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Publisher's version / Version de l'éditeur:

<https://doi.org/10.1002/bit.23127>

Biotechnology and Bioengineering, 108, 8, pp. 1936-1941, 2011-03-09

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Cell to Aperture Interaction in Patch-Clamp Chips Visualized by Fluorescence Microscopy and Focused-Ion Beam Sections

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Received 21 January 2011; revision received 22 February 2011; accepted 28 February 2011

Published online 9 March 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23127

ABSTRACT: Patch-clamp is an important method to monitor the electrophysiological activity of cells and the role of pharmacological compounds on specific ion channel proteins. In recent years, planar patch-clamp chips have been developed as a higher throughput approach to the established glass-pipette method. However, proper conditions to optimize the high resistance cell-to-probe seals required to measure the small currents resulting from ion channel activity are still the subject of conjecture. Here, we report on the design of multiple-aperture (sieve) chips to rapidly facilitate assessment of cell-to-aperture interactions in statistically significant numbers. We propose a method to pre-screen the quality of seals based on a dye loading protocol through apertures in the chip and subsequent evaluation with fluorescence confocal microscopy. We also show the first scanning electron micrograph of a focused ion beam section of a cell in a patch-clamp chip aperture.

Biotechnol. Bioeng. 2011;108: 1936–1941.

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KEYWORDS: ion channels; planar patch-clamp chip; giga-seal; fluorescence microscopy; focused ion beam; scanning electron microscopy

Introduction

Nervous system function 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 that span cell membranes and regulate the trans-membrane flow of specific ions. Due to their key role in regulating physiological functions, ion channels are recognized as

important therapeutic targets for pharmacological intervention. Indeed, numerous drugs have been developed to modify their activity.

Ion channel currents can be measured using a technique called patch-clamp (Neher and Sakmann 1976; Walz 2007). This technique requires that a small-tipped glass pipette filled with an electrochemically conductive solution be sealed with high resistance ($>1\text{ G}\Omega$; giga-seal) to a patch of cell membrane. In the conventional patch-clamp technique, the giga-seal is achieved by applying a negative pressure through the pipette. Once the giga-seal is obtained, the potential of the cell membrane under the pipette tip is clamped and small currents resulting from ion channel activity across the membrane recorded. The giga-seal between the cell membrane and the pipette is therefore fundamental to minimize shunt currents and allow detection of the small conductance currents resulting from the flow of ions through channels spanning the cell membrane under the pipette tip.

Despite the remarkable resolution of the conventional patch-clamp technique, it is a difficult and laborious process that is impractical for the high-throughput necessary for pharmacological screening in drug development studies (Dunlop et al., 2008). Consequently, considerable effort has been invested in the development of automated patch-clamp interfaces: planar patch-clamp chips (Behrends and Fertig, 2007; Sigworth and Klemic, 2005). In these, the apex of the pipette is replaced by a microscopic aperture in a self-supported film on which the cell is placed. The chip is mounted in a two-chamber set-up, the top one serving as the culture dish and the bottom one as the equivalent of the inside of the glass pipette. The cell is patched on the micrometer-sized aperture and probed by placing the recording electrode in the bottom chamber and the reference electrode in the culture dish. The proof of concept

for the first planar patch-clamp devices was obtained using suspended cells from cell lines over-expressing specific ion channels (Fertig et al., 2000; Schmidt et al., 2000). Quartz or glass (Fertig et al., 2003), polyimide (Stett et al., 2003), and poly(dimethylsiloxane) (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. The technology has been integrated in automated patch-clamp systems employed for rapid drug screening (Celletricon, <http://www.celletricon.com/>; Molecular-Devices, http://www.moleculardevices.com/pages/instruments/electrophys_main.html#automated; Nanion, <http://www.nanion.de/>; Sophion, <http://www.sophion.dk/>).

