Polydimethylsiloxane microfluidic films for in vitro engineering of small-scale neuronal networks

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Polydimethylsiloxane microfluidic devices have become standard tools in cell engineering research. However, through-holes where cells access the microchannels are usually fabricated manually using biopsy punches, making it difficult to create a large array of sub-mm sized through-holes. Here, we present a fabrication process for a thin-film microfluidic device containing an array of through-holes, which are as small as 100 μm by 100 μm and span 10 mm by 10 mm. A proof-of-concept application of the device to neuronal patterning experiments shows that spatially complex network dynamics emerge when a non-random connectivity is imposed to cultured neuronal networks. We also demonstrate that the coupling strengths between neuronal modules, a major factor that defines the global network dynamics, can be effectively modulated by varying the microchannel widths. This work opens a new application of microfluidic devices to multicellular systems comprised of several tens to hundreds of neurons. © 2020 The Japan Society of Applied Physics

1. Introduction

Driven by concepts such as the organ-on-a-chip for pharmaceutical sciences, microfabrication technology continues to provide novel applications in cell engineering research.1–3 Neuroscience was among the first fields to adopt cell culture on engineered scaffolds.4–8 Since then, methods including photolithography, microcontact printing, and microfluidic devices have been utilized to provide novel in vitro platforms for fundamental and applied neuroscience studies. Among the multiple approaches that can be used for cell patterning, polydimethylsiloxane (PDMS) microfluidic devices have now been accepted as a highly reproducible and stable platform.9,10 For neuroscience applications, microfluidic devices are used to confine distinct populations of neurons in separate chambers.11,12 Permitting the growth of axons in defined orientations,13–15 and record signal propagation within axons.16–18 However, a limitation of the microfluidics approach is that the reservoirs, or the through-holes via which the cells enter the device, are generally fabricated separately from the microfabrication process, e.g. by manually creating mm-sized holes with biopsy punches.9,14 Although PDMS microstructures with sub-mm through-holes have previously been fabricated using plasma etching,19 capillary filling,20,21 manual air blowing,22 and liquid drop-casting,23 application of the methods to neuronal patterning experiments is rare. This becomes a major issue when designing neuronal networks with a restricted number of neurons, in the range of several tens to hundreds of cells.

Here, we report on the development of a microfluidic film (μFF), a thin free-standing microfluidic device with μm-sized reservoirs, for application in patterning small-scale neuronal networks. A simple and reproducible protocol for fabricating the device, which involves the drop-casting of a thin layer of liquid PDMS on to an SU-8 master mold, is fully described. A challenge in using the μFF for cell culture was air-bubble entrapments in the through-holes, which became more prominent with smaller holes. We show that this issue can be overcome using ethanol-mediated wetting of the PDMS surface. Finally, we demonstrate an application of the newly prepared device to pattern modular networks of primary cortical neurons.

2. Experimental methods

2.1. Device fabrication

The fabrication of μFF involves two steps: fabrication of a master mold and subsequent PDMS structuring. The master mold was fabricated by patterning two photoresist layers on a silicon substrate. A diced silicon (approximately 25 × 25 mm²) was first cleaned in a piranha solution (a 1:1 mixture of concentrated H₂SO₄ and 30% H₂O₂) for 10 min, and the surface oxide was subsequently stripped in 5% HF for 5 s. Afterward, a SU-8 3010 photoresist (Kayaku Advanced Materials) was spin-coated at 3000 rpm and subsequently baked for 1 and 5 min at 65 °C and 95 °C, respectively. Next, photolithography was performed using a mask aligner (Suss MJ-4) and a chromium photomask with both reservoir and microchannel patterns. After a post-exposure bake for 1 and 3 min at 65 °C and 95 °C, respectively, the pattern was developed in SU-8 Developer and rinsed twice in 2-propanol.

The abovementioned procedure was repeated using a thicker photoresist to form the second layer. For this step, the photoresist was replaced with SU-8 3050 (Kayaku Advanced Materials), which was spin-coated at 1500 rpm, and a photomask with only the reservoir patterns was used. A pre-exposure bake was performed for 1 and 20 min at 65 °C and 95 °C, respectively, followed by a post-exposure bake for 1 and 5 min at 65 °C and 95 °C, respectively. Details regarding the pattern geometry are provided in Sect. 3. Individual patterns were arranged in a matrix of 11 × 11 or 12 × 12 to fit in a square region of 10 × 10 mm².

