Cell Clinics: Characterization and In Vitro Testing of the Bio-Amplifier

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ABSTRACT

The Cell Clinics Project at the University of Maryland aims to develop an integrated bio-labs-on-a-chip which can capture, manipulate, and monitor biological cells. This project has many applications such as bio-sensing and whole cell studies, which typically require the infrastructure of a cell-biology laboratory. An integrated CMOS bio-amplifier was designed as a module for this project to measure and amplify weak extra-cellular signals. It is important to be able to monitor cells in vitro over extended periods of time in order to study time-varying cell responses. For this project, I have refined the hardware and software needed to support long-term testing. I also characterized the bio-amplifier in terms of its frequency response and operating point. Finally, I evaluated signals from several long-term tests with electrically active cells cultured directly on the input electrodes of the bio-amplifier.

1. INTRODUCTION

The integration of living cells with electronics is becoming increasingly important and has many potential applications in physiological studies [1], pharmaceutical tests [2], environmental monitoring, and hybrid bioelectronic computational engines [3]. However, the monitoring and manipulation of cells typically requires the complex infrastructure of a cell biology laboratory. By creating a bio-electronic interface with single cells, these measurements can be taken efficiently in remote places. The Cell Clinics Project at the Electrical and Computer Engineering department at the University of Maryland aims at developing this bio-electronic interface by creating an integrated bio-labs-on-a-chip system which can capture, contain, and take measurements from individual cells [4].
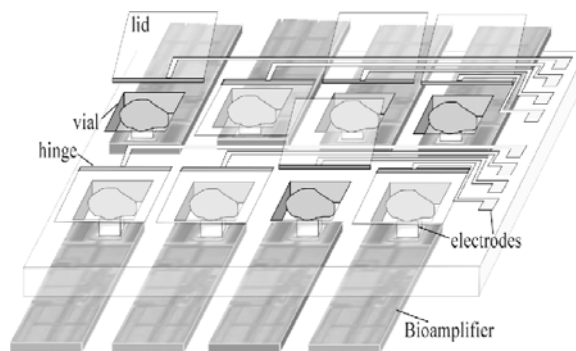

Figure 1. Illustrated Cell Clinics [4]

The capability to monitor individual cells over time as opposed to cell populations allows for the study of individual cellular responses without the need for averaging the responses of cell populations. This is particularly important in providing insight into studying individual cell responses such as metabolism or secretion [1].

2. COMPONENTS

The Cell Clinics prototype consists of micro-electro-mechanical systems (MEMS)

structures which are fabricated upon custom-built sensor chips. The MEMS structures include a lidded microvial which will contain the cell and an actuated hinge that will close in response to electrochemical potentials [5]. The hinge itself is actually a bilayer polymer actuator made of polypyrrole (PPy) and gold, and bends due to volume changes of the PPy [4]. At the bottom of the vials, exposed electrodes come into direct contact with the captured cells and can be used to take measurements. These electrodes are the key to the bio-electronic interface as they will serve as the inputs to sensors or outputs to control devices [3]. For the current bio-labs on-a-chip system, the electrodes are the inputs to bio-amplifier modules designed to measure and amplify action potentials directly from cells. This VLSI amplifier is designed to be integrated with the MEMS structures and is fabricated using a 0.5μm, 3-metal, 2-poly CMOS process. The bio-amplifier is an operational transconductance amplifier in a capacitive feedback configuration [6].
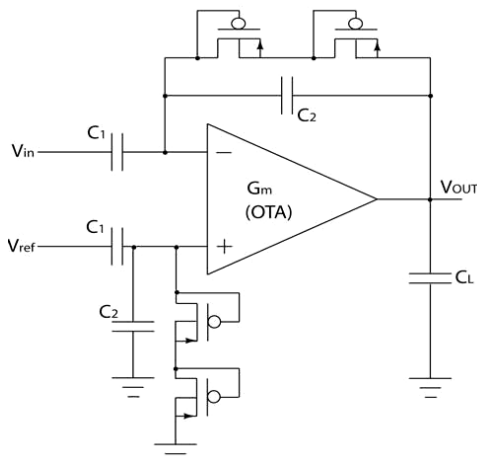

