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Interfacing SH-SY5Y human neuroblastoma cells with SU-8 microstructures

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Abstract

Microwell structures were fabricated using SU-8 photoresist for engineering a quasi-three-dimensional (quasi-3D) microenvironment for cultured neuronal cells. SH-SY5Y human neuroblastoma cells were successfully integrated into microwells of a nominal diameter of 100 μm, with or without 10-μm wide microchannels connecting neighboring microwells, in an aspect ratio (ratio of structure depth over width) of approximately 1. With the help of polyethylene glycol stamping and laminin coating, a neuronal-like network was achieved by integrating populations of SH-SY5Y cells with a microwell network pattern. Resting membrane potential establishment was evaluated with confocal microscopy and the potentiometric fluorescent dye tetramethylrhodamine methyl ester. It was found that the intra/extracellular fluorescent intensity ratio (R) was 2.4 ± 1.4 [n (number of cells measured) = 112] for SH-SY5Y cells on flat SU-8 substrates on day 5 into differentiation, which was not significantly different from the ratio on day 13 into differentiation, 2.0 ± 1.8 ($n = 104$) ($P > 0.05$). For cells in the microwell network structures, R was 4.8 ± 4.7 ($n = 51$) and 3.9 ± 3.2 ($n = 62$) on days 5 and 13 into differentiation, respectively ($P > 0.5$). Cells within the network structures had higher R ratios than on flat substrates, for either day 5 or 13 into differentiation ($P < 0.01$). These results demonstrated that the well network structures, or topographically patterned substrates, were more suitable formats for promoting SH-SY5Y cell resting membrane potential establishment than flat substrates, suggesting the potential to control cellular function through substrate topography engineering.

Keywords: Photolithography; Quasi-three-dimensional; Resting membrane potential; Potentiometric; Confocal microscopy

1. Introduction

In the fields of tissue and cellular engineering, one of the most rapidly advancing sectors is that of cell-based microdevices [1,2]. For these devices to serve their purposes, it has been expected that the response of the integrated cells should mirror physiologically or pathophysiologically what happens *in vivo*. In the *in vivo* condition, cells of a tissue compartment normally live in an extracellular matrix (ECM) meshwork with three-dimensional (3D) and high aspect ratio topographical textures. Three-dimensionality provides cells with characteristic topographical cues in the cellular microenvironments and thus enables cells to differentiate into specific phenotype and maintain specific functions that are usually impossible under two-dimensional (2D) culture conditions [3,4]. To this end, a simple 2D flat substrate may not be regarded as accurately representing the *in vivo* situations and consequently may not be an ideal format for cell-based microdevices.

Microfabrication techniques offer unique approaches for patterning cells and engineering cellular microenvironment. In the field of neuron-based microdevices, microwell structures have been fabricated for patterning neuronal networks or positioning neurons on top of embedded electrodes, with glass [5], silicon [6,7], polyester photoresist [8], polymer elastomer [9,10] and agarose gel [11]. However, in most of these studies, the scale of the networks and the number of addressable cells are small due to the limited number of probing electrodes. Therefore, the systems are not well suited for high-throughput screening with microscopic systems. Additionally, most of these structures were characterized by low aspect ratios (less than one) or depths (of only 1–2 cell diameters) [5–8]. In a few cases where a large

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pattern thickness was used [9–11], the microchannel design for guiding neuronal extensions between microwells made the structures merely the boundaries for 2D growing neuronal cells, and less representative of a 3D microenvironment. Also, comparative functional characterization of cells cultured in engineered microstructures and conventional 2D substrates are lacking.

In this paper, quasi-three-dimensional (quasi-3D) microwell patterns were fabricated using SU-8 photoresist with depths of ∼100 μm or aspect ratios equal to or greater than one. In some cases, to facilitate the extension of neurites, microchannels connecting the microwells were added, forming a microwell network pattern. SH-SY5Y human neuroblatoma cells were interfaced with microwell patterns of either 50 or 100 μm in diameter. Resting membrane potential establishment for SH-SY5Y cells cultured on both the microwell network patterns and flat SU-8 substrates was evaluated with confocal microscopy and a potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRM). The results showed that SU-8 microwell network structures were a more suitable format for promoting SH-SY5Y cell resting membrane potential establishment than the flat SU-8 substrates, suggesting the potential to control cellular function through substrate topography engineering.