Although critical for high-quality patch-clamp monitoring, the mechanism underlying cell-to pipette tip or cell-to-aperture seal formation is poorly understood. In the conventional pipette format, it is known that gentle suction through the pipette attracts the cell membrane inside the glass pipette, forming a “bleb” which increases the surface area of contact between the cell membrane and the probe, hence reducing leak currents (Ruknudin et al., 1991). In planar patch-clamp chips, the tip of the glass pipette is replaced by a micrometer-sized aperture on a flat probe. This surface offers a larger contact area to the cell membrane compared to the pipette and can be treated to enhance cell adhesion. This larger surface contact area should lead to higher seal quality: however, the technique has consistently resulted in $<1\text{ G}\Omega$ seal formation on cells from a suspension sucked on the aperture.

Recently, we utilized the adhesion properties of cultured cells to achieve giga-seals and reported the first recording of action potentials elicited in individual neurons cultured directly on the surface of a silicon patch-clamp chip (Py et al., 2010). The high quality of those recordings is due to an optimized design of the chip, which minimizes the shunt capacitance and access resistance and allows a high resistance seal formation. There are indications that smooth and rounded apertures result in high resistance seals for several types of materials (Chen et al., 2009; Curtis et al., 2008; Lehnert et al., 2007; Sordel et al., 2006) because they offer the best possible conformation for the cell to come into tight contact with the aperture. Apertures on the silicon patch-clamp chip were designed with those characteristics in mind.

To date, there has been no publication on visual methods to study the cell-to-aperture interaction in planar patch-clamp chips. This lack of visual evidence is due to the relative novelty of patch-clamp chips, first reported a decade ago, and to the inherent difficulty in observing micron-scale features on the underside of cell cultures. In this paper, we describe the configuration of a multiple-aperture “sieve” chip that allows this assessment in statistically significant numbers with reduced fabrication and cell culture efforts. We report fluorescence microscopy and scanning electron microscopy (SEM) of focused ion beam (FIB) sections methods to visualize the intimate cell-to-aperture interactions.

Device Design and Fabrication

Silicon patch-clamp chips were fabricated according to a method described in detail in Py et al. (2010) and summarized in Figure 1. The fabrication, undertaken in a commercial foundry (Canadian Photonics Fabrication Centre, www.cpf.ca), started with the deposition of silicon nitride (SiN) on both sides of 3 or 6 in. silicon wafers with a (100) crystalline orientation. SiN is an insulating film with strong mechanical properties and high resistance to potassium hydroxide etching, which makes it ideally suited to this device. Micro-apertures were dry-etched in the front side SiN film and large $600 \times 600\text{ }\mu\text{m}$ square openings, aligned with the apertures, in the backside SiN film. The SiN served as a mask for etching silicon in potassium hydroxide which revealed [110] facets in the bulk of the substrate and resulted in a truncated inverted pyramidal shape which we call the well; the etch left the SiN film at the front of the wafer self-standing. Finally, a silicon dioxide (SiO₂) film was deposited by plasma-enhanced chemical vapor deposition at the back to passivate the [110] silicon facets of the well; SiO₂ was also deposited at the front since it provides a better surface for cell adhesion. Both depositions also partially fill the apertures, smoothing their edges and affecting their shape. The SiO₂ deposition was substantially thicker at the back than at the front, resulting in an aperture, which is funnel shaped (see Py et al. (2010) for SEM of apertures).

Figure 1. Fabrication process: **(A)** $1\text{ }\mu\text{m}$ SiN is grown on wafers and micro-apertures are etched in top layer. **(B)** $600\text{ }\mu\text{m}$ by $600\text{ }\mu\text{m}$ windows are opened in the back, aligned with the apertures. **(C)** The silicon is etched in 30% KOH, revealing [110] facets, until the SiN membrane is left self-standing. **(D)** SiO₂ is deposited at the bottom, passivating the sidewalls of the well and partially in-filling the aperture, as well as at the top.

Chips were diced from the wafer and either used for culture in 24-well plates or packaged to allow localized perfusion through the aperture as described in Py et al. (2010), but with a larger 3 mm diameter hole connecting the chip to subterranean fluidic feeds. Packages surround chips with a 16 mm diameter, 6 mm deep culture vial.

