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Development of Patch-Clamp Chips for Mammalian Cell Applications

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Abstract: We have previously described the designs of two planar patch-clamp neurochips and their application to the electrophysiological study of molluscan neurons cultured on-chip. Neuron attachment and growth over apertures on the neurochip surface permitted the acquisition of whole-cell patch-clamp recordings. To broaden the application of these neurochips from molluscan to mammalian neurons, we conducted a study of cell-to-aperture interaction to optimize conditions for these smaller, more fragile cells. For this purpose, we designed a "sieve" chip having multiple apertures on its surface. Random growth of rat cortical neurons resulted in a 32% (n = 324) probability of cell growth over 2 μm diameter apertures; larger diameters resulted in growth through the aperture. Based on these findings, single-aperture neurochips were fabricated having 2 μm diameter aperture and preliminary electrophysiological recordings from cortical cultures at 14 DIV are presented. The implications of this study for the next-generation neurochips are discussed.

Keywords: Mammalian cells, planar patch-clamp, neurochip, whole-cell recordings.

1. INTRODUCTION

Conventional pipette patch-clamp, schematically presented in Fig. (**1a**), is considered the gold standard for cellular ion-channel monitoring [1]. A high resistance seal between the pipette tip and cell membrane permits high-fidelity monitoring of ion flux across the cell membrane. Conventional patch-clamp experiments are done routinely on isolated cells, cell cultures or tissue slices. However, conventional patch-clamp is complex and labor-intensive, with notoriously low throughput. In an effort to increase throughput, microchip-based planar patch-clamp systems were introduced (Fig. (**1b**)): the glass pipette was replaced by a membrane having microfeatures (called apertures) mimicking the tip of the pipette [2]. Apertures have been fabricated in silicon dioxide (SiO_2) [3-5], silicon nitride (Si_3N_4) [6], quartz/glass [7, 8], poly(dimethylsiloxane) (PDMS) [9, 10] and polyimide (PI) [11]. However, the available planar patch-clamp systems have only been applied to isolated cells in suspension. Important advantages of the conventional patch-clamp experiment are therefore lost using planar patch-clamp chips: the ability to probe cells in culture conditions as well as the probing of cell-to-cell interactions.

We described in two recent publications the fabrication of neurochips for planar patch-clamp monitoring of cells cultured on-chip. The silicon-based [12] and polymer-based [13] neurochips are presented in Fig. (**2a**) and Fig. (**2b**), respectively. While the silicon-based neurochip contains a single aperture on a silicon nitride membrane, the polymer neurochip has two apertures on its polyimide surface. The two apertures are independently accessible through dedicated underlying microfluidic channels in the PDMS layer, a design for simultaneous dual recording of cells forming synaptic connections.

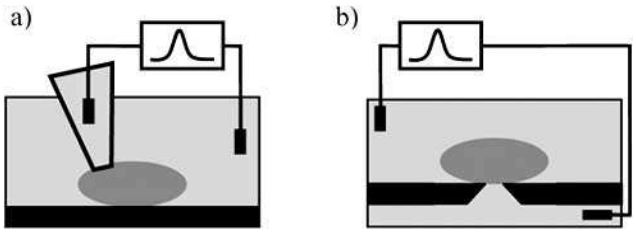

Fig. (1). Patch–clamp techniques.

(**a**) Cartoon showing conventional patch-clamp. The tip of a glass pipette forms a high resistance seal with the cell membrane allowing electrophysiological measurements. (**b**) Scheme for a single-site planar patch-clamp chip. The pipette tip is replaced by an aperture microfabricated on the surface.

Using both neurochip designs we have shown, for the first time, the recording of high fidelity patch-clamp recordings from molluscan neurons grown on-chip. The recording of action potentials from single, isolated neurons demonstrated functional physiological responses from cells grown over apertures. Furthermore, using pre- and post-synaptic molluscan neurons, we have recorded evidence of synaptic activity [14], making it the first report examining synaptic function using planar patch-clamp chips.

The choice of molluscan neurons for these initial studies emanated from a simple fact: for electrophysiological data to be recorded, a neuron has to grow directly on top of the aperture. The use of large *Lymnaea* neurons (40 to 80 μm diameter) allowed manual positioning of cells over the aperture using a dissecting microscope as a visual aid. However, we do realize the importance of obtaining electrophysiological data from mammalian cells. This would offer a more relevant on-chip model of human neurological physiology. The average rat cortical neuron has a diameter of 10 μm, too small to deposit at the aperture site through visual aid. Also, while molluscan neurons are robust, rat cortical neurons are more difficult to establish in culture.