2. Materials and methods

2.1. SU-8 processing

Flat SU-8 substrates or microwell structures were fabricated on 25-mm coverslips (Fisher Scientific, Pittsburgh, PA, USA). Prior to fabrication, the coverslips were cleaned with 20% sulfuric acid and then baked at 110 °C for at least 3 h. SU-8 (2025, MicroChem, Newton, MA, USA) was spun onto the glass substrate at a speed of 1000 rpm for 30 s. To achieve flat SU-8 surfaces, 25% (w/v) photoresist was used and this resulted in a coating thickness of approximately 1.5 μm, as measured with an XP-1 stylus profilometer (Ambios Technology Inc., CA). For fabricating microwell structures, 69% (w/v) SU-8 was used, which resulted in a coating thickness of 70–150 μm, depending on the processing conditions. The SU-8 coating was soft baked, first at 65 °C for 3 min and then at 95 °C for up to 30 min. SU-8 was then exposed in soft contact mode with a Karl Suss MJB 3 HP Mask Aligner using 365 nm UV at 10 mW for four successive 10 s, interrupted for at least 20 s, which corresponded to a total UV exposure intensity of 400 mJ/cm^2. No mask was used to expose the whole surface area of the flat SU-8 substrates while a chromium mask was used to fabricate the microwell patterns. Patterns and their nominal structure dimensions used in this study included: 50-μm wells with a center-to-center spacing of 55 μm, 100-μm wells with a center-to-center spacing of 110 μm, and 100-μm wells with a center-to-center spacing of 190 μm connected by 10-μm wide microchannels. The SU-8 coating was baked again, first at 65 °C for 3 min and then at 95 °C for 9 min before development. Patterns were developed with SU-8 developer (MicroChem, Newton, MA) for 14 min and then briefly immersed in isopropyl alcohol (Fisher Chemicals, Fairlawn, NJ) before drying with nitrogen. Finally, the patterns were hard baked at 150 °C for 20 min.

2.2. Cell culture

SH-SY5Y human neuroblastoma cells were routinely cultured in 75-cm^2 tissue culture flasks (Costar, Corning, NY) with the growth medium in a 10% CO_2 humidified air at 37 °C. These cells have frequently been used as models for studying neuronal function and sensor cells in neuron-based devices [12–16]. The growth medium was made with Eagle Minimum Essential Medium (MEM) containing 10% heat inactivated fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate, 2 mM L-glutamine and 1 mM sodium pyruvate [16–18]. At 75% confluence, the cells were detached by mechanically pipetting and re-suspended in growth medium for plating. Before plating, SU-8 microwell patterns were sterilized with 70% ethanol over night, washed twice with phosphate-buffered saline (PBS) and twice with growth medium. Specifically, microwell network patterns were stamped with 10% (w/v) polyethylene glycol (PEG) (MW 200, Sigma–Aldrich, St. Louis, MO) in water using a flat PDMS stamp (Sylgard 184, Dow Corning, Midland, MI). This was followed by washing with deionized water for 2 min. The patterns were sterilized with 70% ethanol overnight, washed twice with PBS and then coated with human placenta laminin (Sigma–Aldrich, St. Louis, MO, USA) of 3.3 mg/L in PBS for 4 h. The patterns were then washed twice with PBS and twice with growth medium. These treatments were aimed at coating microwells and microchannels with laminin while deterring coating on the top surface of the pattern. Flat SU-8 substrates were treated along with the microwell network patterns except no PEG was stamped before laminin coating. Approximately 5×10^5 cells were plated on each patterned substrate or flat substrate in growth medium, which was contained in a 35 mm Petri dish (FALCON, Becton Dickinson Labware, NJ). On the second day after plating, referred to as day 0 into differentiation hereafter, the medium was changed from growth medium to differentiation medium. The differentiation medium was comprised of MEM with 5% FBS, 2.2 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM dibutyryl cAMP (dcAMP) and 2.5 μM 5-bromodeoxyuridine (BrdU) [16–18]. Differentiation medium was changed daily. Phase contrast images were taken on a Nikon ECLIPSE TE300 inverted microscope (Nikon, Melville, NY, USA) with a Nikon D100 digital camera.

