Extensions upon the Versatile BioLab-On-A-Chip

Eric Chen, Harneet Khurana
Marc Dandin, Alfred Haas, Nicole Nelson, Somashekar Prakash, Pamela Abshire

Department of Electrical and Computer Engineering
University of Maryland
College Park, MD 20742, USA

ABSTRACT

The Integrated Biomorphic Information Systems Laboratory at University of Maryland strives to realize a BioLab-On-A-Chip that investigates biological cells to replace the significant laboratory infrastructure presently required in applications of whole cell studies, physiology, environmental monitoring, and remote biosensing. One aim is to verify the methodology through short-term and long-term monitoring of PC12 cells differentiated with NGF and of bovine aortic smooth muscle cells chemically modulated with sodium nitroprusside dihydrate. To aid in the verification process, data acquisition software has been refined using modules provided by National Instruments. In addition, improved packaging methods are being developed by transitioning from Loctite™ to SU8 biocompatible encapsulation. Meanwhile, extended applications of the technology are being explored; new sensors for high-density monitoring of neurite outgrowth have been designed, simulated, and submitted for fabrication.

1. INTRODUCTION

Applications of whole cell studies, physiology [1], environmental monitoring and remote biosensing are becoming increasingly important, especially with the threat of chemical and biological agents [2] and the recent rise in the number of global pandemics such as the avian flu. These applications, however, typically require use of significant cell biology laboratory infrastructure to accomplish their tasks. We at the University of Maryland strive to eliminate this overhead cost through the realization of a low cost and low maintenance BioLab-On-A-Chip that can efficiently interface with biological cells and conduct various experiments through a series of measurements for a wide array of purposes. One avenue being explored toward this goal is the use of CMOS bioamplifiers integrated with micro-electro-mechanical systems forming lidded microvials to contain cells [3].

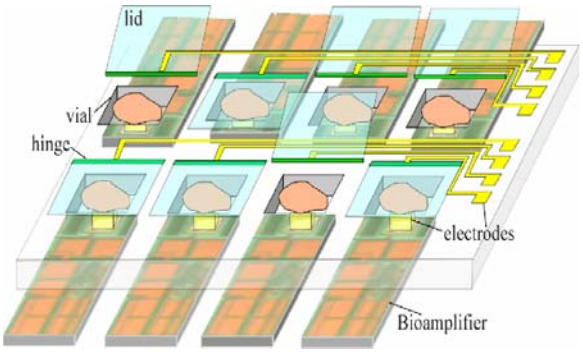

Figure 1. BioLab-On-A-Chip Illustration [3]

2. SENSOR DESIGN AND APPLICATIONS

To begin design of our sensor, we take advantage of the fact that cells, neuronal cells in particular, use action potentials to

communicate within and across the cell membrane. These action potentials are variations in voltages across the cell membrane that can be measured extracellularly, allowing for the ability to electronically interface with biocompatibly-packaged silicon chips [4]. Using the principal component of a CMOS bioamplifier, we have designed, simulated, and submitted nine different chips geared toward the monitoring of neurite outgrowth. All of the new designs implement a vast increase in spatial resolution over previous amplifier arrays, enabling far greater spatial precision and new applications such as the monitoring of developing cell processes.

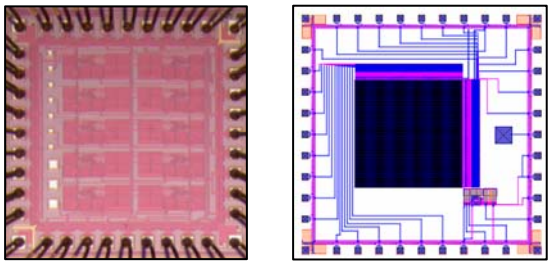

Figure 2. Legacy amplifier design (left); new neurite outgrowth monitoring design (right)

Common to each chip is a 128-by-128 array of pixels, with each pixel comprising electrodes coupled to an individual low-noise preamplification unit and routed to a single chip-level bioamplifier module. This is a vast improvement over previous legacy designs, of which an example is shown above. These designs comprise an array of 10 bioamplifiers, each connected to a single electrode, whose smallest overall dimension measures 24.5µm by 24.5µm [5]. For the new chip it was necessary to develop nine different designs for exploration of different in-pixel circuit configurations and investigation of commercial versus in-house techniques for patterning the passivation layer to define electrodes.

