

Development of a Novel Chip-Based Platform for Long-Term Neuronal Culture, Stimulation, and Imaging

By

John Gerald Vincent II

Submitted to the Department of Mechanical Engineering in Partial Fulfillment of the Requirements for the Degree of

Bachelor of Science

at the

Massachusetts Institute of Technology

June 2009

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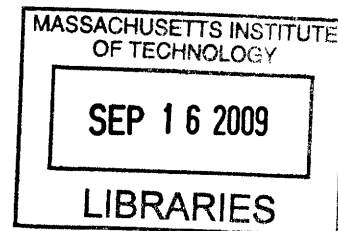
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ABSTRACT

High-throughput cell-based screening has recently been emerging as a feasible technology for drug testing and discovery. However, currently available tools for electrical assaying are expensive, have limited reproducibility, and are unable to sustain long term studies.

The Bioinstrumentation Laboratory at the Massachusetts Institute of Technology has developed a novel, 3D MEA for use in long term study of neuronal cells. These arrays will be cheap and disposable, allowing integration into a fixed stimulation and imaging setup. Neuronal cell activity will be monitored using a Ca^{2+} sensitive dye that can be visualized via the imaging setup.

In the past year, the Bioinstrumentation Laboratory has worked to refine the parameters required to generate replicable, conductive, biocompatible columnar polypyrrole posts using a polydimethylsiloxane mask. In addition, these posts have been shown to support neuronal cell growth.

However, proof of concept requires that the neurons growing on these disposable arrays be able to be selectively stimulated via a graphical interface. The work presented in this thesis describes the development of this interface in a LabVIEW environment. The interface uses the channels of a data acquisition board to individually control the electrodes comprising the MEA. Unlike currently available electrode stimulators, ours allows for the selection of multiple electrodes, rather than just one or two, and a user selected patterning firing sequence. The proposed design was demonstrated using an array of LEDs setup on a bread board. In addition to the development of an electrode stimulator, reliability testing of the electrical contact between the posts and electrodes on a commercially available MEA was tested by seating the array on top of

the MEA. However, to receive better results, the laboratory will plasma treat the surfaces to allow better contact between the surfaces.

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ACKNOWLEDGEMENTS

I would like to first thank Professor Ian Hunter for giving me the opportunity to work in the Bioinstrumentation Laboratory. It had always been my goal to work in his laboratory since my sophomore year at MIT. He and the Bioinstrumentation Laboratory have continued to stimulate my interest in the intersection of biology with mechanical engineering. I am grateful for the kindness and understanding he has shown me.

I would also like to thank Dr. Cathy Hogan for her help, guidance, and patience throughout this project. She perpetually conveyed a sense of excitement and importance with regard to my research project. Dr. Hogan has taught me the value of taking ownership.

In addition, I would like to acknowledge Scott McEuen and Adam Wahab for their help with the LabVIEW aspect of my project. Without their help, I would have probably spent more than enough hours banging my head against a wall.

A special thanks to all my friends, specifically Michael “Krack” Kerekes, Edgar Twigg, and Yuta Kuboyama. The four of us spent many long nights that turned into early mornings in the dining room working on problem sets and our respective theses. Without each others company, many of those problems sets would not have been finished.

I would also like thank my parents for supporting my endeavors and goals of becoming an engineer. I can never do enough that will repay them for the opportunities they have given me.

Lastly, I would like to thank the Department of Mechanical Engineering and the Massachusetts Institute of Technology. Without the carefully crafted curriculum and resources provided to me during the past four years, I would not have had the skill set to take on this project with competence. The “Institvte” has given me an experience the past four years that will be sincerely missed.

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1. INTRODUCTION

The overall goal of the project is to develop a novel three-dimensional MEA (3D MEA) for the study of neuronal cell cultures with an associated imaging system to allow for visualization post stimulation using Ca^{2+} sensitive dyes

Polypyrrole (PPy, Figure 1) is a conducting polymer, a recently developed class of materials. Conducting polymers are an attractive material due to their wide range of applications (sensors, actuators, LEDs, 3D co-micro-fabrication, batteries) (Madden 2000). In addition to polypyrrole being a conducting polymer, it is biocompatible (Madden 2000) and can be found in the biotechnology field (*e.g.* it is used as a scaffold for nerve regeneration [Madden 2000]).

Because polypyrrole is biocompatible and conductive, it is an excellent choice for our application of cell growth and stimulation. Columnar polypyrrole electrodes will allow the growth of neuronal cells atop a glial cell bed, which is essential for long-term culture. In addition, the porous nature of the columnar polypyrrole electrodes will be conducive to a more reliable electrical contact between the cells and the electrodes than that of current MEAs.

This thesis is a continuation of previous work and describes the development of the electrical stimulation and interface system.

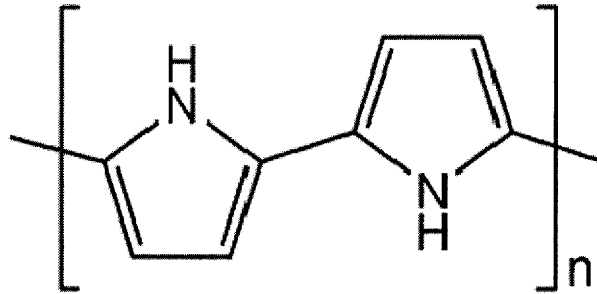


Figure 1: Chemical structure of polypyrrole where “n” is any number, taken from Talei-Franzesi 2008.