2.3. Scanning electron microscopy (SEM)

Cells on SU-8 patterns were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h before rinsing in cacodylate buffer (without glutaraldehyde) three times, 15 min each. This was followed by post-fixing with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h and rinsing in cacodylate buffer (without OsO_4) three times, 5 min each. The samples were then dehydrated successively in 30, 50, 70, 80, 95 and 100% ethanol for 10 min each and dried in a SAMDRI-780A critical point drier (Tousimis Research Corporation, Rockville, MD, USA). Patterns were sputter-coated with gold for 60 s to achieve a coating thickness of about 15.3 nm. SEM images were captured with LEO 982 scanning electron microscope (LEO

Electronenmikroskopie GmbH Korporation, Germany) with an acceleration voltage of 5 kV, using either the regular detector or in-lens detector.

2.4. Fluorescent staining and fluorescence microscopy

The cellular pattern in the SU-8 microwell network was stained with calcein AM (Biotium, Hayward, CA). This fluorescent dye stains living cells and their extensions by the presence of intracellular esterase activity, which converts the non-fluorescent cell-permeant calcein AM to intensely fluorescent calcein. Cells on well network patterns were washed with 2 mL PBS, three to five times. Patterns were then covered with sufficient amount of 2-μM calcein AM in PBS and left at room temperature for 45 min before the staining solution was replaced. Fluorescent micrographs were viewed and captured with a B-2E/C FITC filter block (Nikon, Melville, NY, USA), which has an excitation bandwidth of 465–495 nm and a filter pass range of 515–555 nm.

2.5. Evaluation of resting membrane potential establishment

Resting membrane potential establishment for cells both on flat SU-8 substrates and in microwell network structures was evaluated using confocal microscopy with the potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRM, Molecular Probes Inc., Eugene, OR). Resting membrane potentials were derived by the intra/extracellular fluorescence intensity ratios according to the Nernstian distribution of the dye across plasma membrane [19,20]. Fluorescence intensities were expressed as average gray level readings, which range from 0 to 255 artificial gray level units with 0 and 255 indicating highest darkness and brightness, respectively. The gray level reading was calibrated to be linearly proportional to TMRM concentration within a range of 5 nM to 100 μM ($R^2 = 0.9962$) [16]. For an extracellular dye concentration of 0.5 μM, this linear concentration range corresponds to an effective potential range of +116 to −133.5 mV for the method.Mao and Kisaalita [18] have previously introduced corrections for errors due to background fluorescence, measuring extremely low extracellular fluorescence intensities and non-specific fluorescence binding by cellular organelles. According to their method, the following equations were used to calculate the intra/extracellular fluorescence intensity ratio R and membrane potential V_m:

$$R = \frac{(F_{in}^{10\%} - B^{10\%}) \times (F_{out\_free}^{100\%} - B^{100\%})}{(F_{out}^{100\%} - B^{100\%}) \times (F_{in\_free}^{10\%} - B^{10\%})}, \quad (1)$$

$$V_m\,(\mathrm{mV}) = -58 \log_{10} R, \quad (2)$$

in which $F_{in}^{10\%}$ is the intracellular fluorescence intensity with ND filter set at 10%, $B^{10\%}$ the background value with ND filter set at 10%, $F_{out}^{100\%}$ the extracellular fluorescence intensity with the ND filter removed, $B^{100\%}$ the background value with the ND filter removed, and $F_{in\_free}^{10\%}$ and $F_{out\_free}^{100\%}$ are the intra- and extra-cellular fluorescence correction factors with the ND filter set at 10% or removed, respectively. R and V_m values were expressed as mean ± S.D. Student's t-test was used for statistical comparison of the mean values of R and V_m. V_m values were also presented as frequency distribution histograms. The nonparametric two-sample Kolmogorov–Smirnov test was used for comparison of V_m frequency distributions.

3. Results and discussion

3.1. Interfacing SH-SY5Y cells with SU-8 microwell patterns

The epoxy-based SU-8 photoresist is a widely used bioMEMS or lab-on-a-chip material. The rationale for choosing SU-8 as structure material lies in its mechanical strength, chemical resistance, optical transparency and simplicity in fabrication of high aspect ratio structures [21–23]. With these properties, the resultant neuron-based devices are rendered compatible with both electrical and optical recording. To interface SH-SY5Y cells with microwell structures, we started with 50-μm (diameter) by 100-μm or more (depth) microwell patterns. The well diameter was considered large enough for cells to easily fit during plating.

