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SHORT COMMUNICATION

A drop array culture for patterning adherent mouse embryonic stem cell-derived neurospheres

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Abstract

New therapeutic approaches for repairing an injured or degenerating nervous system have accelerated the development of methods to generate populations of neurons derived from various stem cell sources efficiently. Many of these methods require the generation of neurospheres. Here a simple technique is described for creating an array of adherent mouse embryonic stem cell (mESC)-derived neurospheres using a conventional plastic culture dish and a patterning template. mESC-derived neurospheres are confined to circular (4-mm diameter), gel-coated regions within an array. The adherent neurosphere arrays require 3 days to prepare from an mESC source; they can be maintained in 15 μl drops of medium, and exhibit extensive neurite elaboration after 8 days of cultivation. Additionally, the potential of treating the adherent neurospheres in selected drops of an array is demonstrated with a variety of differentiation-inducing reagents and subsequently individually analysing such neurospheres for gene expression, protein levels and morphological development. Copyright © 2016 John Wiley & Sons, Ltd.

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Self-renewal and tissue-specific differentiation are signature traits of stem cells that researchers have capitalized upon to devise cell-based therapies for the treatment of an assortment of neurodegenerative disorders including amyotrophic lateral sclerosis, Parkinson's disease and age-related macular degeneration (Steward *et al.*, 2013). The promise of stem cells for replacing damaged or diseased cells in the nervous system has spurred an ongoing refinement of methods to generate homogeneous populations of *neurons* efficiently from embryonic, multipotent or induced pluripotent stem cell sources (Torisawa *et al*., 2007; Steward *et al.*, 2013).

Some methods for obtaining populations of embryonic stem cell (ESC)-derived neurons require the formation of embryoid bodies, or stem cell aggregates, which are grown with or without retinoic acid (RA). Since RA directs stem cells towards a neuroectoderm lineage (Ying *et al*., 2003), spheroids grown in a medium devoid of RA must be further propagated in a serum-free defined neuronal differentiation medium, which discourages growth of non-neural cells (Stavridis and Smith, 2003). Neurons can be derived from these primed spheroids (Torisawa *et al*., 2007), from dissociated spheroids, or directly from a monolayer of ESC under medium conditions conducive to neuronal differentiation. Other procedural elements, such as substrate coating, defined medium components and longevity of culture can greatly vary (O'Shea, 2001).

Researchers have demonstrated that neurospheres can be cultured in an array format to create high-throughput assays (Shofuda *et al*., 2013) and even complex neural networks for implantation (Kato-Negishi *et al*., 2010). Traditionally, round bottom 96-well plates and hanging drops are the standard platforms for generating neurospheres. Microfluidic approaches have been used in the past to engineer uniform neurospheres efficiently (Torisawa *et al*., 2007). Shofuda *et al*. (2103) custom-designed a polymethylmethacrylate microchip array, dubbed microsphere array, to gain precise control over neurosphere homogeneity and enable bulk production. Neurosphere size can be controlled with different sized wells, and a polyethylene glycol surface coating permits ease of neurosphere retrieval by hindering cell adhesion (Shofuda *et al*., 2013). Alternatively, a polyethylene glycol-based hydrogel microwell array can be used to form neurospheres, with each neurosphere arising from a single founding cell (Cordey *et al*., 2008). Additionally, polydimethylsiloxane (PDMS) microchambers have been used to culture grid-like arrays of neurospheres that are interconnected with neuronal processes, and the flexible chambers have been used to *stamp* intact neuronal networks directly onto damaged brain regions in rats (Kato-Negishi *et al*., 2010).

Here a quick, simple, cheap and efficient method is presented for patterning an array of adherent neurospheroids on tissue culture dishes. The spheroids can be maintained in individual drops or in a flood of medium. Prior to creating this array, mouse embryonic

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stem cells (mESCs, D3 line) (Doetschman *et al*., 1985) are suspended in neuronal differentiation medium [50:50 mix Dulbecco's modified Eagle's medium (DMEM)/F12 and neurobasal medium, supplemented with 1% B27, 0.5% N2, 1% sodium pyruvate, and 1% antibiotic–antimycotic] for 24 h. During this time, the mESCs are primed for differentiation through removal of trace amounts of leukaemia inhibitory factor that preserved self-renewal and pluripotency, and exposure to a neuronal differentiation promoting medium. This neuronal differentiation medium (NDM) is enriched in B27 and N2 (Lorincz, 2006), which have been demonstrated to be effective in guiding the early stages of differentiation of mESCs into neurons. The resulting mESC heterogeneously-sized aggregates are subsequently dissociated into another single cell suspension that is used to create hanging drops for the formation of more uniformly sized spheroids.

