNs fifteen days after printing at 100x. The top electrode contains hSN cells, while the bottom electrode contains hMN cells. R: Location of left image on its cMEA chip at 25x.

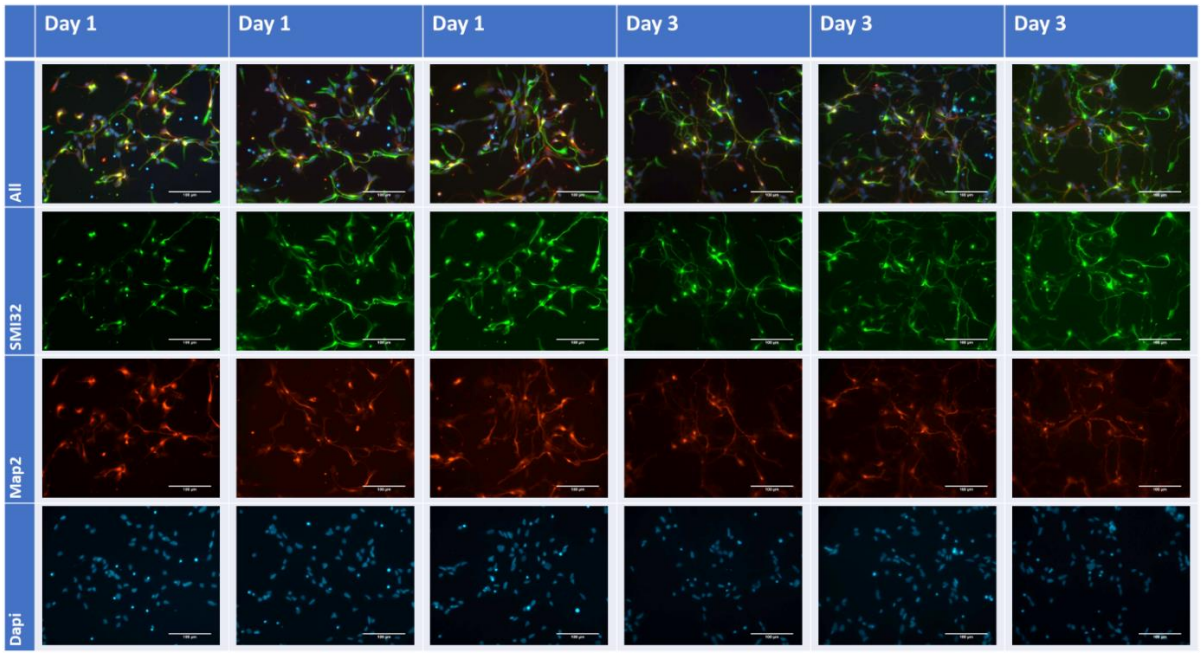
# CHAPTER 8 CONCLUSION

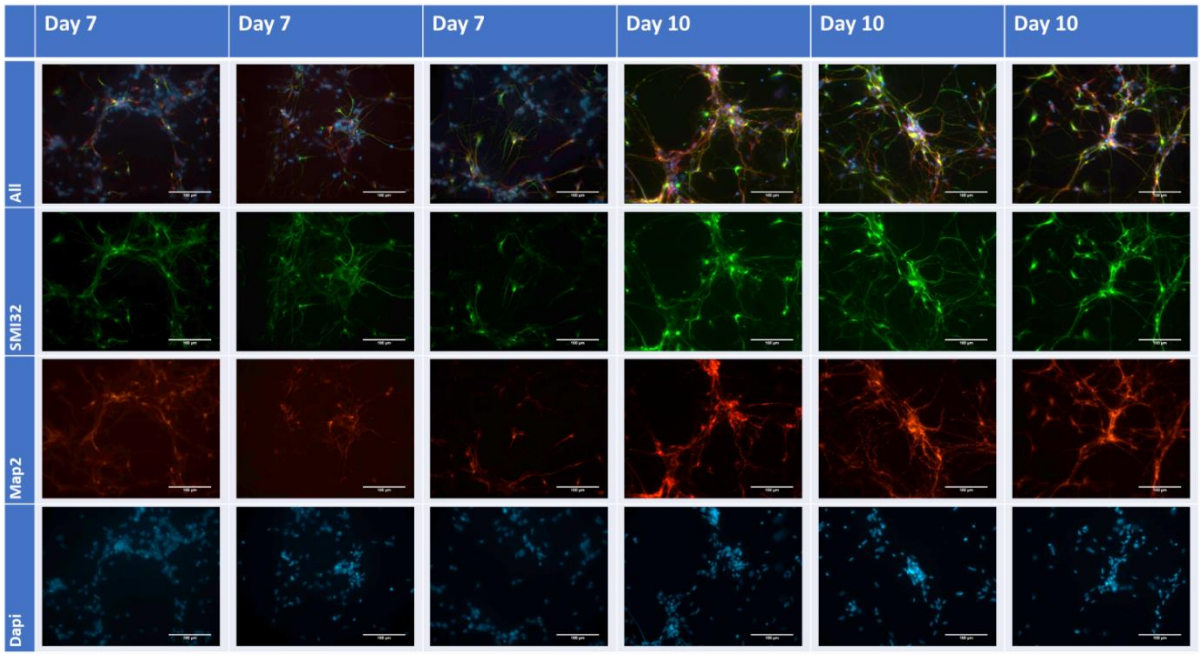
It has been demonstrated that the utilization of a 3D printer, adapted to perform as a cell printer, allowed for the placement of cells in specific locations on surfaces and was shown to be a viable means of plating cells for BoaC applications. This technique can be utilized without negatively affecting the cells, as shown through both visual and electrical analysis methods, using the conventional hand plating technique as a control standard. Cell printing opens a path for a reduction of cell usage per surface and scale up possibilities through automation.

Both printed hMN and printed cardiac cells were shown via microscope imaging and functional recordings to be comparable to or more functional than their hand plated controls. Cell printing can go one step further in creating multi-cell surfaces that the conventional hand plating technique was incapable of fabricating due to the cell printer's increased accuracy and resolution.

It was also shown that it was possible to create a model of hSNs innervating hMNs; the next step in this project is to create a model of the reflex arc [21]. The classic example of this phenomenon is when your knee is tapped at the doctors office, and your leg kicks automatically. In the body, when a sensory neuron encounters a stimulus, it sends a signal to the brain, which tells the motoneuron to move. The motoneuron is connected to a muscle which physically moves the body part that encountered the signal. In order to re-create this in vitro, a third cell type would be introduced: skeletal muscle. The sensory neuron would innervate the motoneuron, and then the motoneuron would innervate skeletal muscle [22]. Testing this would involve measuring the contraction of the skeletal muscle upon electrical stimulation of the sensory neuron.

# APPENDIX A: EXTRA HMN IMMUNOSTAINING IMAGES


Day 1
Day 1
Day 1
Day 3
Day 3
Day 3
All
SMI32
Map2
Dapi


Day 7
Day 7
Day 7
Day 10
Day 10
Day 10
All
SMI32
Map2
Dapi

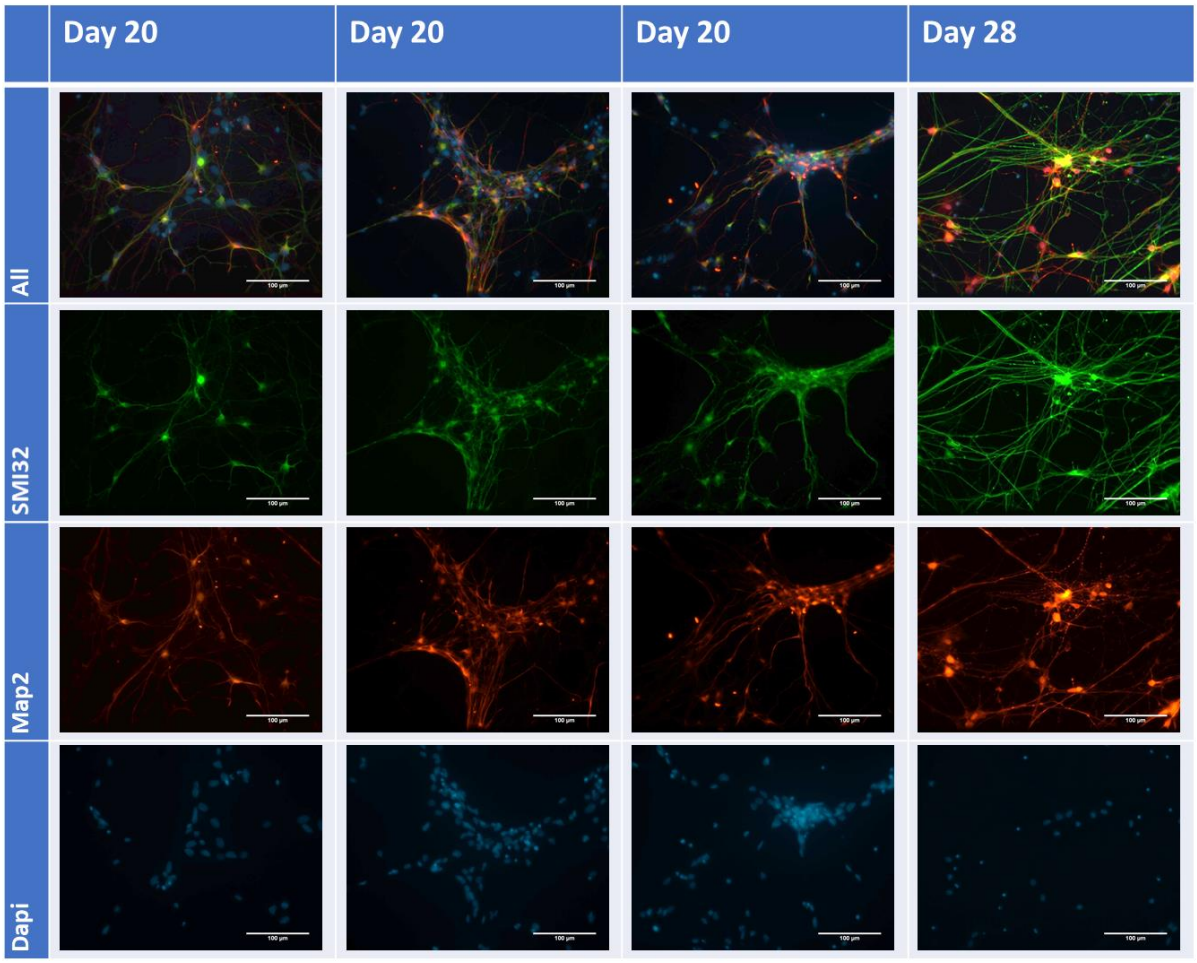
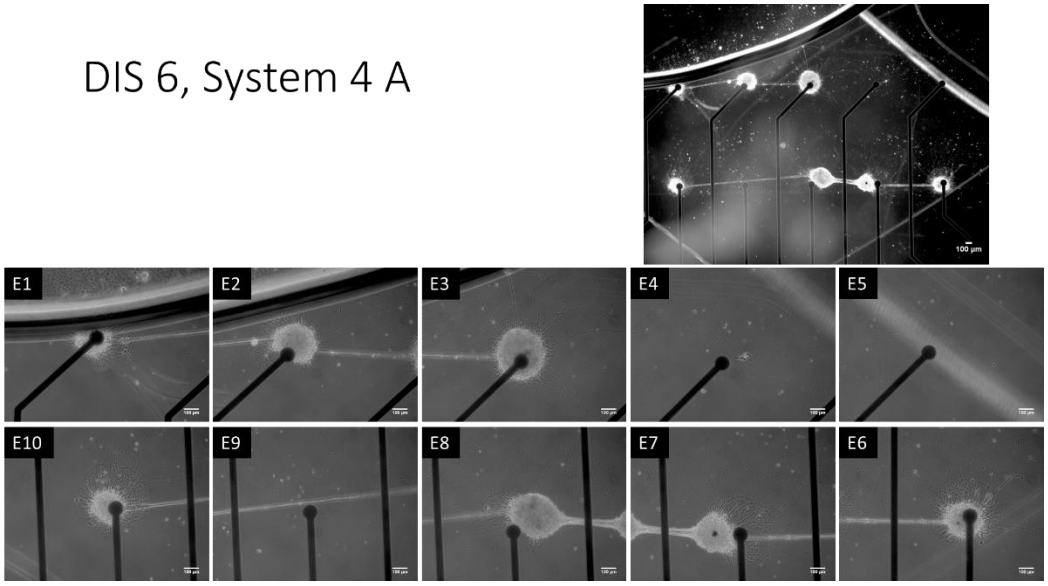



Figure A0-1: Immunostaining images of printed hMNs out to twenty-eight days

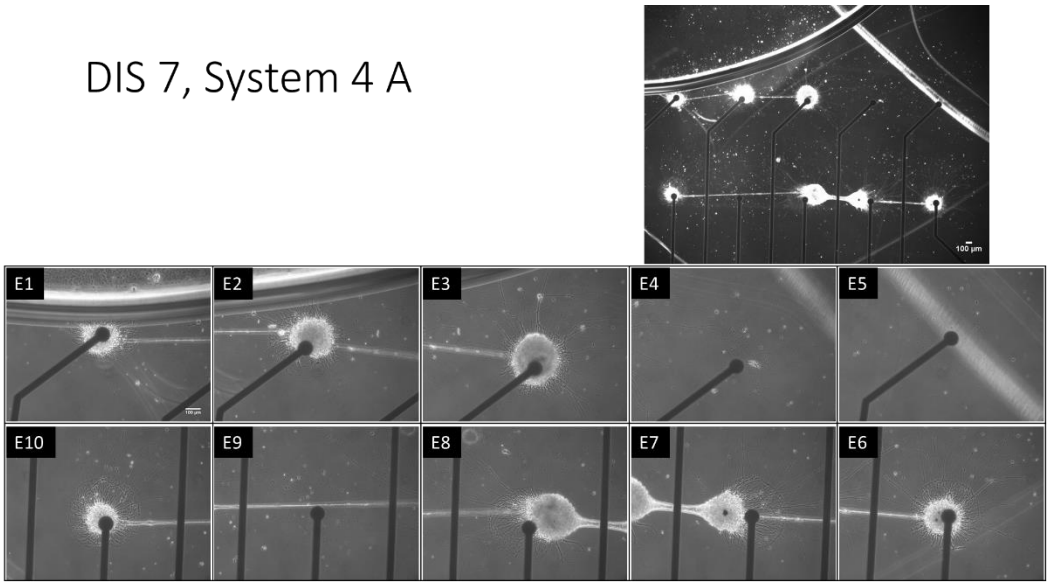
# APPENDIX B: IMAGES FOR RECORDINGS OF PRINTED CMEAS OVER TIME

The following sets of images in this appendix show the progression of cell growth for the hMN coated cMEAs assembled in the system housings for recording of electrical signals. Two cMEAs were in each system, denoted A and B, and three systems were recorded, Systems 4, 23, and26. They were imaged at 6,7, 10, 13, 14, 16, 17, 20, 22, 23, 24, 28, and either 65 or 66 days in system (DIS). One cMEA, System 23 A, was only imaged until 7 DIS since an air bubble formed over the cells, drying out the electrodes and killing the cells.

