A MEMS fabricated cell electrophysiology biochip for in silico calcium measurements

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Abstract

For the last 50 years the state-of-the-art for studying electrophysiological activity of single cells has been based on an investigator using a single microprobe, and attempting to make relevant recordings, one cell at a time. Here we report the design, fabrication and characterization of a MEMS-based lab-on-a-chip system for measuring Ca2+ 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 16 Ceratopteris richardii fern spores in microgravity flight experiments and ground studies. The 16 microfluidic cell holding pores are 150 μm x 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 onto a custom PCB for easy interface with a state-of-the-art data acquisition system. The electrodes are coated with a Ca2+ ion-selective membrane based on ETH-5234 ionophore and operated against an Ag/AgCl reference electrode. Characterization results have shown Nernst slopes of 30 mV/decade that were stable over a number of measurement cycles, and actual fern spore Ca2+ measurements have been recorded with high repeatability and reproducibility. While this work is focused on technology to enable basic research on C. richardii spores, we anticipate that this type of cell electrophysiology 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.

Keywords: Ca2+; Ion-selective electrode array; Silicon; Ceratopteris richardii; Microgravity

1. Introduction

The last 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 [1]. 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 [2,3].

The most popular method for fabricating these μTAS systems utilizes silicon micro fabrication techniques commonly employed in the microprocessor industry. Uhlig et al. [4] 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 (20, 600 and 10 nm) formed by sputtering and thermal evaporation with the chloriding being performed by immersing in 0.1 M NaCl solution. The electrodes were then coated with poly(hydroxyethyl methacrylate) (p-HEMA) 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. Guenet et al. fabricated and characterized a Ca2+ ion-selective microelectrode array platform based on silicon nitride micropipettes and platinum electrodes on a silicon substrate [5]. 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 the 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 onto 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. During early development in the spores of this fern plant, 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 [6]. This irreversible process drives polar axis development within the cell and was discovered using the self-referencing calcium microelectrode. The self-referencing technique allows for the measurement of dynamic flux from individual cells based on computer controlled micro-positional translation and data acquisition of a single ion-selective microprobe [7]. While this technique has proven to be a significant tool to study electrophysiology in cells, it does have inherent limitations that restrict our ability to conduct further research to understand these ion-current signaling events. Specifically we need to continually measure Ca2+ fluxes from multiple positions around each cell, as well as monitor multiple cells at the same time.

The device needed to be small in size, but readily interfaced with a fully functional and dynamic data acquisition system for it to be truly useful as a generic cell physiology analysis platform for discovery in biosystems. These efforts culminated in the development of an in silicon cell electrophysiology biochip (CEL-C). The device was microfabricated to include 16 pyramidal pores on a silicon substrate, 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. Characterizations have been performed using different concentration CaCl2 solutions and have shown stable results which were reliable and repeatable over multiple cycles. Recently actual fern spore Ca2+ ionic gradient measurements were performed successfully using the CEL-C biochip and the results are also reported in this article.

2. Materials and methods

2.1. Design of the CEL-C device

The basic design of the CEL-C biochip is depicted in Fig. 1, where the overall dimensions of the chip are 9 mm × 11 mm. The silicon substrate microchip has 16 pyramidal pores fabricated in it, each of which acts as measurement chamber for measuring trans-cellular Ca2+ ion currents around individual cells. The dimensions of each pore are 150 μm × 150 μm, each with four Ag/AgCl electrodes which extend approximately 14 μm inside each pore. The electrodes are coated with a thin Ca2+ ion-selective membrane to impart ion selectivity. The geometric dimensions of the chip were designed based on the general size of the target cell for the research application. Here it is important that when a fern spore sits inside the pore, the maximum distance from the pole of a fern spore to the electrodes should not exceed 10 μm, which is the maximum allowable distance for an electrode to detect the signal generated by the spore. The entire chip is covered with an SU-8 structural layer which provides insulation to the electrodes, except for a very small portion of the length of the electrodes inside the pores. The SU-8 layer also acts as an enclosure for the pores, sealing the fern spores inside the pores.