Fig. 1 shows representative cross-section profiles of the 50-μm microwell patterns, as well as the patterns with SH-SY5Y cells cultured on day 4 into differentiation. The pattern thicknesses were 146 μm [Fig. 1(a)] and 97 μm [Fig. 1(b)]. The cell density was higher on the 97-μm than on the 146-μm pattern. Neuronal extensions were observed with the 97-μm pattern [Fig. 1(b′)], but not with the 146-μm pattern [Fig. 1(a′)]. It was noted that even with the 97-μm pattern, only one or occasionally two cells were found inside each microwell and these cells did not attach or spread. It was also noted from the cross-sectional profile of the 146-μm pattern that there were signs of underexposure—the thickness of the walls towards the glass base were thinner than the thickness at the well opening [24]. The extent of cell integration and differences observed between the 146- and 97-μm patterns were probably due to the aspect ratio and/or presence of uncross-linked SU-8.

Fig. 2 shows SH-SY5Y cells cultured in a 100-μm (diameter) by 97-μm (depth) SU-8 microwell pattern. In contrast to 50-μm microwells, cells exhibited morphological characteristics of attachment to the sidewalls, forming cellular communities inside each 100-μm microwells. Taken together, the results presented in Figs. 1 and 2 provide a combination of microwell structure aspect ratio and accompanying processing conditions that supported cell integration.

To facilitate the extension of neurites, microchannels connecting the microwells were added to the structures, forming a microwell network pattern. Fig. 3 shows SH-SY5Y cells cultured in a 100-μm (diameter) by approximately 125-μm (depth) SU-8 microwell network pattern on day 5 (a and b) and day 13 (c and d) into differentiation. For cells in the microwell network structures, neuronal extensions could not be visualized in phase contrast images [Fig. 3(a) and (c)] due to the depth of the pattern. With fluorescent staining by calcein [Fig. 3(b)], neurite extensions along the microchannels were visible, sug-

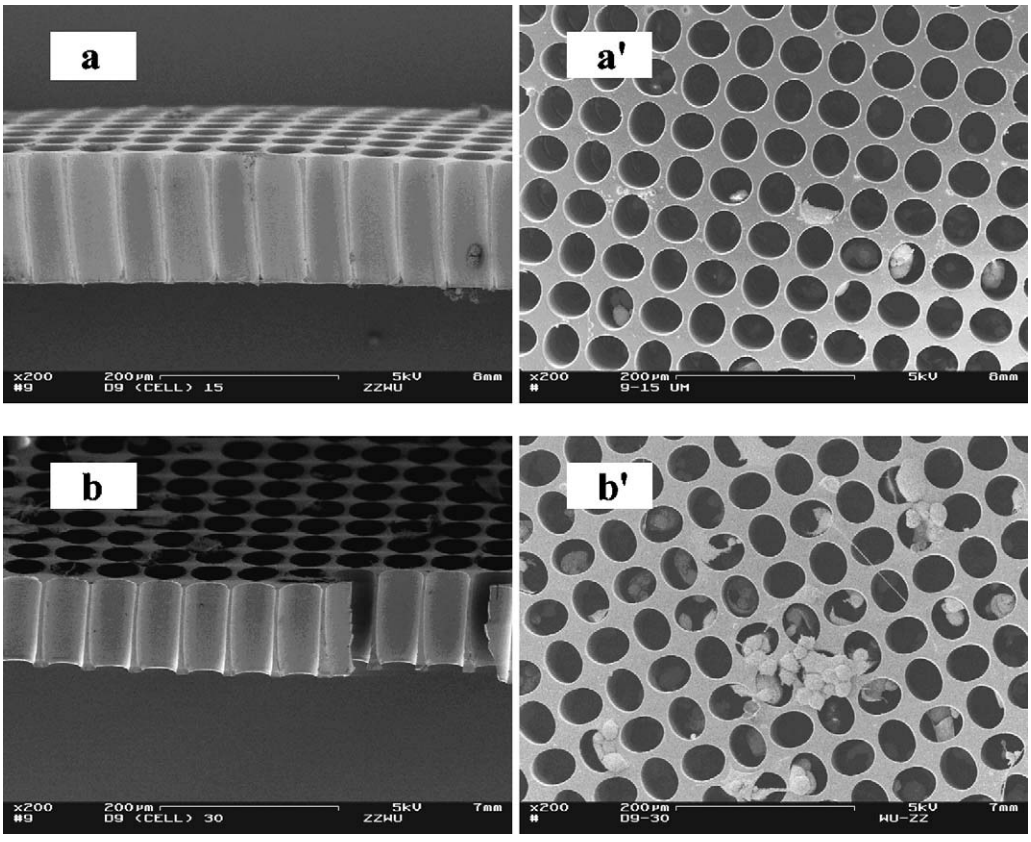

Fig. 1. Scanning electron microscopy (SEM) images showing cross-sectional profiles of the 50-μm diameter microwell patterns (a and b) and SH-SY5Y cells cultured on day 4 into differentiation on the patterns (a′ and b′). Pattern thicknesses were 146 μm (a and a′) and 97 μm (b and b′). Bar = 200 μm.