The simplest in-pixel preamplification circuit configuration we employed was that of a single-transistor common source amplifier to be used as a baseline structure for circuit verification and control purposes. Two more complex circuit configurations we employed were that of NMOS-only differential amplifiers – with local references between electrodes compared against a global reference among all electrodes [6]. We utilize the differential amplifier circuitry to eliminate noise present in the attempt to ensure transmission of an undistorted signal from the cells due to the low expected peak-to-peak input on the order of 100µV. Meanwhile, we restrict the components to NMOS-only to ensure maximal utilization of spatial resolution. The overall dimensions of the electrode elements range from 5.25µm by 5.25µm to 7.5µm by 7.5µm – significantly smaller than previously targeted [5]. Readout from each element of the electrode array is processed by column and row decoders, each optionally coupled to a counter unit to enable sequential element scanning of the array.

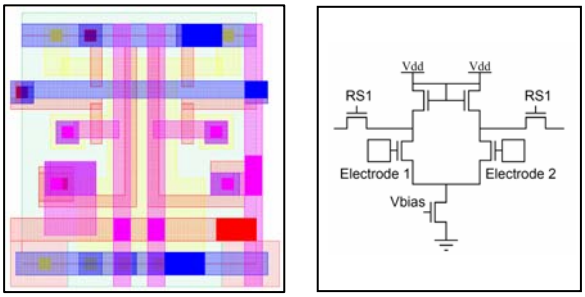

Figure 3. Lay out of in-pixel NMOS-differential amplifier unit (left); schematic (right)

In collaboration with Dr. Elisabeth Smela, we are comparing the performance advantages of in-house glass cuts over commercial window cuts for our application purposes. To that extent, three design combinations were laid out for each of the three configurations. Designs were first laid

out under a 3-metal process constraint using commercial window cuts. The designs were laid out again under 3-metal process with windows cuts to be patterned using techniques developed at the University of Maryland. The third design laid out was under a 2-metal process constraint with in-house window cuts, as the metal 2, but not metal 3, passivation layer is commercially planarized, enabling enhanced deposition of photoresist for future biocompatible encapsulation processes.

As we have submitted the nine designs on July 11, 2006, we are still awaiting their fabrication and receipt for testing.

3. TESTING AND CELL CULTURE

In the interim, we have conducted further experimentation using the legacy bioamplifier chip designs.

Before using cells for on-chip testing purposes, we culture cells for healthy and sustained growth. We have currently cultured two cell lines for evaluation, bovine aortic smooth muscle cells (BAOSMC) for their electrical activity [5] and rat pheochromocytoma cells (PC12) for the ability to differentiate neuronal processes [7]. Both cell lines are stored in sterile flasks, placed in an incubator at 37° C, 5% CO_2. To prevent adverse affects of overcrowding, we subcultured the populations upon reaching 60-80% confluence under a Laminar flow hood. Subculturing procedures under aseptic conditions for each of the cell line are detailed following.

BAOSMC:

1. Remove old media from flask
2. Rinse flask with 5 mL of Hank's Balanced Salt Solution (HBSS)
3. Add 2 mL of trypsin and monitor under microscope until all cells have lifted from the surface to enable transport
4. Add 2 mL of fresh growth media
5. Count cells using haemocytometer
6. Place desired amount of cell suspension into a new flask, bringing volume to 5 mL for a T-25 flask

PC12:

1. Remove old media from flask
2. Add 5 mL of new media
3. Scrape cells from flask surface with scraper, enabling transport
4. Titrate cell suspension 15 times
5. Count cells using haemocytometer
6. Place desired amount of cell suspension into a new flask, bringing volume to 5 mL for a T-25 flask

Figure 4. Flasks containing BAOSMC

When population densities reach levels adequate for testing, we perform the following procedures for administering cells to the chip surface under aseptic conditions.