1.1. Brain Plasticity

Brain plasticity is responsible for the wiring of the neural network during the life time of an organism. Most neural network formation occurs early in the developmental stage of life. However, plasticity also occurs at any stage in the life cycle in the form of synapse strengthening.

1.1.1. Spike Time Dependent Plasticity

Spike Time Dependent Plasticity is believed to be the process responsible for the strengthening or weakening of synapses. By activating synapses within 20 ms to 40 ms before a postsynaptic spike event, the synapse is strengthened. Similarly, to weaken a synapse, the neuron is activated in a similar time window after a spike event. (Constantine-Paton 2006).

1.2. Current Technology and Limitations

Current commercially available MEAs (www.multichannelsystems.com) only allow two types of electrophysiological data collection: field potentials and single neuron action potentials (spike events) in cell culture. While these MEAs allow for a layer of glial cells to adhere to its surface, the planar design causes the glial cells to form an insulation layer that prevents the electrodes from stimulating the neurons growing on top. Higher

voltages can be applied to the electrodes to overcome the problem; however, the increased current will most likely kill the glial cells which are necessary for long-term study. In addition, the software provided by Multichannel Systems only allows for the stimulation of a few electrodes at a time.

The cost of current MEAs also proves to be too high to be pragmatic for screening environments that require a large quantity of tests to be performed. Neurosilicon offers packages, with controller and array, starting at \$2,275 and Multichannel Systems entire system, including arrays, costs \$57,000.

1.2.1. Conducting Polymers

Conducting polymers are an unusual class of organic materials materials that have a high electrical conductivity that can be used in a wide range of applications. Specifically, conducting polymers can be used as actuators, sensors, and in the fabrication of micro mechanical devices (Madden 2000).

Conducting polymers have a high degree of conjugation – alternating single and double bonds in the backbone of polymer (Madden 2000). This conjugation allows delocalization of electrons throughout the π -orbital network, giving rise to the semi-conductive nature of these polymers (Madden 2000). To make these polymers more electrically conductive, the oxidation state of the polymer is changed either chemically or electrochemically through a process called doping (Madden 2000). Similar to the doping process used for silicon, the polymers are reduced or oxidized to become negatively or positively charged, respectively (Madden 2000).

1.2.2. Conducting Polymers in Biotechnology

In addition to the many roles that conducting polymers can play in actuators and sensing elements, these polymers have made their way into the field of biotechnology. More specifically, polypyrrole is the most ubiquitous of the conducting polymers in biotechnology. Its electroactive properties and its potential for incorporation of biomaterials can help control cell morphology and behavior *in vitro* (Talei-Franzesi 2008).

1.2.3. Applications

Cell-based screening is becoming increasingly more popular in drug discovery (Dragunow 2008; Dove 2003). Arrays of living cells that mimic *in vivo* behavior are more biologically relevant predictors of how an organism might respond when exposed to a particular drug. In particular, neuronal cell arrays have the ability to respond to a range of neuroactive compounds. As such, they provide a system that can be used by researchers to identify relevant neuropharmacological compounds in drug research when combined with the appropriate electronic interface.

While many of the cell based screening methods measure multiple parameters of individual cells within wells or plates (Dove 2003) our system will enable network level effects to be evaluated. This is particularly important for the study of brain plasticity (ie. the organization of the neuronal networks). By allowing the user to select specific electrodes and firing sequence, synapses can be strengthened or weakened, allowing subsequent study.

1.3. Previous Work

Current MEAs have limited reproducibility, are expensive, and are unable to sustain long-term studies. In addition, when a co-culture of glial cells is used for long-term studies, the glial cells form an insulating layer and degrade the electrical contact between the electrodes and neurons.

Several studies have shown that surfaces modified topographically affect the growth of neurons and their orientation (Dowell-Mesfin, *et al.*, 2004; St. John, P.M. *et al.*, 1997) and neurite growth is augmented when electrically stimulated through oxidized polypyrrole film (Schmidt *et al.*, 1997). It has also been studied that polypyrrole can be coated with various biomolecules, such as laminin, poly-lysine, and polyethyleneimine to improve contact with the glial cells (Morin, F.O. *et al.*, 2005).

Because of the current problems that planar MEAs present and the advantages of using conductive polymer posts, the Bioinstrumentation Laboratory has been developing an inexpensive, disposable 3D MEA.

Since the preliminary work on the development of columnar polypyrrole by Giovanni Talei-Franzesi, 2008, the Bioinstrumentation Laboratory has refined the fabrication process. Briefly, a bilayered mask is fabricated by reversibly bonding two sheets of implant-grade polydimethylsiloxane (PDMS) either 250 μm , 125 μm , or 50 μm thick – (Figure 2, taken from Talei-Franzesi, 2008). The mask is laser patterned using a standard CO₂ laser. The laser micromachining results in a slightly (approximately 5°) conical cavity, which ensures mechanical stability of the final electrode array. The mask is adhered to a glassy carbon electrode, and polypyrrole electrodeposited through it. The pillars are hollow because of interfacial phenomena that cause the sides of the pillar to grow faster than the center.

