Design, Fabrication and Characterization of an In Silico Cell Physiology lab for Bio Sensing Applications

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Abstract. In this paper, we report the design, fabrication and characterization of an in-silico cell physiology biochip for measuring Ca\(^{2+}\) ion concentrations and currents around single cells. This device has been designed around specific science objectives of measuring real time multidimensional calcium flux patterns around sixteen Ceratopteris richardii fern spores in microgravity flight experiments and ground studies. The sixteen microfluidic cell holding pores are 150 by 150 µm each and have 4 Ag/AgCl electrodes leading into them. An SU-8 structural layer is used for insulation and packaging purposes. The in-silico cell physiology lab is wire bonded on to a custom PCB for easy interface with a state of the art data acquisition system. The electrodes are coated with a Ca\(^{2+}\) ion selective membrane based on ETH-5234 ionophore and operated against an Ag/AgCl reference electrode. Initial characterization results have shown Nernst slopes of 30mv/decade that were stable over a number of measurement cycles. While this work is focused on technology to enable basic research on the Ceratopteris spores, we anticipate that this type of cell physiology lab-on-a-chip will be broadly applied in biomedical and pharmacological research by making minor modifications to the electrode material and the measurement technique. Future applications include detection of glucose, hormones such as plant auxin, as well as multiple analyte detection on the same chip.

1. Introduction

During early development in fern spores (Ceratopteris richardii) the single haploid cells sense and respond to gravity through a mechanism that involves a trans-cellular calcium ion current which opposes the gravity vector [1]. This irreversible process drives polar axis development within the cell and was discovered using the self-referencing calcium microelectrode to measure transmembrane Ca\(^{2+}\) flux during this period of gravimorphogenesis. The self-referencing technique allows for the measurement of dynamic flux from individual cells based on ETH-5234 ionophore and operated against an Ag/AgCl reference electrode. Initial characterization results have shown Nernst slopes of 30mv/decade that were stable over a number of measurement cycles. While this work is focused on technology to enable basic research on the Ceratopteris richardii spores, we anticipate that this type of cell physiology lab-on-a-chip will be broadly applied in biomedical and pharmacological research by making minor modifications to the electrode material and the measurement technique. Future applications include detection of glucose, hormones such as plant auxin, as well as multiple analyte detection on the same chip.
The recent decade has seen a drive towards miniaturization of sensors, especially the fabrication of miniature bio-sensing devices. This has resulted in extensive research being conducted in the fabrication of what are now called as µTAS (micro or miniaturized total analysis systems), commonly referred to as lab on a chip devices. The advantages of miniaturization include reduced size, small sample volumes, multi analyte detection, reduced analysis times and reduced reagents use in devices that are highly uniform and composed of geometrically well defined structures [3]. These properties of µTAS systems are significant in considering fabricating miniature ion selective electrode platforms, which recently were limited to glass electrodes filled with ion selective ionophore solutions for imparting ion selectivity [4,5]. The most popular method for fabricating these µTAS systems utilizes silicon micro fabrication techniques commonly employed in the microprocessor industry. Uhlig et al. [6] fabricated a Valinomycin based potassium-selective planar sensor array on a Silicon substrate. The device was fabricated on a (100) double sided silicon wafer using anisotropic Silicon etching with tetramethyl ammonium hydroxide (TMAH), reactive ion etching (RIE) and metal evaporation. The electrodes were Ag/AgCl/Ag (20nm, 600nm, 10nm) formed by sputtering and thermal evaporation with the chloriding being performed by immersing in 0.1M NaCl solution. The electrodes were then coated with p-HEMA (poly(hydroxymethyl methacrylate)) to form the Ag/AgCl reference electrode. They used four different types of membranes including a high molecular weight PVC (PVC-HMW), carboxylated PVC (PVC-COOH), silicone rubber, and aliphatic polyurethane (Tecoflex) based K+ selective membranes. The potentiometric micro sensor was then tested in aqueous solution, human serum, urine and whole blood samples. Guenat et al. fabricated and characterized a Ca2+ ion selective microelectrode array platform based on Silicon Nitride micropipettes and platinum electrodes on a Silicon substrate [7]. The fabrication steps involved a DRIE process followed by an LPCVD silicon nitride deposition process to form the micropipettes, a KOH etch followed by an RIE process to open the pipette tips, remove extra Silicon Nitride and subsequently thin the Silicon surface to enable to pipettes to protrude above the Silicon surface. Finally a thermal oxide layer is grown on the Silicon. Ti/Pt electrodes are then patterned on the bottom of the channels through lift off and finally the Silicon substrate is anodically bonded on to the Pyrex platform to make the final device. The micropipettes were then filled with a Ca2+ selective membrane to form the ion selective electrodes. The calibrations were done by filling the reservoir with CaCl2 solutions using an Ag/AgCl reference electrode. Preliminary results show a near Nernstian response and biocompatibility of the electrodes was also demonstrated.