gesting the potential for the structure to support and control the formation of neuronal networks when interfaced with cells that can form synapses in vitro. The neuronal-like network of SH-SY5Y cells remained patterned until day 5 into differentiation [Fig. 3(a and b)]. On day 13 into differentiation, cells were observed on the top surface of the pattern [Fig. 3(c)]. Fig. 3(d) is a scanning electronic microscopy (SEM) image showing the cross-sectional profile of the pattern. Although most cells were lost during SEM sample preparation, the remaining cells confirmed that the cells attached not only at the bottom but also on the sidewalls of the microwells, and neuronal extensions extended along the sidewalls of microwells and throughout the microchannels, suggesting a quasi-3D microenvironment for the integrated cells.

3.2. Resting membrane potential establishment for SH-SY5Y cells on flat SU-8 substrates and in microwell network structures

Resting membrane potential (V_m) establishment is an important electrophysiological property of neuronal cells that marks functional differentiation. The rationale for choosing the fluorescence method for V_m measurement in the present study lies in this method's access to cells deep in the microstructures. In

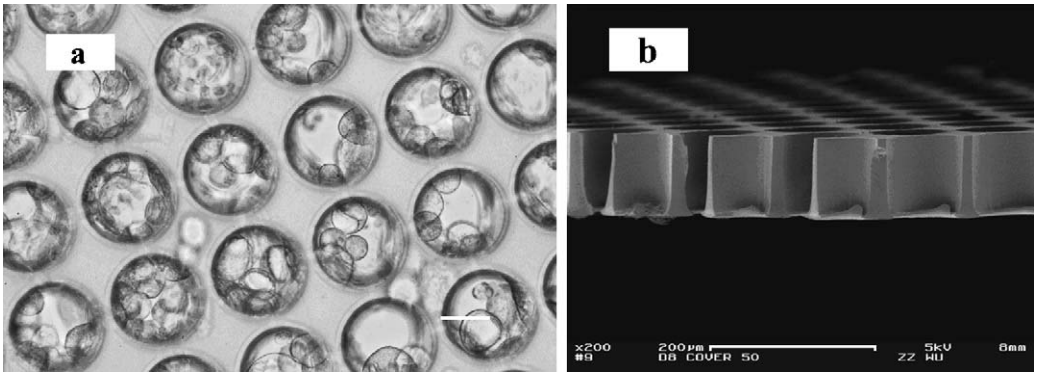

Fig. 2. (a) Phase contrast image showing SH-SY5Y cells cultured on SU-8 microwell patterns with a well diameter of 100 μm on day 6 into differentiation. (b) SEM image showing the cross-sectional profile of the pattern shown in (a). Bar = 50 μm (a) or 200 μm (b).