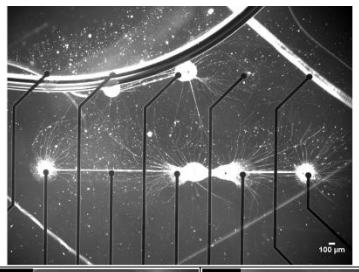
DIS 6, System 4 A

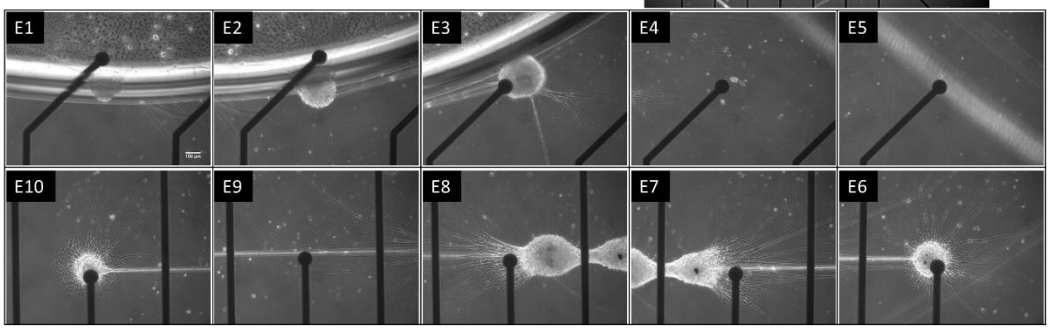



DIS 7, System 4 A

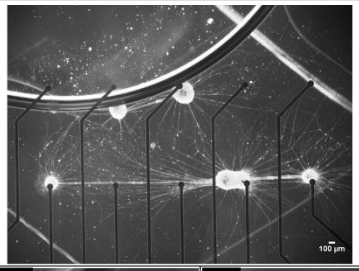


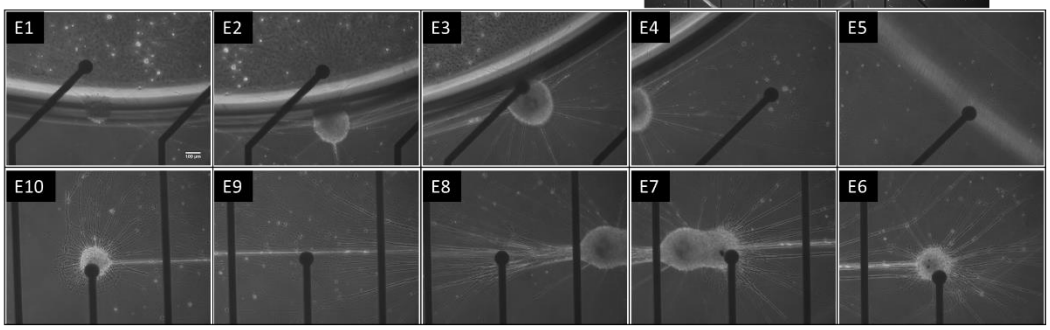
DIS 10, System 4 A



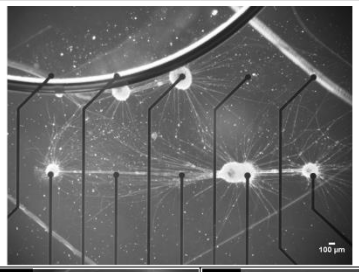


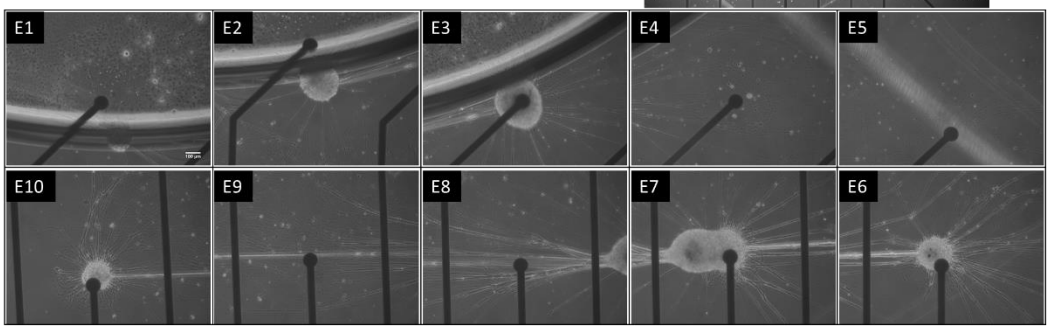

DIS 13, System 4 A



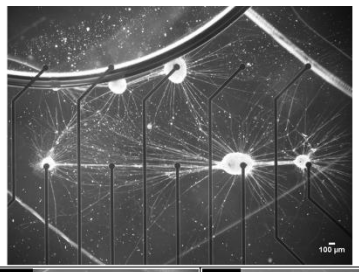


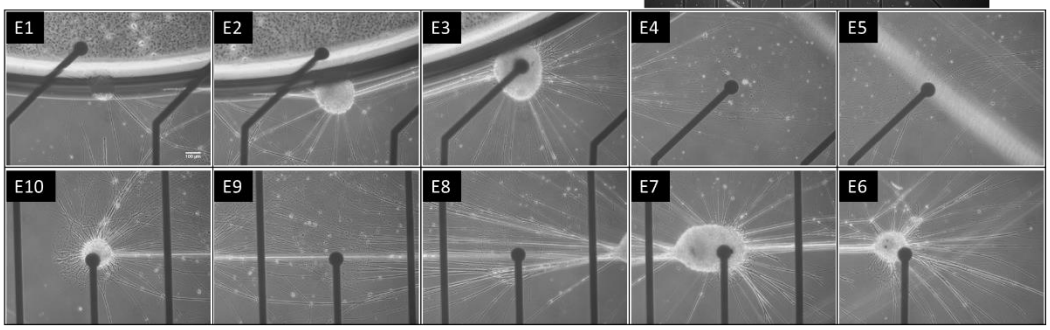

DIS 14, System 4 A



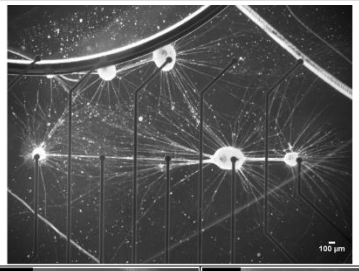


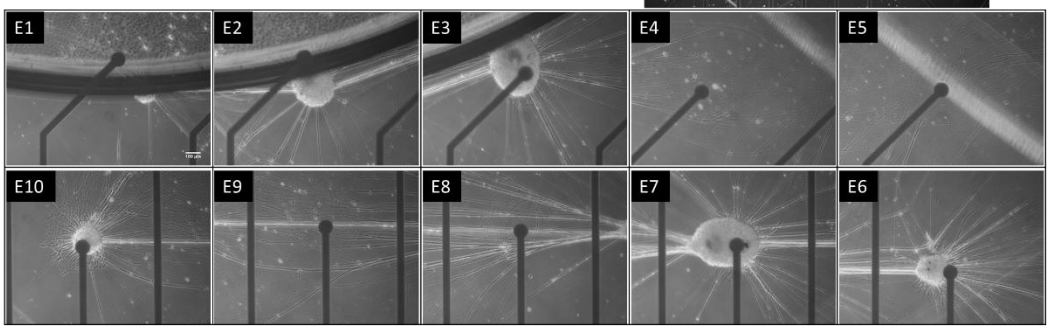
DIS 16, System 4 A



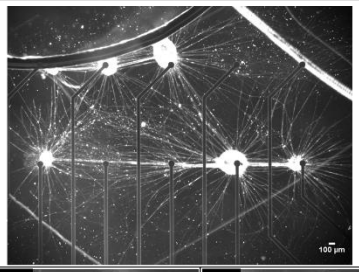


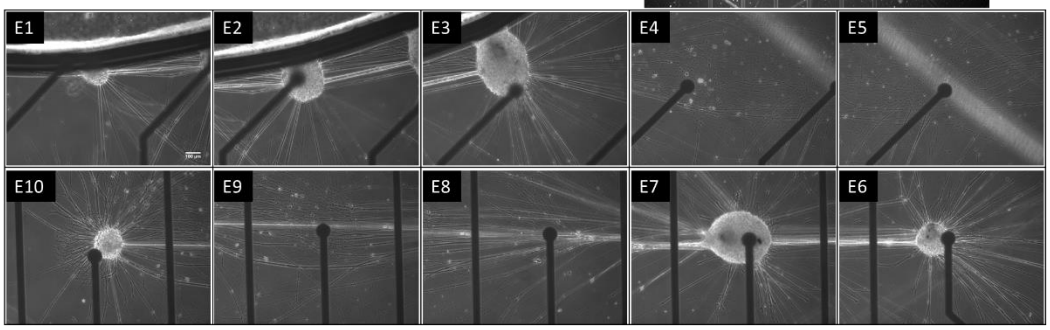

DIS 17, System 4 A



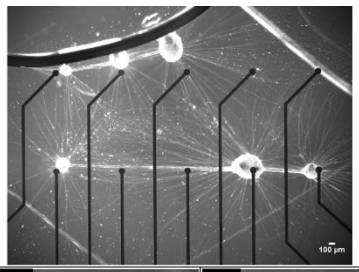


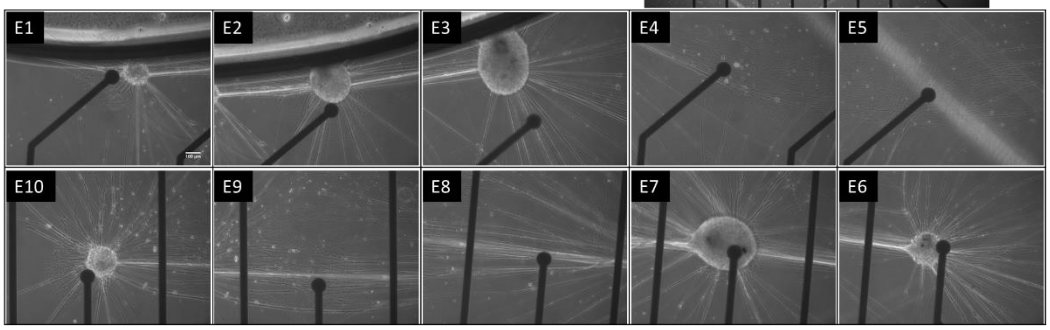

DIS 20, System 4 A



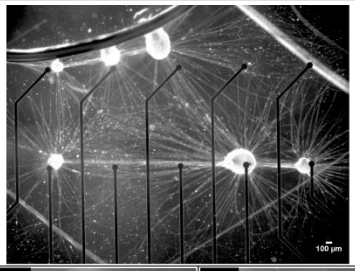


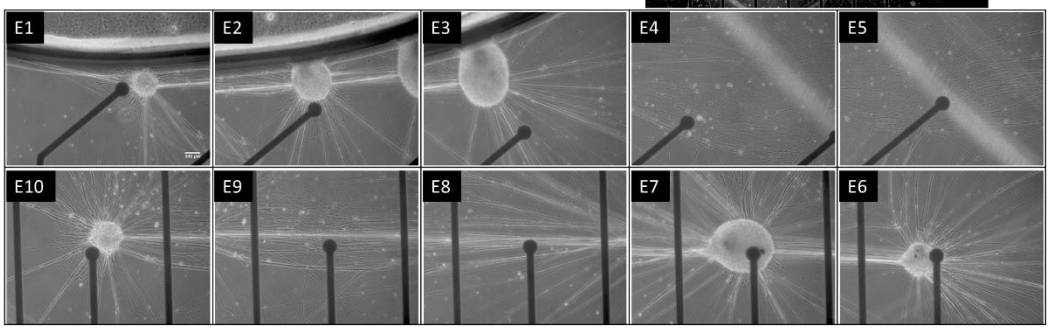
DIS 22, System 4 A



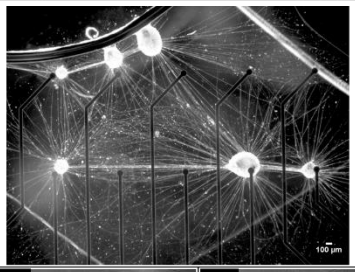


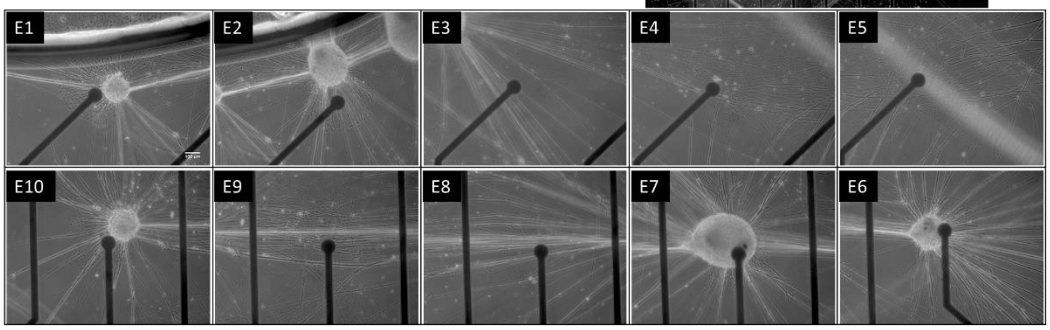

DIS 23, System 4 A



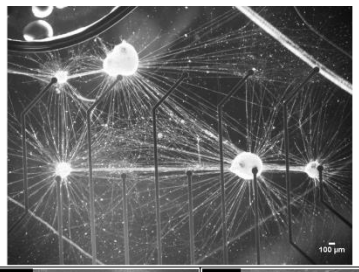


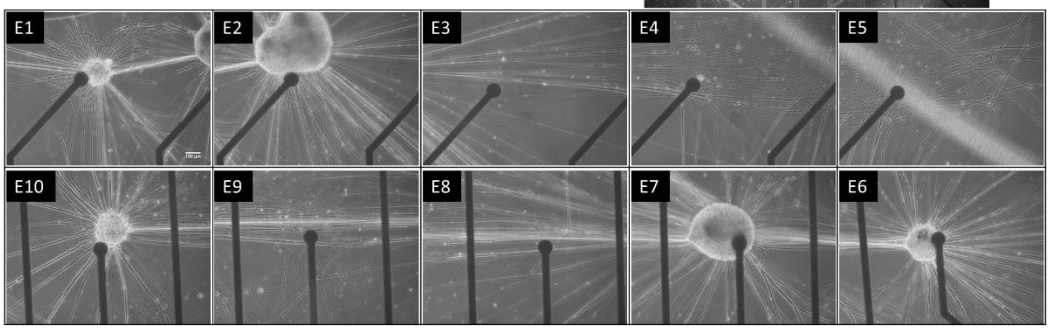

DIS 24, System 4 A





DIS 28, System 4 A

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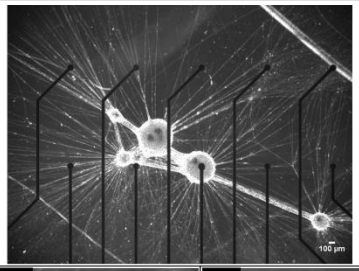
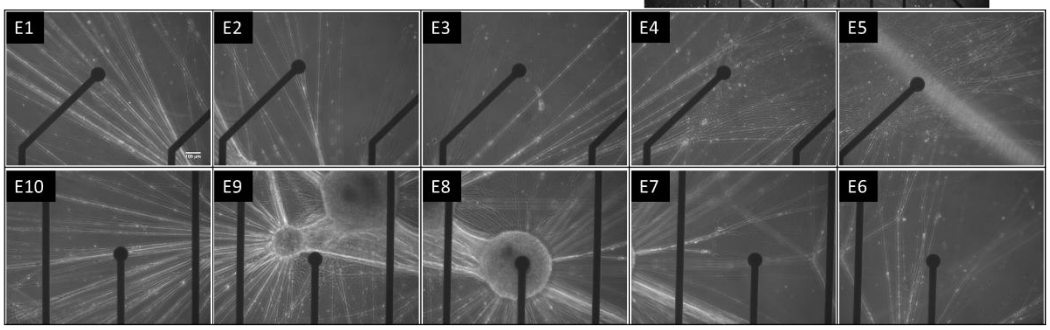
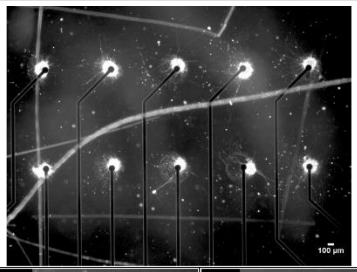
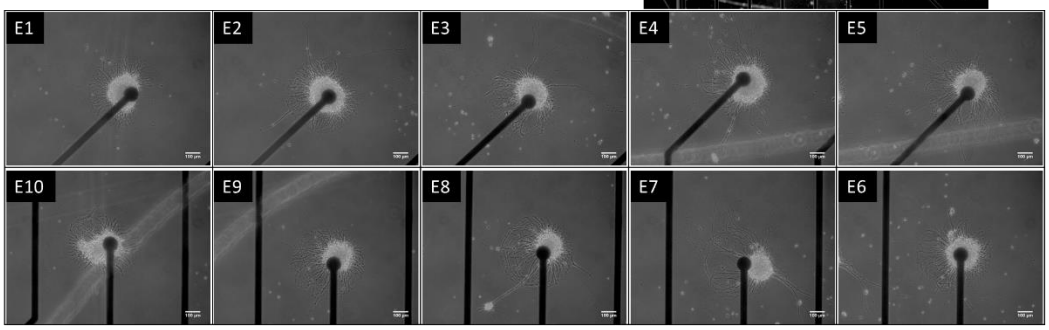