The construct is removed from the deposition bath and the upper, sacrificial mask layer is carefully peeled away. The relative rates of lateral and central polymer growth depend on monomer concentration, current density and deposition time, allowing for control over the thickness of the structures. The same approach may be used to fabricate layered structures with varying composition.

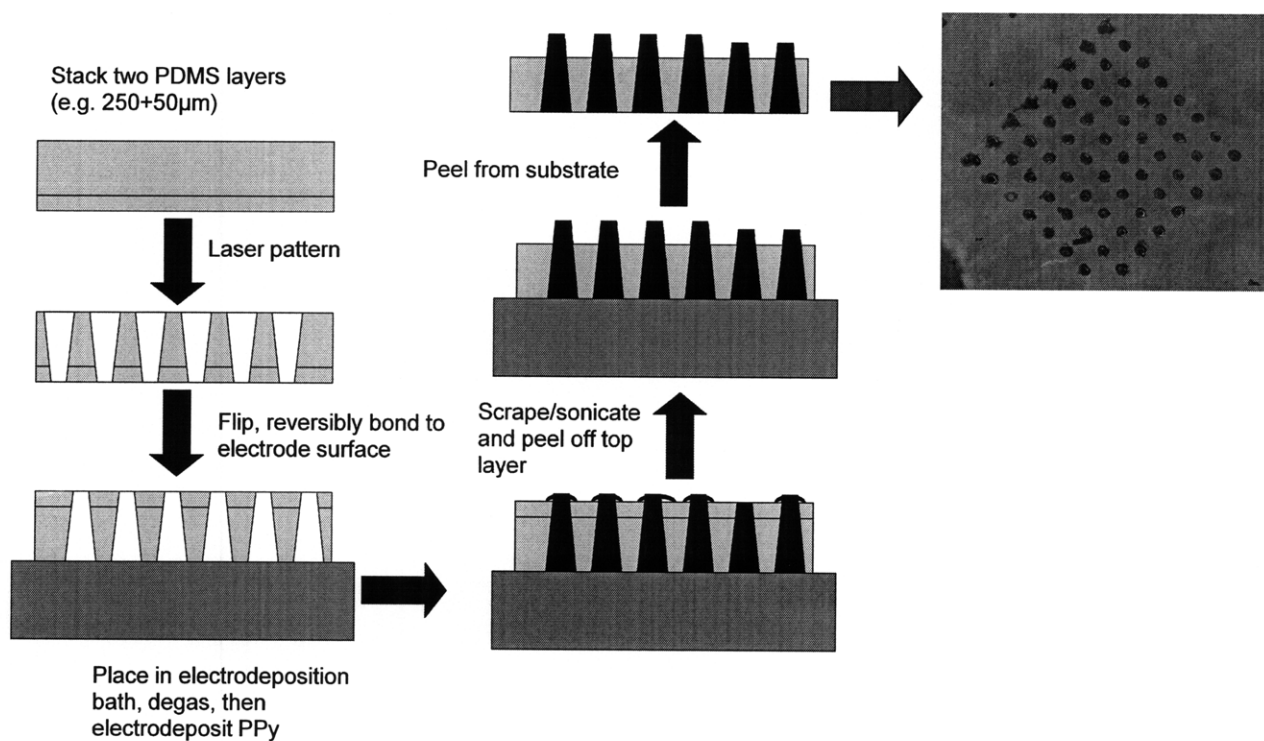


Figure 2: Schematic of fabrication of the polypyrrole columns. The polypyrrole columns are made by stacking two layers of PDMS together. A laser patterns the shape of the columns into the PDMS. The PDMS is flipped and allowed to adhere to an electrode, which allows for the electrodeposition of polypyrrole. Lastly, the top layers of PDMS are removed. Figure taken from Talei-Franzese, 2008

1.4. The Need for an Electrode Stimulator

Multichannel Systems currently offers software packages that can be used with their MEAs to stimulate user selected electrodes. The commercially available software has been designed with an emphasis on electrical recording and stimulation being limited to one, or at most, a few electrodes. However, the Bioinstrumentation Laboratory requires that the design be a highly parallel stimulation system (*i.e.* be capable of selecting multiple electrodes at once), have the ability to pattern electrode firing, and be capable of updating electrode selection in real time. As the system will rely on optical methods to record neural activity, it must be integrated into an optical recording system.

Initial work on a stimulator was based on work by Wagenaar and Potter, relying on an 8-bit Digital-to-Analog Controller (TLC7628, Texas Instruments) (Talei-Franzesi, 2008). This design would allow for the selection of any electrode in an array of sixty-four and rapid switching (approximately 10 μ s) between different ones. However, it was decided this system had many draw backs: unreliability, unfriendly user interface, and crash prone. As we decide to develop a custom system for our application.

