ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

A Novel Silicon Patch-Clamp Chip Permits High-Fidelity Recording of Ion Channel Activity From Functionally Defined Neurons

Christophe Py,[1] Mike W. Denhoff,[1] Marzia Martina,[2] Robert Monette,[2] Tanya Comas,[2] Tarun Ahuja,[2] Dolores Martinez,[1] Simon Wingar,[3] Juan Caballero,[3] Sylvain Laframboise,[2] John Mielke,[2] Alexei Bogdanov,[3] Collin Luk,[4] Naweed Syed,[4] Geoff Mealing[2]

[1]Institute for Microstructural Sciences, National Research Council of Canada, 1200 Montreal Rd., Ottawa, Ontario, Canada K1A0R6; telephone: 613-990-3598; fax: 613-990-0202; e-mail: christophe.py@nrc.ca

[2]Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada

[3]Canadian Photonics Fabrication Centre, Ottawa, Ontario, Canada

[4]Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada

Received 17 February 2010; revision received 14 May 2010; accepted 3 June 2010

Published online 20 July 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.22834

ABSTRACT: We report on a simple and high-yield manufacturing process for silicon planar patch-clamp chips, which allow low capacitance and series resistance from individually identified cultured neurons. Apertures are etched in a high-quality silicon nitride film on a silicon wafer; wells are opened on the backside of the wafer by wet etching and passivated by a thick deposited silicon dioxide film to reduce the capacitance of the chip and to facilitate the formation of a high-impedance cell to aperture seal. The chip surface is suitable for culture of neurons over a small orifice in the substrate with minimal leak current. Collectively, these features enable high-fidelity electrophysiological recording of transmembrane currents resulting from ion channel activity in cultured neurons. Using cultured Lymnaea neurons we demonstrate whole-cell current recordings obtained from a voltage-clamp stimulation protocol, and in current-clamp mode we report action potentials stimulated by membrane depolarization steps. Despite the relatively large size of these neurons, good temporal and spatial control of cell membrane voltage was evident. To our knowledge this is the first report of recording of ion channel activity and action potentials from neurons cultured directly on a planar patch-clamp chip. This interrogation platform has enormous potential as a novel tool to readily provide high-information content during pharmaceutical assays to investigate in vitro models of disease, as well as neuronal physiology and synaptic plasticity.

Biotechnol. Bioeng. 2010;107: 593–600.

KEYWORDS: patch-clamp; silicon planar patch-clamp chip; low capacitance; cultured neurons; electrophysiological recordings; whole-cell recordings

Introduction

Nervous system function and processing of information in the brain is dependent on the intrinsic and synaptic properties of networks of neurons, and these in turn are contingent upon ion channel activity. Ion channels are highly specialized proteins embedded in cell membranes that regulate the flow of specific transmembrane ionic currents. Due to their key role in regulating physiological function, ion channels are recognized as important therapeutic targets for pharmacological intervention, and numerous drugs have been developed that interact with specific ion channels to modify their activity.

Ion channel activity is directly measured by a technique called patch-clamp (Neher and Sakmann, 1976; Walz et al., 2007), which requires that a small-tipped glass pipette filled with electrochemically conductive (physiological saline) solution be sealed to a patch of cell membrane by suction and its voltage potential clamped; small currents resulting from ion channel activity across the membrane are then recorded. Despite the remarkable resolution of this technique, it is a difficult and laborious process that is

Correspondence to: C. Py

Contract grant sponsor: Canadian Institute of Health Research (CIHR) Grant

impractical for high-throughput pharmacological screens which are important tools for drug development (Dunlop et al., 2008). Consequently, considerable effort has been invested in automating patch-clamping and has recently resulted in the development of planar patch-clamp chips (Behrends and Fertig, 2007; Sigworth and Klemic, 2005), where the apex of the pipette is replaced by a microscopic aperture in a self-supported film on which the cell is placed. The fabrication process typically involves patterning a microscopic aperture in the thin film on a wafer and excavating the bulk of that wafer from the back to form the self-supported film. The chip is then mounted in a two-chamber setup, the top one serving as the culture dish and the bottom one as the equivalent of the inside of the glass pipette, containing the physiological saline. The cell is patched on the micrometer-sized aperture and probed by two electrodes, one in each bath (Fig. 1).

The first planar patch-clamp devices were demonstrated on silicon chips using suspended cells from cell lines over-expressing specific ion channels (Fertig et al., 2000; Schmidt et al., 2000). Later, quartz or glass (Fertig et al., 2002, 2003) and polydimethyl siloxane (PDMS or silicone; Klemic et al., 2005) patch-clamp chips were designed to overcome the high capacitive coupling between the culture media and the measuring media resulting from the semiconductive nature of the silicon bulk. Still, the advantages of silicon as a material are considerable. Silicon micromachining is extremely well understood thanks to its pervasive use for microelectronics applications and its offshoot in micro-electro mechanical systems (MEMS) (Gad-el-Hak, 2001), so large quantities of chips can be produced with very high yield and critical process control. This is a significant advantage compared to competing technologies which to date have not demonstrated the same degree of manufacturability. Additionally, silicon is attractive for the integration of multiple functionalities, among which on-chip amplification has already been demonstrated (Aziz et al., 2007; Kaul et al., 2004) and which could extend to other co-located sensors using conventional MEMS technology. To overcome the high coupling capacitance problem, restricting the chamber volume of the chip has been proposed (Matthews and Judy, 2006; Pantoja et al., 2004). However, the quality of signals is still low perhaps because PDMS is highly permeable to water.

