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Studying the formation of large cell aggregates in patterned neuronal cultures

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Abstract

Patterned neuronal cultures could be produced by plating cells dissociated from rat cortices on glass coverslips, the surface of which was printed with poly-L-lysine (PLL)-positive micropatterns. Large cell aggregates, which greatly disrupted the patterned distribution of neurons, were also generated. To investigate how large cell aggregates were formed, dissociated rat cortical neurons were plated on PLL-coated coverslips in a Petri dish, the surface of which was non-adherent to cells. The cell and cell aggregate densities found later on the coverslip surface increased significantly when larger dishes were used. Most of the neurons not attaching to substratum were able to survive for at least 24 h without entering apoptosis. During this time they formed floating spherical aggregates in the medium. These aggregates subsequently were able to attach to PLL-coated coverslips and produced large aggregates resembling those found within our patterned neuronal cultures. The results suggest a causative relationship between the generation of large numbers of neurons unattached to substratum and the formation of large cell aggregates on the patterned neuronal cultures. It was further demonstrated here that patterned neuronal cultures free of large cell aggregates could be prepared by a procedure employing both stencil patterning and microcontact printing technologies.

Keywords: Patterned neuronal cultures; Microcontact printing; Stencil; Cell aggregate

1. Introduction

Neuronal cultures are used as an important model in various neuroscience studies, such as those investigating cell–cell interactions, synaptic transmission and neuronal development. Conventional neuronal culture techniques produce randomly distributed neurons on the surface of a substratum and then neurons are selected for various analyses. With the aid of MicroElectroMechanical Systems technologies, particularly soft lithography, cultures consisting of neurons arranged in patterns designed for special purposes can now be prepared and maintained under *in vitro* conditions for periods up to weeks (Park and Shuler, 2003; Quist et al., 2005; Xia and Whitesides, 1998). Patterned neuronal cultures are potentially useful as a model for studying the behavior and development of highly ordered neural networks and can be used as a tool for high throughput drug screening (Weibel et al., 2005).

It has been recognized that the attachment of neurons to a substratum is one of the prerequisites for the survival and growth of neurons in cultures (Freshney, 2000; Higgins and Banker, 1998; Li et al., 2003). Thus, when neurons are exposed to a substratum whose surface consists of cell-adherent and non-cell-adherent areas, neurons will attach and grow in the former area, while neurons over the latter area will die after a short time. A variety of techniques, including photolithography (Lom et al., 1993; Stenger et al., 1998; Wyart et al., 2002), microcontact printing (Branch et al., 2000; Chang et al., 2003; James et al., 2004; Oliva et al., 2003; Tiebaud et al., 2002; Vogt et al., 2003, 2004, 2005); microfluidic microchannels (Rhee et al., 2005) and stencil patterning (Bain-Yaghoub et al., 2005), have been used to

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make a portion of the substratum surface, in the shape of various micropatterns, cell-adherent, while leaving the remaining surface area non-cell-adherent. When neurons dissociated from brain tissue are plated on such substrata, the attachment and growth of the neurons are confined to the patterned cell-adherent area on the substratum surface. This can be used to produce a wide variety of patterned neuronal cultures.

Patterned cultures of rat cortical neurons on glass coverslips with poly-L-lysine (PLL)-positive micropatterns printed on the surface by microcontact printing techniques could be conveniently prepared. Neurons and their processes were found to be largely confined to the PLL-positive area. However, in addition, large cell aggregates with sizes ranging from tens to above one hundred micrometers in diameter were also frequently found in these cultures. Similar cell aggregates were also found when neuronal cultures were made on glass coverslips whose surface contained Extracellular Matrix (ECM) gel-, collagen- or laminin-positive micropatterns. The presence of such aggregates produced great distortions in the neuronal arrangement of the pattern, thereby rendering patterned neuronal cultures useless either as a model for studying neural network function or as a tool for drug screening. Aggregates of similar morphology have been reported previously in other studies (e.g., Branch et al., 2000; Chang et al., 2003; Gabay et al., 2005; Nam et al., 2004), where the cell-adherent micropatterns on glass coverslips were also made by microcontact printing.

We thus sought to investigate why large cell aggregates were formed in our patterned neuronal cultures. The results indicate that neurons dissociated from rat cortices do not become apoptotic even after being kept for 24 h in containers where the surface is non-adherent to cells. Rather, these neurons form spherical aggregates that float in the medium. These spherical aggregations are able to attach to cell-adherent surfaces of a substratum at a later time, and this leads to the formation of large cell aggregates on the substratum surface. The results thus suggest a causative relationship between the presence of large numbers of unattached neurons initially and the final formation of large cell aggregates during the process of preparing patterned neuronal cultures. On the basis of this knowledge, a procedure employing stencil patterning and microcontact printing techniques has been developed here for preparing patterned neuronal cultures free of cell aggregates.

2. Materials and methods

2.1. Materials

Pregnant Sprague–Dawley rats were obtained from National Animal Laboratory, Taipei, Taiwan. HEPES, DAPI (4′,6-diamidino-2-phenylindole), glucose, poly-L-lysine (70–150 kDa MW), cysteine, Triton X-100, bovine serum albumin, papain and DNaseI were purchased from Sigma (St. Louis, MO, USA). The antibodies to β-III-tubulin and GFAP (glial fibrillary acidic protein) were purchased from Chemicon (Temecula, CA, USA). Syto24, 5-(and-6)-carboxytetramethylrhodamine sccinimidyl ester and Cy5-conjugated goat anti-mouse IgG antibody were obtained from Molecular Probes (Eugene, OR, USA). FITC-conjugated goat anti-mouse IgG antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and Cy3-conjugated goat anti-rabbit IgG antibody was purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). B-27 serum-free supplement, Hank's balanced salt solution (HBSS), minimum essential medium (MEM), neurobasal medium and horse serum were purchased from Gibco (Frederick, MD, USA). Fetal bovine serum was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). TUNEL (TdT-mediated dUTP-X nick end labeling) staining kits (in situ cell death detection kits, fluorescein) were purchased from Roche Applied Science (Mannheim, Germany). Polydimethylsiloxane (PDMS) prepolymer and the curing reagent (Sylgard 184 silicone elastomer kit) were obtained from Dow Corning (Valley Spring, CA, USA). Negative photoresist SU-8 and SU-8 developer were obtained from MicroChem (Newton, MA, USA). Other reagents were obtained from Merck-Schudardt (Darmstadt, Germany).