Figure 2. Bio-amplifier schematic [6]

The input to the amplifier is taken differentially between the electrode and common ground to reduce noise which is critical in measuring weak extracellular voltages. Furthermore, this amplifier has the advantage of operating at low voltage

supplies of $^+$/- 1.5 volts for low-power operation. Extracellular voltages in the range of microvolts to millivolts are expected to be the primary inputs to the bio-amplifier [4]. Because the bio-amplifier needs to produce a substantial output from these weak signals, the amplifier circuit is designed to have a low-pass voltage gain of 100 and a cut-off frequency of 3 kHz. The bio-amplifier test chip is comprised of ten exposed electrodes constructed from layers of aluminum which are connected to ten amplifier modules.
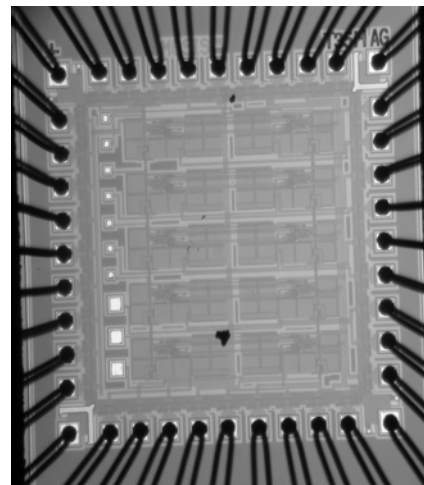

Figure 3. Close view of bio-amplifier: Electrodes are in-line on the left side of the die [4]

Seven of these electrodes have dimensions of 24.5μm by 24.5μm and three electrodes have larger dimensions of 50.4μm by 50.4μm. However, this version of the bio-amplifier is only a prototype for testing purposes. Future versions of the bio-amplifier chip will be designed to have vials with dimensions of roughly 10-20μm so later electrode sizes will actually be smaller.

3. PACKAGING

One of the major obstacles in creating a bio-electronic interface between cells and electronics is the fact that biological samples are deleterious to the materials used in electronics and vice versa. Biological

samples including cells and growth medium are corrosive to materials in the chip and will short out the bond wires connecting the die to the ceramic chip packaging. Conversely, many of the materials used in chip fabrication are toxic to the cells or react with the growth medium. The bio-amplifier must be fully operational while allowing for the long-term viability of cells in order to monitor cells with accurate readings. One of the non-compatible materials used in the chip fabrication is aluminum [7]. In particular, the amplifier electrodes which come into direct contact with the biological samples, are made of aluminum. Aluminum is a reactive metal that easily corrodes and oxidizes. To address this issue, the electrodes are electrolessly plated with gold which is a non-reactive and bio-compatible metal [8]. Electroless plating is more suitable than electroplating for plating the electrodes because it will minimize the noise during measurement. In addition to this, the electroless plating will create a rough surface on the pads which translates into more surface area for the electrodes [4]. The next step in preparing the bio-amplifier for in-vitro testing is to isolate the bond wires from the growth medium and to create a well to contain the biological sample. After many bio-compatibility tests, Loctite™ was chosen as the most suitable material for this interface. This material is a one-part silicone that cures quickly in UV light and bonds to a wide variety of substrates including metal. The bond wires are encapsulated with the Loctite™ and a circular well is formed on top of the bio-amplifier. This completes the physical interface necessary for the testing of the bio-amplifier.
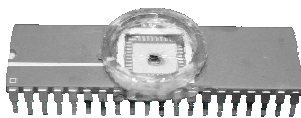

Figure 4. Packaged bio-amplifier chip

4. TESTING & CHARACTERIZATION

Before in vitro testing of the bio-amplifier can commence, the bio-amplifier must be functionally tested and characterized in terms of its frequency response and operating points. The difficulty in conducting functional testing of the bio-amplifier is that there is no pin for a direct input signal. Instead, an input signal must be fed in through the open electrodes on the surface of the bio-amplifier chip. Testing the bio-amplifier in this fashion can also verify that the electrodes are functional.