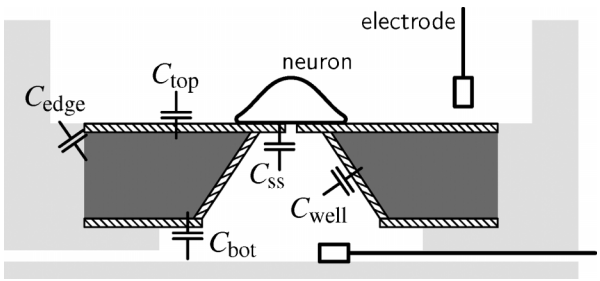

Figure 1. Shunt capacitance model of the packaged chip. The light gray area is the plexiglass package, the dark gray is the silicon wafer, and the hatched areas are silicon nitride/silicon oxide dielectric. The positions of a neuron and two electrodes are shown schematically.

None of the patch-clamp chip devices reported to date have been successfully used to record high fidelity action potentials, either because of the chip's high shunt capacitance, high access resistance, or low cell membrane-to-aperture seal resistance. We report here a chip design that optimizes these parameters and that can be fabricated using a reproducible process capable of high yields. We then demonstrate that these chips are capable of monitoring physiological functionality in neurons by recording whole-cell currents and action potentials elicited by voltage and current depolarization steps, respectively. This is the first demonstration of elicited action potentials in individual neurons cultured directly on the surface of a patch-clamp chip recording device. This represents an important advance over existing chip designs which require that isolated cells in suspension be sealed to the chip substrate using mechanical suction.

Fabrication Process

Wafer Level Fabrication

The fabrication process is schematically represented in Figure 2, and was carried out on 3 or 6 in. double-side polished (1 0 0) silicon wafers at the Canadian Photonics Fabrication Centre (http://cpfc-ccfdp.nrc-cnrc.gc.ca/). The membrane separating the culture media from the measuring media must have a high dielectric rigidity, and the small dimension of the aperture requires the membrane to also be thin and mechanically strong enough so that it can be suspended. We chose a 1 μm thick silicon nitride film rather than silicon dioxide, which has lower mechanical strength and for which we found stress more difficult to control. The same material is used at the back of the wafer as a mask for potassium hydroxide (KOH) bulk micromachining of silicon. A low-pressure chemical vapor deposition (LPCVD) process was optimized for low stress coating of silicon nitride; the 800–850°C process grows dense films that are resistant to the KOH etch and free of pinholes and defects. A Tystar Tylan II LPCVD reactor was used to deposit 1 μm thick nitride film on both sides of the double side polished (1 0 0) Si wafers using dichlorosilane and ammonia as source gases.

In the second step, 3 or 4 μm apertures were opened (scanning electron microscopy image in Fig. 3a) in the silicon nitride layer. The lithography was performed in an ASML 5500/100 i-line stepper, and the silicone nitride was etched in a plasmatherm reactive ion etcher (RIE) using a CF_4 chemistry with photoresist as a mask. A second lithography step at the back of the wafer opened large windows in the silicon nitride, aligned with <1 0 0> Si crystallographic axes and aligned with the apertures on the

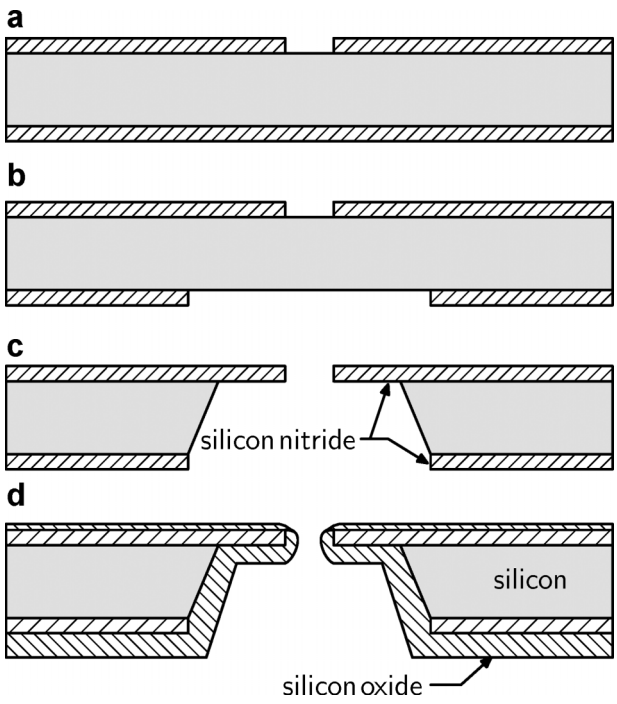

Figure 2. Fabrication process: (**a**) 1 μm LPCVD silicon nitride is grown on wafers and 3 or 4 μm apertures are etched in top layer. **b**: 600 μm × 600 μm windows are opened in the back, aligned with the apertures. **c**: The silicon is etched in 30% KOH, revealing [1 1 0] facets, until the silicon nitride membrane is left self-standing. **d**: 5.4 μm silicon oxide is PECVD deposited at the bottom, passivating the sidewalls of the well and partially in-filling the aperture. This is followed by a 0.1 μm thick coating of silicon oxide on the top surface.