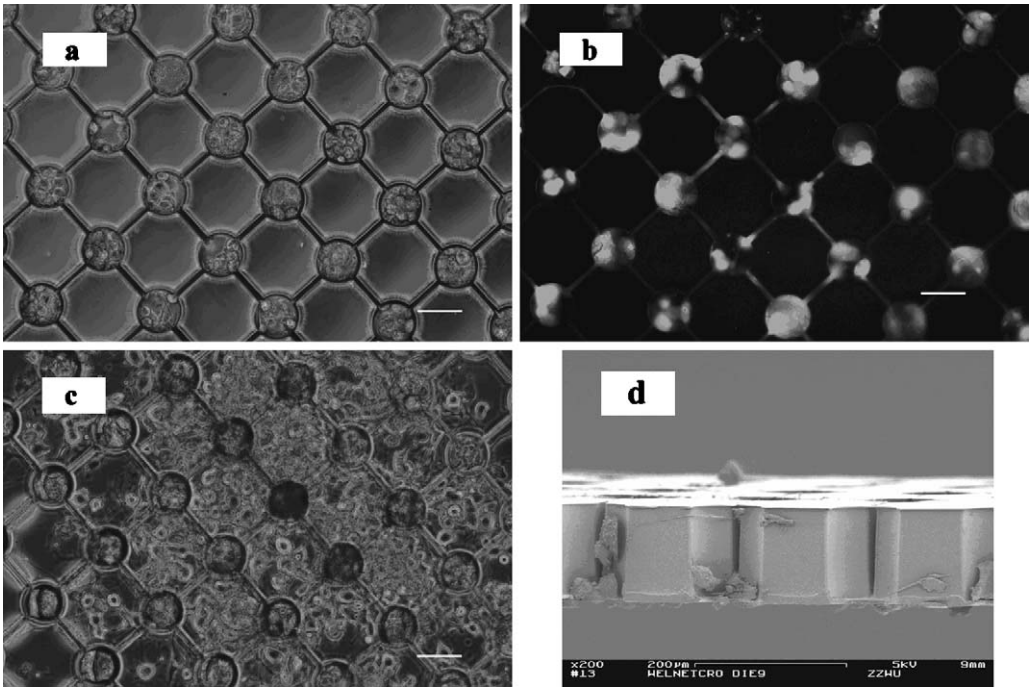

Fig. 3. Phase contrast images showing SH-SY5Y cells interfaced with the microwell network pattern on day 5 (a) and day 13 (c) into differentiation. Image (b) is fluorescent image for the same field as in (a), showing neuronal extensions along the microchannels. Cells were stained with 2 μM calcein in PBS for 45 min before photographing with a FITC filter block. Image (d) is a SEM image showing the cross-sectional profile of the microwell network pattern prepared after cell culture on day 13 into differentiation. The nominal dimensions of the well network patterns are: 100 μm in well diameter, 10 μm in channel width and 90 μm in channel length. Bar = 100 μm (a–c) or 200 μm (d).

contrast to single cell-based techniques (e.g., patch clamp), the advantage of this method lies in its ability to handle large number of cells, providing conclusions that are more representative of the bulk cell population.

Fig. 4 shows confocal images for cells cultured on flat SU-8 substrates (a) and in the microwell network structures (b) on day 5 into differentiation. For microwell network patterns, focal planes were positioned within the depth of the patterns to exclude cells outside the microwell network structures. Table 1 shows the comparison of the means of the intra/extracellular fluorescence intensity ratios (R) along with the estimated V_m values. It was found that R was 2.4 ± 1.4 ($n = 112$) for SH-SY5Y cells on flat substrates on day 5 into differentiation, which was not significantly different from the ratio on day 13 into differentiation, 2.0 ± 1.8 ($n = 104$) ($P > 0.05$). For cells in the microwell network structures, R was 4.8 ± 4.7 ($n = 51$) on day 5 into differentiation, which remained at almost the same level of 3.9 ± 3.2 ($n = 62$) on day 13 into differentiation ($P > 0.5$). Cells within the microwell

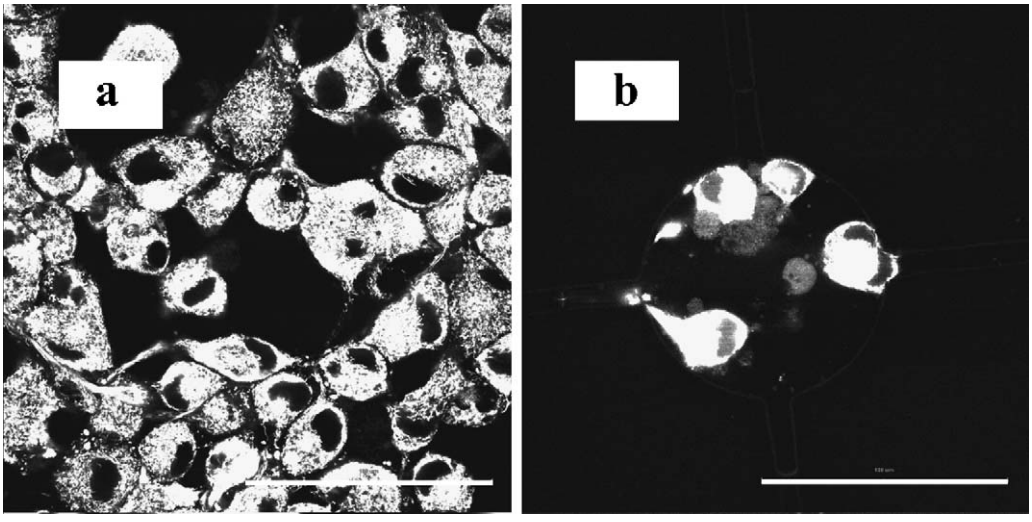

Fig. 4. Confocal images for cells cultured on flat SU-8 substrates (a) and in the microwell network structures (b) on day 5 into differentiation. Cells were stained with 0.5 μM tetramethylrhodamine methyl ester for 20 min before scanning with 543 nm green HeNe laser and a 565 nm long-pass filter. Bar = 100 μm.