2.2. Cortical cell culture

Neuron-enriched primary cortical cultures were prepared from Sprague–Dawley rats as described in the literature (Brewer et al., 1993). In brief, cortices were dissected from rat embryos (18th day of gestation) and treated with papain (10 units/ml). Afterwards, the dissociated cells were washed and suspended in MEM supplemented with 5% horse serum and 5% fetal bovine serum. Cells, at the density of 150–263 cells/mm^2, were then plated on microscopic glass coverslips (18 mm in diameter, Assistant, Germany), pre-coated with PLL uniformly or printed with PLL-positive patterns (see below), in the wells of 12-well culture plates. These were then incubated at 37 °C in a humidified incubator with a 5%CO_2/95% air mixture. Dissociated cells at the density of 150 cells/mm^2 were also plated on PLL-coated glass coverslips, which were themselves placed in PLL-coated or uncoated Petri dishes of 100, 60 or 35 mm diameter and incubated at 37 °C in a humidified incubator with a 5%CO_2/95% air mixture. On the second day (Day *in vitro*, or DIV1), 60% of the medium in the wells or Petri dishes was replaced with the Neurobasal (BN) medium supplemented with 2% B-27. On DIV3, cytosine 1-β-D-arabinofuranoside (final concentration 5 μM) was added to curtail the growth of glial cells. Fifty percent of the medium in the wells or in the Petri dishes was replaced with fresh NB/B27 medium on DIV4 and twice a week thereafter.

2.3. Microcontact printing

Photomasks containing structures of 50 μm × 100 μm grids with cell body adhesion circular sites ∼30 μm in diameter at the intersections of lines and lines of ∼20 μm in width were first produced by a laser plotter on plastic films (FUJI HPB-S 7mil, Taiwan Kong King, Inc., Taiwan). Photomasks containing ladder-shaped structures made up of 100 μm × 500 μm rectangles on either side of 9 or 7 lines 400 μm long and ∼20 μm thick and photomasks containing sets of four lines of 5, 10, 15 and 20 μm in width were also produced in a similar manner. Master

stamps were produced on a layer of negative photoresist SU-8 spin coated on 0.525 mm thick P-type silicon wafer by exposure to UV light (365 nm) through the photomask. After treatment with SU-8 developer and parylene deposition (for narrowing the line width and ease of the stamp peeling step), a mixture of PDMS prepolymer and a curing reagent (Sylgard 184 silicone elastomer kit) in a ratio of 10:1 was poured over the stamp master. After being placed under vacuum for 30 min, curing of the PDMS on the stamp master was carried out by heating at 100 °C for 60 min on a heating plate or in a 60 °C oven for 4 h. Afterwards, the PDMS stamps were peeled from the master.

PDMS stamps were first cleaned by sonication in acetone for 5 min, then by sonication in 70% ethanol for 5 min. After rinsing twice in water, the stamps were autoclaved. The autoclaved stamps were kept in PLL solution (100 μg/ml PLL in 150 mM borate solution at pH 8.4) for 20 min. After removing the excessive PLL solution from the stamp and drying with a stream of nitrogen gas, the stamp was pressed onto the surface of glass coverslips for 2 min. The printed coverslips were then placed in 12-well culture plates and used to prepare the pattern neuronal cultures as described earlier. For observing the printed patterns on coverslips, rhodamine-conjugated PLL was used as the ink. Rhodamine-conjugated PLL was generated by incubating PLL (1 mg/ml in 150 mM borate solution at pH 8.4) with 5-(and-6)-carboxytetramethylrhodamine sccinimidyl ester (final concentration 10 μg/ml) at room temperature for 30 min, followed by dialysis against a solution containing 150 mM borate (pH 8.4) at 4 °C overnight.

2.4. Stencil preparation

An array of cuboidal features (1 mm wide, 8 mm long, 1 mm high) were sculpted on one side of a Teflon block (6 cm × 6 cm × 0.6 cm) by machining (Scientific Instrument Center of National Tsing Hua University, Hsinchu, Taiwan). The resultant Teflon block was used as the master for producing PDMS stencils. A sheet of cured PDMS (0.5 cm thick) made in a round plastic Petri dish (10 cm in diameter) and covered with a layer of Scotch tape was used as the base for making stencils. The Teflon master was placed on the base, with the side containing cuboidal features facing the base, and a steel object of ∼1 kg was placed on top of the Teflon master. A mixture of PDMS prepolymer and a curing reagent (Sylgard 184 silicone elastomer kit) in a ratio of 10:1 was poured into the Petri dish to fill in the space between the Teflon master and base. After curing at 80 °C for 1 h and subsequently removing the Teflon master, the PDMS sheet containing rectangular holes (1 mm wide, 8 mm long, 1 mm deep) was peeled off from the base and cut into 1 cm × 1 cm square pieces each containing two rectangular holes 0.3 cm apart by a scalpel. These square pieces were cleaned by sonication, autoclaved and used as the stencils for preparing neuronal cultures.

2.5. Flowcytometric analysis

Dispersed rat cortical cell suspension were placed in 100 mm Petri dishes, which had or had not been coated with PLL (30 μg/ml), at a density of 150 cells/mm^2. Twenty-four hours later, cells on the PLL-coated Petri dishes were washed with HBSS and incubated with 5 ml of Trypsin-EDTA solution (0.05 trypsin in 0.5 mM EDTA) at 37 °C. Five minutes later, 0.5 ml of horse serum was added to the mixture to stop the trypsin reaction. Cells detached from the Petri dishes were collected by centrifugation. Cells in uncoated Petri dishes were also collected by centrifugation. Cells in these two samples were washed once with PBS (phosphate-buffered saline) and fixed with 4% paraformaldehyde in PBS at room temperature for 1 h. After washing once more with PBS, cells of these two samples were treated separately with 100 ml of 0.1% Triton X-100 (in 0.1% sodium citrate) for 5 min. The cells in these two samples were then treated with TUNEL staining reagents according to the procedures provided by the manufacturer for labeling cells entering apoptotic process. Aliquots of cells from these two sample were also treated with 100 μl of 200 μg/ml DNaseI (in PBS) prior to TUNEL staining and these aliquots were used as positive controls. Aliquots of the cells from these samples that had been treated with the TUNEL staining reagents except for terminal deoxynucleotidyl transferase were used as negative controls. Quantification of the TUNEL labeled samples was carried out with a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA) and the software CellQuest.

2.6. Fluorescence immunocytochemistry

Cultured rat cortical neurons were washed three times with PBS, incubated with fixation solution (3.75% paraformaldehyde, 0.25% glutaraldehyde and 4% sucrose in PBS) at 37 °C for 25 min and then permeabilized by treatment with 0.05% Triton X-100 in PBS at room temperature for 15 min. For double fluorescence staining, the cells were incubated with 3% normal goat serum at room temperature for 45 min and then with mouse anti-β-III-tubulin antibody (1 μg/ml PBS) at 4 °C overnight. The cells were then incubated with Cy5-conjugated goat anti-mouse IgG (5 μg/ml) at 37 °C for 1.5 h. Nuclei were further stained with Syto24 for 10 min or with DAPI for 30 min at room temperature. For triple fluorescence staining, the fixed and permeabilized cells were sequentially incubated with 3% normal goat serum at room temperature for 45 min, with mouse-anti-β-III-tubulin (1 μg/ml) and rabbit anti-GFAP (1 μg/ml) antibodies at 37 °C for 2 h, with FITC-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies at 37 °C for two more hours, and finally with DAPI at room temperature for 30 min. The resultant cells were washed three times with PBS, sealed with Prolong Antifade (Molecular Probes) and then examined using a confocal microscope (LSM 5 PASCAL, Zeiss).