top side. The stepper is not equipped with a back-to-front alignment stage, so we relied on the detection of the wafer flat and edge for positioning. This simple method yielded an alignment better than 10 μm over a 3-in. wafer. Silicon nitride windows in the back were etched with the same RIE process as on the top side. The silicon bulk was then etched in a 30% KOH solution heated at 80°C, at a rate of about 1 μm/min. The anisotropic KOH etch reveals {1 1 0} facets in the silicon crystalline bulk, resulting in an inverted truncated pyramid shaped well (Fig. 3b) with 54.74° facets. For 350 μm thick wafers, a 600 μm × 600 μm square opening at the back of the wafer results in a 100 μm × 100 μm square membrane in the silicon nitride film where the aperture has been patterned. The KOH etch leaves bare silicon walls in the well which were passivated by depositing a thick low stress plasma-enhanced chemical vapor deposition (PECVD) silicon dioxide film (PlasmaTherm 7000). Shadowing effects reduce the thickness of the film deposited in the wells. We found by scanning electron microscope (SEM) section that a 5.4 μm film as measured at the back of the wafer results in a 3.5 μm coverage of the walls of the well, 3.2 μm at the back of the membrane. The coating reduced the diameter of the aperture by 2 μm while rounding its edges. Finally, a thin 0.1 μm thick silicon dioxide film was deposited by the same method on the front side of the wafer. We have found that surface functionalization is more successful on that surface than on silicon nitride, and believe this may be due to a higher concentration of OH bonds, as observed by XPS, which increases the hydrophilicity of the surface and therefore the adhesion of polylysine. The resulting surfaces were smooth, with a roughness measured by atomic force microscope (AFM) to be 6–8 Å rms over a 1 μm square area. Figure 3c shows a SEM picture of a focused ion beam section of a 1.5 μm aperture resulting from a 4 μm aperture in the silicon nitride. Aperture size, morphology, and smoothness are important factors for a quality seal (Behrends and Fertig, 2007), and we found the funnel shape to be optimal (Salim, 2009). Our method achieves smooth apertures without the thermal cost of thermal oxidation (Curtis et al., 2008; Matthews and Judy, 2006; Sordel et al., 2006). Other groups (Fertig et al., 2000; Mourzina et al., 2006; Pantoja et al., 2004; Schmidt et al., 2000) have used lower temperature chemical vapor deposited insulators but did not report on recording of action potentials, perhaps because their designs lead to a high shunt capacitance and poor seals.

Each 3 in. wafer produced about thirty-five 1 cm square chips (some 150 for 6 in. wafers), and a yield higher than 90% was routinely achieved in several batches. The numbers could easily be raised, and the cost of fabrication lowered, by halving the size of the chips to 5 mm × 5 mm.

Packaging and Sterilization

Packages were machined from Plexiglas G sheets. This grade is somewhat resistant to solvents like isopropanol and ethanol, transparent to allow inverted microscopy or backside illumination, and was tested to have no cell toxicity. The packages (Fig. 4) consist of a 16 mm diameter, 6 mm deep culture chamber at the bottom of which the chip is glued in a square recess. Under the membrane of the chip, a 1.5-mm diameter hole connects the chip to subterranean fluidic holes, on each side of the chip to allow flushing of the physiological saline solution or other chemicals. Packages were cleaned with isopropanol in an ultrasonic bath and 1.5 mm glass tubes were glued with Stycast 1266 epoxy (Emerson & Cummings, Billerica, MA 0182) at each end of the subterranean fluidic circuit to be fitted with silicone tubing for perfusion. The finished packages were washed in propanol.

Wafers were cleaned in a piranha etch (H_2SO_4:H_2O_2 (3:1)) and then thoroughly rinsed in deionized water and dried with filtered nitrogen. They were easily diced into chips using grooves patterned in the well etching step. A ring of Dow Corning RTV silicone 3140 glue was dispensed manually using a syringe, on the back of each chip and the chip lowered into the recess of the culture dish in the package. The glue wetted the Plexiglas very well, covering most of the contact surface but not entering the well. After the glue was cured, a second ring of RTV was dispensed with