4.1 Determining the Optimal Bias Point
The first bench test of the bio-amplifier was conducted to test the functionality of the bio-amplifier and to determine its optimal operating point. The goal is to bias the bio-amplifier so that it will produce an output with the gain and frequency response it was designed for. The bio-amplifier array was tested using a probe station with micromanipulators.
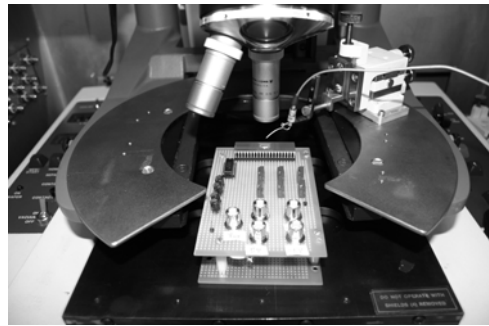

Figure 5. Probe station with custom-built test board

A small signal of 5-10mV was fed into a 0.5 mil or 1 mil (0.0127 or 0.0254 mm) probe tip that was positioned by the micromanipulators. The input signals used were sine waves of various frequencies to approximate the frequency response of the bio-amplifier at the particular bias point. The output of the bio-amplifier was buffered before it was viewed on the oscilloscope. Initial testing of the bio-amplifier with a used, rewired test board was unsuccessful

because the instability of the board caused significant problems. It was difficult for the probe tip to maintain a stable contact with the test board so the output signal was unstable. To remedy this problem, a custom-built wire-wrapped board was constructed which could be screwed into the base of the probe station. This custom built board has a 40-pin DIP socket for the bio-amplifier, an op-amp chip wired in a buffer configuration, 6 screw-controlled $100M\Omega$ potentiometers to adjust bias voltages, and 5 male BNC connectors. This test fixture provided the stability that was needed for the probe tip to maintain a stable contact with the bio-amplifier electrode while the bias voltages were adjusted using a screw driver. An operating point was previously determined for an older version of the bio-amplifier, so this setting was where the testing began. Three bias voltages labeled V_P, V_N, and V_{IB} were adjusted to change the gain and frequency response of the bio-amplifier. Two of the bias voltages were held fixed while the remaining bias voltage was adjusted. The micro-manipulators were then used to feed in the input signal by contacting the open electrodes with the probe tip. Outputs were measured and recorded with input sine waves of various frequencies. This process was repeated to optimize each bias voltage individually with the other two biases fixed. Using this technique, a rough estimate of the frequency response could be determined at the various operating points. From this experimental data, an optimal bias point was chosen based on the specifications of the bio-amplifier. In particular, a low-pass voltage gain of 100 with a cut-off frequency of 3kHz was the ideal response. The bias voltages used for the old prototype of the bio-amplifier was V_P= -0.4V, V_N= 0.4V, and V_{IB}= 0.2V. The new proposed bias voltages that will be used for further testing are V_P= -0.2V, V_N= 0.2V, and V_{IB}= 0.3V. To see where these bias voltages are in the schematic, see [8].

4.2 Frequency Response and Gain

The probe station was useful for determining the optimal bias point but it was difficult to obtain an exact characterization of the bio-amplifier in terms of its gain and frequency response. The smallest, stable input signal that we could measure using the Tektronix® TDS2024 digital oscilloscope was roughly 5mV. Measurements of this small input signal were subject to noise so it was difficult to measure the exact value of the amplitude. In order do determine the exact frequency response and gain of the bio-amplifier, we decided to use a network analyzer. The network analyzer will allow us to use a known small signal of about $707\mu V$ and can measure the gain of the amplifier continuously over a large range of frequencies. Furthermore, the network analyzer can perform this frequency sweep multiple times and average the transfer function measurements up to 16 times. However, the primary problem with using the network analyzer is that the bio-amplifier does not have a pin for a direct input signal. In order for us to use the network analyzer, we had to bond wires from the electrodes to outer pins. A wire bonding machine from the University of Maryland Physics Department was used to bond aluminum wires to our electrodes. Three bonds to the larger electrodes were successfully made and allowed us to feed an input signal in to the bio-amplifier directly. The transfer function of the bio-amplifier was determined for the three different pads at a few bias points to verify that the correct operating point was chosen. For the new, empirically determined bias point, we were able to verify that the bio-amplifier operates very close to the specifications it was designed for. At this operating point, the low pass gain was about 39.5dB (94.4V/V)