2.2. Fabrication of the CEL-C biochip

There were many choices of substrates for fabricating the CEL-C biochip. The most commonly used substrate for MEMS and BioMEMS applications is silicon. Silicon is the standard substrate material for IC fabrication and thus the most common substrate material in the fabrication of MEMS devices [8,9]. An array of devices can be fabricated on a single wafer, which actually defines the yield that is the number of working devices out of the total number of devices on a wafer. Also silicon has excellent mechanical properties, which make it an ideal material for MEMS applications [10]. Glass is another material which is used considerably in MEMS fabrication because of its good dielectric and mechanical properties. Ceramics resist corrosion at high temperatures so that they can operate in harsh environments and also reduce problems in microfabricated devices such as stiction [11]. One of the materials which have recently become the material of choice for MEMS, especially bioMEMS applications is polymers. One such polymer is polydimethylsiloxane (PDMS) whose fabrication principle was first proposed in the paper by Xia and Whitesides, and is termed as soft lithography [12]. Due
to the cost advantage, disposable devices such as microfluidic arrays and microstructures DNA or immuno assays are often fabricated on polymer-based substrates. One example of a simple device is the design and fabrication of silicon micro syringes with a PDMS reservoir for fluid injection into the epidermis [13].

Based on this analysis, the facilities available in the Purdue Microelectronics and Nanotechnology labs, ease of fabrication and the prospect of utilizing the semi conducting properties of silicon to form amplification and other circuits on the base chip in future devices, it was chosen as the substrate material of choice for fabricating the CEL-C biochip. A four mask step process flow was designed to fabricate the CEL-C biochip. Fig. 1 shows in detail the different representative steps in the fabrication of the biochip. The following sections describe in detail the fabrication steps.

Fabrication is carried out on single-sided polished 4 in. (100) silicon wafers. A 1 μm thick oxide is thermally grown on the silicon wafer by inserting in an oxidation furnace at 1050 °C with wet O2. The oxide acts as protective layer or mask for the subsequent anisotropic silicon etch which defines the pyramidal pores. Next, hexamethyl disiloxane (HMDS) adhesion promoter was spin-coated onto the wafer at 4000 rpm for 30 s, followed by AZ-1518 positive phororesist (Micro Chemicals GmbH, Germany) also at 4000 rpm for 30 s. The photoresist is then softbaked for 5 min on a hot plate at 90°C and then exposed in a mask aligner (Karl Suss MA-24) at exposure dose 10 mJ/sec for 20 s. The photoresist is then developed in an AZ 351:DI water solution (1:3) for 45 s. The photoresist acts as a mask for etching the oxide. Since it was desired that the exact pattern be transferred into the oxide from the photoresist, RIE was given preference over BHF to etch the oxide (CHF3:C2F6 at 400 W power and 160 Torr pressure Drytek Triode).

The pores were then formed by immersing in a KOH solution (1905 ml DI water:720 g KOH:595 ml isopropanol at 80 °C) for 3.5 h. Isopropanol is a known addition to KOH solutions for improved surface finish of the silicon surfaces, but it also affects the etch rates of KOH solution [14]. 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 (approximately 106 μm) and its shape would be such that the electrodes leading into it will be within 10 μm of the poles of a 125 ± 5 μm fern spore sitting inside the pore. Tetramethyl-ammoniumhydroxide or TMAH ((CH3)4NOH) which is another silicon anisotropic etchant and is available in the Purdue MicroElectronics and Nanotechnology labs in 25 wt% water solutions commercially available from J.T. Baker, USA was also given due consideration. It gives an etch rate of approximately 40 μm/h when carried out at 90 °C as shown in the experiments carried out by Chen et al. [15]. TMAH has the advantage that it has high selectivity for etching SiO2 as compared to Si, less than 5 nm/h for the same conditions as mentioned before which enables usage of thin oxide layers as mask for etching relatively deep silicon surfaces. However, KOH has a much higher selectivity for the silicon crystal planes as compared to TMAH, approximately 600:400:1 for {110}:{100}:{111} planes [16]. Also a properly controlled KOH etch tends to produce a smoother surface finish as compared to TMAH. Since the most critical part of the pore fabrication process was to maintain a top width of 150 ± 5 μm, it was necessary that side wall etching should be as little as possible so KOH was given preference over TMAH for producing the pores.

