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Highly reproducible quantification of apoptotic cells using micropatterned culture of neurons

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ABSTRACT

The quantification of apoptotic cells is an integral component of many cell-based assays in biological studies. However, current methods for quantifying apoptotic cells using conventional random cultures have shown great limitations, especially for the quantification of primary neurons. Randomly distributed neurons under primary culture conditions can lead to biased estimates, and vastly different estimates of cell numbers can be produced within the same experiment. In this study, we developed a simple, accurate, and reliable technique for quantifying apoptotic neurons by means of micropatterned cell cultures. A polydimethylsiloxane (PDMS) microstencil was used as a physical mask for micropatterning cell cultures, and primary granular neurons (GNs) were successfully cultured within the micropattern-confined regions and homogeneously distributed over the entire field of each pattern. As compared with the conventional method based on random cultures, the micropatterned culture method allowed for highly reproducible quantification of apoptotic cells. These results were also confirmed by using GNs derived from mice with neurodegeneration. We hope that this micropatterning method based on the use of a PDMS microstencil can overcome the technical obstacles existing in current biological studies and will serve as a powerful tool for facilitating the study of apoptosis-involved diseases.

Apoptosis or programmed cell death is frequently quantified by the terminal transferase-mediated nick end labeling (TUNEL)[3] technique. The quantitative analysis of apoptotic cells using TUNEL assay is essential for the study of various factors that influence the neurodegeneration- or neuroprotection-related pathological changes [1–3]. Apoptotic neuron counting seems to be a simple-to-achieve process. However, cultured cells cannot be homogeneously distributed over the field of observation, and for this reason a stereological method has been developed for producing accurate quantification results [4]. This technique uses systematic random samplings to generate unbiased data; however, its execution is extremely time-consuming and labor-intensive.

In contrast to the neurons found in brain sections, which are located within the exquisitely ordered structure(s), neurons grown under normal culture conditions are randomly distributed and are not amenable to classical stereological methods. Hence, most scientists need to continue using the traditional visual enumeration method and manually count all labeled neurons within the selected fields of view [5], although this method suffers from severely limited reliability. Thus, there is an urgent need for the development of reliable and efficient quantification methods to replace the current manual approaches for measuring the events in neurodegeneration.

The micropatterning of live cells in arrays is an emerging powerful technique, allowing the study of single cells or clusters of cells with well-controlled sizes, shapes, and positions in a high-throughput manner [6]. The micropatterning of cells can be achieved in numerous ways such as photolithography, soft lithography, and the stencil technique [7–10]. Among them, the stencil

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[3] *Abbreviations used:* TUNEL, terminal transferase-mediated nick end labeling; PDMS, polydimethylsiloxane; GN, granular neuron; NP-C, Niemann–Pick disease type C; SFM, serum-free medium; PDL, poly-D-lysine; BM–MSC, bone marrow-derived mesenchymal stem cell; DAPI, 4′-6-diamidino-2-phenylindole.

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technique relies on the use of a stencil with well-defined patterns for the localization of cell culture and adhesion. A stencil is a thin membrane consisting of perforated patterns. Direct deposition of cells through the perforated patterns in the stencil allows the patterning to be achieved in a single process. The stencil technique does not require the use of a photoresist-based lithography procedure, and the stencil can be reused for multiple rounds of cell culture [11]. Recently, elastomeric polydimethylsiloxane (PDMS) stencils used for cell cultures have been gaining wide popularity [12,13]. PDMS is inexpensive, mechanically strong, chemically and thermally stable, and (more important) biocompatible [14]. A PDMS stencil provides good adhesion to a glass surface without the need for additional bonding processes. Thus, stencil-assisted cell patterning has shown a unique advantage that cells can be easily grown into a controlled shape and at a predetermined location.

In attempting to develop a rapid and accurate cell quantification method, we applied a PDMS microstencil for the micropatterning of cell cultures and tested its performance in quantifying the apoptotic cells in primary cultured neurons. Micropatterning of cells has been gaining increasing usage for various biological applications [15]. In the traditional culture method, cells are randomly seeded in a culture dish, resulting in many drawbacks in the control of size, shape, and distribution of individual cells, and a wide disparity in the behaviors of cells within the same population may also exist. Thus, the use of the micropatterning method can be helpful for eliminating the variability associated with the conventional cell culture method. By using the PDMS stencil, cells could be cultured at a well-controlled position and with a homogeneous distribution, demonstrating the superiority of the technique over the conventional culture methods.

Another advantage of the current method is its ability to produce highly reproducible results for quantifying apoptotic neurons. Furthermore, the use of inexpensive PDMS stencil indicates the high availability of this technique to many cell biology laboratories without the need to purchase expensive equipment.