1.5. Interface between Columnar Posts and MEA

The feasibility of using the stimulation and imaging interface depends on the ability to obtain good electrical contact between the disposable conducting polymer array and the underlying fixed, patterned electrodes. In order to evaluate the reliability of contact, an array of polypyrrole columnar posts was adhered to a MEA (an EcoMEA) purchased from Multichannel Systems. The EcoMEA was used as a first iteration to show that the concept of stimulating polypyrrole posts with our electrode stimulator worked. In the future, the Bioinstrumentation Laboratory will make its own arrays to adhere the posts to as the MEAs are expensive. The EcoMEA had a spacing of 700 μ m between electrodes

with an electrode diameter of 100 μm . The geometry of the columnar posts will allow for better contact between electrodes and the neuron cells in future studies.

1.5.1. Oxygen Plasma Treatment

PDMS is the most widely used material in the construction of microfluidic devices, primarily due to its rapid prototyping. PDMS' hydrophobic nature makes it difficult to bond to surfaces. A number of efforts have been made to augment the hydrophilicity of the surface (Bodas *et al.* 2006).

The most popular method to make the PDMS surface more hydrophilic is oxygen plasma treatment. However, the hydrophilic characteristics of the plasma treated surface slowly revert back to the natural hydrophobic state of the PDMS over time (Bodas *et al.* 2006). As a first iteration, the Bioinstrumentation Laboratory will adhere the polypyrrole arrays to the Multichannel Systems' EcoMEA to test the reliability of the post-electrode contact. As a result, there is a need to bond PDMS to the surface of the EcoMEA via oxygen plasma treatment. The strongest bond that can be made is PDMS-PDMS, but the Bioinstrumentation Laboratory will look into other PDMS bonding (*e.g.* PDMS-glass), as this will be necessary for the bonding of the polypyrrole array to the EcoMEA surface.

2. THE ELECTRODE STIMULATOR

With the needs of the ideal electrode stimulator in mind, we designed a system to have a simple user interface, allowing the selection of multiple electrodes, and the stimulation of the electrodes in a patterned sequence while also being able to adjust the electrode firing sequence in real time. While the programming and electronics could be very complex, the system was designed with the scientist in mind and made very intuitive.

2.1. The Proposed Design

After making way through our initial iterations, the design approach was based on a three step process: (1) Make a LED on the computer blink, (2) Make a real LED controlled by the computer blink, and (3) Stimulate a post. This approach provided an easy way to design a baseline for the code used in the final program design and an easy way to modify the code as it moved through each stage of the design process.

2.1.1. Initial Iterations

The initial plan was to use an 8-bit microcontroller and interface it with MATLAB to control the selection of electrodes and firing sequence. While it would have been fairly straight forward to program the microcontroller to control the LEDs, it would not have been as trivial to design an associated graphical user interface. As a design requirement, we wanted the program to be very intuitive and simple for someone with no knowledge of computer programming. As a result, this idea was abandoned and it was decided that LabVIEW provided the simplicity in designing an intuitive user interface while not having to sacrifice quality of control obtained from using a microcontroller.

2.1.2. *Blinking Computer LEDs*

A simple graphical interface, shown in Figure 3, was designed to make controlling the electrodes a simple task. This graphical interface could be combined with the two data acquisition cards (NI PCI-6723, 32 analog output channels, connected to act as one 64 channel device) that were being used in the previous study conducted by Talei-Franzesi (2008). This program can control 64 channels independently, allowing the user to select a pattern of electrodes for stimulation and the ability to modify this pattern in real time.