2.7. Quantification

The density of cells on a glass coverslip was calculated from the average number of Syto24-positive spots in five randomly selected fields of the coverslip after the cells growing on it were fixed and stained with Syto24. The density of cell aggregates on a glass coverslip was calculated with the aid of MetaMorph

computer software. An aggregate was defined as a cluster of four or more closely associated Syto24-positive spots. For each coverslip, five fields were randomly selected for counting the number of aggregates in them and the average of these numbers was used to calculate the density of cell aggregates of this coverslip. The sizes of aggregates were expressed as the diameters of circles with sizes equivalent to the areas, as determined by the image quantification program MetaMorph Version 6.2r6 (Universal Image, West Chester, PA, USA), occupied by aggregates in photographs. Statistical testing used the Student's *t*-test.

3. Results

3.1. Formation of large cell aggregates in patterned neuronal cultures

PDMS stamps designed for making ladder-shaped and grid-shaped patterns on flat surface were made by photolithographic techniques (Fig. 1A and B). According to the fluorescent rhodamine-conjugated PLL-positive micropatterns printed on the surface of glass coverslips by these stamps (results not shown), the grid-shaped micropatterns consisted of $61.2 \pm 1.1\ \mu m \times 110.2 \pm 0.9\ \mu m$ (mean ± standard deviation (S.D.), $n = 15$) grids, lines of $7.9 \pm 0.6\ \mu m$ (mean ± S.D., $n = 15$) thick and circles of $24.0 \pm 1.2\ \mu m$ (mean ± S.D., $n = 15$) in diameter at intersections, and the ladder-shaped micropatterns consisted of two $105.1 \pm 3.8\ \mu m \times 489.9 \pm 3.7\ \mu m$ (mean ± S.D., $n = 15$) rectangles interconnected by seven or nine $388.8 \pm 5.6\ \mu m$ (mean ± D.S., $n = 15$) long lines of $8.0 \pm 0.6\ \mu m$ thick (mean ± SD, $n = 15$). When neurons dissociated from rat cortices were plated onto the coverslips printed with PLL-positive micropatterns, the growth of cell bodies and processes of neurons were found largely confined to the PLL-positive patterns on the surface (Fig. 1C and D) and, in addition, many large aggregates of cells were also found attached to the PLL-positive pattern (indicated by arrows in Fig. 1C and D). These large aggregates were interconnected among themselves by thick bundles of neurites. Although most of these bundles ran along the PLL-coated lines in the pattern, bundles running across the uncoated areas were also often encountered (indicated by arrowheads in Fig. 1C and D). Replacing PLL by type 1 collagen, ECM gel or laminin in the above experimental system did not avoid the formation of large cell aggregates in the resultant cultures (results not shown). The formation of large cell aggregates was unlikely due to the incomplete dissociation of cells from rat cortical tissues because the dissociated cells were found to be attached to the coverslips at PLL-positive regions as individual cells initially (Fig. 1E). On the other hand, when dissociated neurons at the same densities were plated on glass coverslips that were uniformly coated with PLL and subsequently maintained under the culturing conditions, neurons were found to be randomly distributed across the coverslip surface with many processes extending from the cell body of each neuron (Fig. 1F). Immunocytochemical analyses further indicated that the large cell aggregates consisted of many β-III-tubulin (a neuronal marker)-positive cells and that the aggregates appeared like domes with the cells bodies of these neurons residing on the surface and their processes within the interior (Fig. 1G). In this study, the cortical neurons were cultured under the conditions that astroglial cells accounted for less than 5% of the total cells (Fang, 2002). To examine whether the cell aggregates found in our patterned neuronal culture also contained astroglial cells, patterned neuronal cultures were subject to triple fluorescence staining with DAPI (a nuclear marker), the antibody to β-III-tubulin (a neuronal marker) and the antibody to GFAP (a marker for astroglial cells). The results indicated the presence of a small number of astroglial cells in a fraction of aggregates (Fig. 1H).

3.2. Behavior of dissociated neurons when exposed to a substratum whose surface consisted of distinct cell-adherent and non-cell-adherent areas

To study how large cell aggregates were formed in our patterned neuronal cultures, we first investigated the behavior of dissociated neurons when exposed to a substratum whose surface consisted of distinct cell-adherent and non-cell-adherent areas. A PLL-coated coverslip (18 mm in diameter) was placed in a plastic Petri dish which was 35, 60 or 100 mm in diameter and was either coated or not coated with PLL and thus cell-adherent or non-cell-adherent, respectively (as shown in the scheme found in Fig. 2A). Neurons dissociated from fetal rat cortices were then placed in these coverslip-containing Petri dishes at the same cell density, 150 cells/mm^2. Neurons were maintained under *in vitro* conditions as described in Section 2. At DIV 10, neurons on these coverslips were fixed and stained with a fluorescent nucleus-specific dye, Syto-24 (Fig. 2B–G). Quantitative analyses (Fig. 3A) indicated that the densities of the cells on the coverslips that were kept in uncoated 100 and 60 mm Petri dishes were significantly higher at 213.0 ± 11.4 cells/mm^2 (mean ± standard error of mean (S.E.M.), $n = 3$) and 142.4 ± 20.9 cells/mm^2 (mean ± S.E.M., $n = 3$), respectively, than their counterparts kept in PLL-coated 100 and 60 mm Petri dishes, 107.0 ± 11.4 cells/mm^2 (mean ± S.E.M., $n = 3$) and 104.3 ± 4.7 cells/mm^2 (mean ± S.E.M., $n = 3$), respectively. These results indicated that unattached neurons above uncoated regions in a Petri dish could move, probably by random Brownian movements, to over PLL-coated regions and then attach to that surface. There was no significant difference between the densities of cells on the coverslips kept in PLL-coated and uncoated 35 mm Petri dishes, 70.1 ± 6.1 cells/mm^2 (mean ± S.E.M., $n = 3$) and 66.7 ± 3.2 cells/mm^2 (mean ± S.E.M., $n = 3$), respectively. The reason for this is likely to be that the quantity of unattached cells in these Petri dishes did not reach a threshold level that affected significantly the cell densities over the PLL-coated areas.