Table 1
Resting membrane potential establishment for SH-SY5Y cells cultured on flat SU-8 substrates and in the microwell network structures

	Flat (2D)		Microwell (3D)	
	R (mean ± S.D.) (n)	V_m (mean ± S.D., mV) (n)	R (mean ± S.D.) (n)	V_m (mean ± S.D., mV) (n)
Day 5 into differentiation	2.4 ± 1.4 (112)	−15.0 ± 21.8 (112)	4.8 ± 4.7* (51)	−27.2 ± 26.3* (51)
Day 13 into differentiation	2.0 ± 1.8 (104)	−9.8 ± 20.1 (104)	3.9 ± 3.2* (62)	−26.2 ± 21.8* (62)

R: intra/extracellular fluorescent intensity ratio; V_m: resting membrane potential; n: number of cells measured. Cells were differentiated with 1 mM dibutyryl cAMP and 2.5 μM 5-bromodeoxyuridine.
* $P < 0.01$ compared to that for flat substrates by Student's t-test.

network structures had higher R ratios and more negative resting membrane potentials than those on flat SU-8 substrates, for either day 5 or 13 into differentiation ($P < 0.01$). Fig. 5 shows the frequency distribution of the estimated V_m values for SH-SY5Y cells on flat substrates and in microwell network structures on days 5 and 13 into differentiation. On flat SU-8 substrates, 79.5% of the cells had developed detectable negative membrane potentials on day 5 into differentiation while only 7.1% of the cells were more negative than −40 mV, a value that represents electrophysiological (functional) differentiation [13]. On day 13 into differentiation, these percentages tended to decrease; with 72.1% of the cells developed detectable negative potentials and only 6.7% of the cells were more negative than −40 mV. For cells in the microwell network structures, 86.3% of the cells had developed detectable negative potentials and 39.2% of cells were more negative than −40 mV on day 5 into differentiation. On day 13 into differentiation, 87.1% of the cells had developed detectable negative potentials and 27.4% of the cells were more negative than −40 mV. The V_m frequency distributions, again, showed that more negative V_m values were established for cells in the microwell network structures than for cells on flat substrates, on either day 5 or 13 into differentiation ($P < 0.01$). This