Our patch-clamp chips as described in Py et al. (2010) only have one aperture. For the purpose of studying and optimizing cell-to-aperture interaction, we integrated many apertures on one chip. We therefore designed a “sieve” chip integrating a 3×3 array of wells, each fitted with a 3×3 array of apertures for a total of 81 apertures per chip (Fig. 2). These multiple-aperture chips allow the collection of a large amount of data with reduced fabrication and cell culture efforts. However, since the apertures have only one common subterranean microfluidic, they cannot be used for electrophysiological assessment.

Cell Culture

For this study, we employed the P19 cell line, which is derived from embryonal carcinoma induced in a C3H/He strain mouse. The main advantages of these cells are their rapid division rate and the fact that they grow in a confluent monolayer, which facilitates the covering of the apertures.

Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen Corporation Carlsbad, CA) containing 200 mM + 4.5 g/L glucose +10% fetal bovine serum (FBS PAA Laboratories, Etobicoke, Canada). P19 culture was started from a frozen stock, in a 100 mm culture dish (VWR Canlab, Mississauga, Ontario, Canada). Cells were then passaged at 2-day intervals (cells approximately at 75% or more confluency) using 1/10 of the cells to seed each

new 100 mm culture dish. Cells were cultured in a tissue culture humidified incubator at 37°C and 5% CO₂. For plating of chips and passaging of cells, cells at 75% confluency were dissociated from the culture dish using a solution of 0.05% Trypsin/0.05 mM EDTA (Invitrogen), resuspended in DMEM, pelleted by centrifugation and resuspended in 10 mL of DMEM. The concentration of viable cells was determined using Trypan Blue and a hemocytometer. 1.5×10^6 viable cells were added to each chip, and the remainder was plated at a 1:10 dilution in a 100 mm dish to ensure continuation of cultures. Cells on chips were cultured for 24 h prior to assessment. P19 cells were passaged a maximum of 26 times.

Sieve chips were sterilized in a Harrick air plasma for 15 min then immersed in sterile deionized water to keep them clean and hydrophilic. To promote cell adhesion, the chips were coated with poly-D-lysine solution (1 part poly-D-lysine + 2 parts sterile PBS) for 2 h at room temperature. After 2 h, the coating solution was removed by rinsing the chips with sterile water. P19 cells were then plated on the chips and placed in a tissue culture incubator (37°C, 5% CO₂) for 1 day. The concentration of the P19 cells plated on chips varied depending on the level of confluence of the cells of the original culture.

Confocal Microscopy

Live imaging was carried out on Zeiss LSM 410 inverted confocal laser scanning microscope equipped with a Melles Griot Argon Ion Laser System, Albuquerque, NM (Series 643, 150 mW, 488,568,647 nm, model 400-K06). It was possible to obtain images through the transparent SiN and SiO₂ films, but brighter images were obtained from the top

Figure 2. Layout of a sieve chip. **A:** The sieve chip is 1×1 cm and **(B)** contains a 3×3 array of wells. **C:** The wells are approximately 150 μ m in size and each contains a 3×3 array of apertures, separated by 50 μ m. Each row of wells corresponds to a different aperture size.

of the sample using the LSM inverter. Imaging was performed with 20 $\times$, 40 $\times$ objective lenses, and a 63 $\times$ water-dipping lens. Calcein-AM and RH-237 dyes were employed for fluorescence imaging. Calcein-AM (Invitrogen Canada Inc., Burlington, ON Canada cat. #C-3100) is a green vital dye and an indicator of cell viability. Specifically, it is membrane-permeant until enzymes in the cell cytoplasm cleave it; the resulting Calcein is no longer permeant and therefore trapped in the cell. RH-237 (Molecular Probes cat. #S-1109) is a red membrane dye that attaches to the lipid bilayer and allows the visualization of membranes and membrane-based components. Both Calcein-AM (5 μ M) and RH-237 (50 μ M) were diluted with Normal Bath Medium (NBM) (mM: 140 NaCl; 3.5 KCl; 0.4 KH₂PO₄; 20 HEPES, 5 NaHCO₃; 1.2 MgSO₄, 1.3 CaCl₂; 15 glucose). Cells were stained with Calcein (5 μ M) for 30 min at 37°C in the dark, then with RH-237 (50 μ M) for 10 min. During imaging, the solution containing the dyes was replaced with normal NBM.