The general method for forming arrays of adherent neurospheres involves the transfer of neurospheres from hanging drops onto adhesive regions already configured in a 4 × 4 circle-array on a 35-mm plastic Petri dish. Geltrex, an adhesive and differentiation-promoting agent, guides the placement of the spheres. Geltrex is a commercially available approximation of extracellular matrix, which contains laminin, collagen IV, entactin and heparin sulfate proteoglycan (Loring and Peterson, 2012). Matrigel, an earlier developed version of this matrix, has been shown to promote ESC-derived neuron progenitor survival and processes elaboration (Uemura *et al*., 2010). A schematic of the adherent neurospheroid array creation process is shown in Figure 1A. Two variations of this method exist, and are referred to as *transfer* and *inversion*.

In the *transfer* version of this method, the single cell suspension, obtained from dissociation of the primed free-floating mESC clusters, is used to form hanging drops (500 cells/10 μl) on the *lid* of Petri dishes. Sterile distilled water is added to each dish's base to inhibit evaporation from the drops, and the spheres are allowed to develop for 24 h. Concurrently, arrays of Geltrex are created on the bases of a separate set of dishes (gel arrays incubated at 37°C for 24 h). Each drop of medium, containing a spheroid, is individually removed from the lid and

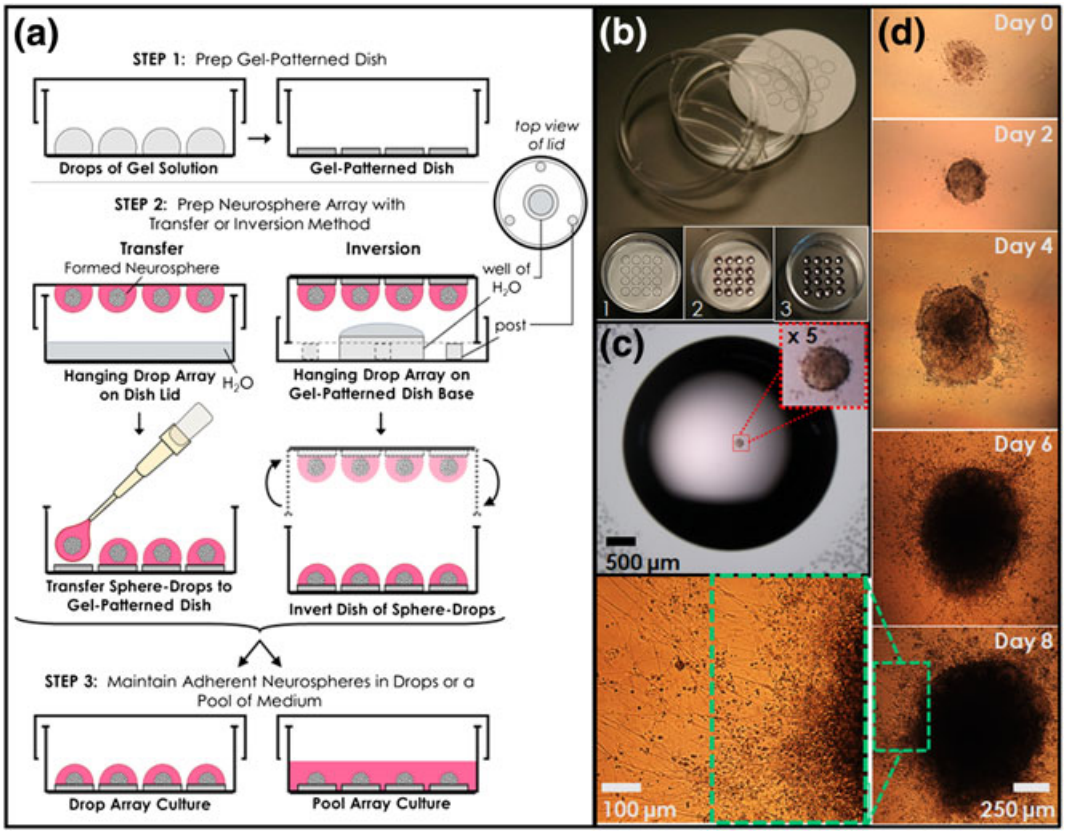

Figure 1. In dish drop array culture of neurospheres. (A) Step 1. Geltrex drops (10 μl) are arranged on the base of an untreated dish and incubated for 24 h at 37°C. Step 2. In the *transfer* method, drops from a suspension of primed mESCs are created on dish lids, and the dish (10-cm diameter) is filled with water (0.1 ml/cm^2) to curb evaporation. In the *inversion* method, hanging drops are formed directly on gel regions covering the base of the dish. A well and three posts, all machined from PDMS slabs, were sealed respectively at the centre and along the perimeter of the Petri dish. The dish containing drops was inverted over the well to rest on the posts and over a well filled with water (~0.5 ml). After 24 h, spheres from both methods are placed in contact with the gel-coated regions pattern. For the *transfer* method, a pipette is used to transfer a sphere-containing drop to a gel coated region of a dish prepatterned with a gel array. In the *inversion* method, wells and posts were removed from the dish lid, and the set-up was inverted, allowing spheres in the array to make contact the existing gel. Step 3. Spheres in both methods were allowed to firmly attach to the gel array for 24 h, after which the cells masses can be maintained in *drop* or *pool* culture formats. (B) A template with a 4 × 4 array of 4-mm diameter circles was created for a 35-mm diamter Petri-dish. The template is placed beneath the dish (1), used to guide deposition of drops (2) and then removed following the completion of drop formation (3). This template also serves as a place-holder when it is realigned beneath gel drops, prior to their removal, and during subsequent formation of drops containing cells. (C) After 24 h, fully formed spheres can be observed inside of the hanging drops that were initially composed of 500 cells in 10 μl neuronal differentiation medium (5 × 10^4 cells/ml). (D) The cell masses were cultured for 8 days. Day 0 was designated as the day the spheres adhered to the gel pattern. At day 8, neurite outgrowth/process formation was clearly visible