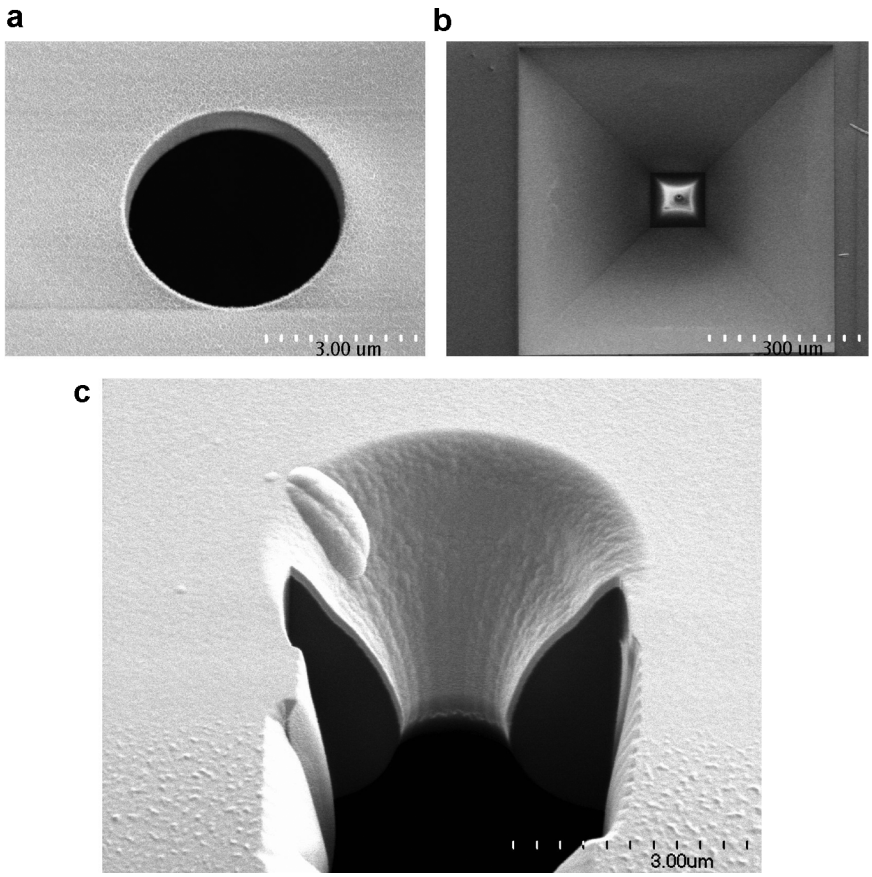

Figure 3. SEM pictures of the aperture chip: (**a**) 4 μm aperture, before passivation. **b**: View of the membrane through the well with a truncated inverted pyramid structure. **c**: Focused ion-beam section of the 4 μm aperture, shown after depositing 5.4 μm SiO_2 passivation from the bottom and 0.1 μm from the top (viewed from the top, i.e., culture, side). The chip is covered with Pt (light color) to facilitate the sectioning and augmenting contrast.

a syringe to cover the gap between the edge of the chip and the recess in the Plexiglas, thereby passivating the bare silicon edge of the chip (see Shunt Capacitance Section). Both the epoxy and the RTV silicone glues were tested for cell toxicity. It was found that cells would happily grow on the glue itself provided it was made hydrophilic.

Packaged chips were sterilized in a Harrick air plasma cleaner for 30 min. We found that using 70% ethanol, while having no substantial effect on the Plexiglas packages, would weaken the glues and cause leaks. The other benefit of this low power plasma is that all surfaces were rendered hydrophilic, and that property was preserved by immediately immersing the chips in sterile deionized water. It is well known that plastic surfaces rapidly revert to a hydrophobic state when exposed to air, and even the wetting properties of silicon dioxide will degrade because of carbon-based pollution. Finally, immersion may protect the aperture from getting plugged during storage. Packaged chips stored in this way were found to remain functional after several months, with their holes still primed with water and open.

Modeling

Electrophysiologists model a patch-clamp probe as an access resistance in series with the cell, and a shunt capacitance in parallel. Capacitance and resistance, associated with the glass pipette in conventional patch-clamp, or with the chip in planar patch-clamp, can attenuate the fidelity (frequency response and amplitude) of biological signals and limit voltage control (clamp) of the cell. Therefore, minimizing both of these elements, while maintaining an appropriate area of the cell membrane over the aperture, is critical to obtaining meaningful biological data (Levis and Rae, 1998). In this section we are summarizing calculations on both those impedance elements.

Shunt Capacitance

The impedance of the mounted chip can be modeled as illustrated in Figure 1. Assuming that the resistance of the silicon wafer itself is negligible and that there are no electrical shorts, the shunt capacitance is the capacitance of

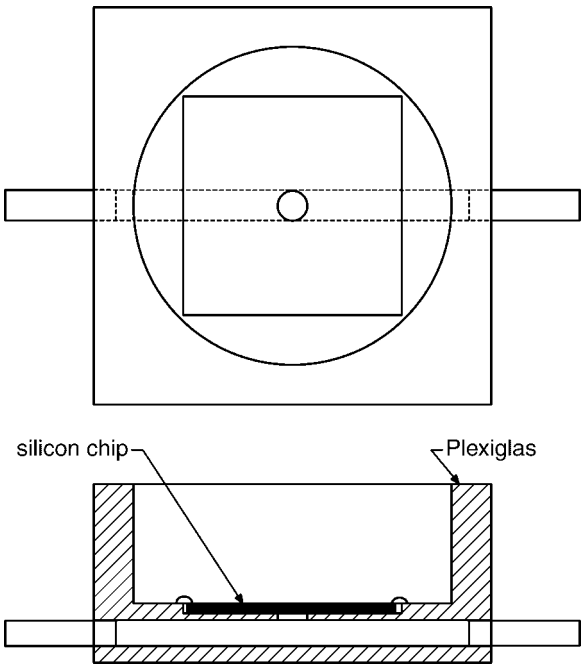

Figure 4. Plexiglas package: The package is machined with a large circular top opening (culture chamber) and a square recess. A subterranean tube is drilled through the bottom section, with glass tubes connected at either end to allow fluid connections. A 1.5 mm aperture connects the tube to the culture chamber. The silicon chip (black in section view) is lowered and glued in the square recess.

the self-supported film in parallel with the capacitance of the dielectric films on the top and bottom of the silicon that interface with the culture and measuring media

$$C_{\text{shunt}} = C_{\text{ss}} + [(C_{\text{top}} + C_{\text{edge}})^{-1} + (C_{\text{well}} + C_{\text{bot}})^{-1}]^{-1}$$

The capacitance of the self-supported film is very small due to the small area (100 μm × 100 μm), and we calculate that $C_{\text{ss}} = 0.2$ pF. Chips are diced from wafers at the end of the fabrication process so the edges of the chip are bare silicon. Consequently, the capacitance of the edges of the chip C_{edge}, where it immersed in the physiological medium, would be very high. Assuming that a 40 Å thick native oxide layer is allowed to grow on the chip by exposure to air for an extended period of time, we calculate $C_{\text{edge}} = 156$ nF, much too high to allow good patch-clamp measurements. In practice, resistive leaks would also be prohibitively high and the edges of the chip are sealed with RTV silicone as described in the Packaging and Sterilization Section. Assuming a dielectric constant of 3 for the glue, a 50 μm glue ribbon and a 0.5 mm gap between the chip and the Plexiglas, we find $C_{\text{edge}} = 1.4$ pF.