1. Expose chip to UV light for 10 min.
2. Remove old media from flask
3. Add fresh 2-5 mL media
4. Scrape cells from flask surface with scraper
5. Titrate cell suspension 15 times
6. Rinse chip with growth media
    a. For PC12, add adhesion promoter collagen to chip surface and let sit to air dry for 1 min

b. For PC12, rinse chip once more with growth media
7. Add cell suspension to cell surface
8. Seal culture well with Breathe-Easy™
9. Place chip into incubator at 37° C, 5% CO_2 overnight

For differentiation of neuronal processes, the PC12 administration has the added overhead of adding media supplemented with nerve growth sfactor (NGF) and bovine serum albumin (BSA) after 24 hours for 2-3 days. Only after allowing five days for complete differentiation are the PC12 cells used in experimental monitoring.

Once the cells are ready for monitoring, we mount the chip onto a custom test board, set biases for the bioamplifier units (V_P= -0.2V, V_N= 0.2V, and V_{IB}= 0.3V), place the entire fixture into a noise-reducing faraday cage, and store the cage within the confines of the incubator. To allow connectivity, a 68-pin ribbon cable is routed through the protective fixture to a data acquisition system external to the incubator, front-ended by a National Instruments DAQCard 6036E PCMCIA data acquisition card.

Figure 5. Data acquisition system components and general setup

Over the course of this summer, we have conducted five sets of experiments, two experimenting with differentiated PC12 cells and the final three using BAOSMC. The BAOSMC trials were performed under four culture conditions for each trial: standard growth media, 50% growth media and 50% HBSS with Ca^{2+} and Mg^{2+} ions, HBSS only, and 10μM sodium nitroprusside in HBSS. Unfortunately, data extracted from all trials have been inconclusive. Even with BAOSMC we were unable to duplicate data that was reminiscent of extracellular action potentials found just one year prior in our experiments [5]s.

Upon review of the chip surfaces after each experimental trial, it was found that predicted levels of cell growth were absent, prompting us to conduct a reevaluation of the biocompatible materials used in the encapsulation process and storage of the chips. Two factors we identified as possible culprits were the Breathe-Easy™ sealing membrane, used to seal the cell culture within the confines of the attached chip well, and Loctite™ 3340, employed for electrical and chemical isolation of the cell culture from non-biocompatible components of the chip package. Evaluating the two materials using their absence as controls, four combinations of cell culture enclosures were studied. The two sets for which Loctite™ was absent produced predicted levels of cell growth, while only two of six samples had appreciable cell growth when only Loctite™ was present. In the presence of both Loctite™ and Breathe-Easy™ no appreciable levels of cell growth were found in any of the samples. The data has fostered support for the hypothesis that the biocompatible reliability of Loctite™ 3340 is time-sensitive, degrading over time. Another issue previously identified with use of Loctite™ was its quality of aqueous absorbtion, which caused the displacement of enclosed bond wire connections when in continual contact with the cell culture for extended periods [5]. Paired with this prior complication, conclusions derived from this

new data prompted renewed impetus in the search for reliable biocompatible encapsulation materials.

4. PACKGING

Before discussing solutions proposed in the renewed encapsulation exploration, we digress into a description of the current encapsulation process and the reasons for its need.

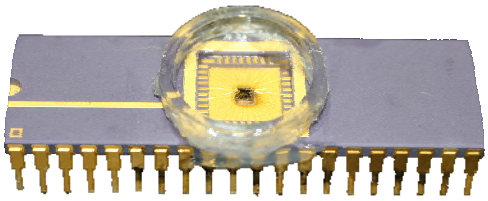

Figure 6. Fully packaged and encapsulated chip

Commercially-available chips are received prepackaged in ceramic 40-pin DIP packages with aluminum used as the electrode material and bond pad metal. Unfortunately, aluminum (Al) is highly oxidizable, corroding upon contact with aqueous solutions and compromising cell viability henceforth [8]. Consequently, we electrolessly plate the biocompatible non-reactant metal of gold (Au) onto the aluminum surfaces [9]. In the process of electrolessly plating Au, we not only benefit from increased electrochemical compatibility but also a reduction in noise. Surface area of the electrodes is also increased due to roughening of the surfaces caused by etching that takes place during the chemical reactions.