It was further noticed that many cells on the coverslips that were kept in the uncoated 100 or 60 mm Petri dishes formed aggregates. Quantitative analysis (Fig. 3B) of the density of aggregates, defined as clusters consisting of more than four cells on these coverslips, indicated that the aggregate densities on coverslips that were kept in uncoated 100 and 60 mm Petri dishes, 13.2 ± 0.6 aggregates/mm^2 (mean ± S.E.M.,

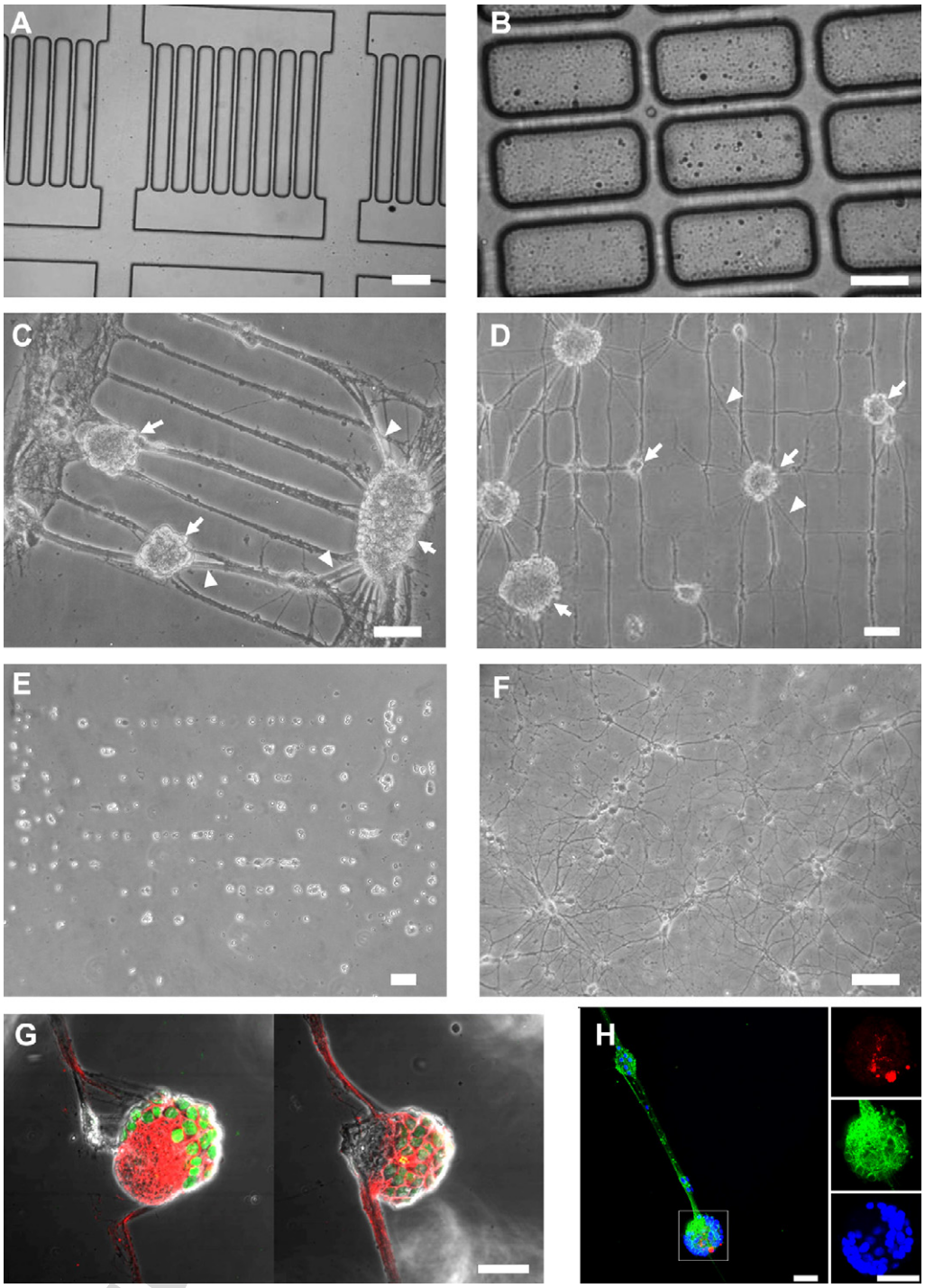

Fig. 1. Large cell aggregates in patterned neuronal cultures. (A and B) Photographs of the ladder-shaped and grid-shaped PDMS stamps, respectively, used for microcontact printing PLL-positive micropatterns on the surface of glass coverslips. (C and D) Photographs of the cells grown on glass coverslips whose surface were printed with ladder- and grid-shaped PLL-positive patterns, respectively. Cells (150 cells/mm^2) dissociated from the cortices of E18 rats were plated on glass coverslips with PLL-positive ladder- and grid-shaped micropatterns printed on their surface. These were maintained in the wells of a 12-well culture plate under *in vitro* conditions for 10 days. Photographs were taken of the unfixed cells. The arrows indicated several cell aggregates and the arrowheads indicated thick bundles not growing along the PLL-coated lines. (E) Photograph of the cultures prepared as described in (C) taken 4 h after plating. (F) Photographs of neurons grown on the surface of a uniformly PLL-coated glass coverslip. Cells dissociated from E18 rat cortices (150 cells/mm^2) were plated on the surface of a glass coverslip uniformly coated with PLL and maintained in wells of a culture plate under *in vitro* conditions for 10 days. (G) Fluorographs of two cell aggregates stained with Syto24 (green, a nuclear marker) and immunolabled with the antibody to β-III-tubulin (red, β-III-tubulin is present in neurons). Neurons dissociated from E18 rat cortices were plated on a coverslip whose surface was printed with grid-shaped PLL-positive patterns. Ten days later, cells on the coverslip were then fixed and stained by Syto24 and immunostained with the antibody to β-III-tubulin. The aggregates appear as domes with cell bodies arranged towards the exterior and processes towards interior regions. The cell bodies of these aggregates are slightly pushed toward right. (H) Fluorographs of two cell aggregates in cultures as described in (G) stained with DAPI (blue, for nuclei) and the antibodies to GFAP (red, an astroglial marker) and β-III-tubulin (green). The lower aggregate, but not the upper one contained a small number of astroglial cells. Insets: the lower aggregate at higher magnification. Scale bars: (A) 100 μm; (B)–(H) 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