Using the fabricated SU-8 mold, the μFF was produced using Sylgard 184 (Dow Corning). The base and curing agents were mixed in a 10:1 ratio and degassed. Subsequently, 6 μl of the mixture was drop-casted to the edge of the 10 × 10 mm² square using a P20 micropipette and allowed to spread for 10–30 min. Finally, PDMS was thermally cured in an oven for 3 h at 60 °C and peeled off from the mold substrate using Dumont #5 forceps.

2.2. Sample characterization

Three-dimensional topography of the photoresist and PDMS microfluidic device was analyzed using confocal microscopy.
All the samples were imaged without prior surface coating. The data were analyzed using MultiFileAnalyzer software (Keyence).

Bubble trapping in reservoirs was quantified under a stereo microscope. The \( \mu \)FF was first sterilized under UV light for 30 min and attached to a glass coverslip (Matsunami C018001; diameter, 18 mm; thickness 0.17 mm) coated with poly-D-lysine (PDL; Sigma P-0899). The sample was subsequently immersed in a minimum essential medium (MEM; Gibco 11095-080) and imaged using a stereo microscope and digital camera. The use of the phenol red-containing MEM, rather than water, substantially eased the observation of bubbles.

### 2.3. Cell culture

Prior to cell culture, the \( \mu \)FF attached to a PDL-coated coverslip was immersed in a neuronal plating medium [MEM (Gibco 11095-080) + 5% fetal bovine serum + 0.6% D-glucose] and stored overnight in a cell-culture incubator. Rat cortical neurons were obtained from the cortices of E18 embryos and cultured using previously published protocols. All procedures were approved by the Tohoku University Center for Laboratory Animal Research, Tohoku University (approval number: 2017AmA-001-1) and the Tohoku University Center for Gene Research (2019AmLMO-001). Briefly, the dissociated cells were suspended in the plating medium and plated at a concentration of \((3.8\text{--}5.0) \times 10^4\) cells cm\(^{-2}\). The coverslip with the \( \mu \)FF was then transferred and placed upside down in a cell-culture dish with glial cells growing in the N2 medium. At 4 days in vitro (DIV), the cultured cells were transfected with the fluorescent calcium probe GCaMP6s (Addgene viral prep #100843-AAV9) using adeno-associated virus vectors. Spontaneous and evoked network activities were recorded at 10 DIV via fluorescence calcium imaging. Statistical analyses of neural correlations were restricted to networks bearing 70–120 neurons to focus on the impact of network morphology.

### 3. Results and discussion

#### 3.1. Fabrication of the PDMS \( \mu \)FF

The fabrication process of the \( \mu \)FF is schematically outlined in Fig. 1. Briefly, the master mold was first created by patterning two photoresist layers on a clean silicon substrate [Figs. 1(a)–1(d)]. The PDMS prepolymer was then drop-casted to the edge of the patterned photoresist [Fig. 1(e)], thermally cured, and peeled off to release the completed \( \mu \)FF [Figs. 1(f) and 1(g)]. Further details of the process are described in Sect. 2.

The \( \mu \)FF fabricated herein was 96.0 ± 19.4 \( \mu \)m (mean ± S. D.; \( n = 15 \) observations from 5 samples) thick and designed to be 10 × 10 mm\(^2\) in size (Fig. 2). In conventional microfluidic devices, the reservoirs are usually created by manually punching mm-sized holes. In contrast, the reservoirs of the \( \mu \)FF were defined by the second-layer photoresist of the master mold, enabling the precise definition of an array of reservoirs as small as 100 × 100 \( \mu \)m\(^2\) [Fig. 2(b)].
minimum feature size of the reservoirs was restricted by the area necessary for the proper growth of neurons, rather than inherent limitations of the lithography process. The micropatterns consisted of reservoirs for cell adhesion (areas: 100 $\times$ 100, 200 $\times$ 200, or 400 $\times$ 400 $\mu$m$^2$) and microchannels for neurite growth (widths: 2, 5, or 10 $\mu$m). Schematic illustrations of representative micropatterns are presented in Figs. 3(a)–3(c) along with confocal micrographs of the prepared $\mu$FF. Dissociated neurons cultured in these structures developed to form a network with modular organization, network connectivity which is evolutionarily conserved in the nervous systems of animals.