To study the growth of the P19 cultures on sieve chips, we loaded the culture dish with Calcein-AM and RH-293. We generally observed strong fluorescence resulting from Calcein uptake, indicating that cells were viable and had typical characteristic morphology. The majority of apertures on the chip were covered with cells (Fig. 3A and B). Stronger fluorescence was seen inside the apertures (Fig. 3D), possibly due to the invasion of the cell into the aperture. Note that all nine apertures in Figure 3B show stronger green fluorescence inside the apertures. Figure 4A and B are X–Z cross-sections obtained by scanning the Z focus (with the very shallow depth of field characteristic of confocal microscopy) and rastering the laser beam in the X-direction. These sections suggest that P19 cells do invade chip apertures, but do not reveal whether there is an intimate contact between its membrane and the sidewalls of the aperture.

In order to determine whether cell-to-aperture seals were tight, P19 cells were cultured on sieve chips, dye-loaded with RH-237 in the culture dish, and with Calcein-AM in the subterranean fluid channel below the apertures. If cells formed a tight cell-to-aperture seal on the chip, Calcein-AM could only enter cells by permeating the membrane patch over the apertures. On the other hand, if close contact between the cell and its substrate was not established, Calcein-AM could leak past and rapidly load neighboring cells. Four sieve chips, containing 81 apertures each, were examined. In the sieve represented in Figure 5A, two apertures are covered with single Calcein-loaded cells, while six apertures are covered with more than 1 Calcein-loaded cell. The last aperture appears to be plugged by debris. Cells over 25–50 μ m away from those apertures only stained with RH-237, indicating that Calcein-AM diffused only to the cells in proximity of the aperture.

Figures 4 and 5 strongly suggest that P19 cells do invade chip apertures and in some cases form a tight seal. To obtain higher resolution and direct evidence of such an intimate contact, we turned to FIB sections and electron microscopy.

Figure 3. Confocal microscopy image of P19 cells. Cells were visualized with 40 $\times$ (A and B) and 63 $\times$ objectives (C and D). A and C: Reflection white light images. The apertures are visible under the cells (white in A, approximate location indicated by white arrows, dark in C). B and D: show the overlay of the reflection and fluorescence images taken with Calcein-AM (green, viability) and RH-237 (red, membrane) dyes. The apertures are visible as brighter green when the cell is directly on top of it. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

FIB Section and SEM Imaging

Sieve chips that demonstrated prospective cell-to-aperture interaction through fluorescence imaging were fixed, milled by FIB and imaged by SEM. FIB techniques are used in the semiconductor industry to characterize 3D nanostructures, and have recently entered into life sciences to visualize below-surface sub-cellular structures (Ballerini et al., 1997; Drobne et al., 2007, 2008; Hayles et al., 2007).

Cells were prepared by aspirating the NBM from the chip vial, fixing in 10% neutral buffered formalin for 15 min, rinsing three times with distilled water and allowing the preparation to dry slowly by evaporation at room temperature in a biology safety cabinet. A thin layer of platinum (15 nm) was then sputtered on both sides of the chip to reduce charging effects from the non-conductive biological material (Hayles et al., 2007). Chips were loaded in a FEI 820 Dualbeam system (FEI Company, Hillsboro, OR) that allows both SEM imaging and FIB milling (Morrissey et al., 2005). Since P19 cells covered the entire surface, imaging from the culture side would give no hint of where the apertures were; consequently imaging was initially performed from the backside of the chip. 10 $\times$ 10 μ m pilot holes were drilled 10 μ m on each side of the aperture of interest for marking (Fig. 6A), employing a gallium ion beam with a dose of 1–1.2 nA and an accelerating voltage of

Figure 4. X-Z section scan image of a P19 cell on a chip aperture stained with Calcein-AM (A). B: P19 cells from the RH-237 channel, which stains cell membrane.