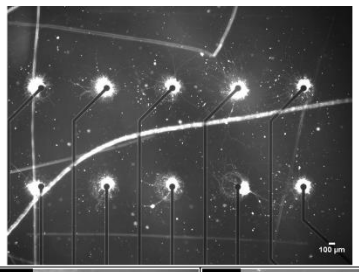



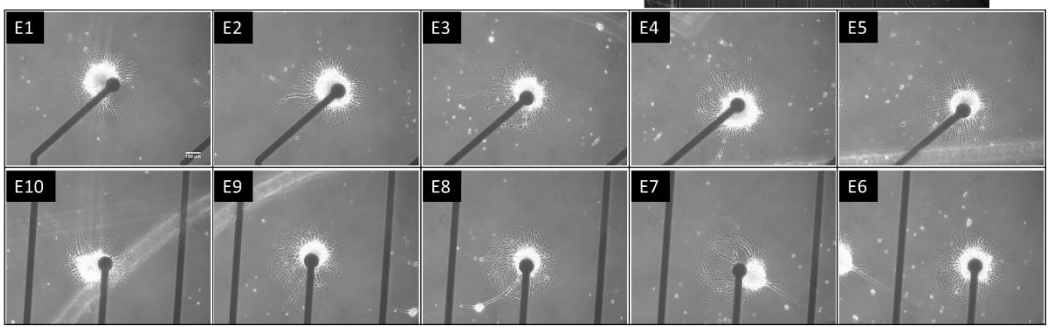

DIS 6, System 4 B



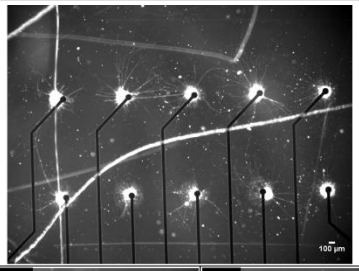



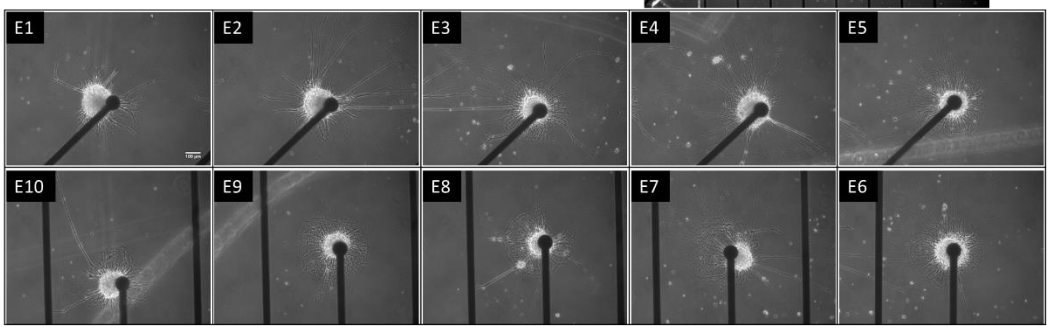
DIS 7, System 4 B



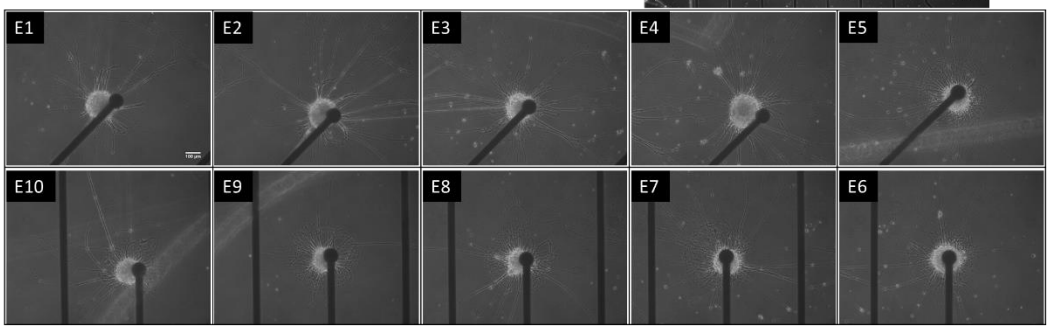



DIS 10, System 4 B

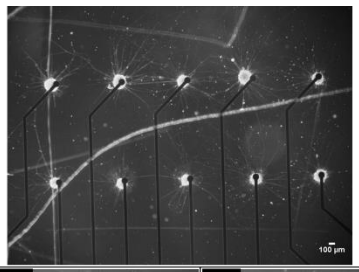


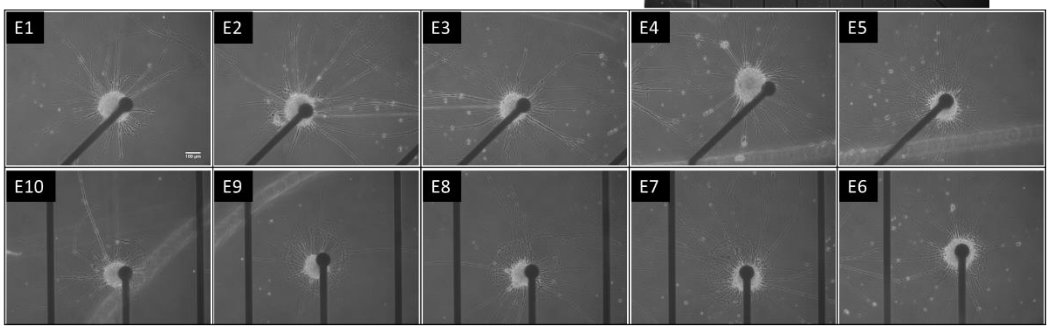



DIS 13, System 4 B

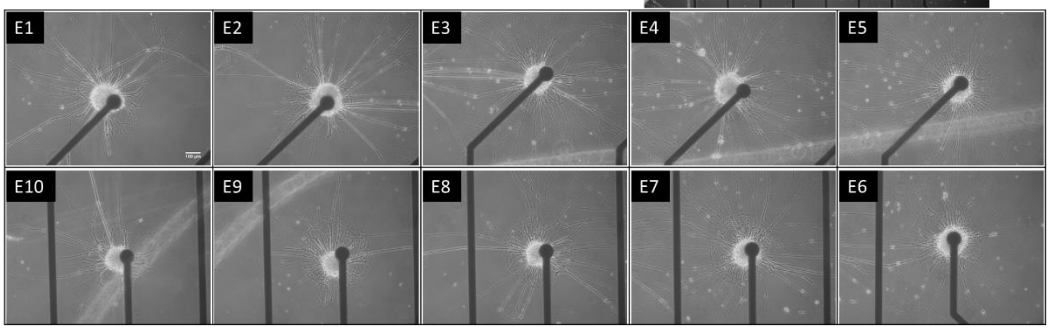


DIS 14, System 4 B

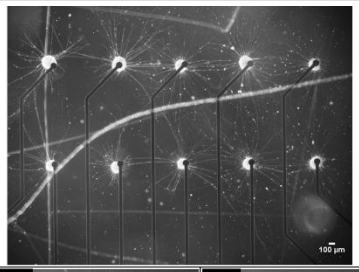


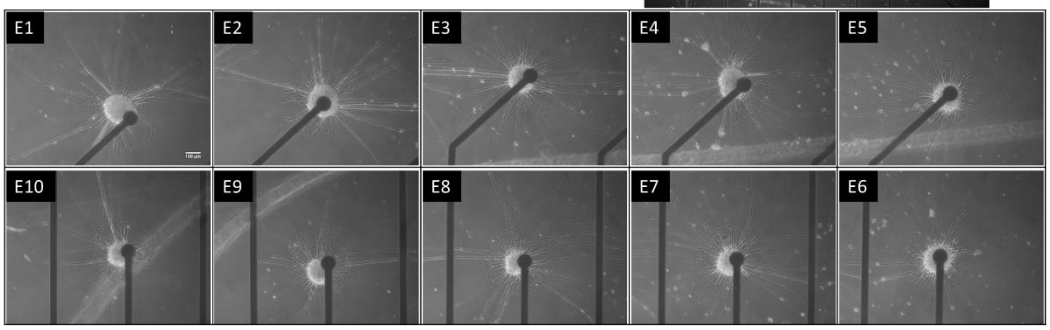




DIS 16, System 4 B

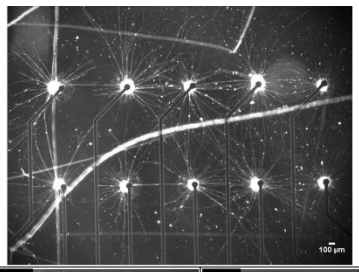



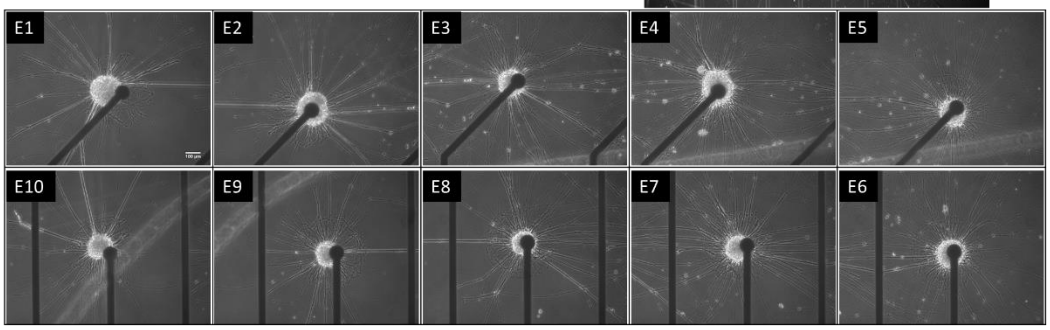

DIS 17, System 4 B



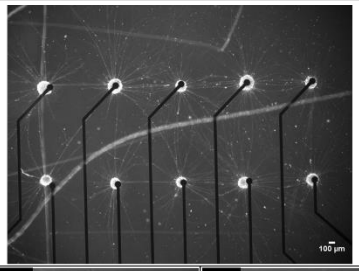



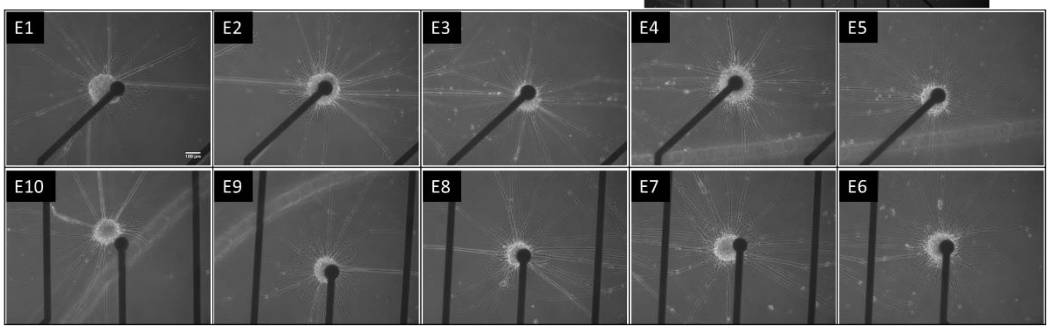