By comparison with the small self-supported membrane, the 1 cm^2 area of the silicon nitride has an enormous capacitance of $C_{\text{top}} = 6$ nF. To reduce the contact area (Matthews and Judy, 2006; Pantoja et al., 2004; Schmidt et al., 2000), we studied the incorporation of thick polymer layers with a 200 μm square opening on top of the membrane, in a process that would still be highly manufacturable. We successfully used SU8 (Microchem, Newton, MA 02464), a photosensitive epoxy resin that can easily be spun in films up to 1-mm thick, and used pervasively as a building material for microfluidic and cell chips. However, we found the film to be mildly cytotoxic, in agreement with Vernekar et al. (2009). We also tested PDMS but found it difficult to manufacture as a spun film at the wafer level: shrinkage makes wafer-level assembly difficult and the film would not be stable upon etching, despite encouraging work (Tserepi et al., 2003; Vlachopoulou et al., 2005). Other materials are presently being studied, but it is worth repeating that this topology and hybrid surface will affect the cell culture conditions.

The formula above, instead, suggests an obvious solution to reduce the capacitance: if C_{top} cannot be reduced, a low shunt capacitance can still be obtained provided C_{well} and C_{bottom} are low enough, and the process is of high enough quality that no resistive leak is present in either the membrane or the glue. C_{well} is the capacitance of the silicon-dioxide-passivated [1 1 0] silicon walls, and C_{bot} is the capacitance of the portion of the back of the chip that is exposed to the measuring media.

The very thick (by microelectronics standards) 5.4 μm silicon dioxide coating is therefore chosen primarily because it results in $C_{\text{well}} = 7.8$ pF. Assuming that the silicone gluing the chip to the package wets all the Plexiglas, we find $C_{\text{bot}} = 8.6$ pF. C_{bot} could be reduced further by reducing the 1.5 mm diameter of the Plexiglas package opening between the chip and the subterranean fluidics, but alignment of the chip in the package would be more difficult. Using the formula above, the resulting net shunt capacitance is $C_{\text{shunt}} = 16.5$ pF.

Series Resistance

The resistance of a cylindrically shaped aperture can be calculated using the formula $R_{\text{c}} = \rho L/A$, where ρ is the resistivity of the solution, L is the length of the aperture, and A is the cross-sectional area. The spreading resistance from the top opening of the aperture into the top fluid reservoir is given by Maxwell's formula $R_{\text{sp}} = \rho/2a$, where a is the radius of the opening. This, plus a similar spreading resistance at the bottom opening, must be added to R_{c}.

The aperture in our chips is not cylindrical as the SEM cross-section in Figure 3c shows. In this case, the aperture resistance can be calculated using

$$R_{\text{m}} = \int_0^L \frac{\rho}{A(z)}\,\mathrm{d}z$$

where z is the distance along the axis of the aperture. The integral can be done numerically using the SEM image to estimate the area, A, as a function of z. Using a media resistivity value of 55 Ω cm, the resistance of the aperture of

Figure 3c (diameter of 1.8 m) would be $R = R_m + 2R_{sp} = 688\,k\Omega + 275\,k\Omega = 963\,k\Omega$ with about a 25% uncertainty.

Chip Characterization

Loading of Fluidic Channels

It is critical to load the subterranean fluidics without trapping air bubbles in the well or aperture, as this would result in an electrical open circuit. The method used was to pressurize the fluid in the subterranean tube and force the air out of the well through the aperture. At this point, if the pressure is high enough to overcome the surface tension, fluid will be forced through the aperture. The pressure needed is about 2.9 atm for a 1 μm aperture and 1.45 atm for a 2 μm aperture (McGuinness et al., 2005). In practice, silicon nitride membrane can withstand pressures over 3 atm; however, this is much too high for standard perfusion systems. The hydrophilization effect of our sterilization process made the loading easier. Verification of a filled aperture was the observation of a small droplet of physiological saline solution forming at the aperture, and microscopic inspection of the membrane for residual bubbles in the subterranean channels. After the air was removed from the well, the top culture chamber was filled with media. There are still some issues with bubbles remaining in the well. We are investigating other methods to remove these, for example by using electrophoresis.

Impedance Measurements

The resistance of the chip was measured by loading the culture well and fluidic channels with physiological phosphate-buffered saline (PBS) solution with a resistivity measured to be 55 Ω cm, immersing Ag/AgCl electrode wires on each side of the aperture and measuring the resistance with a Fluke multimeter. The dc resistance of devices with aperture diameters ranging from 1.3 to 1.8 μm (at the narrowest) were measured to be about 1.5 to 3 MΩ, respectively. The measured values correlate well with size, but there is approximately a factor of 3 between the measured and calculated value. We speculate that the physiological saline may not fully wet the aperture (leaving tiny bubbles) or slight contamination may result in a smaller effective diameter channel. Still, the value is substantially lower than the standard 10 MΩ of a filled glass pipette.