3.2. Neuronal cultures in the $\mu$FF

After attaching the $\mu$FF to a PDL-coated coverslip, the sample was immersed in the neuronal plating medium overnight to prepare for cell seeding. A major issue that arose at this stage was gas entrapment in the reservoirs [Fig. 4(a), left], which became increasingly problematic as the reservoir sizes were reduced [Fig. 4(b)]. To prevent bubble trapping, the sample was soaked in 99.5% ethanol prior to immersion in the neuronal plating medium.\(^{31,32}\) This procedure was highly effective for reducing the number of air bubbles trapped in the reservoirs and microchannels [Fig. 4(a), right]. For instance, the fraction of reservoirs with air bubbles was reduced from 99.9% to 6.1% for reservoirs with a size of 100 $\times$ 100 $\mu$m$^2$. This bubble reduction effect was prominent in other reservoir sizes as well [Fig. 4(b)].

Fluorescent micrographs of the rat cortical neurons grown in 4-module micropatterns are shown in Fig. 4(c). The neurons were stained with the neuronal marker NeuO to better visualize soma and neurites. The pre-plating procedure described above allowed the neurons to occupy almost all the reservoirs. High magnification observation of the microchannel area also showed a presumptive axon protruding from a soma and entering the microchannel [Fig. 4(c)]. For the 10 $\mu$m wide channels, analysis of phase-contrast micrographs revealed that a neuron migrated into 1 out of 28 channels. For the 2 and 5 $\mu$m wide channels, such migration was not observed in any of the microchannels ($n = 20$ and 28 channels, respectively). Therefore, the microchannels used in the present work were predominantly devoid of cell bodies.

3.3. Network activity of the engineered neuronal networks

Finally, to characterize the dynamics of the micropatterned neuronal network grown in $\mu$FFs, rat cortical neurons were transfected with a calcium probe, GCaMP6s. Fluorescence intensity traces of spontaneous neural activity in a 4-module network with a line width of 10 $\mu$m is shown in Fig. 5(a). The data is presented as the relative fluorescence intensity ($\Delta F/F$) of neuron $i$, $f_i$, which was calculated as

\begin{align*}
\Delta F &= F_{i, \text{post}} - F_{i, \text{pre}} \\
F_{i, \text{pre}} &= \text{mean fluorescence before stimulation} \\
F_{i, \text{post}} &= \text{mean fluorescence after stimulation} \\
n_f &= \text{number of fluorescent events} \\
\Delta F/F &= \frac{\Delta F}{F_{i, \text{post}}} \\
\end{align*}
The spontaneous activity pattern of the prepared modular neuronal network was characterized by the coexistence of globally and locally synchronized activity. Globally synchronized activity, or network bursts,\textsuperscript{24,26,27} was observed repetitively, e.g., at 86.0, 143.0, and 233.5 s [Fig. 5(b)]. In addition to the typical activity of cultured neuronal networks, a rich variety of localized synchronizations were observed, e.g., at 92.2, 150.5, and 181.0 s [Fig. 5(b)]. This observation reproduces previous experiments using microcontact-printed protein scaffolds, which showed that the modular organization enriches the variety of spatiotemporal activity patterns exhibited within a single network.\textsuperscript{24}

A comparison of 4-module networks with different channel widths revealed that the dynamical enrichment became more prominent when channel widths were further narrowed. Narrowing of the channel width has been reported to reduce the number of penetrating neurites.\textsuperscript{13} As shown in Fig. 5(c), the functional correlation between a pair of neurons became more broadly distributed between zero (desynchronized) and one (fully synchronous) in micropatterns with 2 \( \mu \text{m} \) and human, and that any rewiring from the original topology decreases the value.\textsuperscript{33} The mean functional complexity derived from the recordings of the 4-module networks is maximized in the network topology obtained from \textit{C. elegans}, cat, macaque, and human, and that any rewiring from the original topology decreases the value.\textsuperscript{34} The mean functional complexity obtained from the recordings of the 4-module networks is maximized in the network topology obtained from \textit{C. elegans}, cat, macaque, and human, and that any rewiring from the original topology decreases the value.\textsuperscript{33}

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\[
r_{ij} = \frac{\langle f_i f_j \rangle - \langle f_i \rangle \langle f_j \rangle}{\sigma_i \sigma_j},
\]

where \( f_i = f(t) \) is the relative fluorescence intensity calculated here using time-dependent background intensity,\textsuperscript{26} \( \langle \cdot \rangle \) designates the time average, and \( \sigma_i \) is the standard deviation of \( x \).