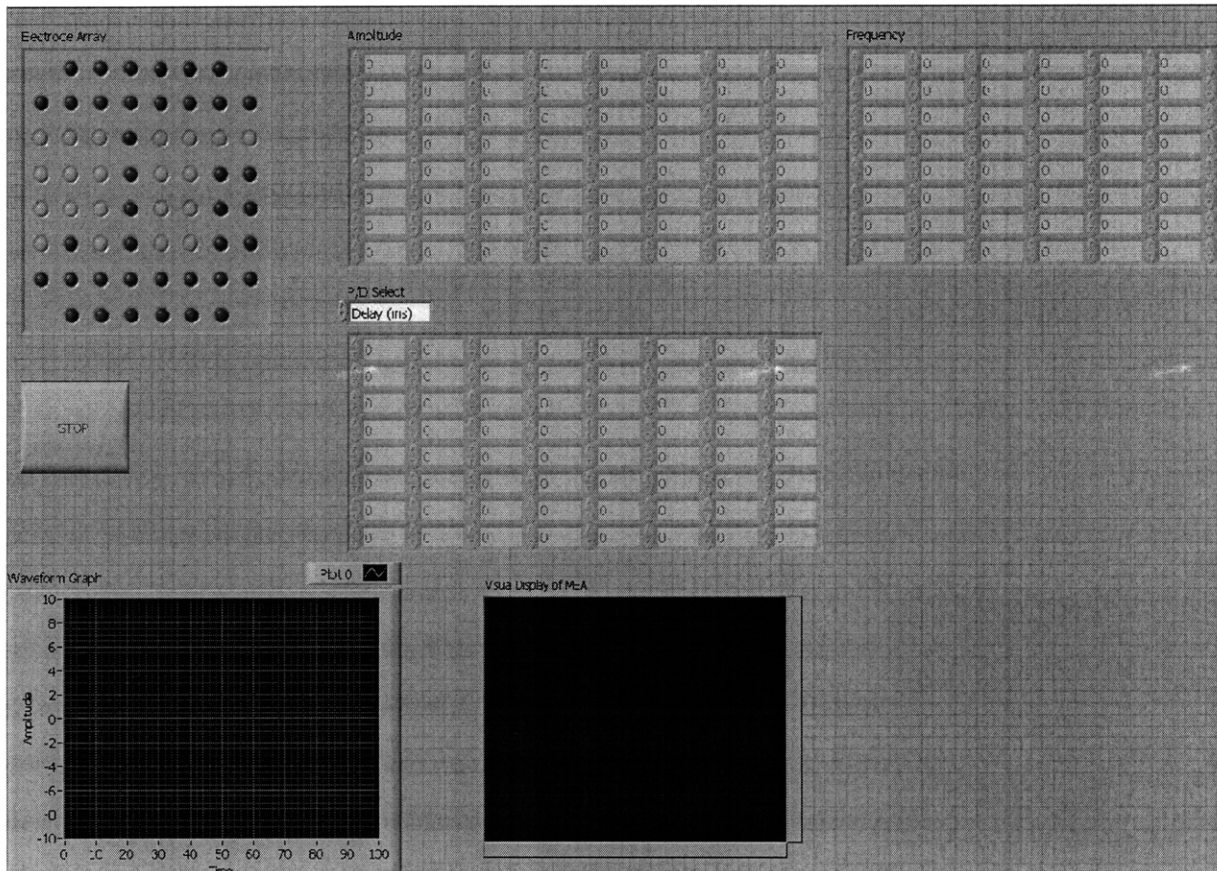


Figure 3: Graphical user interface of the electrode stimulator.

LabVIEW 8.6 was used to design the user interface. The advantages of using LabVIEW are that it is simple, can be adjusted quickly, and can be understood more quickly than reading or writing code.

Figure 4 shows the LabVIEW code that controls the interface in Figure 3. The outer most rectangle with rounded edges (which represents a “while” loop) in Figure 4 allows the program to run continuously; the modifying in real time element. Moving from left to right, four arrays that the user interacts with (amplitude, frequency, phase or delay, and electrode array) enter two rectangles (which represent “for” loops). These two loops scan all four arrays at the same time and correctly match all elements of the arrays with each other (*i.e.* match the electrode selected with the desired frequency, amplitude, and delay). The case structure at the bottom of the inner “for” loop allows the user to select between delay in milliseconds or phase delay in degrees. The case structure at the top of the inner “for” loop controls whether or not an electrode is stimulated. This is based on the electrode selected in the user interface shown in Figure 3.