C_{shunt} was measured on a number of packaged chips using the same electrodes and a Hewlett Packard 4285A precision LCR meter at 100 kHz. Low values of 2–3 pF were correlated to bubbles filling the well below the aperture. Values above 40 pF correspond to a substantial electrical short to the silicon chip. More than half of the chips were measured to have shunt capacitance values between 15 and 27 pF. The variations and difference with the calculated value are attributed mostly to the fact that the silicone gluing the chip to the package does not wet all of the bottom silicon surface directly next to the Plexiglas (as observed by dismounting some chips), enlarging the area of direct contact of the saline to the silicon chip. We are looking at ways to automate the assembly process, which would help to eliminate that variability. Another possible source of leaks is in the glue sealing the edges of the chip from the physiological medium. We are looking at alternate packaging designs where the chip edges would not be immersed in the culture chamber.

Cell Preparation

Snail neurons were chosen because they represent a simple but well-established model to study neuronal electrophysiology (Bell and Syed, 2009) and have proven to be a suitable preparation to use on another type of neurochip (Kaul et al., 2004). Freshwater snails (*Lymnaea stagnalis*) were maintained in an aquarium containing aerated pond water at room temperature (20–22°C), and fed lettuce. Snails, 1–2 months old (10–15 mm in length), were used to isolate neurons for experiments, whereas 2- to 4-month-old animals (20–30 mm in length) were used to prepare conditioned medium (CM: isolated snail brains incubated in defined media for 3–4 days). Detailed cell isolation and culture procedures have been described previously (Syed et al., 1990). In brief, snails were deshelled and anesthetized with 10% listerine solution (ethanol, 21.9%; and methanol, 0.042%) in normal Lymnaea saline (in mM: 51.3 NaCl, 1.7 KCl, 4.0 $CaCl_2$, and 1.5 $MgCl_2$) buffered to pH 7.9 with HEPES. The central ring ganglia (CRG) were then removed and washed with normal saline containing gentamicin (50 μg/mL) three times (10 min each). CRG were then incubated for 20 min in defined medium (DM: serum-free 50% L-15 medium with 20 μg/mL gentamicin and inorganic salts at mM: 40 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, and 10 HEPES, pH 7.9) containing trypsin (2 mg/mL; Sigma, St. Louis, MO), followed by a 15-min wash with DM containing trypsin inhibitor (2 mg/mL; Sigma). After enzymatic treatment, the CRG were pinned down in a Sylgard dissection dish containing high osmolarity DM (750 μL of 1 M glucose was added to 20 mL DM to raise osmolarity from 130–145 to 180–195 mOsm). The outer and inner sheath layers were removed using fine forceps and the Left Pedal Dorsal 1 (LPeD1) interneuron was extracted using gentle suction applied through a Hamilton syringe (Hamilton Co., Reno, NV) and a fire-polished, Sigmacoated (Sigma) glass pipette (60–70 μM tip diameter).

Patch-Clamp Recording

Poly-L-lysine was applied to the chips for 2 h and then rinsed with water. Isolated Lymnaea LPeD1 neurons were plated, within an hour, onto the poly-L-lysine-coated chips in the presence of DM. To monitor electrophysiological measurements, the subterranean microfluidic channels were loaded with a physiological saline solution containing (in mM): 50

KCl, 5 $MgCl_2$, 5 EGTA, and 5 HEPES (pH 7.4 adjusted with KOH; osmolarity 130 mOsm), while the culture well was filled with physiological medium (see the Cell Preparation Section).

A minimum of 2 h after plating the neurons, chips were connected to an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) by placing the recording electrode, coupled to the headstage, in the subterranean microfluidic channels and the reference electrode in the culture well. Signals were filtered at 2 kHz and acquired at a sampling rate of 20 kHz. Test voltage steps (5 mV) were then applied to measure the total resistance (the sum of the cell membrane resistance and total access resistance).

A total of 11 chips with neurons cultured over the apertures were tested. Of these, five chips showed large capacitive transients in response to the test voltage steps, suggesting attainment of the whole-cell patch-clamp configuration in neurons on these chips. Of the remaining six chips tested, four chips with neurons cultured over the apertures had a resistance of $14.2 \pm 6.2\,M\Omega$ but no large capacitive transient. We concluded that neurons on these chips did not form adequate membrane to chip seal resistances to permit recording of biological activity. The last two chips tested indicated infinite resistance, probably because of air bubbles in the subterranean microfluidic channels. The 5/11 success rate is similar to or better than that reported for glass pipette and automated patch-clamping.

We subsequently conducted current-clamp and voltage-clamp recordings on the five neurochips in which the whole-cell patch-clamp configuration was attained in neurons. This unexpected spontaneous entry into the whole-cell configuration was likely due to the prolonged exposure of the patch of cell membrane over the aperture to the physiological saline solution, which differed from the physiological medium in the bath both in terms of ionic composition and osmolarity. Typical whole-cell voltage and current responses to an incremental series of intracellular current or voltage steps are shown in Figure 5A and B, respectively. The observation of robust action potentials in response to depolarizing current steps confirms the acquisition of electrophysiological activity characteristic of Lymnaea neurons (Fig. 5A). This is the first report of such activity from neurons cultured on a planar patch-clamp chip. The quality of the recordings is due to a low shunt capacitance and low access resistance, but must also be the result of a high cell to aperture seal. While spontaneous entry into the whole-cell mode precluded us from measuring the seal impedance, we are currently studying that seal by performing focused ion beam sections of cells on apertures that suggest intimate seals.