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reformed on a circular gel-coated region of the prepared gel-array dish. Minute amounts of medium can be added to each drop to achieve a total drop volume of 15 μl. An array template (Figure 1B), printed on paper, is positioned beneath a culture dish to guide the placement of gel or spheres as well as the removal or addition of solutions.

In the simplified *inversion* variation of this method, drops of a single-cell suspension are formed directly on gel-coated regions that are pre-pattered in an array on the dish *base*. The dish base is then inverted so that it rests over small silicone posts, positioned along the inner rim of the dish lid as illustrated in Figure 1A (Step 2), to ensure sufficient ventilation. The spheres formed in these hanging drops are suspended from, but not touching a gel pattern. After 24 h, the dish is smoothly inverted so that all of the spheres make contact with the gel at once, and this contact is prerequisite for sphere-surface adhesion. This quick inversion process circumvents the time-consuming task of using a pipette to transfer each drop, one-by-one, to a single gel-coated region.

For both methods, it was found that the spheroids adhered firmly to the gel patterns after 24 h, as evidenced by the outward spread of cells (Figure 1D). At this point, designated day 0 for the differentiation of adherent spheroids, the cell masses can be maintained by either adding fresh drops of NDM to spheroids or gently flooding the entire spheroid array with fresh NDM. Spheroids were maintained on gel in either *drops* or a *pool* of medium for a period of 8 days (Figure 1A, Step 3, drop culture left, pool culture right). A portion of the medium was removed and replaced every other day. Specifically, in the *pool* culture, around half of the medium is replaced with fresh NDM medium. In the *drop* culture, 7.5 μl of medium was carefully removed from each drop and replaced with 12.5 μl of fresh NDM medium. In some instances, medium in drops was refreshed daily. Neurite process formation could be observed as early as day 2, and extensive neurite outgrowth was visible at day 8 (Figure 1D).

The technique used to form adherent neurospheroid arrays was categorized as *transfer* or *inversion*, and the maintenance of cultures by the *drop* or *pool*, method. In the figures, d denotes daily medium exchange. The methods compared and evaluated were: transfer-drop, transfer-drop(d), transfer-pool, and invert-pool. No inversion-drop conditions were evaluated.

Cell density and hanging drop volume were optimized to rapidly generate uniform spheres (Figure 1C). In the *transfer* method, the average diameter of spheres obtained from hanging drops was 166 μm $\pm$ 15 μm (n = 80). In the *inversion* method, an average diameter 134 μm $\pm$ 22 μm (n = 153) was found. It is possible that the presence of the gel could affect the sphere formation process. Each user would need to carefully optimize hanging drop formation conditions for a chosen array culture technique.

Here we use a 16-drop template for a 35 mm-diameter dish, but some researchers may desire to form spheres on surfaces with larger areas. An array template could also be designed to match the well geometries and arrangements that are characteristic of common well-plate schemes that are compatible with liquid handling robots (Tung *et al*., 2011).