A thin insulating oxide layer was thermally grown and then a 240 nm Ti/Ag layer was patterned on the wafer via liftoff technique [17] to form the electrodes. 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 photore sist provided excellent surface coverage and repeatable results as is evident from Fig. 2. Eight hundred nanometers thick Ti/Au bonding pads were then patterned also using liftoff. Finally, a thin layer of AgCl was grown on the electrodes by immersing it in 6 wt% NaOCl solution (bleach) for 9–10 s. The chloriding was visually observed by noticing the change in color of the electrodes which turned a dull black from shining silver. Care has to be taken to prevent over chloriding which would occur even if chlorided for 5 s more. The chloriding was purposefully done after gold bonding pads formation, since the Ti/Au layer would not adhere on AgCl. Fig. 2 shows an SEM image of the Ag electrodes leading inside the pyramidal pore, the chlorided electrodes and the gold bonding pads.

The final step in the fabrication of the CEL-C biochip was the formation of the structural layer which serves the dual purpose of insulating the electrodes as well as forming a holding cell for the fern spores to sit inside. PDMS was one material which was considered for this application which is used very frequently in microfluidic packaging applications [18]. The other was SU-8 negative photo resist, which 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 [19]. It can achieve thickness up to 200 μm and has been used to form very intricate structures such as a micro gripper [20] and even a micro wankel rotary engine [21]. 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 s. After soft baking the resist as per Microchem recommendations, the resist was exposed for 65 s 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. Fig. 5 shows images of the SU-8 layer on the chip and the final chip after dicing.

3. Experimentation and results

3.1. Ca²⁺ ion selectivity and packaging

The CEL-C biochip was tested for selectivity towards Ca²⁺ ions. For characterization, the chip was first spin-coated with ETH-5234 calcium ion-selective membrane [22] at 1000 rpm for 30 s, 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-nitrophenoxy 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 (KTP-CIP). Exactly 180 mg of the above mixture was dissolved in 4 ml tetrahydrofuran (THF) and stored in a 4°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 (Fig. 3) 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 CEL-C device is then wire bonded onto the PCB using an Industrial wire bonder (Westbond, UIC) and then the bonding pads are 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 a sophisticated DAQ system through a 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 eight 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. Details of the hardware and software have been reported elsewhere.

For conditioning the ion-selective membrane was soaked overnight in a 10 μM CaCl₂ + 10 mM NaNO₃ solution [23] which is necessary to activate the membrane. The separate solution method was used to characterize and test the CEL-C biochip. Here, a reference electrode was fabricated using a coiled Ag/AgCl wire that was chlorided by driving it in 1 M HCl as the anode of an electrolytic circuit with a 9 V battery. CaCl₂ solutions of concentrations varying from 100 μM to 10 mM 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.