In the whole-cell recordings reported here using the planar patch-clamp chip series resistance was not measurable so no compensation was attempted. The current-clamp recordings in Figure 5A show virtually no current offset in response to voltage steps, confirming that series resistance compensation was not required. To further assess the fidelity

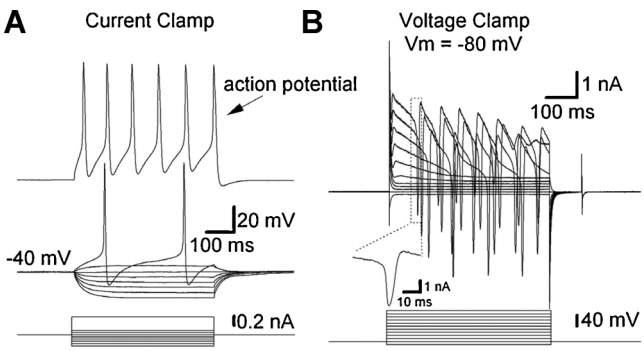

Figure 5. Whole-cell patch clamp recordings from an LPeD1 neuron cultured on a neurochip. **A**: Voltage responses (**top**) of an LPeD1 neuron to graded series of intracellular current pulses (**bottom**). The current pulses were applied at a voltage membrane potential of −40 mV. **B**: Current responses (**top**) of the same LPeD1 neurons shown in (A) to a series of intracellular voltage pulses (10 mV/pulse) from a voltage membrane potential of −80 mV. The inset shows the boxed region with an expanded time scale.

of our recordings, we calculated the rise time of currents evoked during the rising phase of the action potential in voltage clamp (Fig. 5B and inset). Typically such currents are due to a Na+ conductance. We found that these currents had a rise time (10–90%) of 0.69 ± 0.27 ms ($n = 5$). Lymnaea neurons have previously been reported to have Na+ conductance channels (Kostyuk and Krishtal, 1977) and such invertebrate Na+ channels have been shown, using conventional patch clamp, to have activation kinetics ranging from 0.99 to 2.72 ms (Gilly et al., 1997). This suggests that we are capturing the highest frequencies of electrophysiological activity in these neurons.

In conventional patch-clamp the cell-attached configuration is established by the formation of a gigaohm seal between the membrane and the pipette before rupturing the membrane under the pipette to attain the whole-cell configuration. While in the cell-attached configuration, application of a voltage step generates transients due to the pipette capacitance, which can be easily compensated. Giga-seal formation and subsequent membrane rupture are accomplished by suction. By contrast, neurons cultured on our planar patch-clamp chips spontaneously formed membrane to substrate seals and adopted the whole-cell configuration without application of suction prior to initiation of recording. This meant that we could not compensate for the capacitance of the chip and cell membrane alone, so it was important that the chips have low capacitance. The averaged whole cell capacitance was 34.2 ± 3.14 pF ($n = 5$), which includes the capacitance of the cell and the chip.

Prolonged exposure of the cell membrane spanning the micro-hole to the high concentration of K+ (50 mM) present in the subterranean channel may have precipitated "spontaneous entry" into the whole-cell configuration by rupturing the membrane. This was not a serious issue during the time course of the study, as evidenced by the robust and

stable electrophysiological responses observed. However, for longer term studies this would be undesirable, resulting in the diffusion of key cytoplasmic components. It may be possible to avoid this occurrence by initially culturing neurons over the microhole with media in the subterranean channel, and then perfusing the channel with the high K+ solution immediately prior to recording.

Conclusion

This is the first report of recording of whole cell ion channel activity and stimulated action potentials from neurons cultured directly on a planar patch-clamp chip. These results were made possible by the low access resistance and low shunt capacitance of our chip and package design which allows the measurement of fast transients. This interrogation platform is a potentially powerful tool to enable high-information content pharmaceutical assays and to investigate in vitro models of disease. Ion channels are specialized and exquisite sensors of various bioactive parameters, including specific neurotransmitters and metabolites, as well as transmembrane voltage (Dunlop et al., 2008). The true biosensing capabilities of chips monitoring ion channel activity are only limited by the type of ion channels native to the cells being recorded from, or to the type of ion channel overexpressed in the host cell.

The authors wish to gratefully acknowledge the technical support of Reza Dowlatshahi, Christina Elliott, Naksup Kim (CPFC), and Mark Malloy for the microfabrication of chips at the wafer level; Allan Horner and Robert Trepanier for packages fabrication; Hue Tran, Ping Zhao, Kate Sokolina, and Danish Salim for packaging and sterilization; Jeff Fraser for SEM; and Guy Parent for AFM. Naweed Syed was supported by a Canadian Institute of Health Research (CIHR) grant. Collin Luk is the recipient of NSERC and Alberta Heritage Foundation for Medical Research (AHFMR) studentships.

References

Axon Instruments, Inc. 1993. The axon guide: A guide to electrophysiology & biophysics laboratory techniques. Sunnyvale, CA: Molecular Devices.

