Supplementary Material

for

Olfaction on a Chip

by

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1 Technology

1.1 Chip

CMOS neural amplifiers capable of amplifying and recording the action potentials from electrically active cells have been demonstrated. The basic circuit used for the bioamplifers in this work is based on [30]. This circuit is an operational transconductance amplifier (OTA) with its gain set by capacitive feedback. This design was chosen because it presented a good balance between system noise and total power usage. Minor adjustments were made to the circuit, the most notable of which was the addition of a cascode to the current mirror to increase the common-mode rejection capability of the structure. The only other real change to the system was the addition of unity gain buffer stages after the amplifiers, which proved necessary to handle the large capacitive load (100 pF per channel) presented by our data acquisition hardware. The IC was fabricated in a commercially available 0.35 μm CMOS process using the MOSIS service.

1.2 Chip Packaging

The packaging procedure was described in detail in [27]. A polymer handle wafer was formed around the CMOS IC. Electrical connections to the bondpads at the perimeter of the IC were made by patterned thin film metal traces (Ti/Au). The sensing electrodes (formed at the IC foundry using a reactive material such as Al) were also covered with the Ti/Au layer to present a relatively inert surface to the cells. The chip surface was selectively passivated with Parylene C to expose the sensing electrodes while protecting the bondpads and input/output traces from the cell culture medium.

The package redistributes the chip's electrical input/output connection points to the periphery of the handle wafer. This provides the dual benefits of moving the electrical connections away from the fluidics and increasing their pitch, which makes it easier to form electrical connections using non-microfabricated components.

1.3 PCB and DAQ

The packaged CMOS chip was interfaced to the DAQ via a two-level PCB using spring-loaded contact pins (Figure 4). The PCB consisted of a pair of two-layer boards used in a motherboard-daughterboard configuration with the packaged chip clamped between them. An on-board power

source of 2 AA batteries minimized power line noise in the system. Potentiometers set bias voltages, and decoupling capacitors stabilized the power supply and reference voltages for the IC. Headers connected the flat ribbon cables to the DAQ. An access port was milled into the top PCB to allow access to the packaged chip. The port was used to pass through the tubes for stimulant delivery, and also to allow optical assessment of the chip surface. The entire PCB was designed to be placed in either an incubator or a refrigerator, depending on the requirements of the cell culture being performed on the chip surface.

Voltages were recorded from all 20 channels on the chip simultaneously at a sampling frequency of 20 kHz and a resolution of 16 bits. Data conversion with the DAQ (National Instruments, USB-6259) was at a sampling rate of 1.25 Msamples/sec. Data were saved using a Matlab script.