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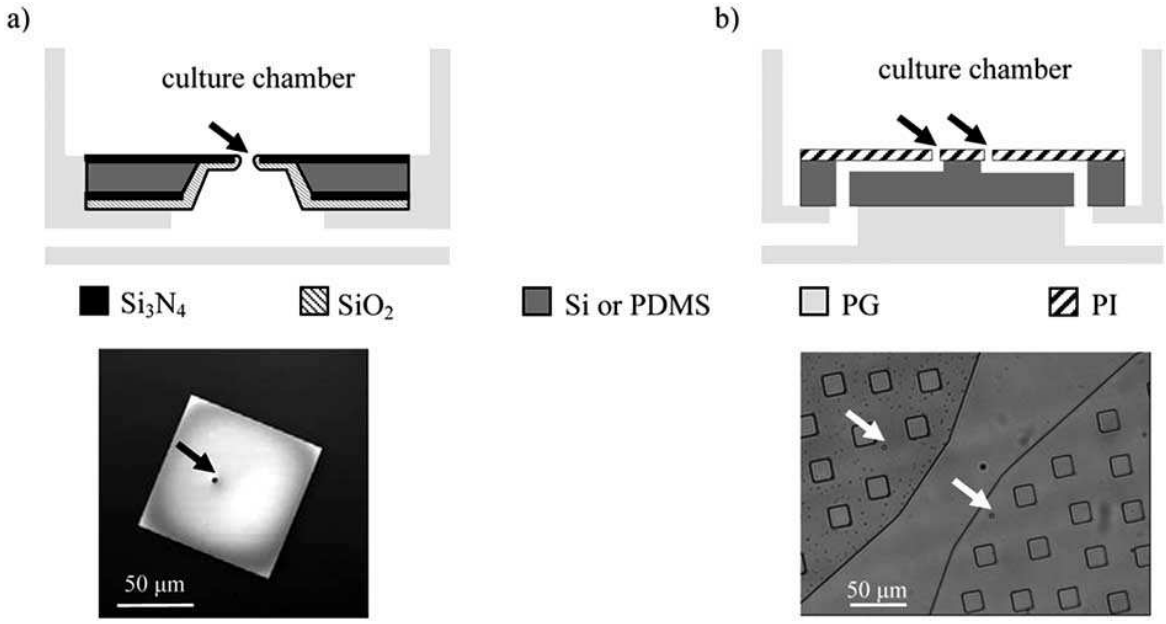

Fig. (2). Planar patch-clamp neurochips.

(**a**) Schema of the single-aperture silicon-based neurochip (top) and micrograph of the aperture region on the silicon nitride (Si_3N_4) membrane (bottom). (**b**) Schema of the two-aperture polymer-based neurochip (top) and micrograph of the aperture region on the polyimide (PI) layer (bottom). Note that the two independent PDMS microfluidic channels are visible. The neurochips are mounted in a Plexiglas package (PG) with an upper opening for cell culture. Arrows point to aperture location.

In this report, we present a "sieve" chip having 81 apertures on its surface and use it to evaluate mammalian brain cell growth over apertures. Using a combination of fluorescence and scanning electron microscopy imaging, we study the optimal aperture geometry allowing for rat cortical neuron growth. Preliminary electrophysiological results obtained on a single-aperture neurochip are shown. Finally, we discuss the implication of this study on future neurochip designs.

2. METHODS

2.1. Design and Fabrication

Sieve Chip

Fig. (**3**) presents the sieve chip. The diagram (Fig. (**3a**)) shows a cross-section; pictures of the sieve chip's surface are shown in Fig. (**3b**). The fabrication procedure was amended from [12]. Apertures were etched in a 1 µm thick silicon nitride film, and inverted pyramid-shaped wells were etched anisotropically in a KOH solution from the back of the wafer. This resulted in 200 x 200 µm self-standing areas in the silicon nitride (referred to as the membrane) containing the apertures. A thick SiO_2 layer was deposited to passivate the silicon walls of the well, and to adjust the diameter and shape of the apertures. To emulate the surface conditions of our two-aperture polymer neurochip (Fig. (**2b**)), the surface was covered with polyimide as described next. An adhesion promoter (VM-651, 0.2% in water, HD Microsystems, Parlin, NJ) was spun at 3,000 rpm on the top side of the wafer followed by polyimide (PI-2610, HD Microsystems) at 2,700 rpm. After an initial pre-bake at 100 ºC for 90 s and 150 ºC for 90 s on a hot plate, the wafer was placed in a diffusion oven for final curing at 350 ºC under nitrogen atmosphere for 30 min. Using the silicon nitride membrane as a mask,