Fig. 4 shows the characterization curves obtained on four electrodes of a pore on the CEL-C device 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, the results are consistent over numerous measurement cycles. Fig. 5 shows the ranked normalized calibrations of all 64 calcium electrodes on the CEL-C biochip. The mean Nernst slope was $34.2 \pm 7.2$ mV, with 50% falling within the $674 \pm \sigma$ error bounds. The most recent batch of chips yielded 12/14 chips that produces 100% calibration yields of all 64 electrodes with results similar to Fig. 5. Real-time calibration plots of selected sensors on the chip show that the response time of the sensor is in the millise-
Fig. 4. Calibration curves from a solid state calcium electrode array in a single fluidic pore on the CEL-C device. Electrode output was measured against an Ag/AgCl reference electrode. All of the electrodes were exposed to each of the standard solutions (0.1, 1.0 and 10.0 mM). Plots A and C are the initial curves, whereas B and D reflect stable calibrations achieved after conditioning. Two runs of calibration curves were done to test the shelf life of the selective calcium selective membrane. Plots A and B are based on data obtained from a newly fabricated chip, whereas C and D are based on data obtained from that chip after being dried down and stored for a week. A slight drift is observed but the same Nernst slope is obtained.

ond time frame shown in Fig. 6, which is more than adequate for the current research applications.

3.2. Fern spore measurements

As part of the final preparations towards the NASA microgravity flight experiments we have been making recordings of the signals obtained from germinating spores on the chip. Several preliminary experiments have been conducted, and these have provided only a glimpse of the dynamics of the intrinsic calcium currents in this cellular system. In one experiment we challenged the spores with a mitochondrial inhibitor (Fig. 7) which deprived the cell of ATP required to drive the calcium

Fig. 5. Fixed interference method calibration curves of all 64 electrodes on the CEL-C device. For clarity the calibration curves were ranked and plotted. The mean Nernst slope was 34.2±7.2 mV, with 50% falling within the 674e error bounds.

Fig. 6. Real-time calibration of solid state calcium sensors on the CEL-C device. At time = 0 the electrodes were exposed to 0.1 mM CaCl₂. Concentrated calcium solution aliquots were subsequently added to the chip to yield the following concentrations: 0.3, 0.65, 2.825 and 6.4125 mM. The individual sensor responses to the concentration changes were nearly instantaneous.
pumps in the membranes of the sarcoplasmic reticulum and the plasmalemma. As a result we can see the cells releasing their calcium stores into the media within the fluidic pore.

Recently, we have been able to demonstrate successfully the full capability of the CEL-C biochip by performing ground experiments on fern spores over a course of almost 20h. Fig. 8 shows the results obtained on one of the spores which strongly corroborate the results first demonstrated by Chatterjee et al. The recordings were started 3.5 h after the spores were first exposed to light, or the onset of germination. This data has been collected using the DEDC mode which measures the potential difference between the top–bottom electrodes in a pore. As is clear, the potential difference goes up until it reaches a maximum at around 8 h of germination. This indicates an increasing Ca$^{2+}$ efflux at the top and influx at the bottom as was demonstrated by Chatterjee et al. using self-referencing microelectrodes. The potential difference then remains steady for a couple of hours and then starts decreasing until it reaches a minimum value around hour 18 and then flat lines. This is where the Ca$^{2+}$ current subsides and any change in the gravity vector does not influence the polarity of the fern spore any more.

4. Discussion and conclusion

Advanced tools for studying cellular development and physiology are required by the life sciences community in order to advance science in areas that are not accessible by invasive/destructive techniques. In this work we have developed this type of technology with the goal of applying it to study cellular calcium signalling in the model system, the Ceratopteris fern spore. Edwards and Roux [24] first demonstrated the gravitropic response by C. richardii fern spores using time lapse images of nucleus migration during the first 24 h of polarity determination. Chatterjee et al. [6] later linked this to a Ca$^{2+}$ ion flux around the fern spores, that correlates to a transcellular calcium current (high efflux at the top and influx at the bottom). This current was shown to correlate with the earth’s gravitational field and directs polarity for the first 24 h of germination. This was measured using the self-referencing Ca$^{2+}$ selective vibrating electrode developed by Kuhntreiber and Jaffe [25]. While this approach did allow the research to document the cell response to gravity, it was limited by the number of locations around the cell that can be monitored, and the research environment in which it can be used. Ultimately there was a need to continuously measure multiple positions around several cells in near real-time, in order to understand the dynamics of the system. The result of this work is CEL-C technology that allows for continuous measurement of 16 cells using four electrodes for each cell. Because the cells are immobilized into the microfluidic pore with the sensor array, the chip itself can be used in dynamic experiments including real-time rotation, and actual microgravity flight experiments.