The broadening of the neural correlation resulted in the increase of functional complexity \( \chi \), a statistical measure of integration-segregation balance in complex networks:\textsuperscript{24,33}

\[
\chi = 1 - \frac{1}{M} \sum_{j=1}^{m} P_r(r_{ij}) - \frac{1}{m},
\]

where \( |\cdot| \) designates the absolute value, \( m \) the number of bins \((=20), M = 2(m - 1)/m \) a normalization factor, and \( p(\cdot) \) the probability. By its definition, \( \chi \) evaluates the deviation of the probability distribution of \( r_{ij} \) from a uniform distribution.

The value of \( \chi \) has been shown to be maximized in the network topology obtained from \textit{C. elegans}, cat, macaque, and human, and that any rewiring from the original topology decreases the value.\textsuperscript{33} The mean functional complexity derived from the recordings of the 4-module networks is maximized in the network topology obtained from \textit{C. elegans}, cat, macaque, and human, and that any rewiring from the original topology decreases the value.\textsuperscript{33}

Fig. 4. (Color online) Neuronal culture. (a) Gas entrapment in the reservoirs, which inhibits the plated cells from entering the device (left), can be substantially reduced using ethanol to mediate PDMS surface wetting (right). (b) The fraction of reservoirs occupied with air bubbles without (grey) and with (white) ethanol-mediated wetting. Data are shown as means \( \pm \) S.D. \( *p < 0.05, **p < 0.01 \) (two-sided Student’s t-test). (c) Fluorescence micrograph of rat cortical neurons cultured in the 4-module micropattern for 3 d. Bird’s-eye, low-magnification (top) and high-magnification (bottom) observations. The left side of the top panel is that of an area without the PDMS where neurons are randomly growing. In the bottom panel, the micropattern geometry is outlined with dashed lines to aid visualization. Scale bars, 1 mm (a), 500 \( \mu \text{m} \) (c, top), and 50 \( \mu \text{m} \) (c, bottom).
patterning applications, the drop-casting approach is the simplest and most reliable way to produce $\mu$FFs. Although cell culture experiments using microfluidic devices with an array of sub-mm sized reservoir holes have been demonstrated,$^{12}$ a step-by-step fabrication procedure of an array of sub-mm sized reservoir holes have been demonstrated,$^{12}$ a step-by-step fabrication procedure of such devices has not been detailed previously. Under the increasing demand to study the structure-function relationships in biological neuronal networks at single-cell resolutions, the newly developed $\mu$FF technology provides a novel platform to engineer neuronal networks.

4. Conclusions

We described herein a reliable protocol for producing $\mu$FFs which can be used to engineer network connectivity in small-scale neuronal networks. The protocol consists of the standard fabrication of a master mold using SU-8, followed by a drop-casting of liquid PDMS. The thermally cured PDMS with a thickness of approximately 96 $\mu$m was sufficiently stable to be freestanding and be transferred to a coverslip pre-treated with poly-lysin. Gas entrapment in reservoir holes, which became an issue with decreased reservoir sizes, was efficiently suppressed by wetting the PDMS surface with ethanol and substituting the ethanol layer with a serum-containing medium. Finally, rat cortical neurons were cultured in a $\mu$FF with a modular structure consisting of four square-islands of $200 \times 200 \mu m^2$ to examine whether the observations on microcontact-printed protein scaffolds could be replicated in the microfluidic device. Because of the high reproducibility, stability, and transferability of PDMS microfluidic devices, expanding the application of microfluidic devices should impact researchers in the field of bioengineering and associated disciplines, including biophysics, medicine, and pharmacology.