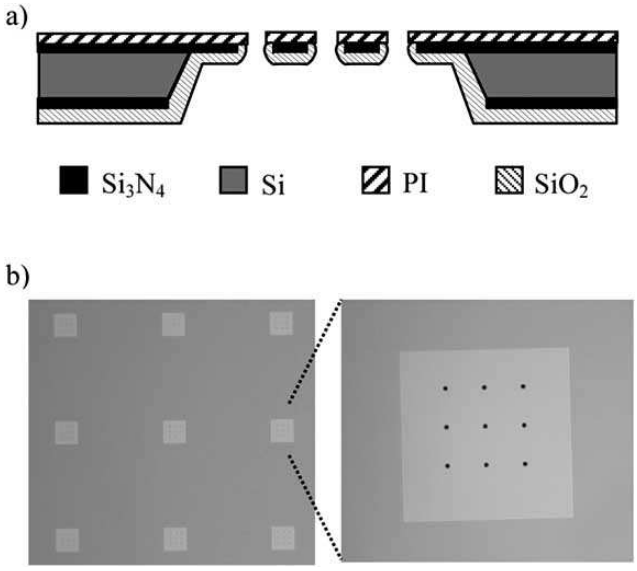

Fig. (3). Sieve chip for assessment of growth of rat cortical cell cultures.

(**a**) Schematic cross section of one membrane on the sieve chip: the bulk silicon has a silicon nitride top membrane with 3, 4 and 5 µm diameter apertures. A SiO_2 layer is deposited from the backside; 2, 3 and 4 µm diameters apertures result. A polyimide layer is deposited and etched on top of the silicon nitride membrane. (**b**) Surface of one chip (left) containing 9 membranes; close-up of one membrane containing 9 apertures, for a total of 81 apertures per chip. Membrane size is 200 µm.

apertures were etched from the backside in a reactive ion etcher under a mixture of oxygen (20 sccm) and CF_4 (4 sccm) flow. As seen in Fig. (**3b**), each sieve chip contained 9 membranes (left image), with each membrane having 9 apertures (right image) for cell-to-aperture interaction studies. In each sieve chip, the top membranes have 2 μm diameters apertures, the middle membranes, 3 μm diameters and the lower membranes, 4 μm. Before use, the wafers were diced into 1 x 1 cm individual sieve chips.

Single-Aperture Neurochip

Fig. (**4**) presents the single-aperture neurochip; a schema and close-up of the aperture region were shown in Fig. (**2a**). Detailed fabrication procedures have been presented previously [12] and follow the procedure described above for the sieve chip. Single-aperture neurochips were made functional, i.e. ready to acquire electrophysiological recordings, by mounting them in a Plexiglas (PG) package. The PG has an upper 16 mm diameter opening for cell culture and a lower fluidic loading region connected to silicone tubing which houses the recording electrode.

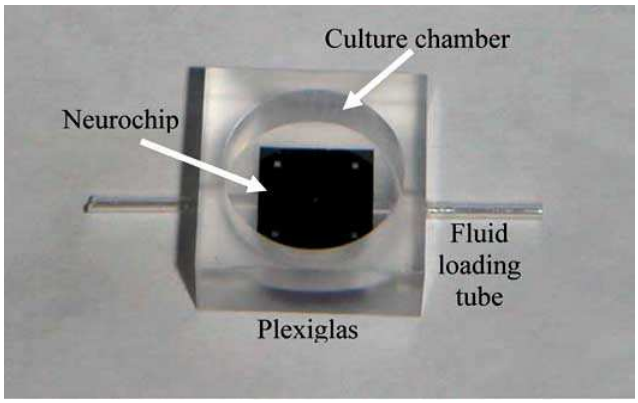

Fig. (4). Single-aperture neurochip used for electrophysiological analysis.