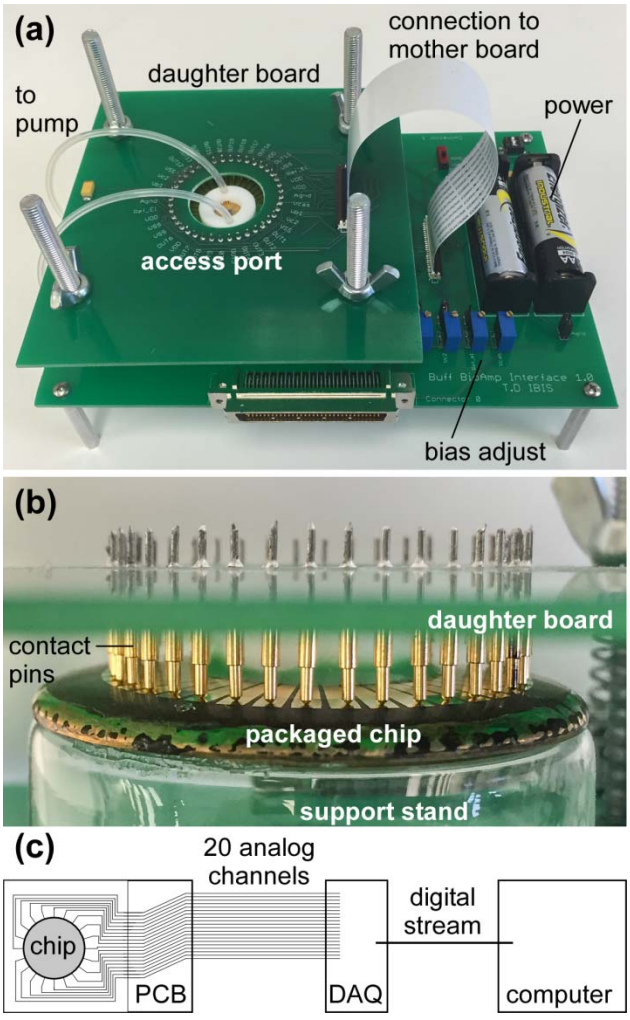

Figure 4. a) Overview of the PCB boards used to contact the chip. b) The daughter board had spring-loaded pins to contact the chip package and a cable to the mother board. The mother board included batteries for power, pots for adjusting the on-chip transistor biases, and a cable connector. c) The PCB was connected to a data acquisition system that converted the analog data to digital signals, which were transmitted to a laptop for recording and later analysis.

1.4 Data Processing

In most cases it was necessary to filter the data to automate detection of spiking events. Significant noise was observed at the 60 Hz power line frequency and its harmonics. Signals were filtered in Matlab using a high-pass filter with 80 dB rejection and a cutoff frequency of 400 Hz. (Filtering with a bandpass filter (400 Hz – 5 kHz) lead to exaggeration of certain frequency components and distortion of the signal.) A peak-finding algorithm (peakfinder.m, Nathanael Yoder, 2013), made an initial identification of action potentials by determining the temporal derivative of the signal as well as relative and absolute thresholds. In cases of small peaks in high noise, this was followed by a weighted-average algorithm.

2 Materials and Methods

2.1 Amphibian Cell Harvesting and Plating

Culture requirements for amphibian cells are less strict than those for mammalian cells, allowing the use of CO_2-free media and storage in a refrigerator. Salamander OSNs are also significantly larger than murine cells, allowing easier identification and handling.

Adult tiger salamanders (Ambystoma tigrinum) were anesthetized by percutaneous application of benzocaine (0.02% solution: 990 mL 48-hour aged tap water, 10 mL ethanol, 200 mg benzocaine (Sigma E1501)). Salamanders were placed into the benzocaine bath to immerse the ventral side for 20-30 minutes [44] and then euthanized.

Olfactory epithelium was dissected from the dorsal and ventral walls of the nasal cavities. The tissue was placed into a modified amphibian Ringer's solution fortified with antibiotics and cut into mm-sized pieces. The modified Ringer's solution had the following composition: 120 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5 mM HEPES, 5 mM glucose, 0.5 mM EDTA, 100 U/mL penicillin, 100 µg/ml streptomycin, adjusted to pH 7.6 using NaOH and HCl and passed through a 200 nm filter. After dissection the tissue was rinsed several times with Ringer's solution to remove mucus and bacteria.

The minced tissue was digested with papain (20 units/ml) activated with L-cysteine (1 mM) in a modified Earle's Balanced Salt Solution (EBSS) containing EDTA (0.5 mM). Before mixing in the enzyme, the EBSS was brought to physiological pH by bubbling 95% O_2:5% CO_2 through it for 5 minutes, then it was filtered for sterilization and fortified with antibiotics (100 U/mL penicillin, 100 µg/ml streptomycin). Lyophilized enzyme (Papain Dissociation System, Worthington Biochemical Corporation) was mixed into the solution by swirling, then the solution was heated to 37 °C in a water bath for 10 min in a capped container in which the air had been displaced by O_2:CO_2.