Materials and methods

PDMS microstencil fabrication

The micropatterns in the PDMS microstencils were designed by using AutoCAD (Autodesk, USA). The overall size of a PDMS microstencil was 10 × 10 mm, within which an array of 500-μm-diameter holes with 1000-μm pitches (center-to-center distance) constituted the functional elements. The fabrication process began with the structuring of a 250-μm-thick SU-8_100 master for PDMS replication by using a typical photolithography process. SU-8_100 photoresist (Microchem, USA) was spin-coated onto silicon wafers, followed by soft baking at 65 °C for 30 min and 95 °C for 90 min. After ultraviolet (UV) exposure through a photomask and subsequent baking at 65 °C for 15 min and 95 °C for 25 min, the wafer was chemically developed in SU-8 developer (Microchem), briefly rinsed with isopropyl alcohol, and then dried with a gentle stream of nitrogen gas. After that, the surface of SU-8 master was silanized by exposure to tridecafluouro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemical Technologies [UCT], USA) to improve the release of PDMS replica from the master mold.

PDMS prepolymer was produced from a mixture of Sylgard 184 silicone elastomer base and curing agent at a ratio of 10:1 (m/m) (Sylgard 184 kit, Dow Corning, USA). The mixture was then placed in a vacuum desiccator for 60 min to remove the air bubbles created during mixing. The PDMS microstencil was fabricated by spin-coating PDMS prepolymer onto the SU-8 master, followed by blowing nitrogen gas across the master surface to remove the thin residual layers of PDMS from the top of the patterns. The subsequent curing was performed in an oven at 65 °C for 60 min. Afterward, the microstencil was carefully peeled off from the master mold.

Animals

A colony of BALB/c Npc1^{nih} mice was maintained for this study by brother–sister mating of heterozygous animals. Polymerase chain reaction was performed to determine the genotype of each mouse [16]. All procedures were performed in accordance with an animal protocol approved by the Kyungpook National University institutional animal care and use committee. Animals were housed in a temperature-controlled room on a 12-h light/dark circadian cycle.

Culture of primary granular neurons

Mouse primary granular neurons (GNs) were generated by using the procedure described previously [3]. Briefly, GNs were obtained from the cerebella of wild-type and Niemann–Pick disease type C (NP-C) mice [16] at postnatal days 5 to 7. The cerebella were collected in a Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) supplemented with glucose (6 mg/ml, Sigma). Then, single cells were obtained by dissociating the cerebella from the tissues by using the SUMITOMO Nerve-Cell Culture System and resuspended in serum-free medium (SFM) containing 10% heat-inactivated fetal bovine serum (FBS). SFM was composed of Neurobasal A-Medium (Gibco) supplemented with GlutaMAX I (2 mM, Gibco), penicillin–streptomycin (20 U ml^{-1}, Gibco), and KCl (250 μM, Sigma). To obtain GN-enriched monolayer culture, cells were preplated twice for 30 min in 35-mm dishes coated with poly-D-lysine (PDL, Sigma) and then strained through a 70-μm nylon cell strainer (BD Biosciences) before final cell suspension and counting. For viable cell counting, 20 μl of cells was transferred to an Eppendorf tube with an equal volume of 0.4% trypan blue solution (Sigma). Cells were counted using a dual-chamber hemocytometer, adjusted to 5 × 10^4 cells per ml in SFM containing B-27 supplement, and seeded 1 ml of a cell suspension to each well into 24-well tissue culture plates with PDL-coated glass coverslips (12 mm). The culture medium was replaced every 3 days.

Patterning of GN culture using microstencil

The microstencil method used for localizing the culture of GNs is briefly schematized in Fig. 1A. All of the microstencils and glass coverslips used in this study were autoclaved at 121 °C for 15 min. The PDMS microstencil was placed on top of a glass coverslip, which was then treated with PDL. GNs (5 × 10^4) were seeded on the microstencil and incubated for another 24 h, and the stencil was carefully removed afterward.

Isolation and coculture of bone marrow-derived mesenchymal stem cells

Tibias and femurs were dissected from 4- to 6-week-old mice. Bone marrow was harvested, and single-cell suspensions were obtained using a 40-μm cell strainer (BD Biosciences). Approximately 10^6 cells were plated in 25-cm^2 flasks containing MesenCult MSC Basal Medium and Mesenchymal Stem Cell Stimulatory Supplements (Stem Cell Technologies) with antibiotics according to the method described in our previous study [17]. The cells were cultured for 1 week, and the population of plastic-adherent bone marrow-derived mesenchymal stem cells (BM–MSCs) was used for subsequent experiments.

For indirect three-dimensional coculture experiments, Millicell Hanging Cell Culture Inserts (Millipore) with a pore size of