Our efforts have been aimed towards developing a device that would enable us to measure the Ca2+ flux patterns around numerous Ceratopteris richardii spores simultaneously in real time. The device needed to be small in size, but readily interfaced with a fully functional and dynamic data acquisition system for it to truly useful as a generic platform for studying cell physiology for discovery in biosystems. These efforts culminated in the development of an in-silico cell physiology-lab-on-a-chip. The device was microfabricated to includes 16 pyramidal pores on a silicon substrate, with each having 4 Ag/AgCl electrodes leading into them at the four poles. An SU-8 structural layer was used to form the insulating layer as well as form a microfluidic cell holding port, to secure the individual cells within the 4 electrode matrix. The entire chip is wire bonded onto a custom PCB for easy interface with a custom data acquisition unit. Preliminary characterizations have been performed using different concentration CaCl2.
solutions and have shown stable results which were reliable and repeatable over multiple cycles.

2. Design and fabrication

2.1. Design of the *in-silico* cell physiology lab.

The basic design of the *in-silico* cell physiology lab is depicted in Figure 1 (a) and (b). The chip is 9 mm x 11 mm in dimension and has 16 pyramidal pores on it. The top dimensions of each pore are 150 µm x 150 µm ± 2 µm. This pore width was designed based on the requirement that the fern spore should sit inside the pyramidal pore, the Ag/AgCl electrodes, which lead into each fluidic pore on four sides to a depth of 14 micrometers, should be within 10 µm from the surface of the cell. Based on previous microsensor experiment we found this is the optimal spatial range for measuring the Ca$^{2+}$ current generated around the fern spore. The 16 pores were arranged in a symmetric format and staggered slightly to ensure easy routing of the bonding pads. Finally an insulating structural layer is formed to expose only a small portion of the Ag/AgCl electrodes, and to structurally build up the walls of the fluidic pore. The exposed electrodes are then coated with the Ca$^{2+}$ ion selective membrane to impart ion selectivity. The whole chip was then wire bonded onto a custom PCB, that includes an array of 64 amplifiers, to transfer electrode signals from each electrode to a data acquisition (DAQ) system. Once connected the bonding pads were covered with a transparent epoxy to completely insulate all electrical connections. The calibration and characterization tests were done using 0.1 mM, 1.0 mM and 10 mM CaCl$_2$ solutions with a Ag/AgCl reference electrode.

2.2. Fabrication of the *in-silico* cell physiology lab.

There were many options available for fabricating the *in-silico* cell physiology lab. Numerous lab on a chip devices are being fabricated using softlithography [8] because of its simplicity and cost effectiveness, and this option was given due consideration. Fabricating the device on a glass substrate was also considered. However, the expertise available in Silicon micro fabrication and the prospect of utilizing the semi conducting properties of Silicon to form amplification and other circuits in future devices on the base chip made this a natural choice for fabricating the *in-silico* cell physiology lab.