Describing the electroless plating process, we first treat the Au surfaces with Techni Acid Salt TAS-3Z to remove the native oxide and then immediately deposit with nickel (Ni) through use of Techni EN 2600 before oxidation occurs again. Once Ni has coated the Al surfaces, we use a final chemical reaction involving Oromerse SO to deposit Au onto the Ni coatings, readying the chips for the next step in the biocompatible encapsulation process. All chemical reactions performed during the electroless plating are conducted under a fume hood with protective personal equipment worn.

With complications concerning corrosive metals now resolved, we turn our focus to insulating the bond wires from contact with cell media to prevent the solution from shorting the bond wires together. Using Loctite™ 3340, as mentioned in the previous section, we create a two-level encapsulation that chemically and electrically isolates the packaging and circuitry from any contact with the cell culture. As Loctite™ is a negative photopatternable UV-curing material, we use photolithographic techniques to form the desired geometries of our encapsulation and employ two masks in the process. The first mask, used to target finer resolution, covers a smaller area to enhance the exposure area of the electrodes while completely encapsulating the ends of the bond wires connected to the die. The second larger mask is used to accommodate the exposure area imprinted by the first while encapsulating the remaining exposed area where the bond wires connect to the package. The procedure of the process is as follows:

1. Drop onto the chip an amount of Loctite™ just enough to fully cover the die
2. Coat the contact surface of the preliminary mask with PDMS and align over the die using microscope and tweezers
3. UV-cure the chip for 4-6 minutes, until the Locitite™ coagulates
4. Remove the initial mask

5. Administer an additional amount of Locitite™ to cover the entire die cavity and accompanying bond wires
6. Coat the contact surface of the final mask with PDMS and align over the die using microscope and tweezers
7. UV-cure the chip for 6-10 minutes, until the Locitite™ coagulates
8. Remove the final mask
9. Wash away the excess Loctite by alternating between a series of short bursts of DI H_2O and light rinsing with ethyl acetate aimed over the die
10. Dry with pressurized N_2 and review encapsulation under microscope
11. Repeat steps 10 and 11 as necessary

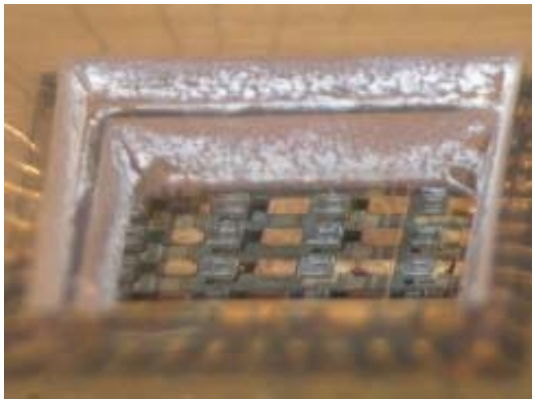

Figure 7. Two-level Loctite™ encapsulation

Due to reuse of standardized masks and varying chip designs, the electrodes occasionally are covered by coagulated Loctite™. As the Loctite™ is not fully cured after the initial two sessions, we use a needle to scrape away undesired encapsulation from the surface of the electrodes between successive cleanings of the chip. Any potential damage to the underlying circuitry is prevented by the presence of a passivation layer. Once the electrodes are completely cleared of excess Loctite, the chip is exposed to a final UV-cure for 12-30 minutes to fully harden the encapsulation.

The final step of the encapsulation process is to append a well onto the package to contain an appreciable amount of cell culture to enable monitoring. We use commercially available RTV to attach a circular plastic structure to complete this task.