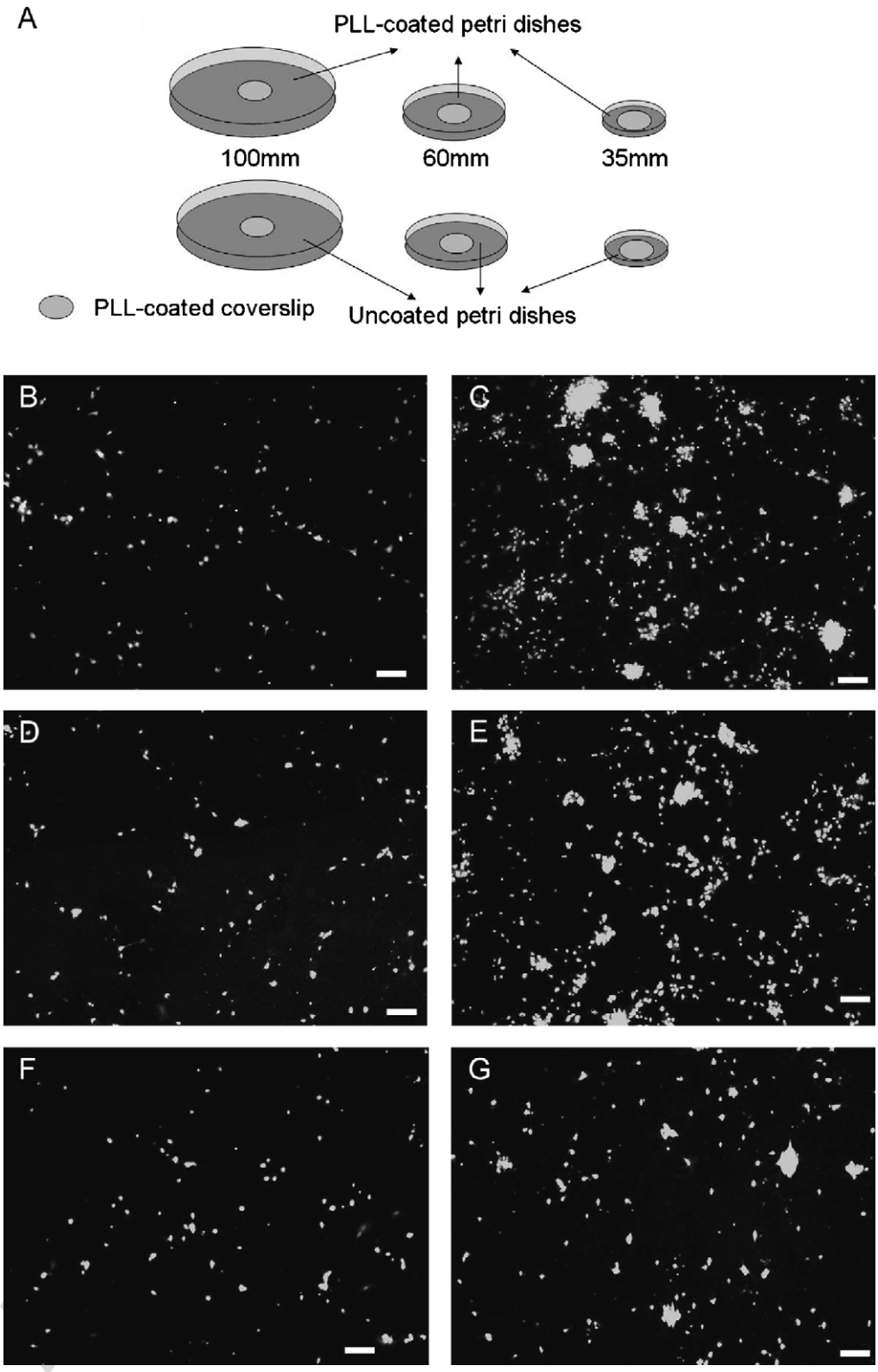

Fig. 2. The behavior of cells dissociated from rat cortices after plating on a substratum whose surface is only partly adherent to cells. (A) The experimental scheme. A PLL-coated glass coverslip was placed in each of the plastic uncoated Petri dish whose surface was non-adherent to cells and consisted of three sizes, namely 35, 60 or 100 mm diameter (lower dishes). A PLL-coated coverslip was also placed in each of the plastic Petri dish whose surface was coated with PLL, rendering it adherent to cells (upper dishes). Cells dissociated from E18 rat cortices at a density of 150 cells/mm^2 were placed in these coverslip-containing dishes. After being maintained under *in vitro* conditions for 10 days, the coverslips were removed from these dishes, fixed and then stained with Syto24, a fluorescent nuclear marker. (B), (D) and (F) were the fluorographs of the coverslips placed in PLL-coated dishes of 100, 60 and 35 mm in diameter and (C), (E) and (G) were the fluorographs of the coverslips placed in uncoated dishes of 100, 60 and 35 mm in diameter. The results are from a representative experiment out of a total of three experiments. Scale bars: 100 μm.

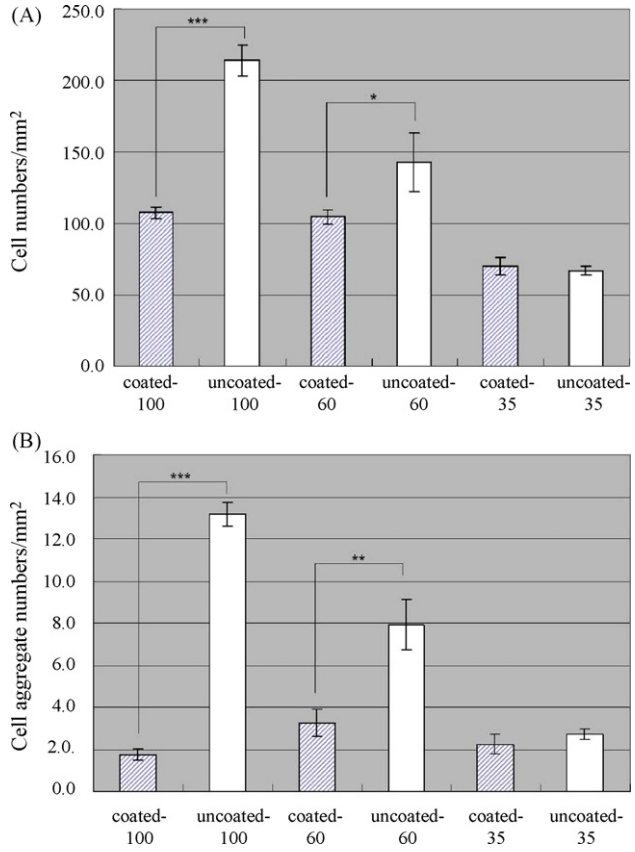

Fig. 3. Quantification of the cell densities (A) and cell aggregate densities (B) on the glass coverslips obtained in the experiments shown in Fig. 2. The densities of cells and aggregates were calculated from the fluorographs of Syto24-stained nuclei on glass coverslips obtained from the experiments shown in Fig. 2 according to the procedures described in Section 2. Data are from three different experiments. Columns and error bars indicate mean ± standard error of mean, respectively; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, significantly different. The densities of cells and aggregates in coverslips kept in coated 35 mm-dishes are not significantly different ($p > 0.05$) from those kept in uncoated 35 mm-dishes. Labels, uncoated-100, -60 and -35 indicate that these results were obtained from coverslips that were kept in uncoated Petri dishes of 100, 60 and 35 mm in diameter, respectively. Labels, coated-100, -60 and -35 indicated these results were obtained from the coverslips that were kept in PLL-coated Petri dishes of 100, 60 and 35 mm in diameter, respectively.