DIS 20, System 4 B



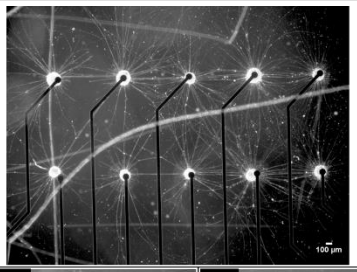



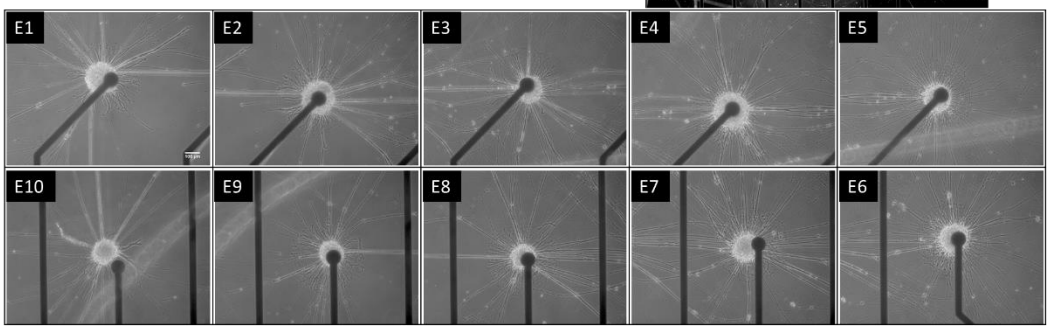

DIS 22, System 4 B



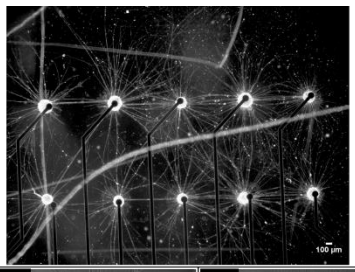



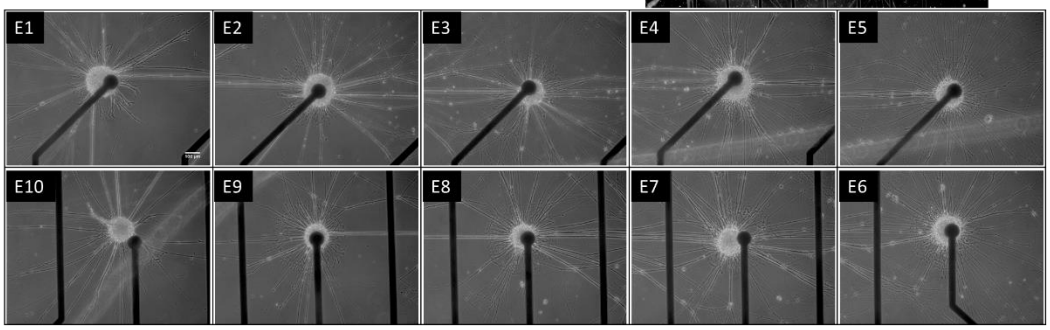

DIS 23, System 4 B



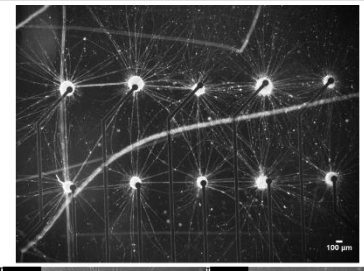



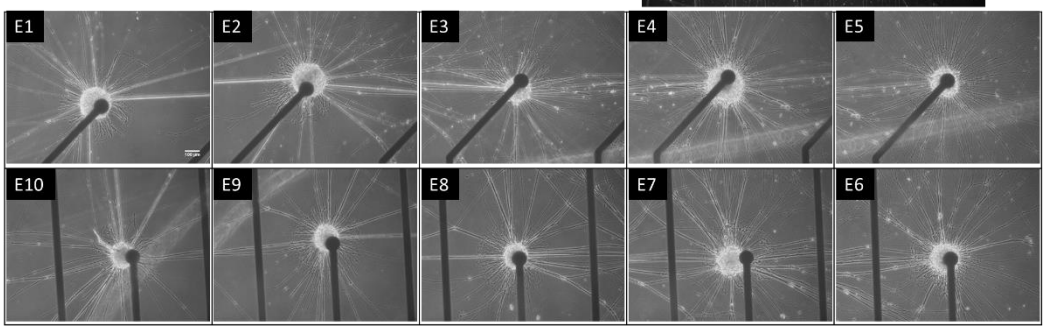
DIS 24, System 4 B



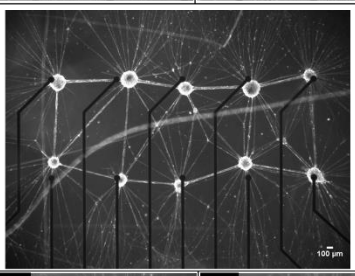



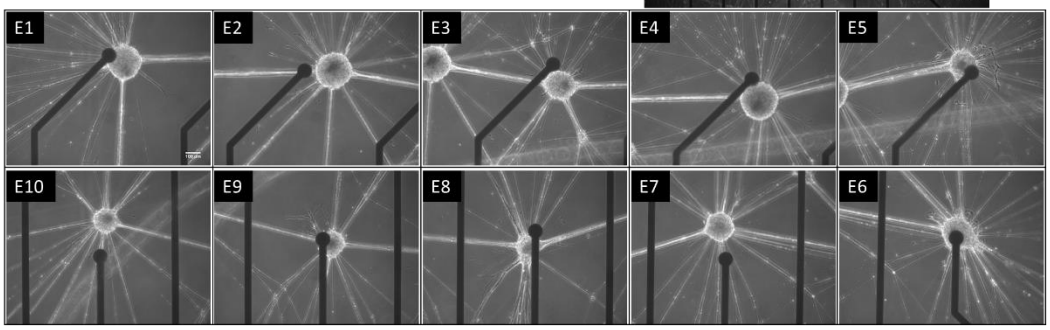

DIS 28, System 4 B



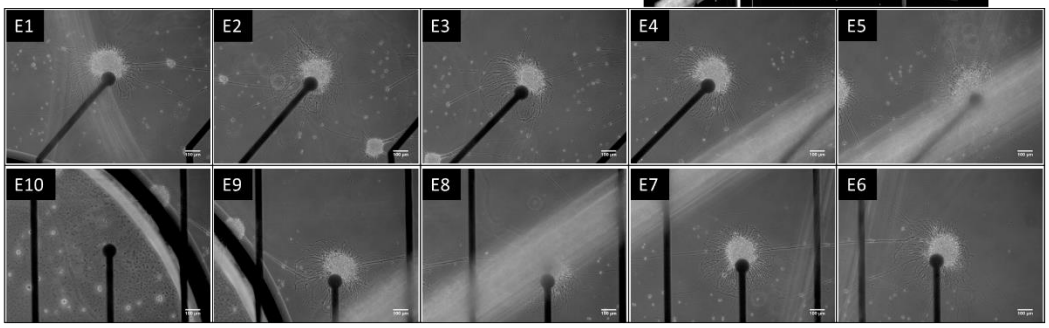




DIS 66, System 4 B

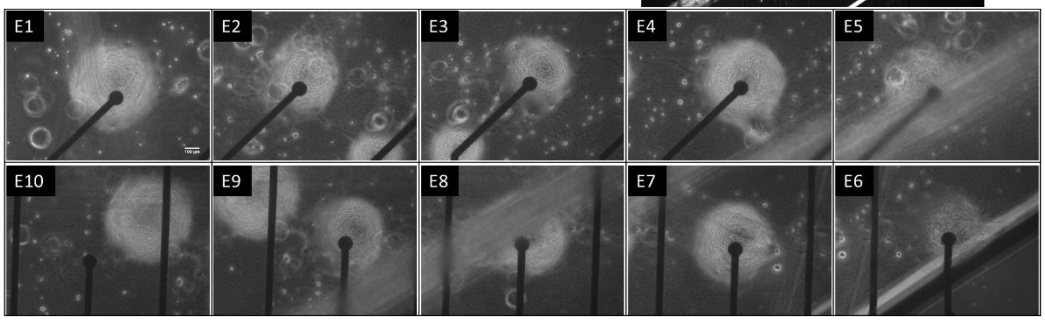


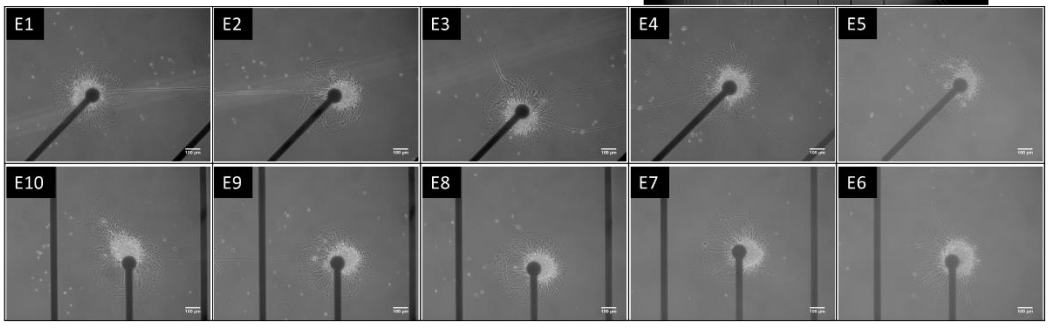
DIS 6, System 23 A



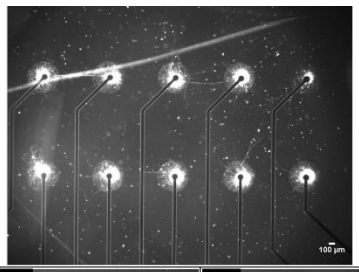

DIS 7, System 23 A
(Dried out, last set of images for this chip)