Recalling that the biocompatible reliability of Loctitie™ 3340 is under review with a renewed investigation of biocompatible materials and methods presently underway, a proposed method to simplify the lengthy and tedious encapsulation procedure was presented for evaluation.

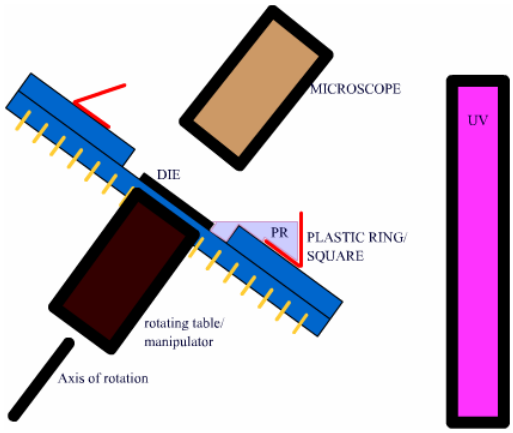

Figure 8. Gravity-assisted encapsulation proposal

The proposed process would employ the assistance of gravity to form an exposure cavity in a relatively few number of simple steps. A plastic frame encompassing the die cavity would be appended to the package, forming a well cavity. The package would then be placed into an inclined rotatable mount. A low viscosity negative photoresist would then be administered into the cavity until the bond wires and pads are encompassed. UV-curing would then proceed, encapsulating the contained bond wires and pads. Rotations of the mount along with successive administrations of photoresist and UV-curing would then commence until all bond wires have been encapsulated. As we are still assessing the

equipment infrastructure required to enable this process, we are pursing a promising alternative method using a biocompatible and hydrophobic SU-8 photoresist [10] and HumiSeal®, which has a high degree of biocompatible and hydrophobic properties as well. Employing photolithography, we will place a SU-8 perimeter around the desired electrode cavity area of an unpackaged chip and then backflow HumiSeal® towards the SU-8 perimeter to encapsulate the bond wires after packaging. It is expected that surface tension along with appropriate dimensions and geometries of the perimeter will prevent overflow of the HumiSeal® into electrode areas reserved for exposure to enable electrical interfacing. We plan to experiment with lateral thicknesses of the perimeter wall in the range of 15-30μm and vertical heights ranging up to 200μm. Masks have already been submitted for printing, designed with a number of geometries for trial as well.

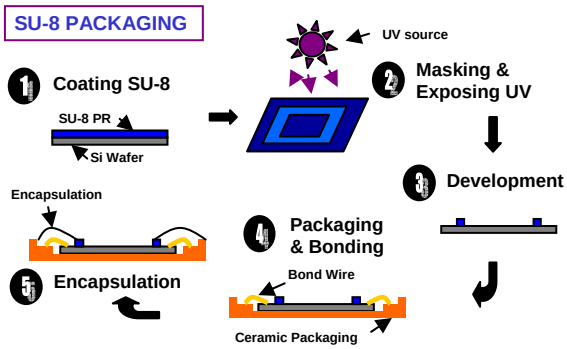

Figure 9. SU-8 packaging flowchart

5. DATA ACQUISITION

A key final component to the BioLab-On-A-Chip system is that of the data acquisition software and supporting hardware used to monitor test subjects. Last year marked the transition to utilization of better hardware, the National Instruments DAQCard 6036E in particular. This year we focused on optimizing the software capabilities.

We begin with a brief overview of the overall functionality of the legacy data acquisition software. Written in MATLAB® and incorporating using functions from the MathWorks Data Acquisition Toolbox, the software program takes as its input the sampling rate, sampling duration, and number of files into which to store the acquired data. Once a monitoring session is initialized, the program will subdivide the duration by the number of files prescribed and run an acquisition session for the fractional duration calculated. Upon completion of this acquisition session, others are sequentially run until the full duration of the monitoring session has elapsed. After the full monitoring session is completed, the software can load and graph acquired data from the stored files, zooming into specified sections accordingly.