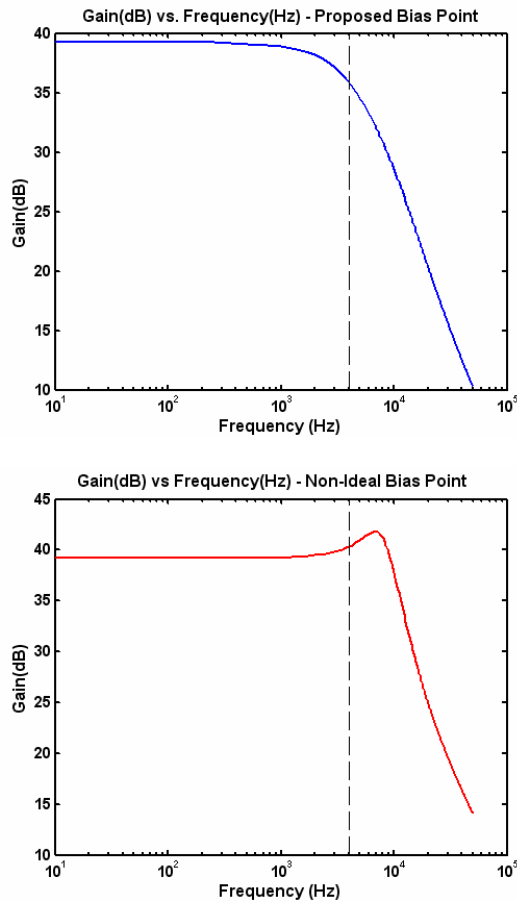and the 3dB frequency was close to 3.01 kHz.

Gain(dB) vs. Frequency(Hz) - Proposed Bias Point

Gain(dB) vs Frequency(Hz) - Non-Ideal Bias Point

Figure 6. Transfer function of the same electrode and bio-amplifier at two bias points (dotted line denotes ideal cut-off frequency of 3 kHz)

The transfer function plots show that the proposed bias point provides for the frequency response that we desire.

5. IN VITRO TESTING

In vitro testing of the bio-amplifier is necessary before the lidded microvials can be fabricated. The bio-amplifier must be able to monitor cell activity over extended periods of time (24+ hours). Therefore, a reliable and accurate testing process must be developed to monitor cell activity using the bio-amplifier. Also, the signals from the bio-amplifier must be evaluated to validate the accuracy of the bio-amplifier.

5.1 Cell Culture
Bovine aortic smooth muscles cells (BAOSMC) are used for in vitro testing because they are electrically active and easy to culture. These cells are plated onto the custom packaged bio-amplifier chip with sterile growth medium. The chip is then taken into a humidified incubator which maintains a constant temperature of 37°C and CO_2 level of 5%. The cells are left to adhere to the electrode surfaces and non-sterile Hank's balanced salt solution (HBSS) is added to the growth medium before testing in order to induce cell activity.

5.2 Previous Attempts at Testing
The earliest attempts in using the bio-amplifier to measure extracellular signals used basic, straightforward methods. Cells were loaded onto the bio-amplifier which drove an op-amp in the unity gain buffer configuration. The signals were monitored using an oscilloscope and the data could be acquired using a GPIB interface. However, the cells would only be viable for a certain period of time because they are exposed to the environment outside of the incubator. A portable and long-term system of monitoring was necessary for the bio-amplifier to be used as a sensor. This system was developed to test the bio-amplifier while it was still in the incubator. A test board was constructed and placed in a noise reducing faraday cage. To test the bio-amplifier in an incubator, a data acquisition card and laptop were used to collect data. In addition, a data acquisition program was written in MATLAB to collect and view data. Using this new system, only one long-term in vitro test of the bio-amplifier was conducted. Some activity was observed, but no conclusive results could be drawn out of this test. The voltage activity recorded did not clearly resemble typical action potentials and premature package failure brought the results into question. Significant chemical

reactions occurred within the well containing the growth medium and it is probable that the bond wires and biological sample were affected in some way. Since then, packaging has improved significantly and bio-amplifier testing can resume.