2.2. Rat Cortical Cultures

After a 5 min sterilization process in an air plasma (Harrick Plasma, Ithaca, NY), sieve chips were transferred to a 24 well plate (VWR Canlab, Mississauga, ON, Canada) and immersed in a poly-L-lysine solution for a minimum of 2 hours. Following a water rinse, rat cortical cells were plated. Similarly, functional single-aperture neurochips were sterilized, loaded with distilled water in the lower fluidic channel and poly-L-lysine in the upper culture chamber for a minimum of 2 hours. Following a water rinse, culture media was loaded in the lower fluidic channel and rat cortical cells were plated on the surface.

Cultures of E18 rat cortical cells were prepared as described previously [15]. Briefly, timed-pregnant Sprague-Dawley rat (Charles River Canada, St.Constant, QC, Canada) were anesthetized with halothane and killed by cervical dislocation. After dissection of the cortical region of the fetal brain, cortical cells were dispersed by trituration, centrifuged at 2,400 rpm for 5 min at 4 °C, and suspended in a medium containing Minimal Essential Medium (MEM, Wisent Canadian Laboratories, St-Bruno, QC, Canada) supplemented to 25 mM glucose, 10% horse serum, 2 mM L-glutamine (all from Sigma Aldrich, Oakville, ON, Canada), 10% Fetal Bovine Serum (FBS, PAA Laboratories, Etobicoke, ON, Canada) and 100 units of penicillin-100 μm streptomycin (Introvigen Corporation, Carlsbad, CA). A 1.3 x 10^6 cells/mL suspension was passed through a 40 μm cell strainer (Fisher Scientific, Nepean, ON, Canada) to remove cell clumps.

A 500 μL aliquot of filtered cell suspension was added to each well of the 24 well plate containing sieve chips. A 500 μL aliquot was added to the functional single-aperture neurochips. Cell cultures were maintained at 37 °C in a 5% CO_2 humidified incubator (NuAire Inc., Plymouth, MN) for a minimum of 14 days. After 6 days *in vitro*, cultures were treated with 15 μg/mL 5-fluoro-2'deoxyuridine and 35 μg/ml uridine (both from Sigma Aldrich) to minimize glial growth. Media change was performed at 7 days *in vitro*: half of the medium was replaced with medium consisting of MEM, 10% horse serum and 100 units penicillin-100 μg streptomycin. All procedures involving animals were approved by the Institute of Biological Science Animal Care Committee.

2.3. Imaging

Normal Bath Media (NBM) was prepared as follows: 140 mM NaCl; 3.5 mM KCl; 0.4 mM KH_2PO_4; 20 mM HEPES, 5 mM $NaHCO_3$; 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$; 15 mM glucose.

Calcein-AM (Molecular Porbes, Carlsbad, CA) was used as a cell viability indicator. A 5 mM stock solution was prepared in DMSO and further diluted to 40 μM in NBM. Cells were subsequently stained using 5 μM Calcein-AM.

RH-237 (Molecular Probes) was used to stain cell membranes. A 10 mM stock solution was prepared in ethanol and further diluted in NBM to 50 μM. Cells were subsequently stained using 50 μM RH-237.

Prior to sieve chip imaging, a 500 μL aliquot of 5 μM calcein was placed in each well and incubated in the dark at 37 °C for 30 min. Calcein was then replaced by the same volume of 50 μM RH-237 solution and left in the dark at room temperature for 10 min. Finally, sieve chips were placed in NBM solution and ready for imaging.

Fluorescence and reflection images of cultures on sieve chips were obtained using a LSM-410 Zeiss (Thornwood, NY) confocal microscope equipped with a krypton/argon laser (Melles Griot, Carlsbad, CA) and a LSM Tech Inc. (Etters, PA) objective inverter. For each dye, excitation wavelength and emission filter were appropriately selected and images of both dyes were collected sequentially. Calcein was excited at 488 nm and emitted fluorescence collected using an emission filter with a bandwith of 515 to 540 nm. For RH-237, the excitation wavelength of 488 nm and a long-pass emission filter with a cut-off at 590 nm were used. Reflection image were collected using the 568 nm line of the laser with no filter in front of the photomultiplier tube. Fluorescence and reflection images were merged using a Northern Eclipse Software (Empix, Mississauga, ON, Canada).