$n = 3$) and 7.9 ± 1.2 aggregates/mm^2 (mean ± S.E.M., $n = 3$), respectively, were significantly higher than their counterparts kept in PLL-coated 100 and 60 mm Petri dishes, 1.7 ± 0.3 aggregates/mm^2 (mean ± S.E.M., $n = 3$) and 3.3 ± 0.6 aggregates/mm^2 (mean ± S.E.M., $n = 3$), respectively (Fig. 3B). This raised the possibilities that the neurons migrating from uncoated areas to over the PLL-coated areas had a tendency to form aggregates or that these neurons could promote the formation of cell aggregates upon attachment to the PLL-coated surface. On the other hand, the densities of cell aggregates on the coverslips kept in PLL-coated (2.2 ± 0.5 aggregates/mm^2, mean ± S.E.M., $n = 3$) and uncoated (2.7 ± 0.2 aggregates/mm^2, mean ± S.E.M., $n = 3$) Petri dishes of 35 mm in diameter were not significantly different. This is consistent with variation in cell density described above, because the number of unattached cells in this latter experiment might be too low to induce the formation of large cell aggregates on the PLL-coated areas.

These results indicated that the unattached neurons in the medium could not only migrate from the medium over the uncoated area to the medium over the PLL-coated areas and subsequently attached to the latter areas on the substratum surface, but that these reattached cells could also form or promote the formation of cell aggregates there. This observation is consistent with earlier reports (Maar et al., 1997; Segev et al., 2003) that cells dissociated from rat cortices would form clusterized networks, consisting of large cell aggregates interconnected to each other by thick neurite bundles, when plated at high cell densities on the SiN surface of glass or in microwells.

3.3. Unattached neurons are able to survive for at least 24 h without entering into apoptosis and meanwhile form floating spherical aggregates in the medium

In our procedure for preparing patterned neuronal cultures, the medium containing the unattached neurons was partly replaced with fresh medium 24 h after plating. We thus tested if neurons could survive a period of 24 h without forming attachment to the substratum. Dissociated neurons were kept in uncoated Petri dishes for 24 h, subjected to TUNEL staining that specifically labels cells undergoing apoptosis and quantified by flow-cytometry. Neurons similarly treated except for the incubation with the terminal deoxynucleotidyl transferase were used as the negative control and neurons treated with DNase1 were used as the positive control. Neurons grown on PLL-coated coverslips for 24 h were detached by treating with trypsin and also used as a positive control. It was found that only a small fraction of the unattached neurons, $15.1 \pm 4.9\%$ (mean ± S.D., $n = 3$), were apoptotic (the portion under the horizontal line labeled by apoptosis in Fig. 4). It was found that only $8.6 \pm 3.3\%$ (mean ± S.D., $n = 3$) of neurons grown on PLL-coated coverslips were undergoing apoptosis (the portion under the horizontal line labeled by apoptosis in Fig. 4). This latter number could however be an overestimation due to the damage inflicted by the trypsin treatment. Nevertheless, these results indicated that the majority of neurons are able to survive for at least 24 h in the medium without forming attachment to the substratum.

We then examined the morphology of the unattached neurons in the culture medium over a period of 24 h. Neurons dissociated from fetal rat cortices in culture medium were placed in plastic Petri dishes, which were not coated with PLL. Most neurons still remained as separate individual cells or small aggregations of cells ~7–10 μm in diameter 4 h later (Fig. 5A). However, after 24 h, most neurons formed spherical aggregates 26.1 ± 4.5 μm (mean ± S.D., $n = 52$) in diameter floating in the medium (Fig. 5B).

3.4. Neurons kept unattached to substratum for 24 h re-attached and grew on PLL-coated coverslips

Upon exposure to a PLL-coated coverslip with neurons growing on it, the spherical aggregates formed from neurons that were kept in an uncoated Petri dish for 24 h were found to attach to the glass surface as aggregates 32.3 ± 5.0 μm (mean ± S.D., $n = 88$) in diameter (Fig. 6A). After 10 days, many large cell aggregates

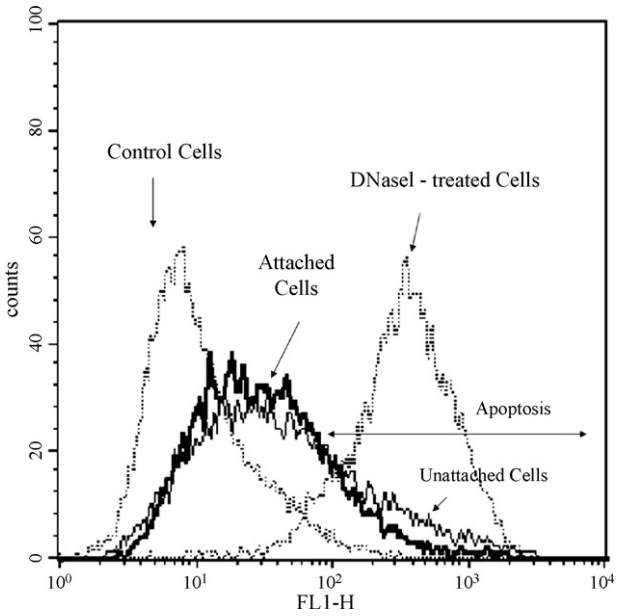

Fig. 4. Analysis of apoptotic cells in dispersed rat cortical neurons after being kept in a non-cell-adherent dish for 24 h. Cells dissociated from E18 rat cortices were kept in an uncoated Petri dish for 24 h. Cells were then collected by centrifugation, fixed by paraformaldehyde, treated by TUNEL staining reagents and subjected to flowcytometric analysis (the curve labeled by Unattached Cells). An aliquot of these cells were treated with DNase1 prior to the TUNEL staining (the curve labeled as DNase1-treated Cells). Another aliquot of these cells were treated similarly except that treatment with terminal deoxynucleotidyl transferase was omitted (the curve labeled by Control Cells). Cells dissociated from fetal rat cortices were also plated onto a PLL-coated glass coverslip, detached from coverslip by treatment with trypsin 24 h later, collected by centrifugation and analyzed similarly (the curve labeled by Attached Cells). Cells under the horizontal line labeled by Apoptosis were considered to be cells undergoing apoptosis. The results are from a representative experiment of a total of three experiments.