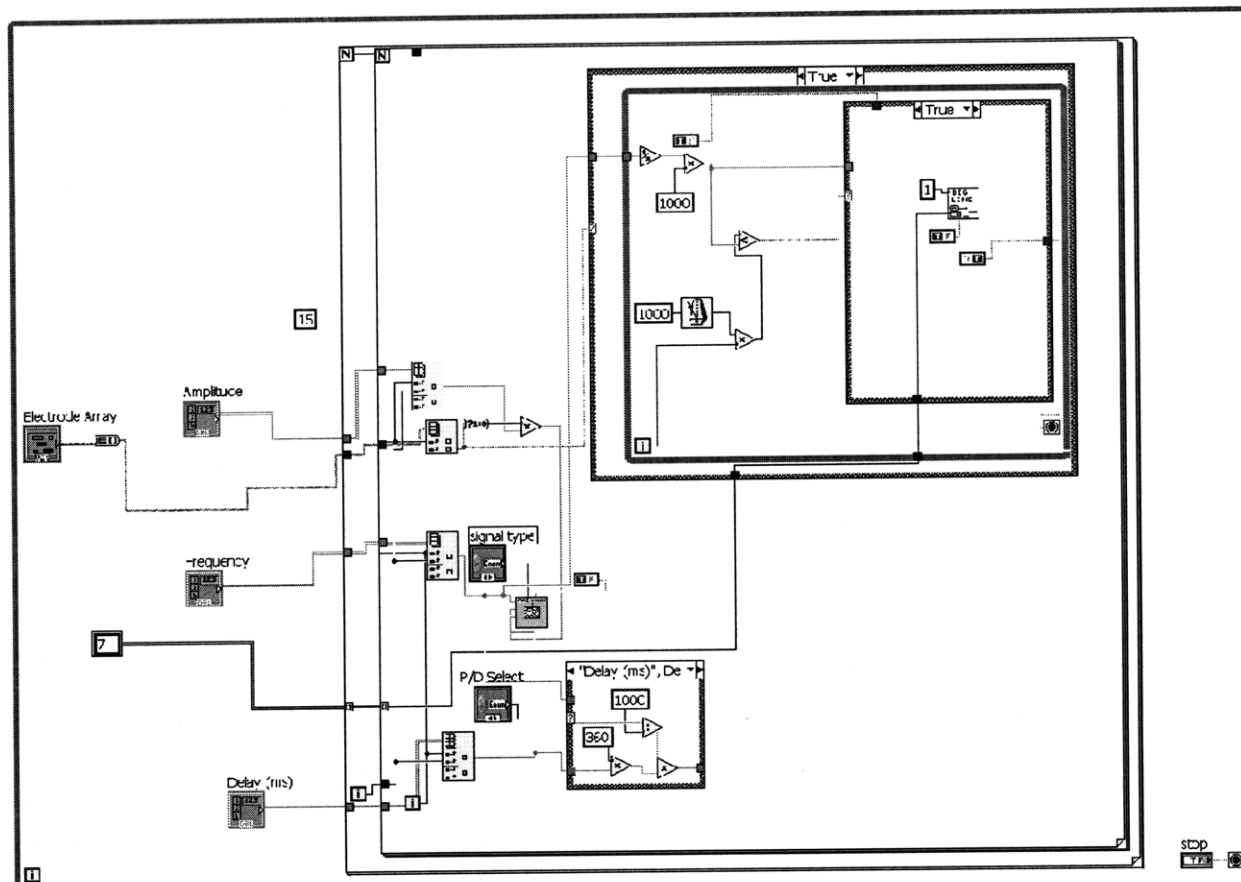


Figure 4: Block Diagram that controls the graphical user interface in Figure 3.

2.1.3. Controlling Real LEDs

After successfully making on screen LEDs blink and a satisfactory user interface was designed, an array of real LEDs was setup on a bread board to test the proposed design, Figure 5. A data acquisition board (DAQ) (DAQPad -6052E, with eight digital output channels) was interfaced with the computer and bread board. For this test, a simpler interface and block diagram were used to prove the concept, Figure 6 and Figure 7, respectively. After confirming the LEDs could be properly controlled, this simple design was then use to test on the contact between the polymer posts and the Multichannel Systems MEA.