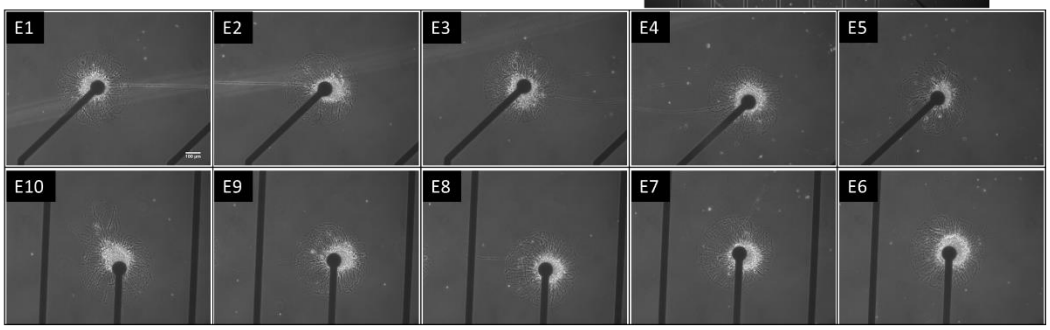



DIS 6, System 23 B

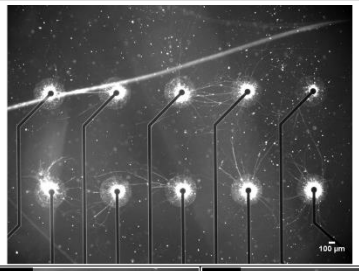


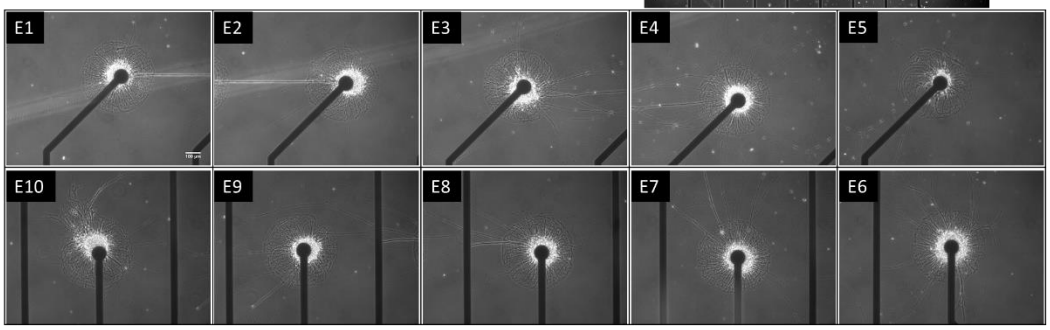
DIS 7, System 23 B



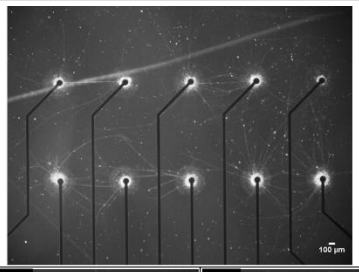



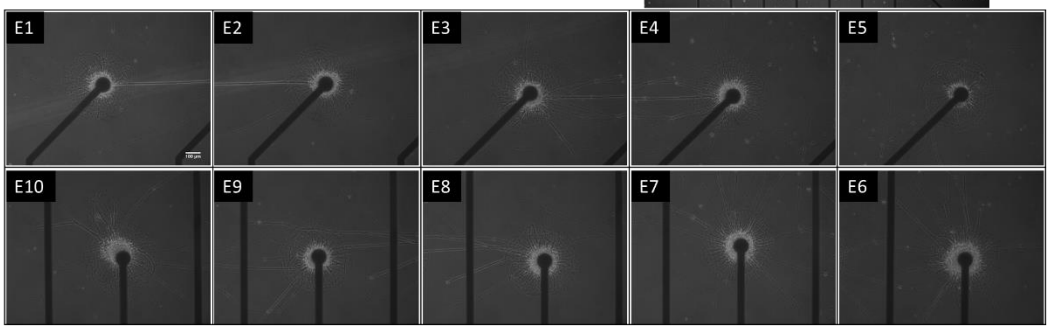

DIS 10, System 23 B



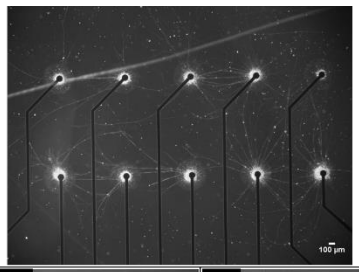



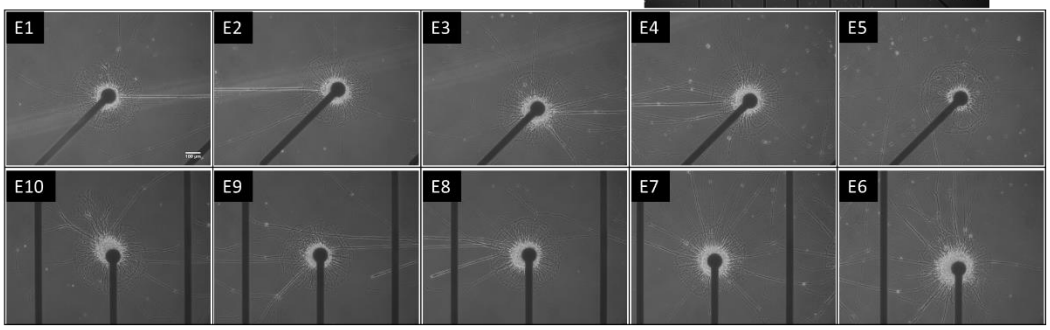

DIS 13, System 23 B



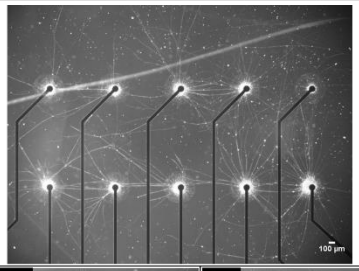



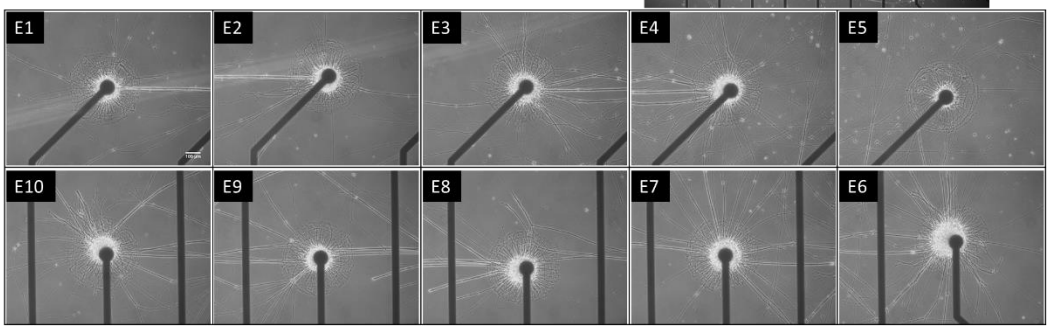
DIS 14, System 23 B



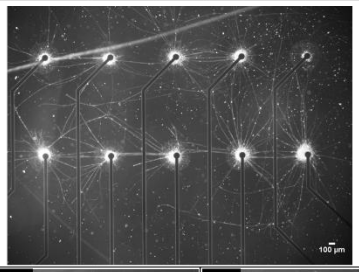



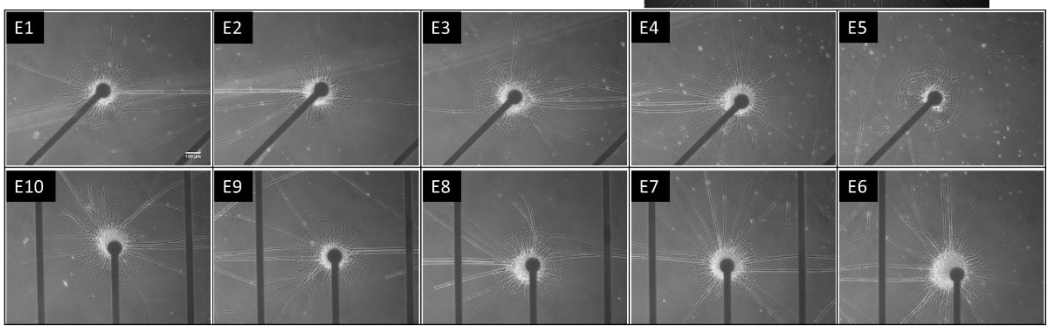

DIS 16, System 23 B



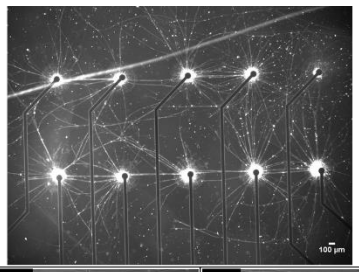



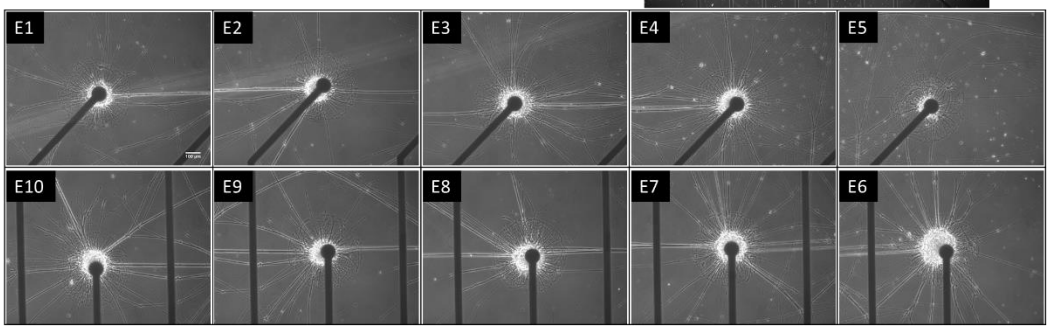

DIS 17, System 23 B



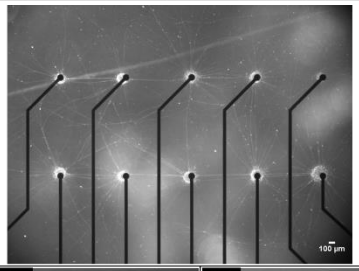



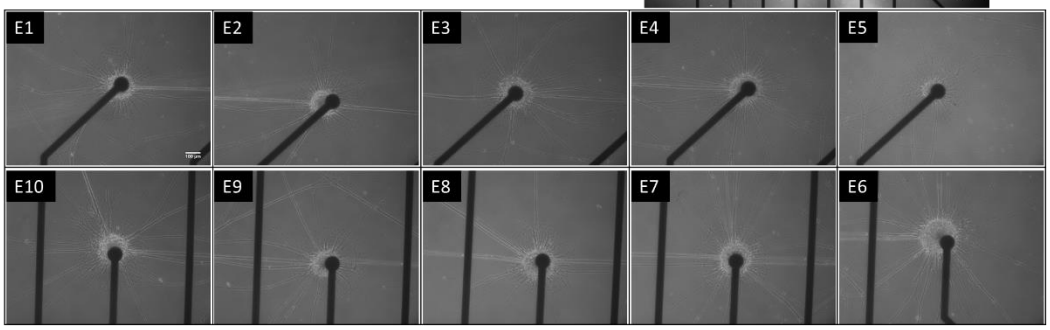
DIS 20, System 23 B



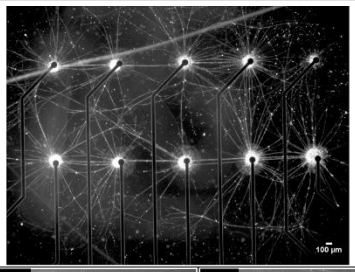


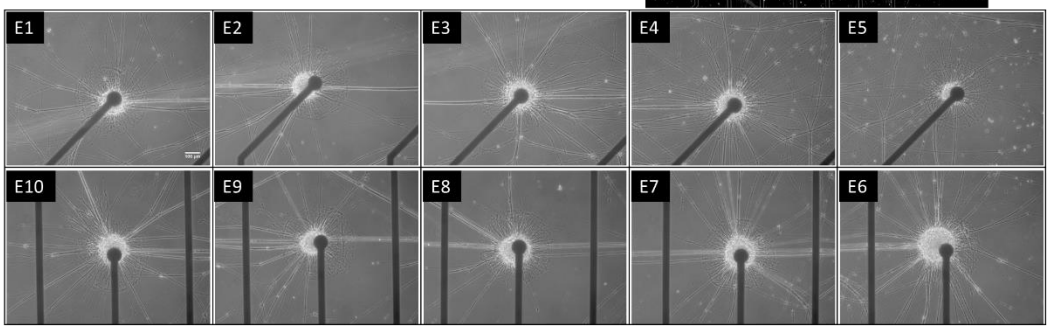

DIS 22, System 23 B



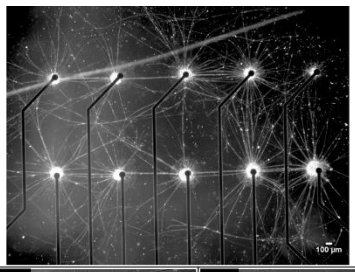


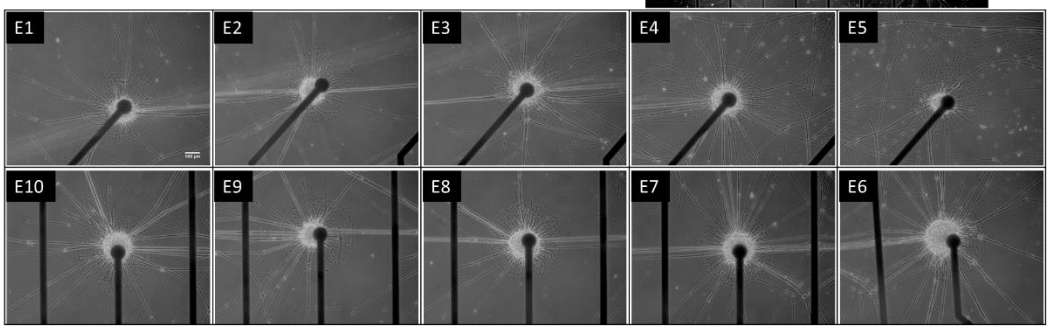

DIS 23, System 23 B



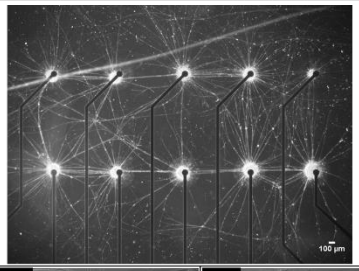


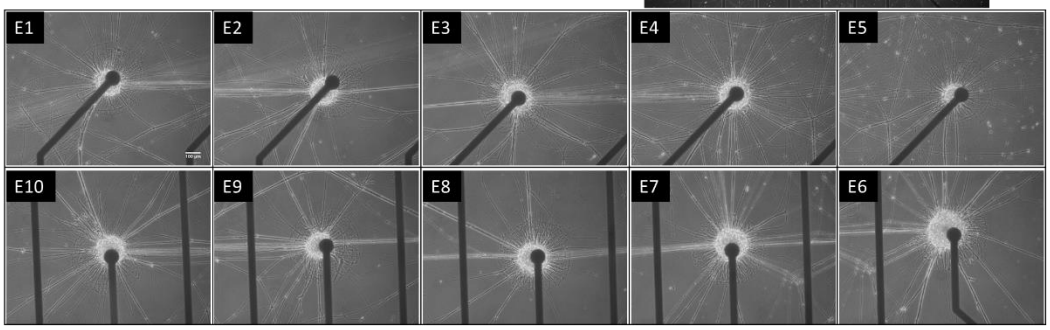
DIS 24, System 23 B



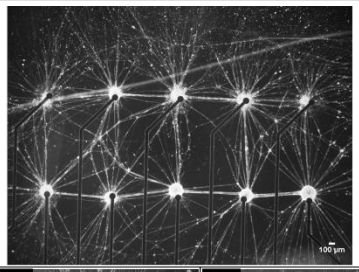