2.4. Electrophysiology

Recordings from rat cortical cells at 14 days *in vitro* (DIV) were obtained using functional single-aperture neuro-

chips having a 2 μm diameter aperture. As described previously [12], functional neurochips were connected to an Axopatch 200B amplifier (Axon Instrument, Foster City, CA) by placing the electrodes in the silicone tubing and a reference electrode in the upper culture dish of the Plexiglas package. Signals were filtered at 2 kHz and acquired at a sampling rate of 20 kHz. Analyses were done off-line with IGOR software (Wavemetrics, Lake Oswego, OR) running on a personal computer.

3. RESULTS AND DISCUSSION

3.1. Cell-to-Aperture Interaction Study

To study cell-to-aperture interactions, sieve chips containing apertures with three different diameters (2, 3 and 4 μm) were fabricated. While nanometer-sized surface topography may promote cell-to-surface adhesion in some cell types [16, 17], apertures are not inherently conducive to cell attraction, suggesting that minimizing their diameter could encourage cell growth at the aperture site. By contrast, two considerations favor larger apertures. First, for electrophysiological measurements to be made on the cell through the underlying fluidics, continuous liquid contact must be maintained between the cell, the fluid and the electrode. A small diameter aperture makes this more challenging due to the difficulty in purging microscopic air bubbles forming at the aperture site. Second, smaller aperture diameters increase access resistances, decreasing signal-to-noise ratio and response time during electrophysiological recording [13].

A total of 12 sieve chips were plated with rat cortical cells. Figs. (**5a, 5c** and **5d**) show these cultured cells (14 DIV) on the three different aperture sizes: 2, 3 and 4 μm, respectively. A close-up of a cell at a 2 μm aperture position is shown in Fig. (**5b**). Cell viability was assessed using calcein (green fluorescence on the figure); cell membranes were stained by RH-237 (red fluorescence). White arrows indicate viable cell growth at the aperture position and apertures are circled. We found a 32% (n = 324) probability of cell growth at the 2 μm apertures, a 33% (n = 324) probability of growth at the 3 μm apertures and 27% (n = 324) probability at the 4 μm apertures. These results suggest that rat cortical cell growth was independent of aperture size in the range tested.

However, further investigation of cell-to-aperture interaction using scanning electron microscopy (SEM) of focused ion beam (FIB) sections (described elsewhere [18]) revealed that cells growing at 3 μm diameter apertures actually extended through the aperture and along the underside of the sieve chip's membrane. Fig. (**6**) shows an SEM image of a

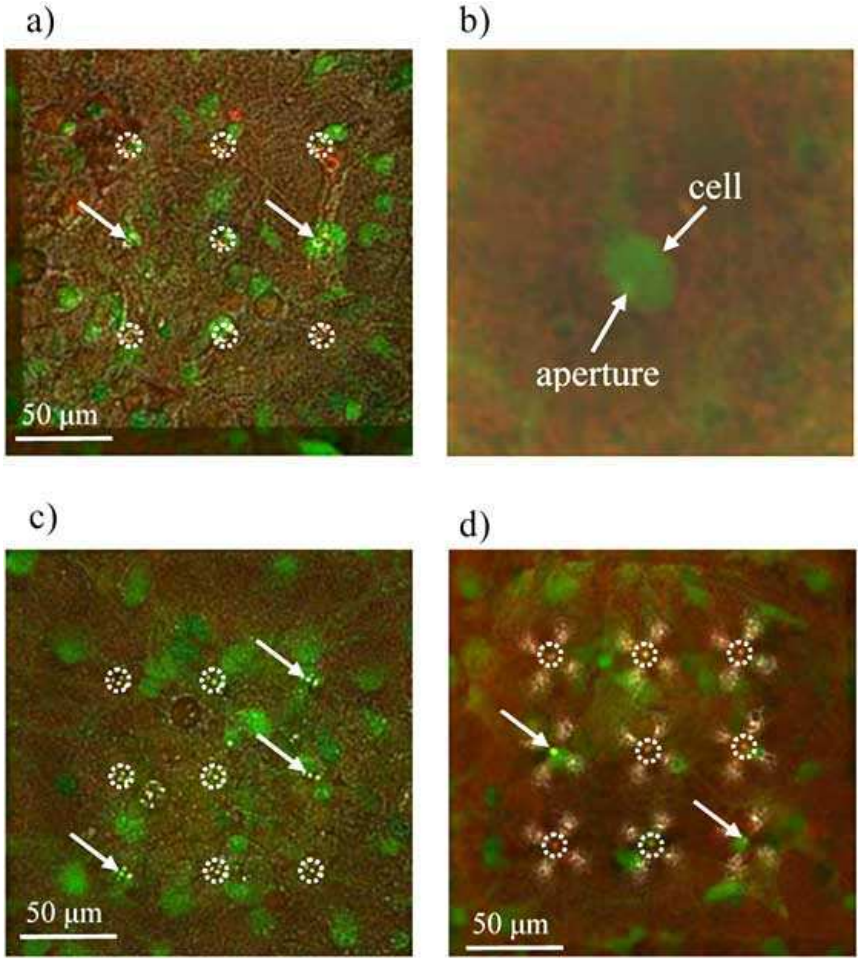

Fig. (5). Imaging results of cell growth on the sieve chip.