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1) S. N. Bhatia and D. E. Ingber, Nat. Biotechnol. 32, 760 (2014).
2) T. Tanii, K. Sasuki, K. Ichisawa, T. Demura, Y. Beppu, H. A. Vu, H. T. Chi, H. Yamamoto, and Y. Sato, Jpn. J. Appl. Phys. 50, 06GL01 (2011).
3) K. Shimba et al., Front. Neurosci. 13, 890 (2019).
4) T. Hiroto, K. Torimitsu, A. Kawana, and J. Fukuda, Brain Res. 446, 189 (1988).
5) D. Kleinfled, K. H. Kahler, and P. E. Hockberger, J. Neurosci. 8, 4098 (1988).
6) A. Offenhausser et al., Soft Matter 3, 290 (2007).
7) I. Suzuki and K. Yasuda, Jpn. J. Appl. Phys. 46, 6398 (2007).
8) M. J. Aebersold, H. Dermutz, C. Forró, S. Weydert, G. Thompson-Steckel, J. Vörös, and L. Demkó, Trends Anal. Chem. 78, 60 (2016).
9) J. W. Park, B. Vahidi, A. M. Taylor, S. W. Rhee, and N. L. Jeon, Nat. Protoc. 1, 2128 (2006).
10) L. J. Millet and M. U. Gillette, Trends Neurosci. 35, 752 (2012).
11) M. Bisso, A. Bossca, V. Pasquale, L. Berlendini, and M. Chiappalone, PLoS One 9, 107400 (2014).
12) C. Forró, G. Thompson-Steckel, S. Weaver, S. Weydert, S. Bhe, H. Dermutz, M. J. Aebersold, R. Pitz, L. Demkó, and J. Vörös, Biosens. Bioelectron. 112, 75 (2018).
13) J.-M. Peyrin et al., Lab Chip 11, 3663 (2011).
14) L. Pan, S. Alagapan, E. Franca, S. S. Leoncoudou, T. B. DeMarre, G. J. Brewer, and B. C. Wheeler, Front. Neural Circuits 9, 32 (2015).
15) R. Renault, J.-B. Durand, J.-L. Viovy, and C. Villard, Lab Chip 16, 2188 (2016).
16) L. Pan, S. Alagapan, E. Franca, T. DeMarre, G. J. Brewer, and B. C. Wheeler, IEEE Trans. Neural Syst. Rehab. Eng. 22, 453 (2014).
17) M. K. Lewandowska, D. J. Bakum, S. B. Rompani, and A. Hierlemann, PLoS One 10, e0118514 (2015).
18) K. Sakai, K. Shimba, K. Kotani, and Y. Jimbo, Integr. Biol. 9, 678 (2017).
19) H. Le-The, M. Tibbe, J. Loessberg-Zahl, M. Palma do Carmo, M. van der Helm, J. Bemer, A. van den Berg, A. Leferink, L. Segerink, and J. Eijkel, Nanoscale 10, 7711 (2018).
20) J. Choi, K.-H. Lee, and S. Yang, J. Micromech. Microeng. 21, 097001 (2011).
21) T. Masters, W. Engl, Z. L. Weng, B. Arasi, N. Gauthier, and V. Viasnoff, PLoS One 7, e44261 (2012).
22) J. H. Kang, E. Um, and J.-K. Park, J. Micromech. Microeng. 19, 045027 (2009).
23) K. Zhou, X. G. Zhu, Y. Li, and J. Liu, RSC Adv. 4, 31988 (2014).
24) H. Yamamoto et al., Sci. Adv. 4, eaa4914 (2018).
25) T.-W. Chen et al., Nature 499, 295 (2013).
26) H. Yamamoto, S. Kubota, Y. Chida, M. Morita, S. Moriya, H. Akima, S. Sato, A. Hirano-Iwata, T. Tanii, and M. Niwano, Phys. Rev. E 94, 012407 (2016).
27) T. Sumi, H. Yamamoto, and A. Hirano-Iwata, Soft Matter 16, 3195 (2020).
28) J. C. Er et al., Angew. Chem. Int. Ed. 54, 2442 (2015).
29) M. P. van der Heuvel, E. T. Bullmore, and O. Sporns, Trends Cogn. Sci. 20, 345 (2016).
30) S. Moriya, H. Yamamoto, H. Akima, A. Hirano-Iwata, S. Kubota, and S. Sato, Chaos 29, 013142 (2019).
31) H. Yabu, Y. Nakamichi, Y. Hirai, and M. Shimomura, Chem. Lett. 40, 597 (2011).
32) I. Pereiro, A. F. Khartchenko, L. Petrini, and G. V. Kalgala, Lab Chip 19, 2296 (2019).
33) G. Zamora-López, Y. Chen, G. Deco, M. L. Kringelbach, and C. Zhou, Sci. Rep. 6, 38424 (2016).
34) H. Kawano et al., PLoS One 7, e48034 (2012).
35) K. Ganguly, A. F. Schinder, S. T. Wong, and M. Poo, Cell 105, 521 (2001).
36) D. A. Wagenaar, J. Pine, and S. M. Potter, BMC Neurosci. 7, 11 (2006).
37) Y. Yada, T. Mita, A. Sanada, R. Yano, R. Kanzaki, D. J. Bakkum, A. Hierlemann, and H. Takahashi, Neuroscience 343, 55 (2017).