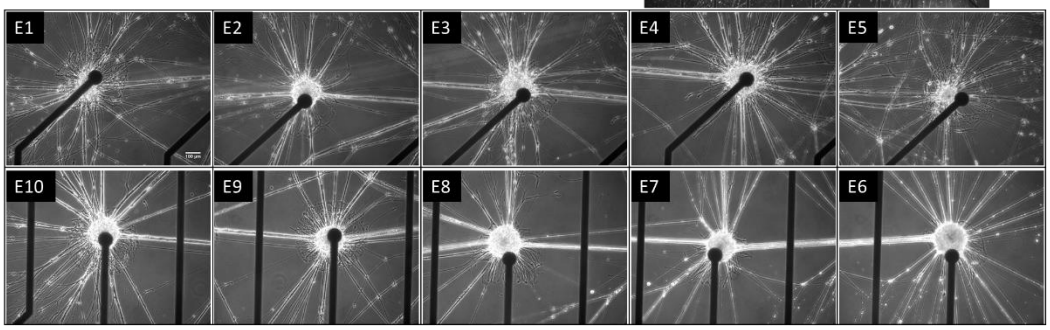

DIS 28, System 23 B



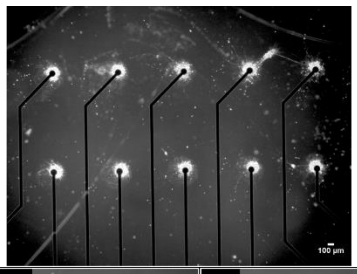


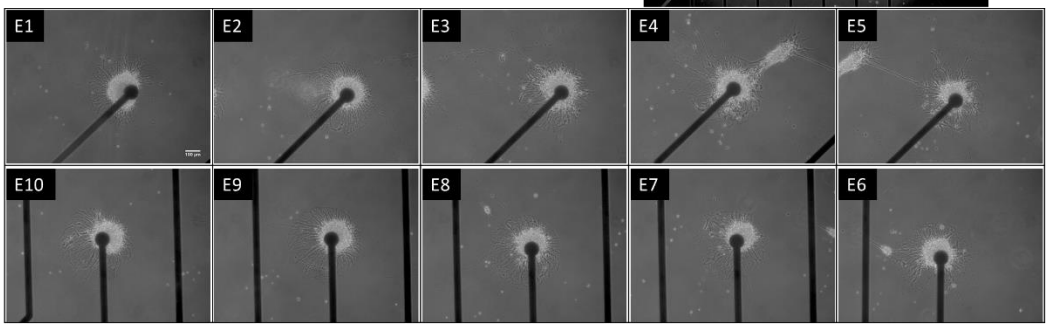

DIS 65, System 23 B



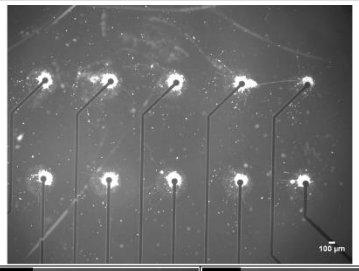


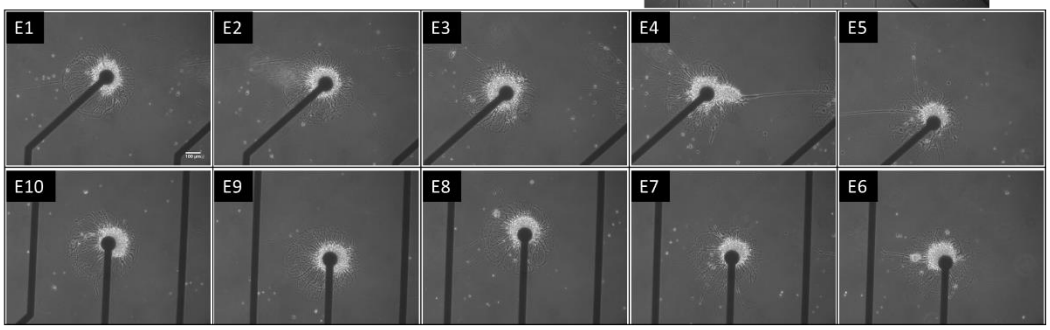
DIS 6, System 26 A



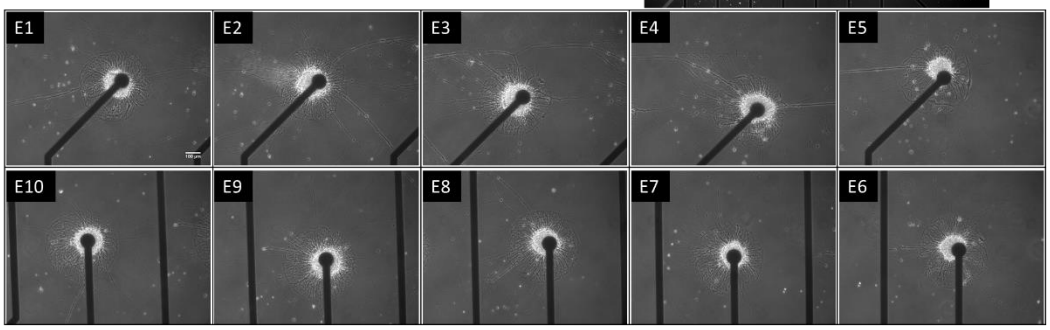




DIS 7, System 26 A

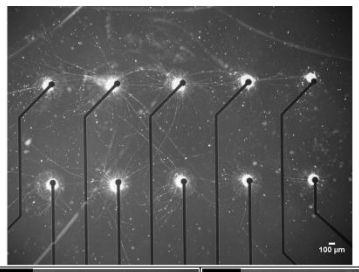


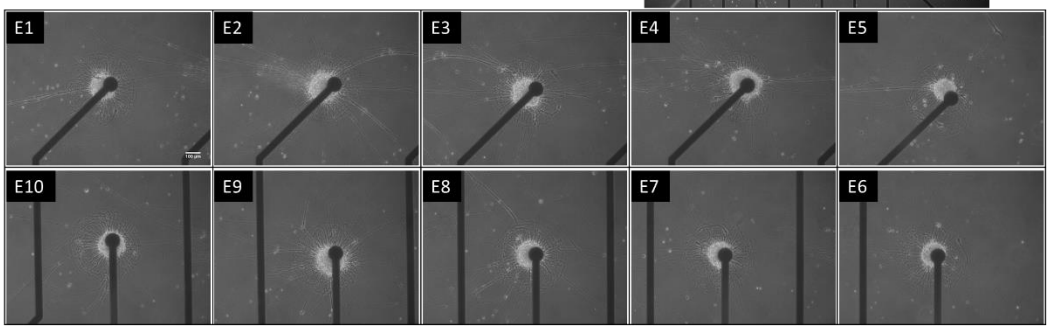




DIS 10, System 26 A

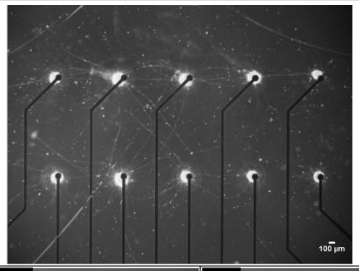



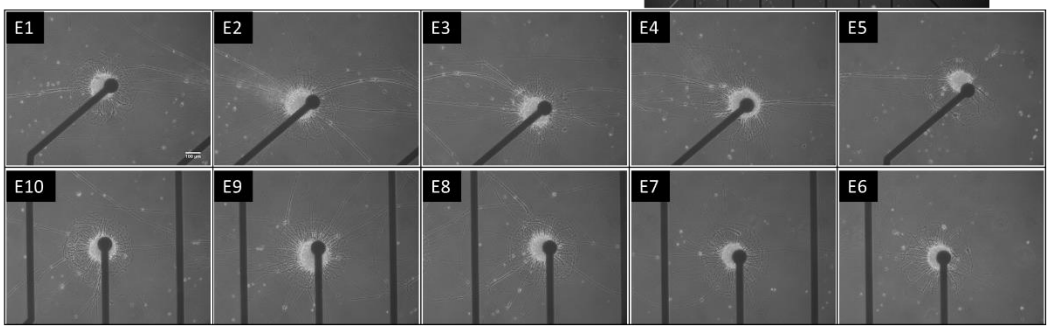
DIS 13, System 26 A



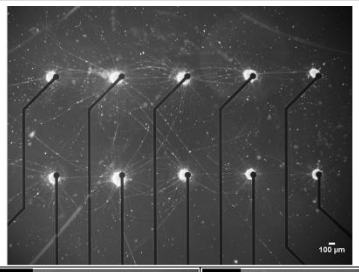



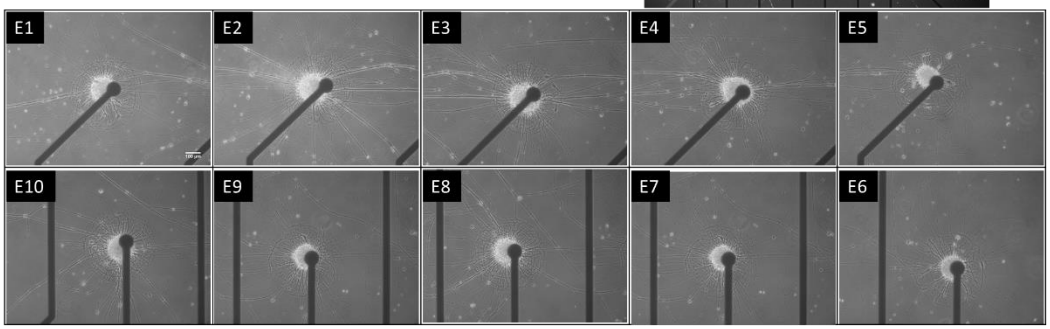

DIS 14, System 26 A



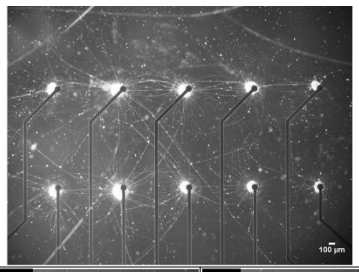



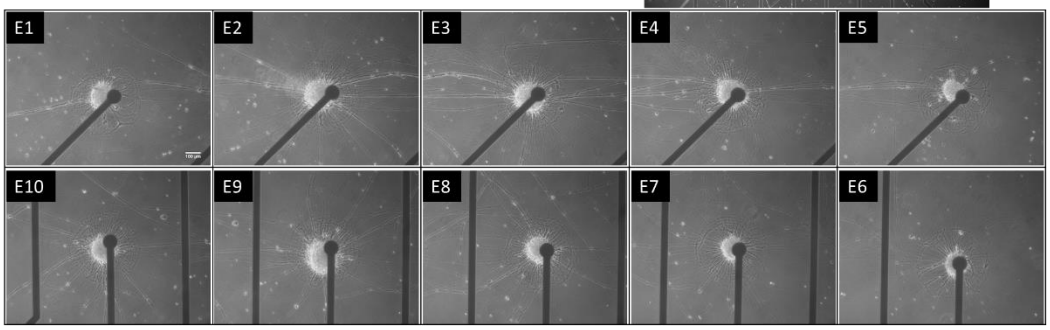

DIS 16, System 26 A



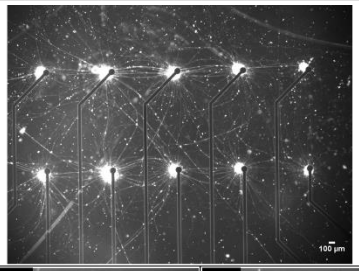



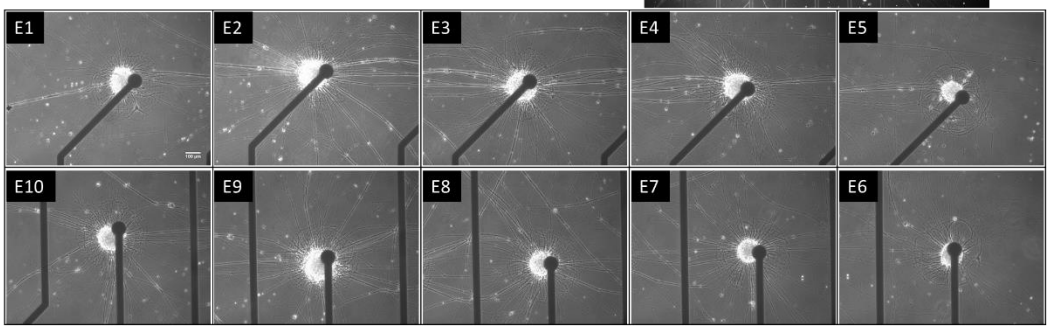
DIS 17, System 26 A



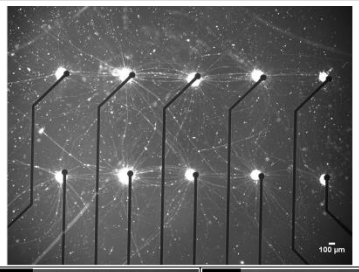



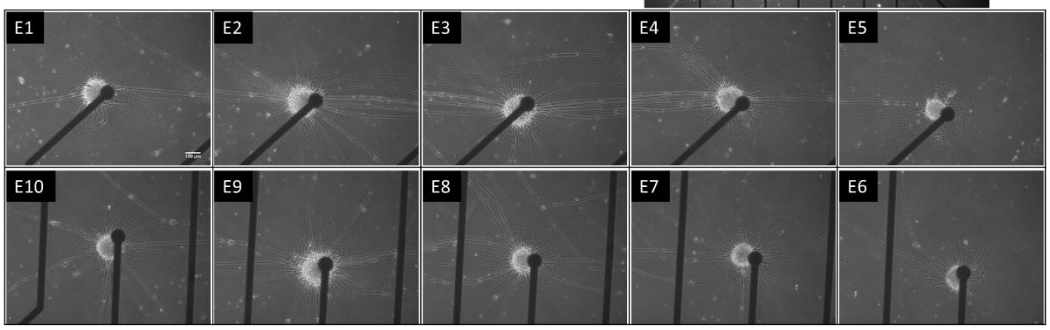

DIS 20, System 26 A



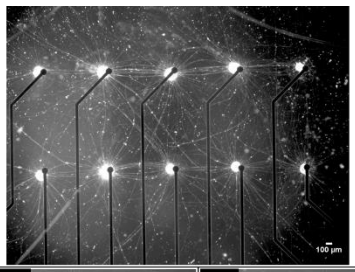



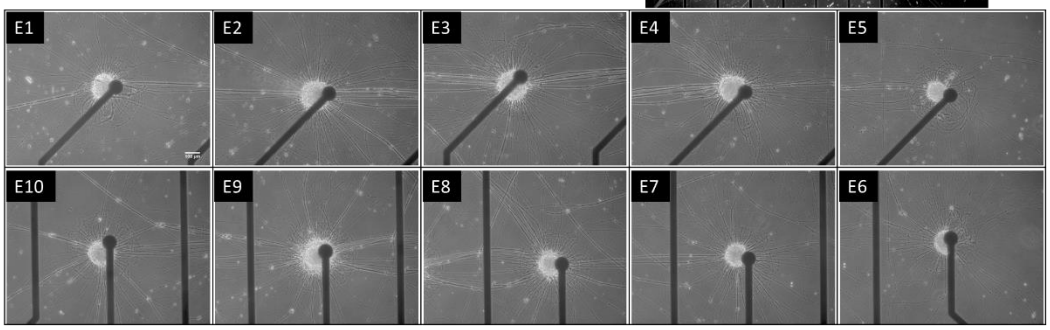

DIS 22, System 26 A



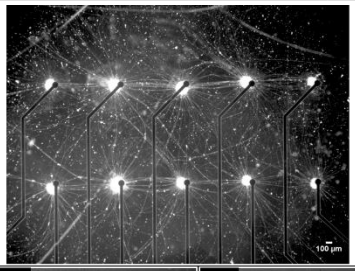



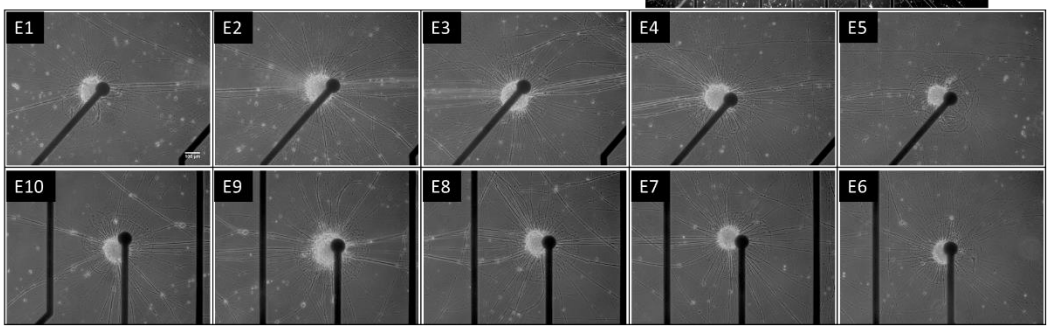