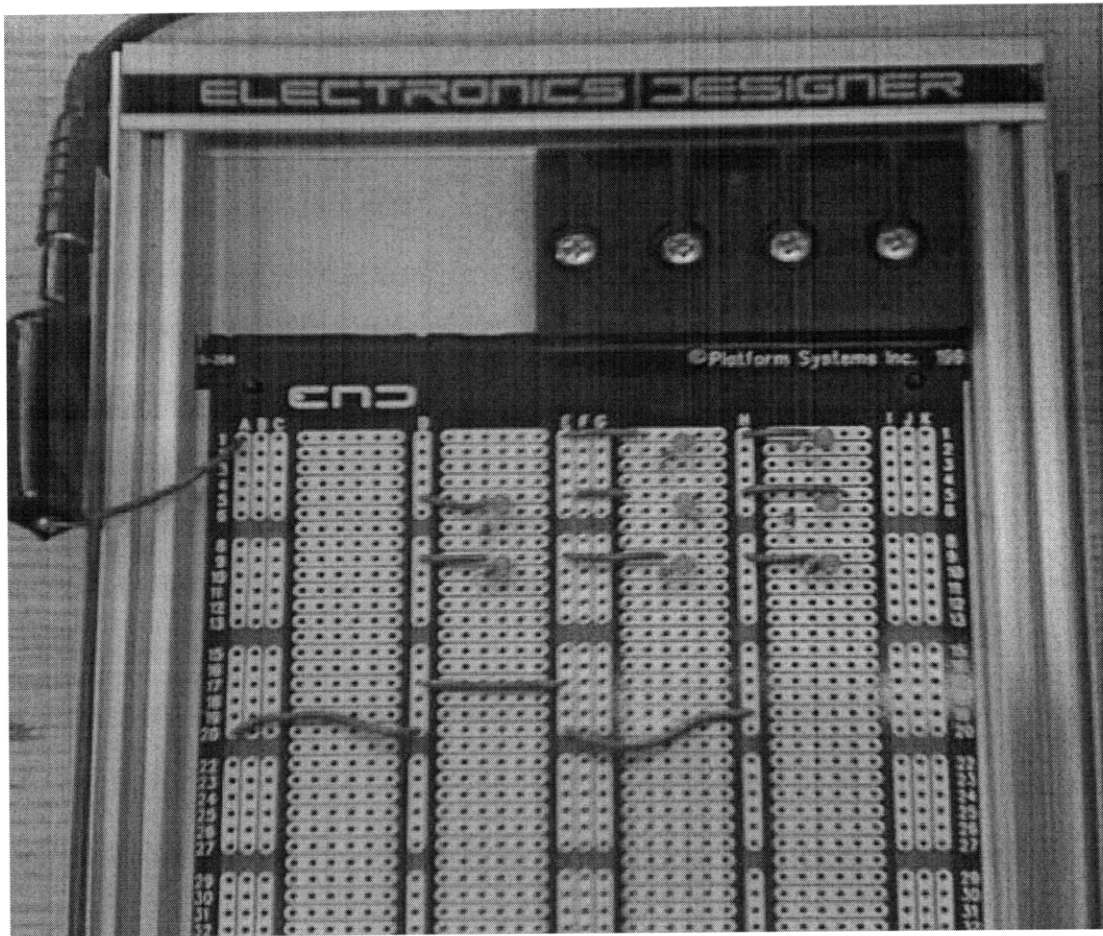


Figure 5: LED array setup

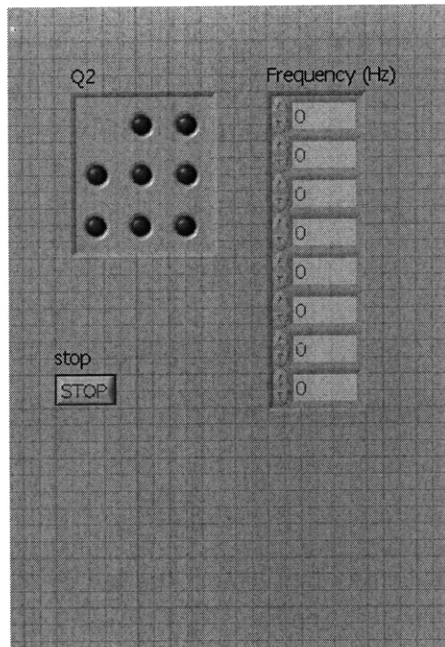


Figure 6: The simplified user interface used to test the proposed design.

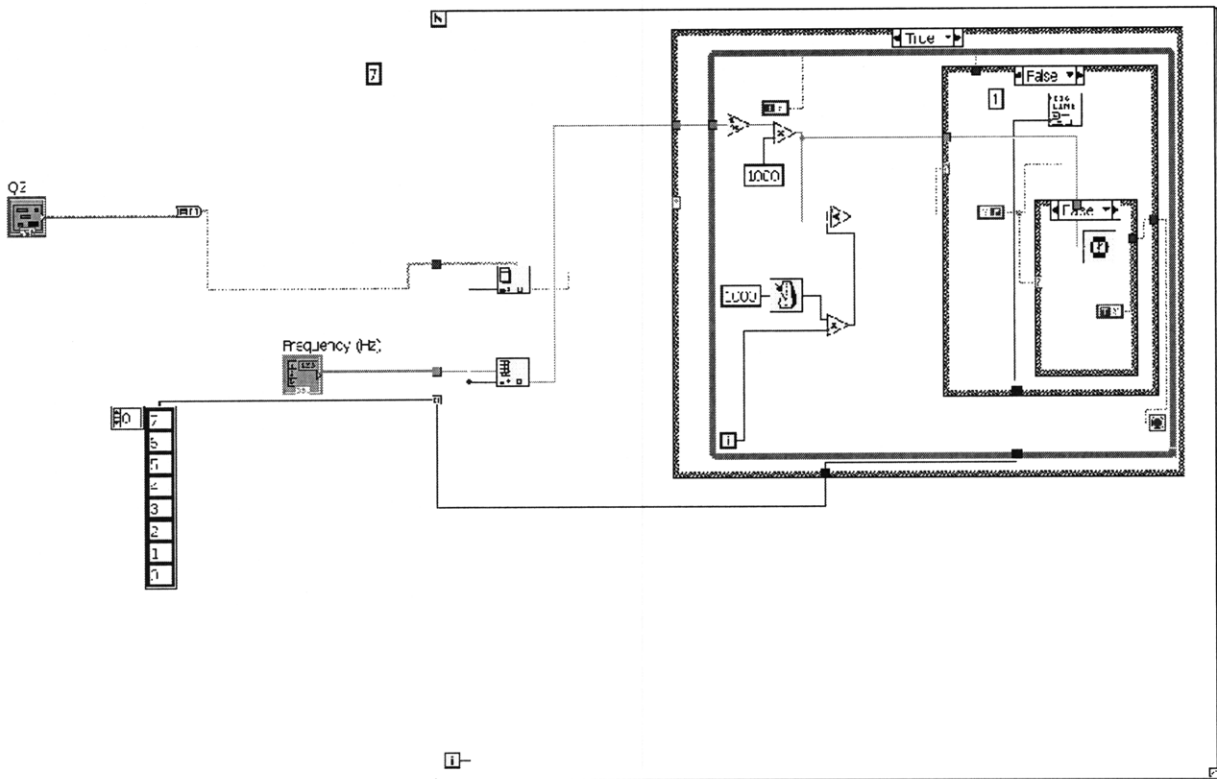


Figure 7: Simplified Block Diagram used for testing the proposed design.

3. INTERFACE BETWEEN MEA AND POLYMER POSTS

An important step in the assembly of the stimulation interface is to ensure that reliable contact can be made between the disposable conducting polymer arrays and the underlying fixed, patterned electrodes. In order to test reliability of contact, preliminary resistivity tests of the columnar posts were conducted.

3.1. Interfacing Polypyrrole Posts with the MEA

The final step in this study was to test the quality of the electrical contact between the columnar, polypyrrole posts and the EcoMEA purchased from Multichannel Systems. This was attempted initially by adhering the array to the micro electrode such that the posts appeared to be in contact with the electrical contacts on the MEA (Figure 8).