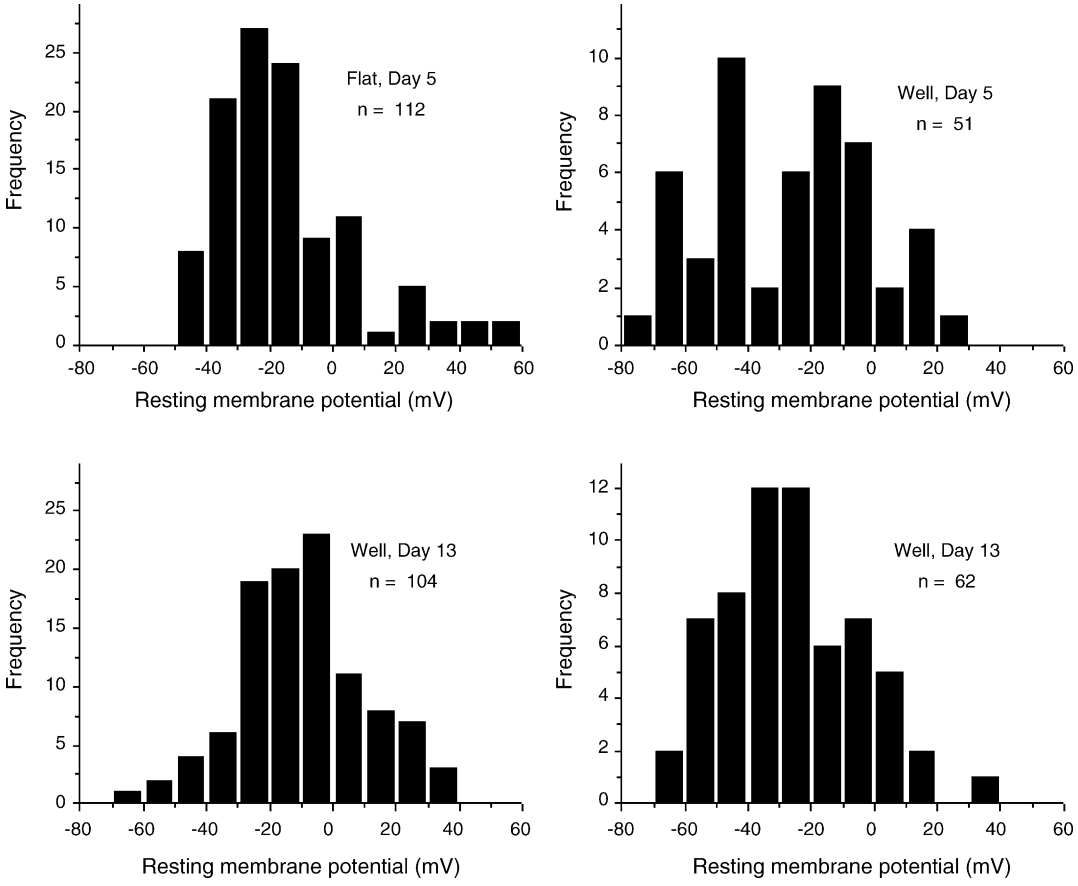

Fig. 5. Frequency distribution for the estimated resting membrane potentials of SH-SY5Y cells on flat SU-8 substrates and in the microwell network structures. Statistical analysis with two-sample Kolmogorov–Smirnov test showed that cells in the microwell network structures established more negative resting membrane potentials than cells on flat substrates on either day 5 or 13 into differentiation ($P < 0.01$). The frequency distribution for cells on flat substrates on day 5 into differentiation was significantly different from that for day 13 into differentiation ($P < 0.01$). The frequency distribution for cells in the microwell network structures on day 5 into differentiation was not different from that for day 13 into differentiation ($P > 0.20$).

result is in agreement with our previous study, which showed that topographical scaffolds are more favorable for V_m establishment than flat substrates [25]. Also, from the mean values shown in Table 1, we found no significant V_m establishment on day 13 as compared to day 5 into differentiation for cells both on flat SU-8 substrates and in microwell network structures, although the V_m frequency distribution for cells on flat substrates on day 5 into differentiation was significantly different from that for day 13 into differentiation ($P < 0.01$). This contrasts with a previous study using NIE-115 murine neuroblastoma cells, where cells gradually and fully developed V_m until day 13 into differentiation [26]. The lack of V_m establishment by SH-SY5Y cells on day 13 as compared to day 5 can be most likely explained by the well-known death of differentiated cells and replacement by proliferating cells for long differentiation time [16].

To correct for the non-specific fluorescence dye binding, the correction factors in Eq. (1) are averages from the bulk cell population rather than being derived from the exact cell to be evaluated. This explains the "positive" resting membrane potential readings shown in Fig. 5 and previously reported with the same method [16–18,25]. Using the patch clamp technique, V_m values of −25 mV [12] and −32 mV [13] have been reported for undifferentiated, −55 mV [14] for dcAMP-differentiated and −49 mV [13] for retinoic acid differentiated SH-SY5Y cells. These V_m values are comparable to the values from our study, attesting to the validity of our technique.

4. Conclusions

Microwell structures were fabricated using SU-8 photoresist for engineering a quasi-3D microenvironment for neuronal cells. SH-SY5Y human neuroblastoma cells were successfully integrated into the microwells of a nominal diameter of 100 μm and of an aspect ratio of approximately one. With the help of PEG stamping and laminin coating, we achieved a neuronal-like network by integrating populations of SH-SY5Y cells with a microwell network pattern, of 100 μm in well diameter, 10 μm in channel width and more than 100 μm in pattern thickness. With confocal microscopy and the potentiometric fluorescent dye TMRM, we demonstrated that SU-8 microwell network structures were a more suitable format for promoting SH-SY5Y cell resting membrane potential establishment than the flat SU-8 substrates, suggesting the potential to control cellular function through substrate topography engineering.

Acknowledgements

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