DIS 23, System 26 A



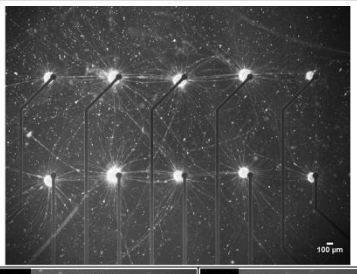



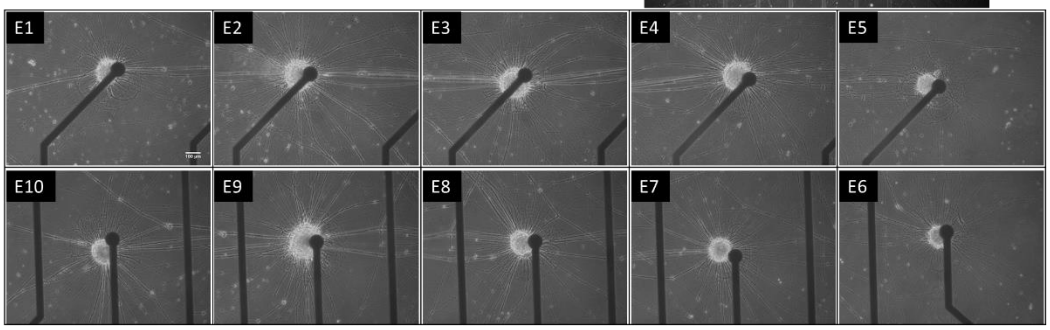

DIS 24, System 26 A




DIS 28, System 26 A



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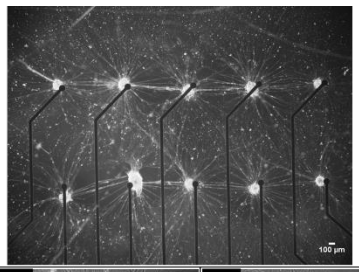


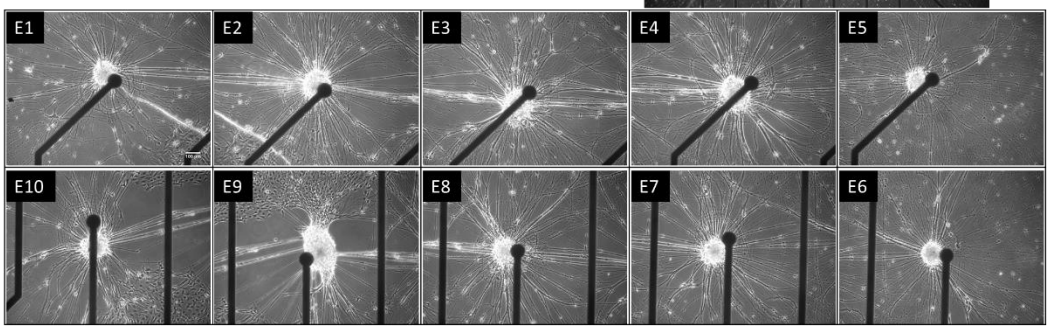


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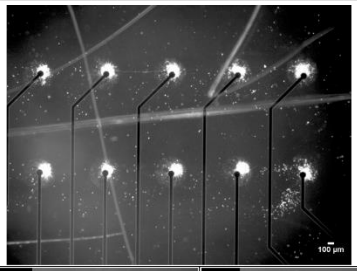


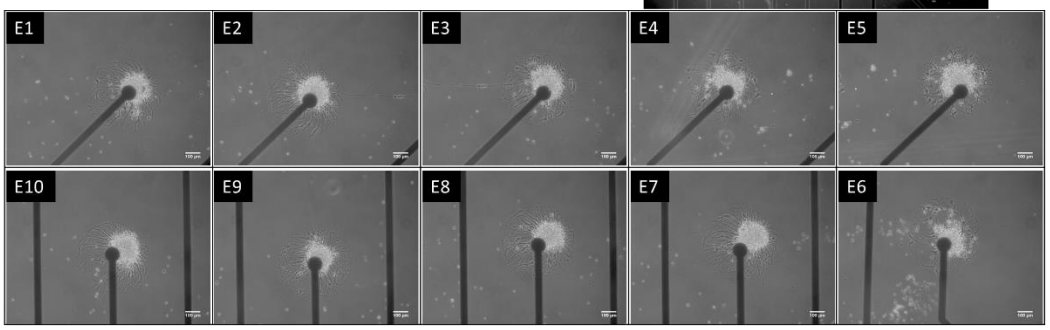


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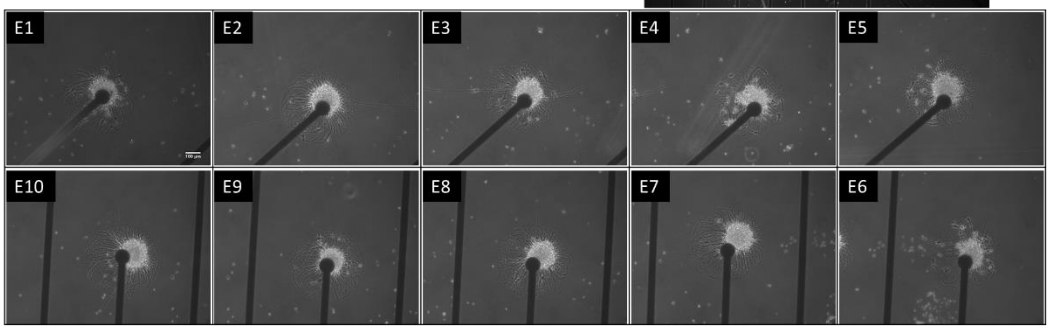

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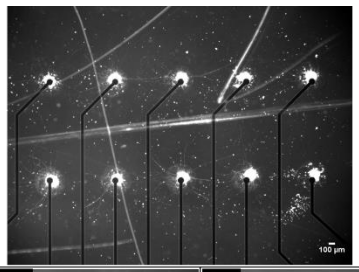

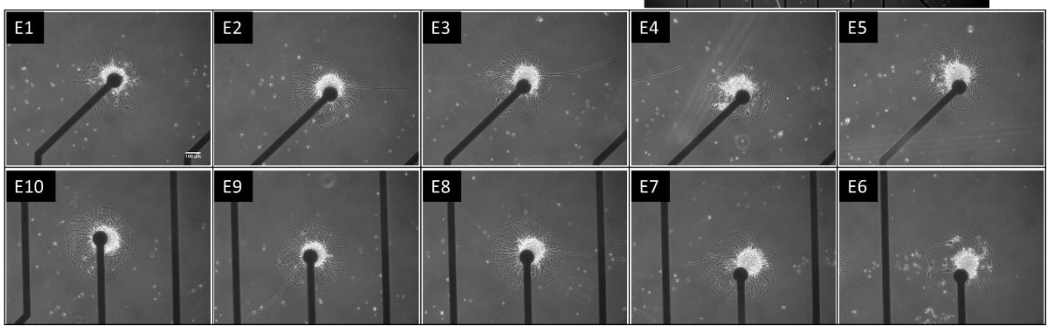


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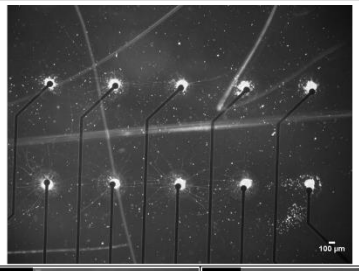

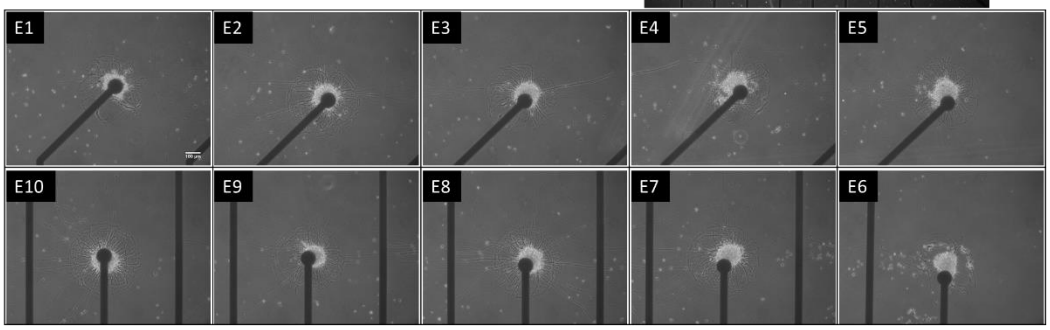


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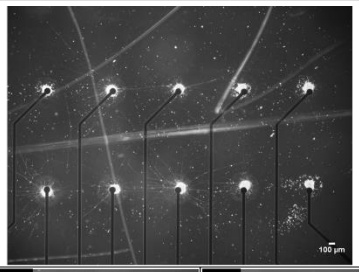

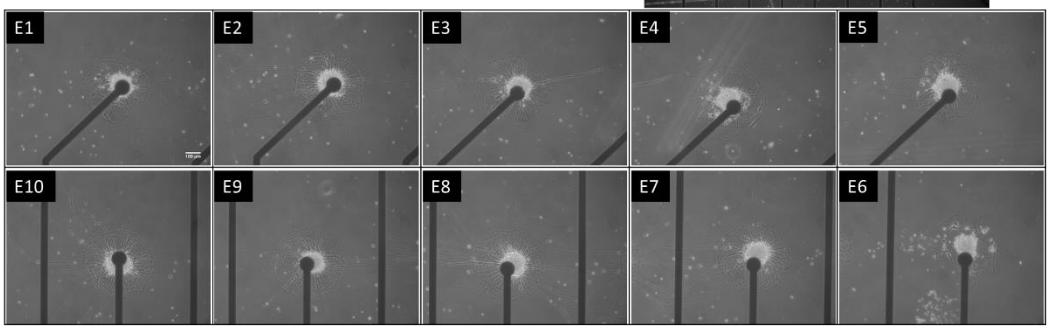

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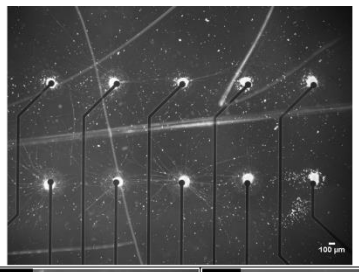


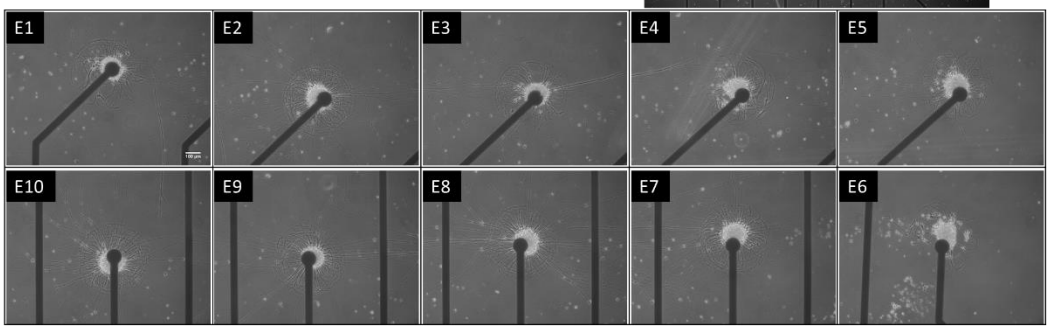


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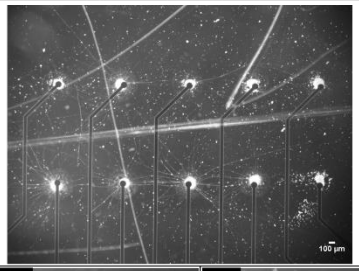