Here we extended the pioneering work of microfabricated ion-selective electrode array platforms, previously demonstrated by Uhlig et al. [4] and Guenat et al. [5]. In our project we have been able to extend this work to the level where it has become an essential tool for basic cell biology research. The CEL-C biochip has demonstrated the capability as a reliable cell electrophysiology device, which can be extended to encompass a wide range of ion detection. Calibrations on a wide range of devices show output and general behaviour (Fig. 4) which is in agreement with expected Nernst slopes of Ca$^{2+}$ selective electrodes. The demonstrated repeatability in the CEL-C calibrations, where Nernstian calibrations were stable over periods of weeks, is the first indicator of device reliability and repeatability of the fabrication process. The bonding and uniformity of the calcium selective membrane over the chip is promoted by the hydrophobic nature of the SU-8 layer and the organic membrane solution. Because of the relatively high surface area of the SU-8 material relative to the exposed metal and silicon materials, a fairly even and
complete layer of material was deposited and cured. The bonding and adhesion between the membrane and the SU-8 materials effectively provides an electrically insulating barrier the separates each of the sensor circuits from one another. The additional advantage of the calcium membrane is it effectively changes the bulk surface of the chip to a hydrophilic surface, once it is cured through solvent volatilization, and this subsequently promotes wetting and loading of the device.

The response time of the chip to any changes in Ca\textsuperscript{2+} concentration (Fig. 6) is in the millisecond time frames, and this response is repeatable over a number of cycles. Coupled with a state of the art amplification and DAQ system, equipped with flexible software designed specifically for this system, we can measure this response on all the 64 electrodes at the same time with minimal noise. In Fig. 7 we first demonstrated the capability of the CEL-C biochip by measuring the Ca\textsuperscript{2+} released by germinating fern spores loaded on one biochip in response to a mitochondrial inhibitor (KCN). This was followed by actual ground measurements of Ca\textsuperscript{2+} ionic flux around germinating fern spores measured over a period of 24 h indicated in Fig. 8. Throughout these experiments, the CEL-C device is encased in a Faraday box to nullify the effect of external artefacts. Since these measurements were done in the DEDC mode, explained in the previous section, we measured the total voltage difference measured across the top and bottom of a germinating fern spore. The results are interpreted in terms of the average Nernst slope of the top and bottom electrode (32 mV/decade) which is an indicator of the changing Ca\textsuperscript{2+} ionic flux across the fern spore. The result shows an increase in the calcium concentration across the fern spore for the first 8–10 h after which it decreases gradually and completely disappears around hour 18–19. These results are very similar to the results obtained by Chatterjee et al. [6] with the only difference being that now the entire experiment can be done at multiple points around a single fern spore and on 16 fern spores at the same time.

In conclusion the CEL-C biochip has demonstrated the capability of becoming an all around cell electrophysiology tool for cell biologists. 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. Initial ground studies have already been performed on fern spores and we are currently integrating this system and testing cell viability and basic responses 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 out of Johnson Space Center. While this version of the in silico CEL-C biochip has been designed targeting analysis of transcellular ionic calcium currents in *C. richardii* fern spores, the device is actually much more versatile and can be adapted to a variety of cell physiology and other biomedical applications to become the ultimate tool for systems biology. Replacement of the Ag/AgCl electrodes with platinum electrodes and coupled with an amperometric circuit converts the device into a platform for amperometric detection of oxygen, nitric oxide, ascorbate and various neurotransmitters. Amperometric detection of hydrogen peroxide is also a bridge to oxidase based 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. This would also allow us to expand to do multiple analyte detection on the same chip.

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References

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