The tissue was incubated in the digestion solution at 35 °C for an hour, with constant agitation. The tissue was then triturated gently 10 times, and remaining large tissue pieces allowed to settle to the bottom of the tube. The turbid cell solution was removed and centrifuged (300 G, 5 min). The supernatant was removed and replaced with 3 mL of the modified EBSS, with added protease inhibitors, ovomucoid inhibitor (1 mg/mL), and bovine serum albumin (1 mg/mL). The

cell pellet was re-suspended and incubated at 4 °C for 15 min. Because the osmolarity of amphibian medium is lower than that of EBSS, the solution was changed slowly. Cell viability was maximized when amphibian storage medium (120 mM NaCl, 3 mM KCl, 1mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose at pH 7.2, fortified with 50 U/mL penicillin and 50 µg/ml streptomycin) was added drop by drop over approx. 5 min to the cell suspension. This solution was centrifuged (300 G, 5 min), the supernatant removed, and the cell pellet re-suspended in 1 mL of amphibian storage medium.

The number of cells and their viability were determined by Trypan blue staining (Invitrogen) and a hemocytometer (Fisher, Neubauer hemocytometer). Typical yields from two animals were on the order of 10^6 viable cells.

2.2 Cell Plating

The packaged amplifier chips were treated with O_2 plasma (30 sec. 100 W, 500 mTorr, 2 sccm O_2) to render the parylene and chip surfaces hydrophilic. A cell culture well was created by gluing (bio-compatible silicone adhesive Kwik-Cast Sealant, World Precision Instruments) a chamber (Teflon, 1 cm dia.) to the surface. The plating area was incubated overnight at 4 °C with a polyethyleneimine (PEI) solution (PEI (Sigma P3143) diluted to a final concentration of 0.1% in borate buffer (155 mg boric acid (Sigma-Aldrich B6768), 237 mg sodium tetraborate (Sigma 71997), 50 mL sterile filtered water (Sigma W3500), pH adjusted to 8.4 with HCl). After incubation, the PEI solution was removed by aspiration and the chip surface rinsed 4x with sterile water.

The well was filled with 100 µL of a solution containing concanavalin A (Sigma L7647) 10 mg/mL in phosphate buffered saline (PBS) (D8662 Sigma-Aldrich). The solution was allowed to settle for 1 hour at room temperature, after which the liquid was removed.

The dissociated cells were plated at a density of 2000 cells/mm^2, and the total media volume was brought to 200 µL. The cells were allowed to settle overnight. Between experiments the cells were stored at 4 °C, and half the medium was replaced daily to compensate for evaporation.

2.3 Odorants and Perfusion

2.3.1 Odorant Solutions

Odorant solutions were prepared by mixing stock solutions of odorants into the culture medium. Each odorant was first mixed at a concentration of 20 mM in dimethyl sulfoxide (DMSO). Three stock odorant mixtures were used, each containing three odorants. Just before use, the odorant solutions were diluted in cell culture medium by a factor of 100, yielding 200 µM odorant in a 1% DMSO solution (total 600 µM in 3% DMSO). Due to the volatility of these odorants, new solutions were prepared every two days.

Mixture	Syringe	Odorant	Conc.	Sigma #
1 (60 mM)	1	α-pinene	20 mM	147524
	2	geraniol	20 mM	163333
	3	amyl acetate	20 mM	W504009
2 (60 mM)	1	eucalyptol	20 mM	C80601
	2	acetophenone	20 mM	A10701
	3	isopentyl acetate	20 mM	79857
3 (60 mM)	1	2-isobutyl-3-methoxypyrazine	20 mM	297666
	2	linalool	20 mM	L2602
	3	l-carvone	20 mM	W224901

2.3.2 KCl Control Solution

The culture medium was modified to contain 50 mM KCl, with NaCl concentration reduced to maintain osmolarity. The final solution contained 73 mM NaCl, 50 mM KCl, 1mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose at pH 7.2, fortified with 50 U/ml penicillin and 50 μg/ml streptomycin.

2.3.3 Perfusion

A PTFE perfusion chamber with machined paths was specially designed that limited the fluid volume to 200 μL and permitted optical assessment using an immersion lens (Figure 5). The design of the perfusion chamber was informed by a COMSOL simulation of fluid flow. Fluid volume in the perfusion chamber during storage was 200 μL, but during experiments it was reduced to 100 μL to enable faster solution exchange. Three syringe pumps were used for perfusion (NE-1000 New Era Pump Systems): two for infusion and one for withdrawal at rates of 200 and 1000 μL/min, respectively. Using two separate pumps for infusion allowed for quick exchange of the perfusion solution, with the second perfusion line primed and ready to be plugged into the port once the first pump finished. Syringes (5 mL) were placed into the pumps, with needles (18 gauge) connecting the syringes to silicone tubing. The tubing was primed before use and plugged into ports on the perfusion chamber.