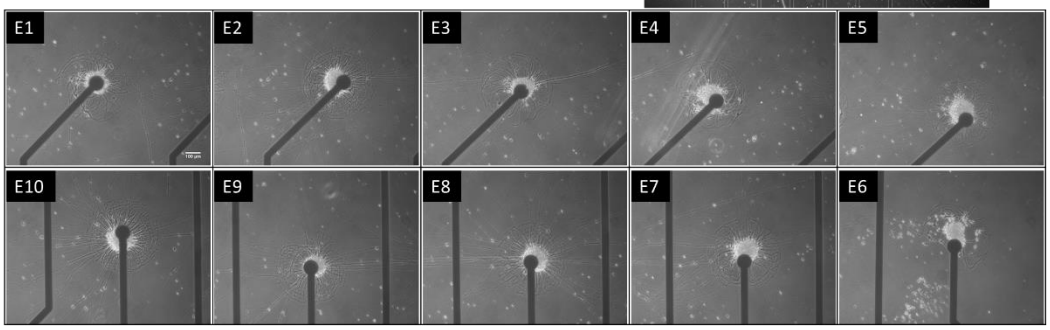


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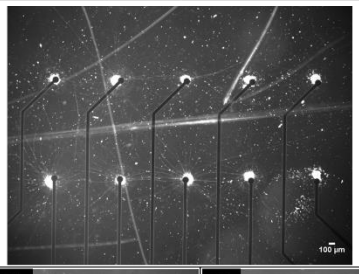


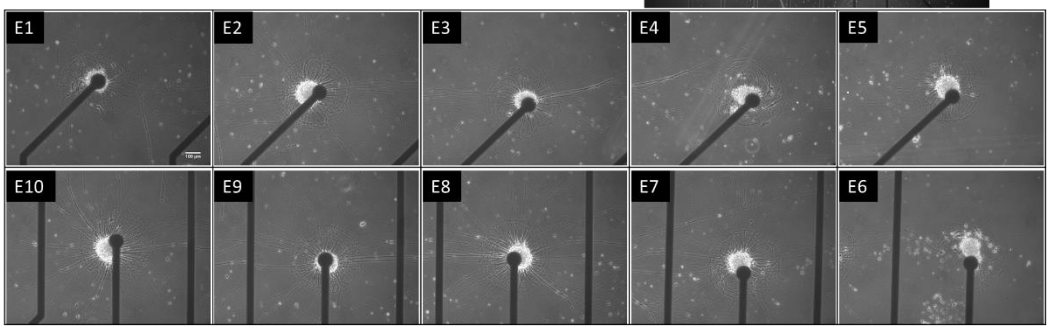

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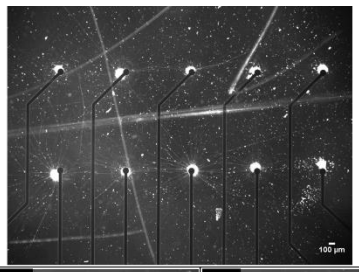

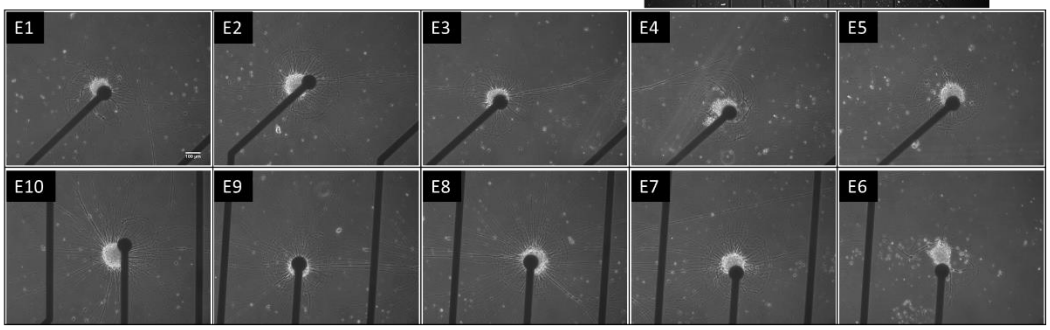


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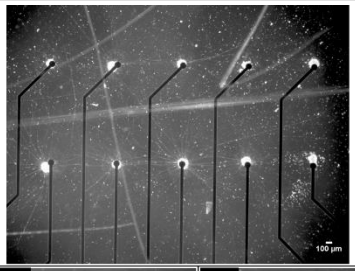

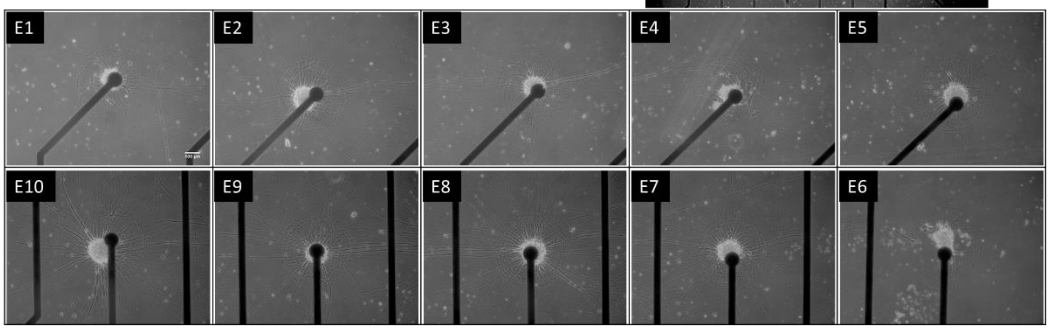


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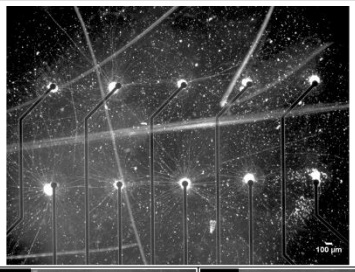

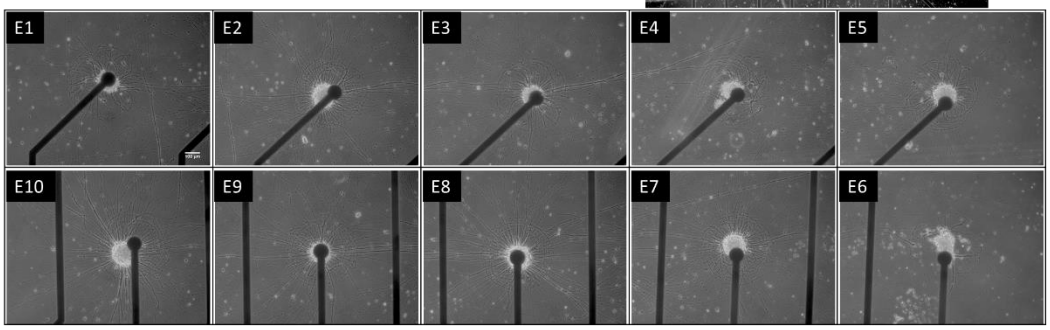

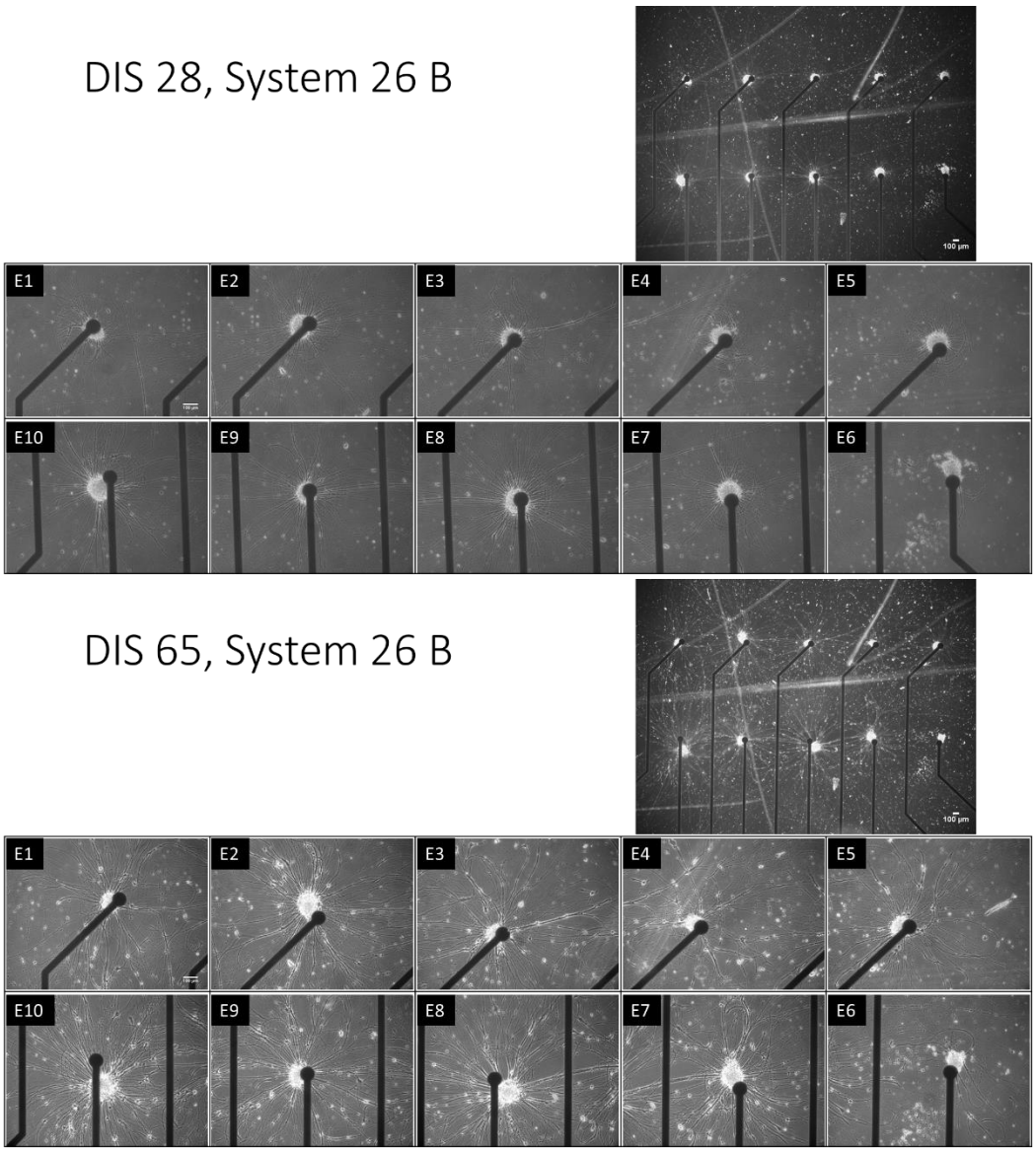


Figure B0-1: hMN coated cMEAs up to 66 days, assembled in system housings for electrical signal recordings

# APPENDIX C: PLOTS FOR INDIVIDUAL CMEA RECORDINGS

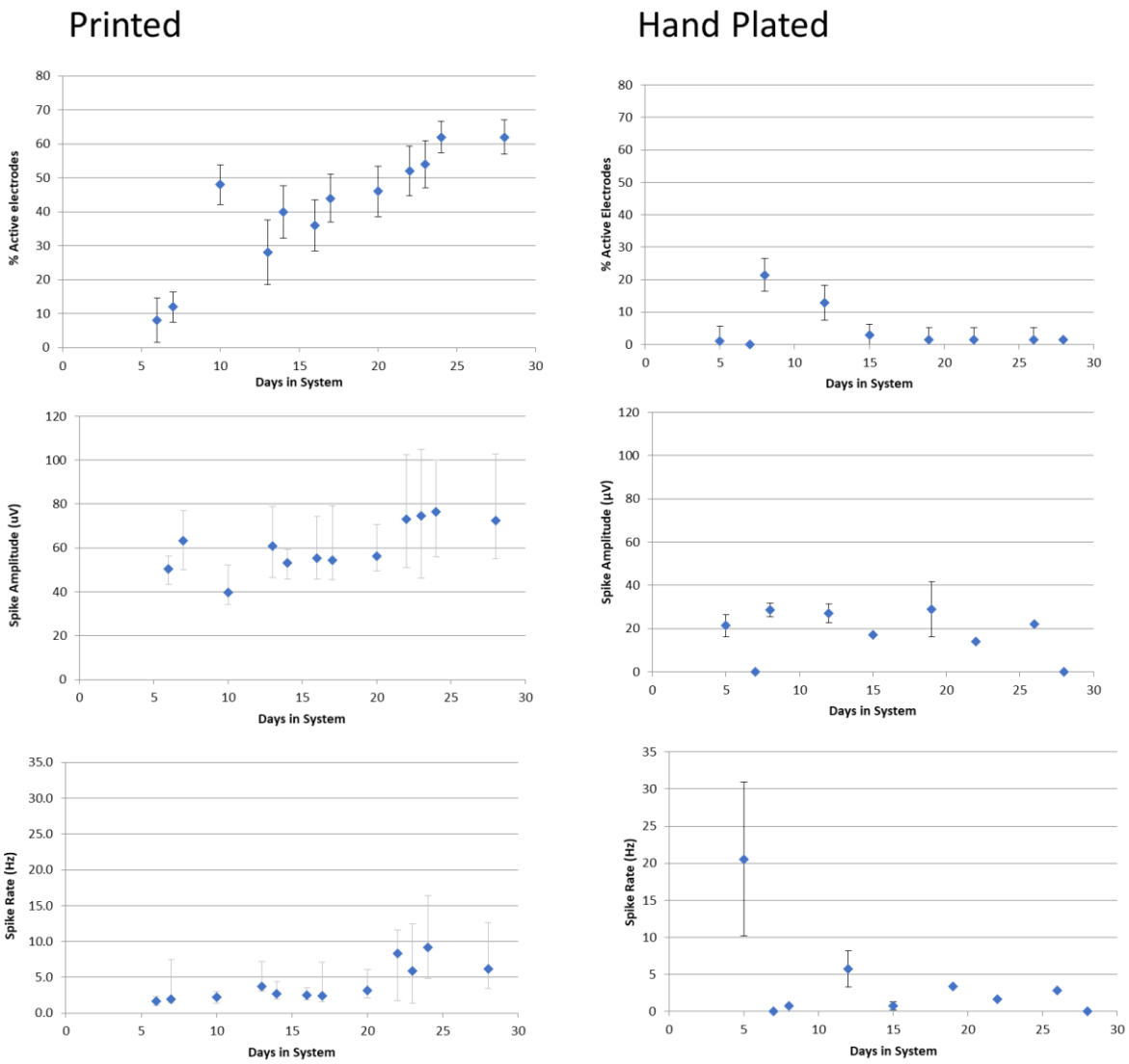


Figure C0-1: Recordings from cMEA chips in systems

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