Reconstructed images of membranes containing (**a**) 2 μm, (**c**) 3 μm and (**d**) 4 μm apertures. (**b**) Close-up of a cell on the 2 μm aperture from (**a**). Apertures are circled, arrows point to the apertures where viable cell growth occurred.

fixed mammalian cell grown at the 3 μm aperture site following a FIB section: cell extension through the aperture, away from the culture surface is seen. These findings suggest that 2 μm diameter apertures are more appropriate than larger ones as candidates for future neurochip development.

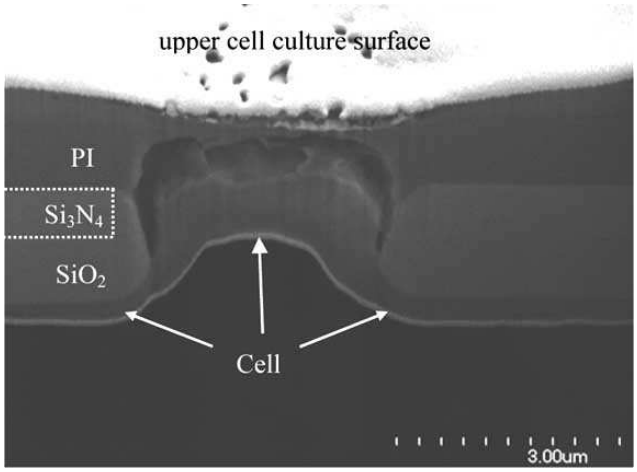

Fig. (6). SEM of focused ion beam cross section of a 3 μm aperture, showing a 10 μm diameter mammalian cell growing on the underside of the sieve chip's membrane.

3.2. Preliminary Electrophysiological Results

Rat cortical cells were subsequently plated on functional single-aperture neurochips such as the one presented in Fig. (**4**). Aperture diameter was set at 2 μm. Preliminary electrophysiological data is shown in Fig. (**7**). Fig. (**7a**) presents a micrograph of the membrane region: a cell (circled in the image) is over the aperture site. Whole-cell patch-clamp experiments were run in current-clamp mode: input intracellular current pulses (Fig. (**7b**), lower trace) triggered membrane potential responses (Fig. (**7b**), upper traces). In Fig. (**7c**), spontaneous spiking activity is observed. This demonstrates that the single-aperture neurochip could both stimulate and record membrane activity from rat cortical cell established in long-term culture (14 DIV) over an aperture recording site. This preliminary result is encouraging and suggests that neurochip application to mammalian cells is a realistic objective that could be reliably achieved provided further refinements to the neurochip design.

3.3. Next Generation Neurochip

Our overarching goal is to produce a neurochip with multiple aperture recording sites, each individually addressable through underlying microfluidic channels, for the electrophysiological interrogation of mammalian cells forming synaptic connections. While the single-aperture neurochip used in section 3.2 can only record from a single cell, the two-apertures neurochip shown in Fig. (**2b**) is our first generation polymer neurochip for multi-cellular patch-clamp monitoring: the two independently-addressable apertures were built to allow simultaneous two-cell recording. Preliminary patch-clamp data from the single-aperture neurochip are encouraging and suggest that application to mammalian cells is a realistic goal. It follows that refinements to the first-generation