5.3 Hardware Refinement

The test equipment developed in the first long-term test of the bio-amplifier was quickly assembled and was slightly damaged in storage. In order to ensure smooth testing of the bio-amplifier, the hardware needed some refinement as well as repairs. The first step was to use a more reliable and faster data acquisition card. The National Instruments PCMCIA 6036E data acquisition card along with a 68-pin ribbon cable was purchased for this purpose. The faraday cage could be reused but the test board itself had to be reassembled. Many wires and components had become loose or disconnected. The board was completely reconstructed with a design similar to the test board used for the probe station testing. However, this board required an extra op-amp buffer because data would be collected differentially from 8 channels simultaneously. The 8 electrodes selected for outputs were the electrodes that would least likely be covered by the Loctite packaging.
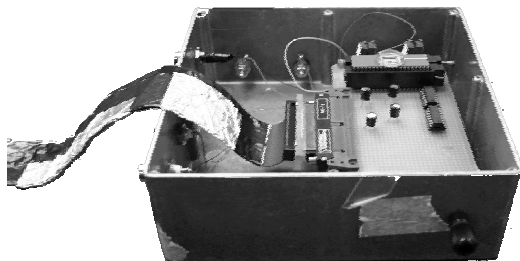

Figure 7. Test board in Faraday cage connected to 68-pin ribbon cable

New, thin BNC cables were also purchased to connect the supplies of the test fixture to the power supplies which were outside of the incubator. An external hard drive was also used for data logging to fit the copious amounts of data amassed from extended tests.

5.4 Software Refinement

The MATLAB user interface previously developed for data acquisition and viewing had many problems, especially in terms of its user friendliness. Because its graphing capabilities had so many problems, this function of the user interface was not frequently used. The user interface was revamped and many new functions were added to make this software user-friendly.
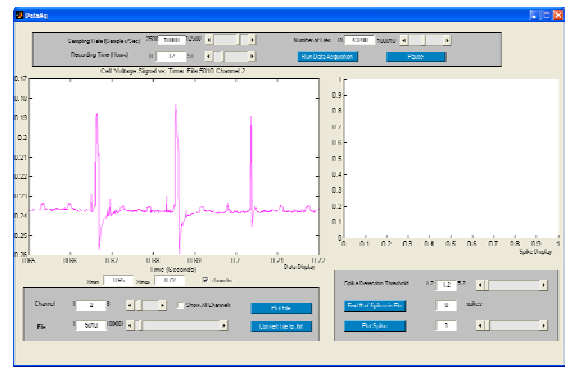

Figure 8. Revamped graphical user interface with improved user functionality

With new modifications, the user interface can now plot logged data with increased user control to view and manipulate data. The display and controls configuration were also optimized to save space and increase the plot area. The data acquisition portion of the user interface was improved to be able to run quick user tests with notifications to update the status of the data acquisition. This portion of the software was also modified to be compatible with the new data acquisition card. The spike sorting and display function is in the process of being updated. Overall, the user interface was made to contain all the functions necessary for acquiring, viewing, and manipulating data.

5.5 Data Collection

Two in vitro tests of the bio-amplifier have been conducted and one more test is pending. During the first in test run, premature packaging failure within the first day caused many of the bond wires on the chip to disconnect. The Loctite™ 4304 seems to pull out the delicate bond wires due to long term contact with the growth medium. As a result, supply voltages were disconnected and the chip was no longer functional.

Figure 9. Incubator located in Bio-process Scale-up Facility (BSF)

We did not observe any meaningful data or any electrical activity of cells before the packaging failed. Upon further inspection, a look at the chip under the microscope showed that no cells adhered to the electrodes or chip surface. Presumably, the cells did not survive the multiple day test or the cells may not have been extracted onto the chip properly. In the second test of the bio-amplifier, we did not expect the packaging to hold for an extended period of time. The two bio-amplifier chips used in both tests were packaged using the same material, Loctite™ 4304. Future packaging will be done using Loctite™ 3340, which appears to be less prone to disconnecting the

bond wires. Nonetheless, we were able to collect data for a few hours before the packaging failed. As we expected, the bond wires were disconnected within the first day, but some electrical activity was recorded by the bio-amplifier before this happened. Beginning from about the third hour of the test run, voltage spikes similar to cell activity appeared on many of the electrodes.