2.2.1. Pyramidal pore formation through anisotropic etching

The major steps in the fabrication of the *in-silico* cell physiology chip are indicated in Figure 2. Fabrication is carried out on 4'' {100} Silicon wafers. A 1 micron thick oxide is first thermally grown (oxidation temperature 1050º) to act as a mask for the subsequent KOH etch which forms the pores. The wafer is first spin coated with HMDS (Hexamethyldisiloxane) for adhesion promotion and then AZ-9260 positive photoresist (Microchemicals GmbH) at 2500 rpm for 30 seconds to form a 10 micron thick layer. The wafer is exposed in a mask aligner (KarlSuss MA-24), exposure dose 10 mJ/sec for 80 secs, developed and then subjected to RIE (step i) to transfer the pattern in the oxide (CHF$_3$:C$_2$F$_6$ at 400W Power and 160 torr pressure). The pore were then formed by immersing in a KOH solution (1905 ml DI water: 720 grams KOH: 595 ml isopropanol at 80ºC) for 3 ½ hours. The top width of the pore was optimized so that when subjected to an anisotropic KOH etch (step ii), which etches the crystal planes at an angle
of 56.74°, the depth of the pore (approx. 106 microns) and its shape would be such that the electrodes leading into it will be within 10 microns of the poles of a 125 ± 5 µm fern spore sitting inside the pore. KOH was given preference over TMAH, which also is an anisotropic Silicon etchant like KOH [9], but KOH was chosen because of its higher selectivity for the Silicon crystal planes [10] which minimizes chances of over-etching. Figure 3 shows an SEM of the pores formed on the Silicon wafer.

2.2.2. Electrode definition
A thin insulating oxide layer was thermally grown and then a 240 nm Ti/Ag layer was patterned on the wafer via lift off technique to form the electrodes (steps v and vi). One of the major problems faced was patterning of the electrodes on the inclined wall of the pyramidal pore, as this portion is out of plane while focusing in the mask aligner. However, the AZ-9260 thick positive photoresist provided excellent and repeatable results. 800 nm thick Ti/Au bonding pads were then patterned using the liftoff technique. Finally, a thin layer of AgCl was grown on the electrodes by immersing it in 6% weight NaOCl solution (bleach) for 9-10 seconds. The chloriding was visually observed by noticing the changing the colour of the electrodes which turned a dull black from a shining silver. Care has to be taken to prevent over chloriding which would occur even if chlorided for 5 seconds more. The chloriding was purposefully done after gold bonding pads formation, since the Ti/Au layer would not adhere on AgCl.

2.2.3. SU-8 structural layer
The final step in the fabrication of the in Silico cell physiology lab involved the formation of an insulating layer to insulate the length of the electrodes outside the pores as well as form measurement chambers to secure the fern spores or any other biological entity. SU-8 negative photo resist has played a significant role in forming high aspect ratio, highly inert structures for MEMS applications due to its low absorption near optical UV range [11]. It can achieve thickness up to 200 µm and has been used to form very intricate structures such as a micro gripper [12] and even a Wankel rotary engine [13]. Due to its inert nature and ease of fabrication, SU-8 was used to form the insulating layer. SU-8 50 (Microchem), which can form a 100 µm thick layer, was used for the purpose. SU-8 50 was first static dispensed onto the wafer, and then spun at a 1000 rpm for 40 seconds. After soft baking the resist as per Microchem recommendations [14], the resist was exposed for 65 seconds and then post baked. The resist was developed but was not hard baked. This process yielded an SU-8 layer ranging from 90 to 100 µm in thickness which had very good adhesion and aspect ratio. Figure 4 shows images of the SU-8 layer on the chip and the final chip after dicing.
3. Characterization of the in-silico cell physiology lab

3.1. Characterization setup
The in-silico cell physiology lab was tested for selectivity towards Ca²⁺ ions. For characterization, the chip was first spin coated with ETH-5234 Calcium ion-selective membrane [15] at 1000 rpm for 30 secs, and then was allowed to dry at room temperature until it formed a uniform layer all over the chip. The membrane is composed of plasticizer (65.3% w/w): o-nitrophenyl octyl ether (o-NPOE), Membrane matrix (33% w/w): Poly (vinyl chloride) (PVC), Ionophore (1% w/w): ETH-5234, and cation selector (.7% w/w): Potassium tetrakis (4-chlorophenyl) borate (KTPClP). Exactly 180 mg of the above mixture was dissolved in 4 ml tetrahydrofuran (THF) and stored in a 4º C refrigerator. The membrane was wiped off from the bonding pads using a cotton bud dipped in Acetone and the chip was stuck on a custom PCB using a commercially available epoxy. The custom PCB allows for onboard amplification of the signal greatly reducing noise as well isolation of the data acquisition unit (DAQ) from the ion sensing electrodes. The amplification is done by a set of 64 low noise and low drift op-amps (Analog devices). The in-silico cell physiology lab was wire bonded on to the PCB using an Industrial wire bonder (Westbond) and then the bonding pads were covered with another commercially available epoxy such that all the wire bonds were totally protected by the epoxy, which allows electrical insulation and mishandling protection to the wire bonds, and only the top of the chip where the tests have to be conducted is open. This entire setup is then interfaced with the DAQ system through an National Instruments (NI PXI-6289) 18 bit, 32 channel DAQ card. The DAQ system consists of a connector block through which the electrodes are connected to a switching matrix which allows us to measure different potentials across any 8 pairs of electrodes in real time. This data is then accessed through a program written in Labview (Version 7.0) that allows us to control the switching matrix, and measure/display the data.