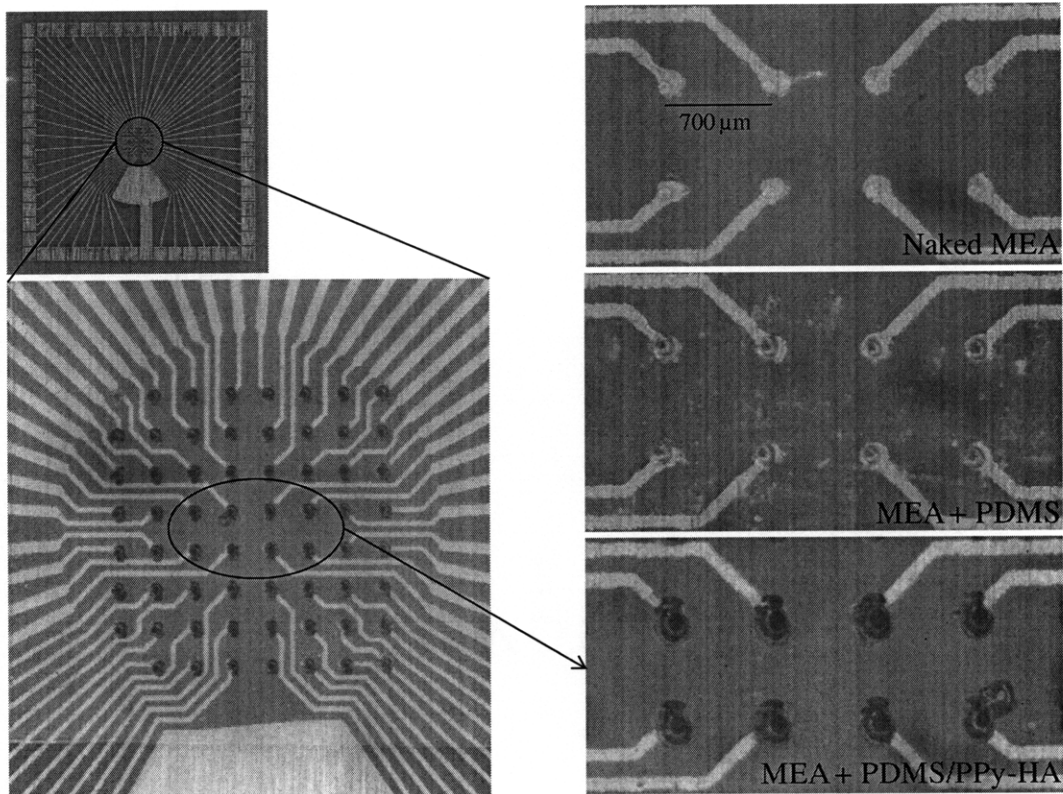


Figure 8: EcoMEA from Multichannel Systems to test reliability of electrical contacts.

3.2. Preliminary Resistivity Measurements

The resistance of a post was measured by using a needle-like electrode placed in a micromanipulator. The micromanipulator was used to make the needle contact a single post. The circuit was completed by contacting another lead to the small square on the perimeter of the EcoMEA that corresponded to the electrode the post was sitting on top of. Using a handheld Fluke 189 voltmeter, the resistance of the post was measured. A total of five posts were measured five times each, Table 1 shows the results.

Table 1: Results from testing the resistance of multiple posts. In the post designation, r=row and c=column. OL indicates a closed circuit was not made.

Post Designation	Test 1	Test 2	Test 3	Test 4	Test 5
R2C4	OL	OL	8.7 M Ω	OL	OL
R3C4	OL	OL	OL	OL	OL
R5C5	OL	OL	OL	OL	6.4 M Ω
R2C5	OL	OL	OL	OL	OL
R1C5	OL	OL	OL	OL	OL

As can be seen from the results, reliable contact was only made twice out of twenty-five attempts. During testing, the needle electrode attached to the micromanipulator had a tendency to puncture the posts and lift the entire PDMS off the EcoMEA. However, this was not noticed until the latter half of the testing as lifting became very noticeable and more frequent. It is possible that lifting was occurring during most of the testing, on a smaller scale, and thus yielded the results shown in Table 1.

To make the contacts more reliable, plasma treatment of the PDMS bottom surface will be required. Plasma treatment converts the surface from hydrophobic to hydrophilic (hydroxyl groups, -OH, substitute for the methyl groups, -CH₃) and is done in the EML located at MIT's Microsystems Technology Laboratory, MTL.

4. CONCLUSIONS AND FUTURE WORK

An electrode stimulator has been developed to control individual electrodes on the MEA, with an intuitive graphical user interface that allows the patterned firing of selected electrodes. Using a 64 channel data acquisition board, up to 64 individual electrodes can be individually selected. The program was demonstrated on an array of eight LEDs, using an eight channel data acquisition board. The current switching time between electrodes is in the millisecond range. This should allow for the evaluation of spike timing-dependent plasticity as this form of plasticity involves stimulation times in the 20 to 40 ms (Constantine-Paton 2006). Firing sequences can be designed to strengthen or weaken specific synapses.

As a first iteration, an array of polypyrrole posts was placed on top of a commercially available MEA to test the reliability of the electrical contacts. However, the seating of the array did not prove to provide a reliable enough contact between the MEA and the posts. Plasma treatment of the PDMS surface will be conducted to further test the post/electrode electrical contact.

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