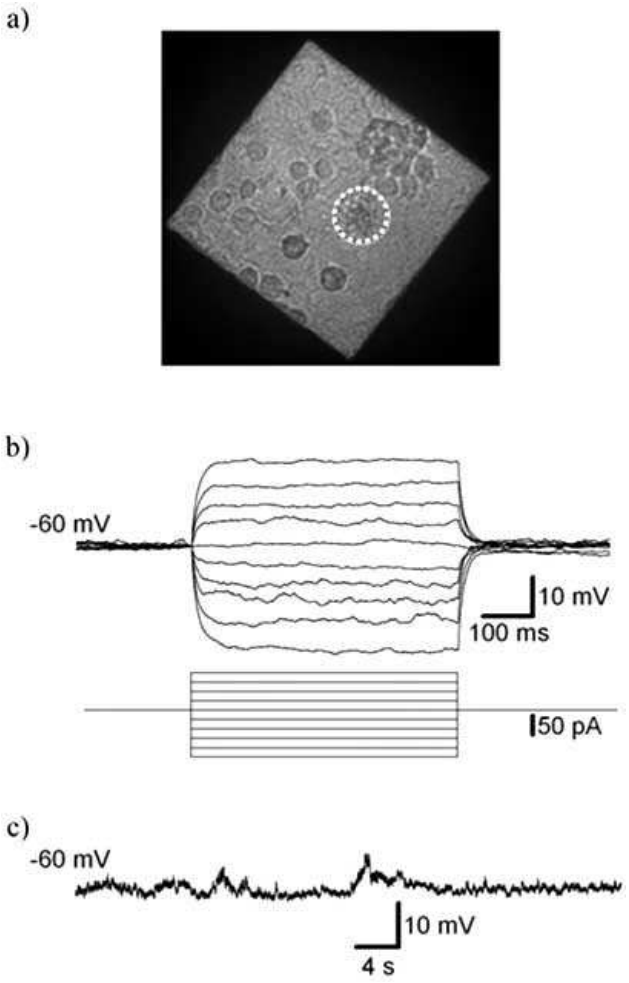

Fig. (7). Whole-cell patch-clamp recordings obtained from a cultured mammalian cell.

(**a**) Transmitted light image of a single-aperture neurochip with rat cortical cells cultured on it (14 DIV). The cell at the aperture location is circled. (**b**) Voltage responses (top) to a series of intracellular current pulses (bottom) of the cell shown in (**a**). The current pulses were applied at V_m = -60 mV. (**c**) Recording of spontaneous activity from the cell shown in (**a**).

polymer neurochip design can be expected to enable recordings of synaptic activity from mammalian neurons.

Given that mammalian neuron growth over apertures occurred with a 32% probability for randomly organized cell networks, the probability of recording synaptic activity in the two-aperture neurochip of Fig. (**2b**) is low. For the neurochip to become a useful tool in the study of synaptic connectivity, rivaling the use of conventional glass pipette, this percentage will have to be improved. We are studying several design refinements to accomplish this. First, reducing the diameter of apertures from its original 4 μm diameter to 2 μm is possible and will increase the neurochip's access resistance from 1.43 MΩ to 1.98 MΩ (see [13] for detailed calculations). This still compares favorably to conventional glass pipette [19]. Another promising approach to enhance growth-over-aperture probability is by chemically patterning cell-adhesion peptides (such as poly-L-lysine) around the aper-

tures [20]. We are actively developing and evaluating patterning strategies to accomplish this critical objective.

To increase the probability of obtaining simultaneous dual patch-clamp recording of synaptically-connected cells, the number of apertures on the neurochip surface will have to be increased. Since each aperture must be singly-addressable through underlying microfluidic channels, channel geometry will have to be reassessed, including the use of PDMS for channel fabrication. Our current fabrication procedure involves the fabrication of PDMS and PI independently on 2 inch carrier wafers, followed by a final bonding step: this produces 9 chips per wafer. To reduce irreproducible shrinking variations during the silicone fabrication, we showed a two-layer PDMS fabrication that reduced the variations in the channel fabrication, from ±13 μm over 1 cm to ±4 μm over 1 cm, thus allowing aligned bonding to the upper PI layer over large areas. Increasing the number of microchannels will impose more geometrical restrictions and higher alignment accuracy will be needed. For this purpose, we are currently studying fabrication processes that would lead to a replacement of PDMS by glass.

4. CONCLUSION

We developed a sieve chip for the assessment of cell-to-aperture interactions. We have shown that 2 μm apertures are superior to larger ones (3 and 4 μm) in terms of supporting noninvasive growth of rat cortical cells over these structures. With a 32% cell-over-aperture probability for randomly organized cell growth, we are actively studying surface functionalization strategies aimed at improving this probability. Using the sieve chip results, functional single-aperture neurochips having 2 μm aperture have yielded encouraging preliminary whole-cell patch-clamp recordings from rat cortical cells 14 days in culture.

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