5.6 Preliminary Evaluation

The spikes recorded in the second in vitro test began at about 3.5 hours after we loaded the cells onto the bio-amplifier. These spikes were consistent for almost 20 minutes and appeared spontaneously and less frequently after that. The data seen over the course of this period contains many significant voltage spikes that look very similar to action potentials.
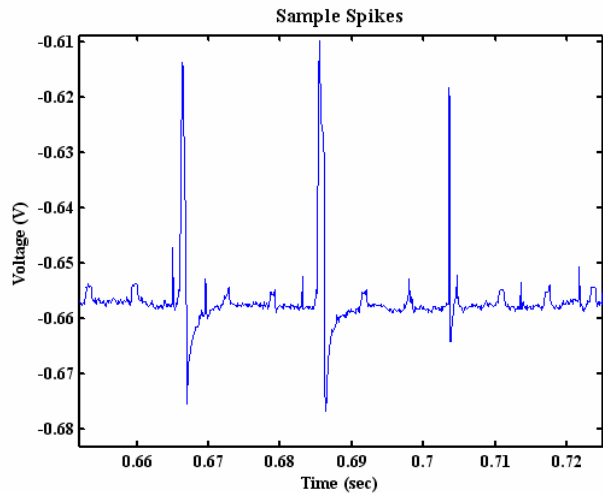

Figure 10. Sample data of output voltage spikes collected during second test

The voltage readings spike quickly and a slower refractory period immediately ensues. The voltage spikes had typical peak values of 500 µV above the DC offset and a refractory potential of about 200 µV below the offset. The total time spanning the spike and the refractory period was commonly around 2ms. Extracellular voltages are commonly several hundred microvolts [6]

and have duration times of 0.2-20 milliseconds. The voltage spikes measured by the bio-amplifier appear to be similar to extracellular voltage spikes in terms of spike amplitude and duration. The other curious property of the voltage activity is that most of the spikes registered on all the electrodes.
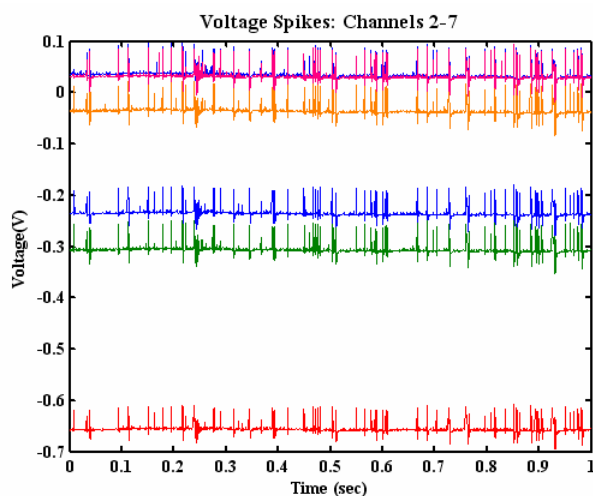

Figure 11. Sample data (multiple channels)

As the plot depicts, the voltage spikes register on each channel with no significant delay. In fact, the spikes are virtually simultaneous. It is likely that because the cells and electrodes are so close together, the propagation time between the electrodes was undetectable at our sampling rate of 10,000 samples per second. When this chip was examined under the microscope shortly after testing, we noticed that there was a large clump of cells plated directly on the electrodes. Cells were densely packed and covered the whole surface of the electrodes. Further investigation into the propagation of action potentials among densely populated cells is underway.

6. CONCLUSIONS

The bio-amplifier chip has been functionally tested and fully characterized in terms of its optimal operating point and frequency response. In vitro testing of the chip is underway and a reliable system of data acquisition and analysis has been developed and refined. Major hardware and software updates were added to improve the long-term in vitro testing of the bio-amplifier. Two in vitro tests of the bio-amplifier have been conducted and it has been shown that the bio-amplifier can successfully measure weak extracellular signals.

7. ACKNOWLEDGEMENTS

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