(indicated by arrows in Fig. 6B) 63.3 ± 19.1 μm (mean ± S.D., $n = 19$) in diameter, but very few individual neurons, were found on the coverslip surface (Fig. 6B), and these large cell aggregates were interconnected by thick bundles of processes (indicated by arrowheads in Fig. 6B). These cell aggregates resembled closely their counterparts found in our patterned neuronal cultures (Fig. 1C and D).

3.5. Preparing patterned neuronal cultures free of large aggregates

The results obtained in this study suggest a causative relationship between the presence of large numbers of unattached neurons at the initial stage of culture and the presence of cell aggregates at the final stage of preparing patterned neuronal cultures. To avoid the initial production of large numbers of unattached neurons, we tried to remove as large a proportion of unattached neurons as early as possible after the neurons over the cell-adherent areas have firmly attached to the substratum surface. This approach led to a significant decrease in the number of aggregates, but did not completely prevent the formation of aggregations in the final patterned cultures (Zeng and Chang, unpublished data).

An alternative approach was taken by using a PDMS stencil to define the region for initial neuronal attachment and making PLL-positive lines by microcontact printing on glass surface to guide the growth of neuronal axons. A PDMS stencil (1 cm wide, 1 cm long, 1 mm thick) containing two rectangular holes (1 mm wide, 8 mm long) was placed on top of a piece of glass coverslip, the surface of which had been printed with PLL-positive lines. After coating the surface of the resultant stencil/glass coverslip with PLL again, dissociated cortical neurons were plated on it. After 4 h, the original medium with unattached cells was replaced by fresh serum-free medium, and the stencil along with neurons attached on it was also lifted off and discarded. The neurons remained on the coverslip were maintained under the culturing conditions for periods up to 3 weeks. The cell bodies of all neurons and their processes were respectively confined to the areas defined by the rectangular holes of the stencil and the PLL-positive lines made by microcontact printing (Fig. 7A and B). Since this procedure did not produce large numbers of unattached cells, no large cell aggregates was found in the resultant cultures (Fig. 7A). In contrast, if the stencil was lifted from the aforementioned PLL-coated stencil/glass coverslip before plating the dissociated cells, many large cell aggregates were found on the coverslip 3–4 days later (Fig. 7C). The results thus indicate that by combining the stencil patterning and microcontact printing technologies, patterned neuronal cultures free of large cell aggregates could be prepared.

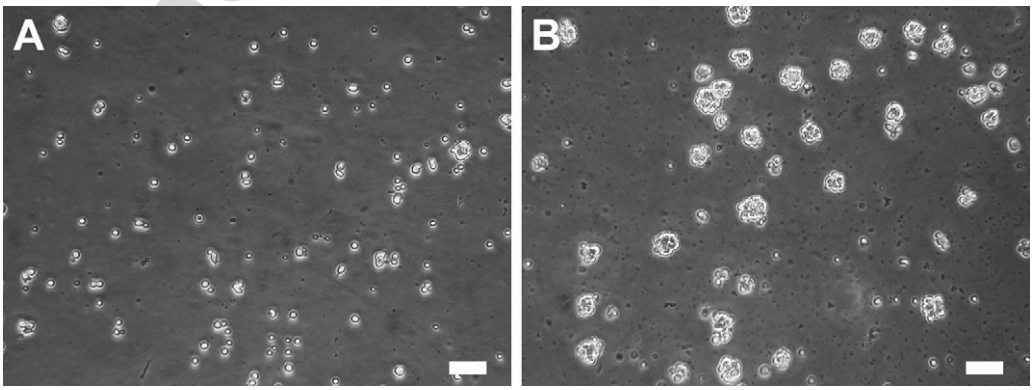

Fig. 5. Photographs of cells kept in dishes whose surface was non-cell-adherent to cells for 4 h (A) and 24 h (B). Cells dissociated from E18 rat cortices at the cell density of 150 cells/mm^2 were placed in uncoated Petri dishes and kept in a CO_2 incubator. Photographs of cells and their aggregates floating in the medium were taken 4 and 24 h later. Scale bars: 50 μm.

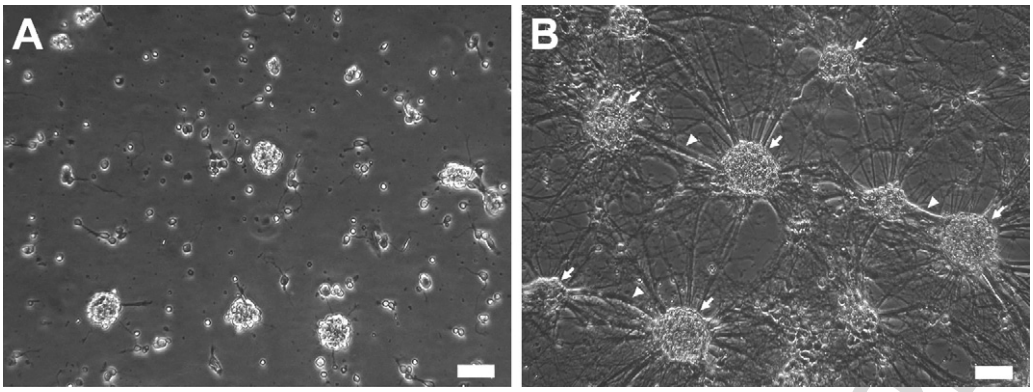

Fig. 6. Formation of cell aggregates on the surface of glass coverslips. Cells were dissociated from E18 rat cortices. A part of these dispersed cells was plated on a PLL-coated glass coverslip, which was itself placed in a culture plate well. A part of these dispersed cells were kept in an uncoated plastic Petri dish. Twenty-four hours later, 60% of the medium over the PLL-coated coverslip was replaced by the cell suspension removed from the Petri dishes. Photographs of cells growing on the glass coverslip were taken 1 day (A) and 10 days (B) later. Cell aggregates and thick bundles of processes are indicated by arrows and arrowheads, respectively. Scale bars: 50 μm.