30 kV. A 5 μm wide, 20 μm long, and 1 μm thick protective strip of platinum was then locally deposited by an in situ chemical vapor deposition from a platinum-containing organic gas precursor. This second thicker and non-directional Pt coating in the FEI system, combined with the relatively low gallium ion dose, resulted in minimal damage of the cells sidewalls and no curtaining effect (Drobne et al., 2007; Hayles et al., 2007). The chip was then taken out and flipped to image and process it from the front side. A second protective strip of platinum was then locally deposited between the two pilot holes employing the same method as on the well side. Finally, a 15 $\mu\text{m} \times 15 \mu\text{m}$ square opening was milled employing the same beam conditions as

Figure 5. Fluorescence microscopy image of P19 cells stained with RH-237 (red, membrane) and Calcein-AM (green, viability). The perfusion is an indication of how intimately the cell is sealed to the aperture, diffusing only among cells neighboring apertures in (A) (20 $\times$ lens) or exclusively in the cell on top of the aperture as seen in (B) (63 $\times$). [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

Figure 6. Scanning electron micrographs of a FIB section of a P19 cell plated on top of a funnel-shaped aperture in a patch-clamp chip. A: shows square pilot holes milled from the back of the chip on either side of the aperture to locate it exactly, and the larger central square opening milled from the top through the layer of cells, with the back end aligned with the center of the pilot holes to cut through the center of the aperture. B: shows the cell lowered in the aperture conforms in shape with the funnel-shape sidewalls of the aperture.

for the pilot holes, with the back end of the square opening aligned with the pilot holes so the section is centered in the middle of the aperture. While the beam can be aligned very precisely (20 nm), the pilot holes are only indicative of where the center of the aperture is. Thus, we resorted to milling 1 μm in front of where we estimated the center of the aperture would be, then milled back in steps of 0.5 μm until the section of the aperture had the full expected diameter as observed with the in-situ SEM. Following FIB milling, higher resolution imaging was performed by SEM in a Hitachi 4700 FESEM (Hitachi High Technologies, Pleasanton, CA). The platinum coating minimized charging, which allowed employing a 3 kV beam, and images were taken with a 45 $^\circ$ incident tilt.

Figure 6B shows the structure of the chip and the cell in exquisite detail, and is the patch-clamp chip equivalent of the freezing and high-voltage (transmission) electron microscopy method developed by Ruknudin et al. (1991) to image blebs in patch-clamp glass pipettes. The clearest top and bottom layers are the protective platinum coating. The self-standing film of the chip is composed of a clearer SiN layer with a sharp-edged aperture resulting from a very directional fabrication process, smoothed in a funnel shape by the subsequent deposition of darker SiO₂ layers, very thin at the top and very thick at the bottom. Sub-cellular structures are visible with the nucleus located right on top of the aperture and details of other organelles in the cytoplasm. The cell in this picture is clearly lowered down the aperture. Because the cell has been fixed and dried, we expect it has not retained the shape it had in culture, and cracks are visible in the biological material. The cell did not fully retain its shape during the fixation and drying processes we employed; post-fixation (Drobne et al., 2007) or cryo-preparation (Hayles et al., 2007) would have provided a better immobilization of the tissue. If anything, however, we expect the process to have shrunk the biological material. Since the contours of the cells still mirror the funnel shape of the aperture, we infer that there must have been close contact between the membrane of the cell and the surface of the aperture.

Conclusion

The design of a multiple aperture sieve chip, visualization of cell-to-aperture interactions using confocal microscopy, and loading of a viability dye through the aperture itself, provides an efficient method to initially evaluate the probability of obtaining intimate cell-to-aperture seals in statistically significant numbers. It offers useful means to assess and refine key chip surface and aperture features critical to successfully developing planar patch-clamp chips for use with cultured cells. We find that our P19 cell culture conditions result in a high proportion of hole coverage, but the loading of a viability dye through the aperture reveals that aperture coverage does not necessarily result in a tight seal.

The FIB sectioning/SEM imaging technique described here brings the first visual evidence that cells forming tight seals lower in the aperture. This brings additional information relevant to determining the optimal aperture shape and in particular whether deep apertures are needed to obtain high impedance seals (Chen et al., 2009; Curtis et al., 2008; Lehnert et al., 2007; Sordel et al., 2006).

The authors wish to thank Alexei Bogdanov and Juan Caballero for fabrication of the patch-clamp chips at the CPFC, and Lilin Tay for advice on cell fixing for SEM imaging.

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