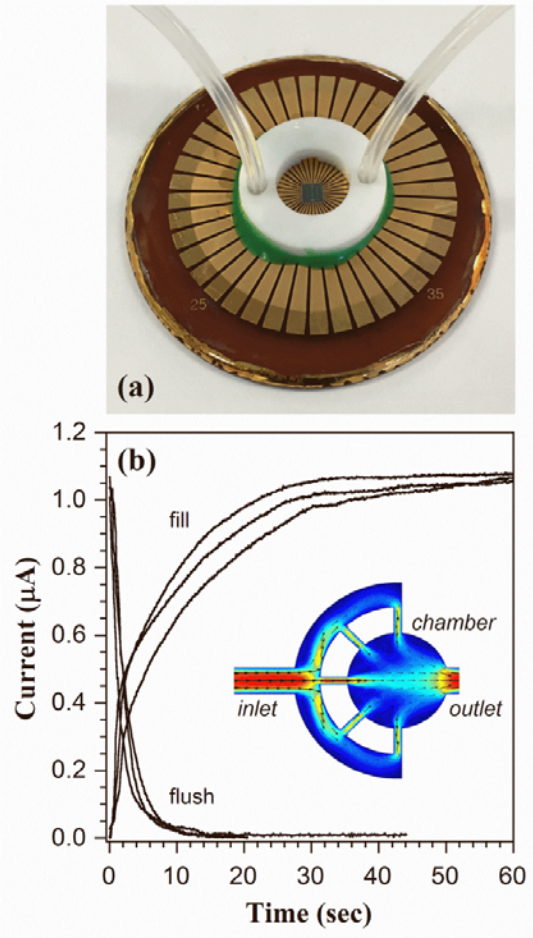

Figure 5. a) A packaged IC surmounted by the perfusion chamber. The chamber outlet can be seen at the top of the inner wall, at the center. b) Current as a function of time as a measure of solution concentration change. Flushing was performed at a five times faster rate than filling. The inset figure shows a COMSOL simulation of the fluid flow along the pathways in the chamber from the five inlets to the one outlet. Color indicates velocity, with dark blue representing the lowest velocity and red the highest.

The perfusion system was characterized by measuring the change in conductivity between two electrodes. The PTFE perfusion chamber was placed on top of a surface on which two thin film gold electrodes had been patterned. The chamber was filled with deionized water and the following perfusion protocol was followed using 1 M KCl solution: 60 seconds KCl at 200 μL/min, 5 seconds wait, 15 seconds deionized water at 400 μL/min, 5 seconds wait; this procedure was repeated 3 times. The resulting change in conductivity is shown in Figure 5. Ninety percent of the maximum concentration was reached after 30 seconds, and 10% of the maximum concentration was reached 5 seconds after initiating flushing.

2.3.4 Odorant Perfusion

Perfusion of odorants in each experiment followed the same protocol. The chamber was initially flushed with medium containing 1% DMSO (15 sec at 400 μL/min with 5 additional seconds to allow for switching the solution lines, for a total time of 20 sec). Then each odorant mixture

solution was perfused (60 seconds at 200 µL/min, with an additional 5 seconds for switching), with a 15 sec flushing step (using regular storage medium modified to contain 1% DMSO) in between odorant solutions. Lastly the modified medium containing 50 mM KCl was perfused for 60 sec after the final flushing step. If an OSN in the cell plating was found to respond to a particular odorant mixture, the next experiment included perfusion of only the constituent odorants of that mixture to determine which odorant had elicited the response. After experiments were performed, the cells were flushed with standard medium and the total volume was brought back to 200 µL for storage. If multiple experiments were performed on the same day, the cells were stored at 4 °C for a period of 1-2 hours between experiments.

References

Numbering follows that used in the main text.

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