3.2. Characterization methods and results
For conditioning the ion selective membrane was soaked overnight in a 10µM CaCl₂ + 10mM NaNO₃ solution [16] which is necessary to activate the membrane. The separate solution method was used to characterize and test the in-silico cell physiology lab. Here, a reference electrode was fabricated using a coiled Ag/AgCl wire that was chlorided by driving it in 1M HCl as the anode of an electrolytic circuit with a 9V battery. CaCl₂ solutions of concentrations varying from 100µM to 10mM were placed on the chip using the protective epoxy as a containing well, and the reference electrode was dipped into this solution. The potential developed by each electrode was then measured and recorded. This method is used to calibrate the chip by measuring the voltage output of each electrode in each of the standard calcium solutions.

Figure 6 Calibration (A=initial, and B=conditioned) curve of solid state calcium electrode array. Electrode output was measured against a Ag/AgCl reference electrode. All of the electrodes were exposed to each of the standard solutions (0.1mM, 1.0mM and 10.0mM)
Figures 6 and 7 show the characterization curves obtained on four electrodes of a pore on the *in-silico* cell physiology chip which are representative of the entire chip. A Nernst slope of 30 mV/decade was observed over repeated characterization cycles, and was consistent with expected results. Though a slight drift is observed but the results are consistent over numerous measurement cycles.

Figure 8 shows the calibrations of 16 of the 64 individual calcium electrodes on the *in-silico* cell physiology biochip. For this calibration 50 µl of 100 µM CaCl₂ was added to the chip and mixed with 50µl DI water to yield a 50µM standard. Next 50µL of this solution was removed and the remaining solution was mixed with 1mM CaCl₂ to yield a 500µM solution. Part of this was then removed and mixed with a sufficient volume of 10mM CaCl₂ to yield a 5mM solution. The calibrations were done in this manner in order to minimize possible introduction of air bubbles in the microfluidic ports, or drying of the membrane that would require substantial reconditioning. Calibrations were obtained for all of the electrodes but for clarity we randomly selected 16 electrode calibrations to be graphed. The mean Nernst slope was 34.2±7.2mV, with 50% falling within the .674σ error bounds.

4. Discussion and Conclusion
In this paper we have reported our efforts in the design and fabrication of an *in-silico* cell physiology device which has currently been calibrated and tested for Ca²⁺ selectivity. The device consists of a set of 64 electrodes leading into 16 pores on a Silicon substrate where each pore acts as a measurement cell in itself. Nernst slope of 30 mV/ decade (average) were obtained and the results were repeatable and stable. We are currently integrating this system and testing cell viability and basic responses (Figure 9) in preparation for microgravity flight experiments to study polar calcium currents that drive cell gravi-morphogenesis. These experiments will be flown on NASA’s Zero-g aircraft operated out of Johnson Space Center. While this version of the *in-silico* cell physiology has been designed targeting analysis of transcellular ionic calcium currents in *Ceratopteris richardii* fern spores, the device is actually much more versatile and can be adapted to a variety of cell physiology and other biomedical applications. Replacement of the Ag/AgCl electrodes with Platinum electrodes and coupled with an amperometric circuit converts the device into a platform for amperometric oxidase enzyme biosensing. Work is already under way to furnish the device with a more sophisticated microfluidics system which will enable faster detection times and more control. Future applications include detection of glucose, plant hormones (auxin), as well as multiple analyte detection on the same chip.

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Figure 9. Micrograph showing individual fern spores integrated into the fluidic ports on the *in-silico* cell physiology biochip. Here we see an individual un-germinated spore (insert) and a germinating spore with it’s growing rhizoid growing out of the pore.

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