Aziz JNY, Genov R, Bardakjian BL, Derchansky M, Carlen PL. 2007. Brain-silicon interface for high-resolution in-vitro neural recording. IEEE Trans Biomed Circuits Syst 1(1):56–62.

Behrends JC, Fertig N. 2007. Chapter 14: Planar patch-clamp. In: Walz W, Boulton AA, Baker GB, editor. Neuromethods, 2nd edn.

Bell HJ, Syed NI. 2009. Hypoxia-induced modulation of the respiratory CPG. Front Biosci 14:3825–3835.

Curtis JC, Baldwin K, Dworak BJ, Stevenson TJM, Delivopoulos E, MacLeod NK, Murray AF. 2008. Seal formation in silicon planar patch-clamp microstructures. J Microelectromech Syst 17(4):974–983.

Dunlop J, Bowlby M, Peri R, Vasilyev D, Arias R. 2008. High-throughput electrophysiology: An emerging paradigm for ion-channel screening and physiology. Nat Rev Drug Discov 7(4):358.

Fertig N, Tilke A, Blick RH, Kotthaus JP, Behrends JC, Bruggencate Gt. 2000. Stable integration of isolated cell membrane patches in a nano-machined aperture. Appl Phys Lett 77(8):1218–1220.

Fertig N, Blick RH, Behrends JC. 2002. Whole cell patch clamp recording performed on a planar glass chip. Biophys J 82(6):3056–3062.

Fertig N, George M, Klau M, Meyer C, Tilke A, Sobotta C, Blick RH, Behrends JC. 2003. Microstructured apertures in planar glass substrates for ion channel research. Receptors Channels 9(1):29–40.

Gad-el-Hak M. 2001. The MEMS Handbook. Boca Raton, FL: CRC Press.

Gilly WF, Gillette R, McFarlane M. 1997. Fast and slow activation kinetics of voltage-gated sodium channels in molluscan neurons. J Neurophysiol 77(5):2373–2384.

Kaul AR, Syed NI, Fromherz P. 2004. Neuron-semiconductor chip with chemical synapse between identified neurons. Phys Rev Lett 92(3): 381021–381024.

Klemic KG, Klemic JF, Sigworth FJ. 2005. An air-molding technique for fabricating PDMS planar patch-clamp electrodes. Pflüg Arch Eur J Physiol 449(6):564–572.

Kostyuk PG, Krishtal OA. 1977. Effects of calcium and calcium chelating agents on the inward and outward current in the membrane of mollusc neurones. J Physiol 270(3):569–580.

Levis RA, Rae JL. 1998. Low-noise patch-clamp techniques. Methods Enzymol 293:218–266.

Matthews B, Judy JW. 2006. Design and fabrication of a micromachined planar patch-clamp substrate with integrated microfluidics for single-cell measurements. J Microelectromech Syst 15(1):214–222.

McGuinness P, Drenckhan W, Weaire D. 2005. The optimal tap: Three-dimensional nozzle design. J Phys D 38:3382–3386.

Mourzina Y, Kaliaguine D, Schulte P, Offenhausser A. 2006. Patterning chemical stimulation of reconstructed neuronal networks. Anal Chim Acta 575:281–289.

Neher E, Sakmann B. 1976. Single-channel currents recorded from membrane of denervated frog muscle fibers. Nature 260:799–802.

Pantoja R, Nagarah JM, Starace DM, Melosh NA, Blunck R, Bezanilla F, Heath JR. 2004. Silicon chip-based patch-clamp electrodes integrated with PDMS microfluidics. Biosens Bioelectron 20(3):509–517.

Salim D. 2009. University of Ottawa Co-Op Report, August 2009.

Schmidt C, Mayer M, Vogel H. 2000. A chip-based biosensor for the functional analysis of single ion channels. Angew Chem 39(17):3137–3140.

Sigworth FJ, Klemic KG. 2005. Microchip technology in ion-channel research. IEEE Trans Nanobiosci 4(1):121–127.

Sordel T, Garnier-Raveaud S, Sauter F, Pudda C, Marcel F, De-Waard M, Arnoult C, Vivaudou M, Chatelain F, Picollet-D'hahan N. 2006. Hourglass SiO_2 coating increases the performance of planar patch-clamp. J Biotechnol 125(1):142–154.

Syed NI, Bulloch AGM, Lukowiak K. 1990. In vitro reconstruction of the respiratory central pattern generator of the mollusk Lymnaea. Science 250(4978):282–285.

Tserepi A, Cordoyiannis G, Patsis GP, Constantoudis V, Gogolides E, Valamontes ES, Eon D, Peignon MC, Cartry G, Cardinaud C, Turban G. 2003. Etching behavior of Si-containing polymers as resist materials for bilayer lithography: The case of poly-dimethyl siloxane. J Vacuum Sci Technol B 21(1 SPEC):174–182.

Vernekar VN, Cullen DK, Nick F, Yoonsu C, Andrés JG, Mark GA, Gregory JB, Michelle CL. 2009. SU-8 2000 rendered cytocompatible for neuronal bioMEMS applications. J Biomed Mater Res A 89(1):138–151.

Vlachopoulou ME, Tserepi A, Vourdas N, Gogolides E, Misiakos K. 2005. Patterning of thick polymeric substrates for the fabrication of microfluidic devices. J Phys 10(1):293–296.

Walz W, Boulton AA, Baker GB, editor. 2007. Patch-clamp analysis: Advanced techniques. In: Neuromethods. 2nd edn. Secaucus, NJ: Springer Protocols 38.