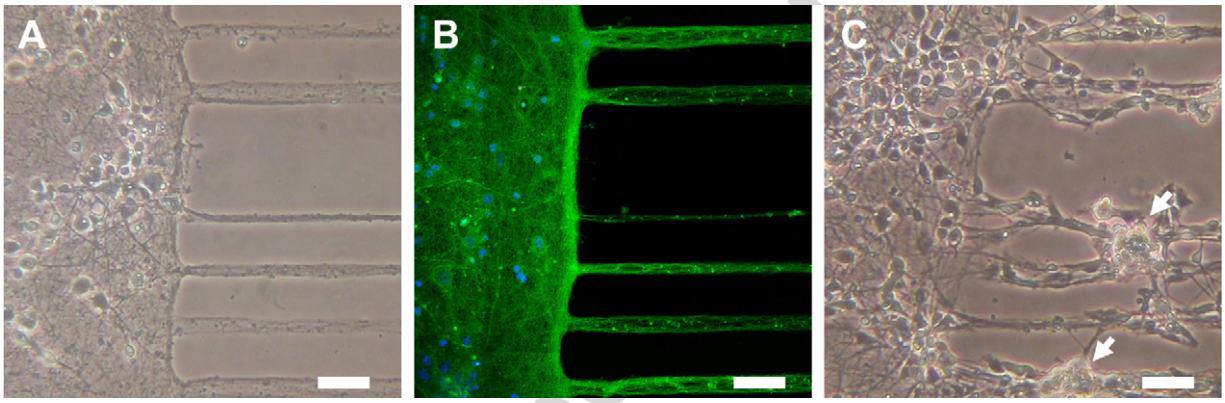

Fig. 7. Preparation of patterned neuronal cultures free of large cell aggregates. A PDMS stencil (1 cm wide, 1 cm long, 1 mm thick) containing two rectangular holes (1 mm wide, 8 mm long, 1 mm deep) was mounted on a piece of glass coverslip (18 mm in diameter), the surface of which had been printed with sets of four PLL-positive lines of 5, 10, 15 and 20 μm in width (from top to bottom). The resultant stencil/glass coverslip was coated with PLL, placed in a well of a 12-well culture plate, and used for the following experiments. (A and B) Dissociated rat cortical cells (263 cells/mm^2) were plated on the PLL-coated stencil/glass coverslip. After 4 h, the original medium was replaced by fresh serum-free medium, and then the stencil was lifted. The resultant cultures were maintained under *in vitro* conditions, and the photograph of the un-fixed cells was taken on 10 DIV (A). Cells on the coverslip were also subject double fluorescence immunostaining with the antibody to β-III-tubulin (green, for neuronal somata and processes) and DAPI (blue, for nuclei) (B). (C) Stencils were first removed from the PLL-coated stencil/glass coverslip, and dissociated cortical neurons (263 cells/mm^2) were then plated over the glass coverslip. The original medium was replaced by fresh serum-free medium 4 h later. The cultures were maintained under *in vitro* conditions, and the photograph of the un-fixed cells was taken on 4 DIV. Cell aggregates are indicated by arrows. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

In this study, we observed that most of the neurons dissociated from fetal rat cortices could be kept in dishes where the surface is not adherent to cells for at least 24 h without entering into apoptosis and that these cells form floating spherical aggregates during this period. These observations are consistent with the known behavior of cells dissociated from brain tissue and the fact that they form spherical aggregations when kept from adhering to substratum for prolonged periods of time. Large aggregates of several hundred micrometers in diameter have been prepared by maintaining dispersed brain cells in a serum-containing medium in agar-coated plates or in flasks that are kept constant rotation (Freshney, 2000; Seeds, 1971). The resulting neural aggregates have then been used as a three-dimensional model for studying neural development (Trapp et al., 1979). Similarly, neurospheres, or floating neural clusters, have been prepared by keeping cells dissociated from different regions of the brain in serum-free medium and in non-cell-adherent plates, then these are used to isolate neural stem cells (Song et al., 2002; Wu et al., 2001).

We further observed that the floating aggregates formed from dissociated rat cortical neurons that were kept in non-cell-adherent dishes for 24 h could still attach to the surface of PLL-coated glass coverslips that already had neurons growing on them. Ten days later, many large cell aggregates were found on the surface of the coverslip, and these aggregates were interconnected by thick bundles of processes. These cell aggregates and thick process bundles resemble closely those found in our patterned neuronal cultures that were produced by plating dissociated rat cortical neurons on glass coverslips whose surface had been printed with PLL-positive micropatterns.

Based on the above described basic properties of dispersed neurons and also on the results obtained in this study, it is deduced that when dispersed neurons are plated over a glass coverslip whose surface consists of distinct cell-adherent and non-cell-adherent regions, neurons residing over the non-cell-adherent regions of the surface will form aggregates hours after plating. Meanwhile, the neurons initially residing over the cell-adherent regions of the same surface will adhere to the glass surface. This will decrease the cell density of the medium over the cell-adherent regions, thereby creating a cell density difference between the media over the cell-adherent and over the non-cell-adherent regions of the coverslip. This cell density difference will drive the migration of cells or cell aggregates from over the non-cell-adherent regions to over the cell-adherent regions. The migration of neurons from the medium over the non-cell-adherent regions to the medium over cell-adherent regions on the substratum surface, which was made locally cell-adherent by coating with carbon nanotubes, has also been reported in a recent study (Gabay et al., 2005). Therefore, the local concentration of cells in the cell-adherent regions on glass surface will increase greatly. This may lead to the formation of large cell aggregates because dissociated rat cortical neurons tend to form clusters when plated on substrata at high cell densities (Maar et al., 1997; Segev et al., 2003). Furthermore, since the serum and other peptide components in the medium could bind to the un-coated regions of glass surface, some dissociated cortical cells might initially bind non-specifically to the non-cell-adherent regions. Over time, these cells may die and also contribute to the formation of aggregates.

The presence of large cell aggregates in a patterned neuronal culture will greatly distort the cell arrangement that should match the original design, and it is thus advantageous to avoid their formation when preparing patterned neuronal cultures. In this study, we have demonstrated that this goal could be achieved by employing both stencil patterning and microcontact printing technologies in preparing patterned neuronal cultures. The recent years have seen a rapid growth in the development of stencil patterning technologies (e.g., Nam et al., 2006; Ostuni et al., 2000; Pal et al., 2006). Our results further point to the opportunity that these newly developed stencil patterning technologies could be employed together with the microcontact printing technologies in the preparation of diverse patterned neuronal cultures, wherein neurons grow faithfully following the original designs for various applications.

The causative relationship between the presence of large numbers of cells un-attached to substratum at the initial stage and the later presence of large cell aggregates in the resultant patterned neuronal cultures was derived from experiments where microcontact printing was employed to make cell-adherent patterns on glass surface. However, it is likely that aggregates may also be formed similarly when patterned neuronal cultures are prepared using other substrata whose surface is patterned into distinct cell-adherent and non-cell-adherent regions by methodologies other than microcontact printing. It is clear that extra steps should be taken to prevent the generation of large numbers of unattached neurons at any stages in the process of producing patterned neuronal cultures. If this is carried out, the yield of producing patterned neuronal cultures with the neuronal growth faithfully following the original designs should be significantly improved.

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