The region of elaborate neurite outgrowth is clearly outlined by labelling cells/processes for neuronal beta tubulin III, TUJ1 (Figure 2A). A morphometric analysis technique was used to characterize process formation of stained cell masses that were fixed at 2-day intervals over a total of 8 days, when using the *transfer-pool* method (Figure 2B) on day 8 for all types of culture methods (Figure 2C). The area of neurite outgrowth was obtained by subtracting the area of a nuclei cluster from the area of an entire cell mass (Figure 2D). FIJI (NIH) image processing software was used to obtain area measurements. Clear formation of intricate neurite elaboration is evident at day 6, where there is a substantial increase in neurite outgrowth area, compared to days 2 and 4 (Figure 2E). There was no significant difference found among the average values of neurite outgrowth areas among the four different culture methods evaluated (Figure 2E).

To confirm neural differentiation, we performed quantitative reverse transcription polymerase chain reaction (RT-qPCR) on complementary deoxyribose nucleic acid (cDNA) made from ribonucleic acid (RNA) isolated from cells in 1-day-old spheroids suspended in hanging drops at day 0, and also those adhered to Geltrex patterns at day 4 and day 8 (Figure 2F). The *transfer-pool* group of cells was used to harvest RNA from day 4 and day 8 spheroids. The neurospheroids were screened for: the temporal expression of the neuron-specific markers, β-tubulin III (Tubb3) and synaptophysin (Syp); the embryonic stem cell marker, octamer-binding transcription factor 4 (Oct-4); and the glial cell marker, glial fibrillary acidic protein (GFAP). The 8-day-old spheroids expressed significantly higher levels of Tubb3 and Syp and lower levels of Oct-4 and GFAP than day 4 and day 0 spheroids. These results suggest that the spheroids are directed toward at least the early stages of neuronal development.

To demonstrate the usability of this method for treating spheres in an array selectively, drops of cells were treated with green fluorescent cell tracker according to a specific configuration (Figure 2G). Ideally, a neurosphere array representing different brain regions could be constructed by including neurospheres, derived from selected brain regions, within different drops at specific locations on the array. These regions could be homogenously or selectively treated with various agents to assess therapeutic or neurotoxic effects. 1,3-Dinitrobenzene is an example of a neurotoxicant that causes brain-region specific damage (Dixon and Philbert, 2015).

The distances between the spheroids could be optimized for specific biological assays. The effects of longer-duration cultures (>1 week) and variable interspacing lengths on neurospheroid development need further experimentation.