In its implementation of dividing acquired data into manageable-sized files for analysis, the legacy software introduces windows of data loss between the completion of an acquisition session and the startup of the following acquisition session. The duration and frequency of these windows are dependent on the file size, a factor dependent on the values of the initial input. The larger the storage file size, the smaller the frequency of the windows yet the longer in duration the windows; vice versa, with a smaller the file size, the duration of the windows would shorten while introducing and increase in frequency. In consequence, limiting the acquired data to a single file would make long-term data unmanageable while using too many files may render the data invalid as the continuous nature of the data is compromised. Without highly specialized I/O functions, overcoming this obstacle is exceedingly difficult problem due to the sequential command execution nature of MATLAB®.

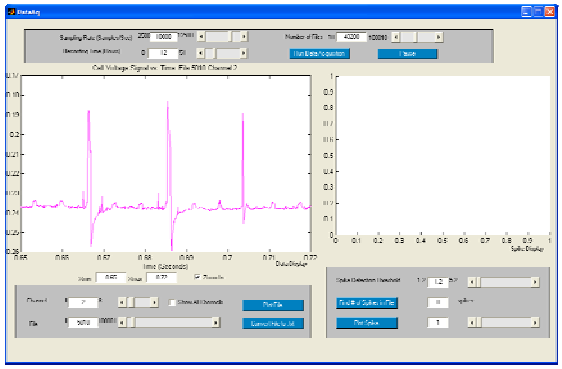

Figure 10. Legacy software graphical interface

Furthermore, the legacy software suffers from an absence of a real-time display of the monitoring session. While this only results in wasted experimentation time when cell viability or hardware setup is initially compromised in trials, the inability to expedite the sampling of data can prove disastrous for more time-sensitive real-world applications of remote biosensing and environmental monitoring.

Hence, we have investigated various third-party software packages, extensively evaluating the NI-DAQmx Tools package from National Instruments in particular. Using a set of LabVIEW VIs translated into MATLAB® MEX-functions, the package allows the desired real-time readout while operating within the MATLAB® framework. As NI-DAQmx Tools is an evaluation package, however, it does not provide the logging of continuous data into multiple manageable-sized files. However, the commercial National Instruments Math Interface Toolkit package, from which NI-DAQmx Tools is derived, allows MEX-function translation of most LabVIEW VIs, including one which enables the desired logging functionality. Consequently, we are currently exploring our options of purchasing this individual software toolkit to enable us to work within the convenient MATLAB framework used for data manipulation and analysis within the lab.

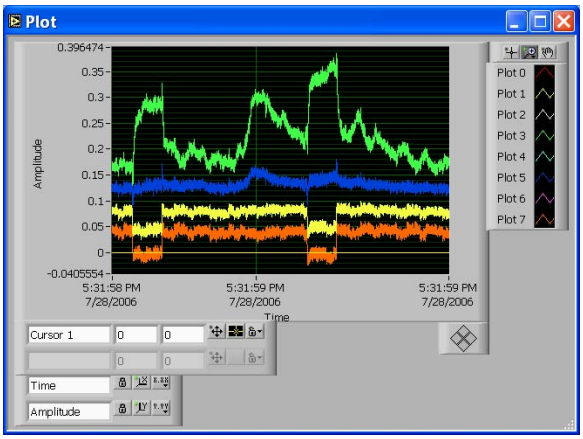

Figure 11. NI-DAQmx Tools Graphical Interface

6. CONCLUSIONS

In our exposure to nearly all aspects involved a developing a bioelectronic acquisition sensor system, we have involved ourselves in enhancing each major component of a BioLab-On-A-Chip. Nine different chips geared toward the purpose of monitoring neurite outgrowth have been recently submitted for fabrication. A complete overhaul of our biocompatible encapsulation process and materials is underway while options to enhance our data acquisition software are being explored. Consequently, we are gradually but surely taking the steps necessary to achieve our goals of revolutionizing the technology infrastructure used in cell biology applications.

7. ACKNOWLEDGEMENTS

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