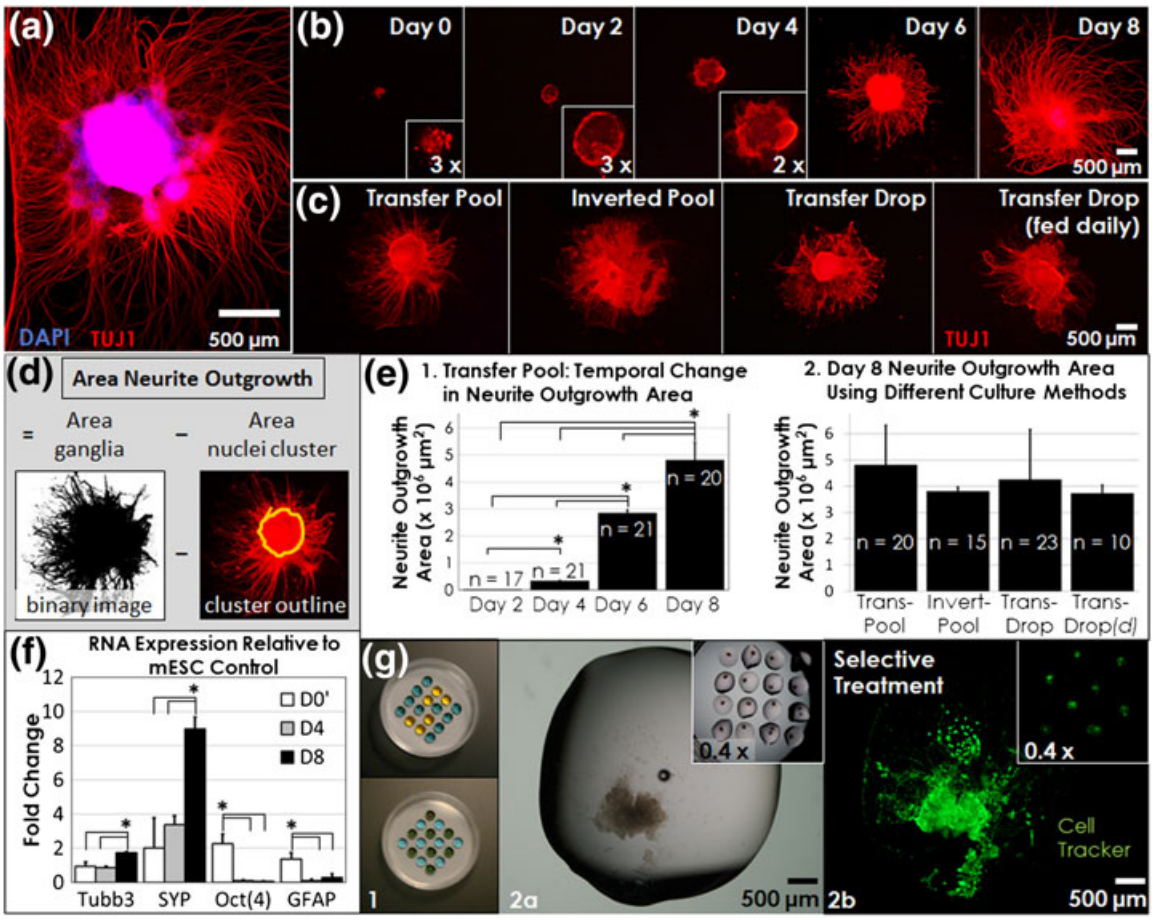

Figure 2. Characterization and patterned treatment of differentiated neurospheres. (A) Fluorescent image of a complex neuritic elaboration from an 8-day old neurosphere-stained with neuron-specific beta-III (TUJ1) and 4′,6-diamidino-2-phenylindole (DAPI). (B) Temporal expression of TUJ1 for neurospheres cultivated with the *transfer-pool* method. (C) Comparison of differentiated neurospheres stained for TUJ1 at Day 8, using the indicated culturing methods. (D) Quantitation for the area of the neurite outgrowth was performed by subtracting the area of a nuclei cluster from the area of the entire cell mass. FIJI was used to create a binary image of the cell body mass, outline the nuclei cluster and perform area calculations. (E) Neurite outgrowth areas on day 2, 4, 6 and 8 for the *transfer-pool* technique and day 8 for the indicated types of culturing techniques. Group means were determined and the analysis done using one-way ANOVA with Tukey's *posthoc* procedures. (**p*-value <0.05; error bars show standard deviation). (F) Relative gene expression, compared to mESC control, of Tubb3, Syp, Oct-4 and GFAP. Data were obtained by taking triplicate measurements from three independent RTqPCR experiments. Group means were analysis using one-way ANOVA with Tukey's *posthoc* procedures. (**p*-value <0.05; error bars show standard deviation). (G) Two of the many possible patterning schemes are shown with coloured drops (Doetschman *et al.*, 1985). A *transfer-drop* culture was selectively treated with cell tracker on day 8 (2a, b). The fluorescent staining demarcates the pattern and cell morphology (2b) of the same treated drop shown in bright field (2a). Insets show the entire neurospheroid array

The novelty of this system lies in its multipurpose features that include the formation of adherent neurospheroids arrays inside drops of solution, maintenance of adherent neurospheroids inside drops or a pool of medium for several days, and the use of this method to pattern the treatment of adherent neurospheroids, selectively. This simple, yet versatile, system for creating adherent spheroid arrays and drop cultures could prove useful not only for neuroscience studies, but also other research areas where prerequisite formation of uniform adherent spheres is required.

A detailed procedure is supplied as supporting information.

Conflicts of interest

The authors declare that there is no conflict of interest.

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A.R.D. designed and performed experiments, analysed the data, and wrote the manuscript. Y.R. performed experiments and wrote the manuscript. K.H. performed experiments, analysed the data, and wrote the manuscript. K.F.B. wrote and edited the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Supplementary Figure 1. *Inversion* method. (A) A PDMS well (inner diameter = 12 mm, outer diameter = 16 mm, height ~ 3 mm), centred aligned on the dish lid, and three PDMS posts (diameter = 4 mm, height ~ 4 mm), equally spaced along the edge of the lid, are affixed via contact adhesion. (B) An array of medium drops, formed on the dish base are inverted over the lid. (C) Side view of the inverted set-up showing hanging drops on dish base, and water well and posts on dish lid. Posts prevent obstruction of gas flow, by propping dish slightly ajar to permit ventilation. (D) The inverted dishes are housed in a larger dish to guard against contamination. Up to three *inversion* array dishes can be arranged in a 100 mm-diameter dish.