

*Short Communication***A Low-Cost Method for Brain Slice Cultures**

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Abstract. Low-cost, simple procedures for organotypic tissue cultures are desirable for high-throughput biological experiments such as large-scale medical/drug screening. We present a practical and economical method to cultivate brain slices using hydrophilic filtration membranes. With a cost reduction of more than 90%, this technique allows us to prepare hippocampal slice cultures that are morphologically and functionally indistinguishable from those obtained by the widely used Millicell-CM[®] method.

Keywords: membrane-interface tissue culture, organotypic culture, hippocampal slice

Organotypic membrane-interface cultures made from slices of developing brain tissue preserve complex multi-cellular circumstances to a considerable extent, though not perfectly, and hence they have been used as model systems to estimate brain function, development, and damage (1). This technique was originally introduced by Yamamoto et al. (2) and largely improved for practical use by Stoppini et al. (3). Stoppini's method uses a Millicell-CM[®] membrane as an interface, on which slices of explanted tissue can be maintained in stationary culture for many weeks to months.

We introduce herein an alternative method. The new method utilizes commercially available Omnipore[®] microporous filters. This membrane is designed to be used for filtration of aqueous solution. According to the manufacturer's specifications, it is made of hydrophilic polytetrafluoroethylene with a pore size of 0.45 μm and a porosity of 80%, which is almost equivalent to the Millicell-CM[®] membrane. Using Omnipore[®] filters, we have prepared more than 2,000 slice cultures for more than a year and found no evidence that these cultures differed from those prepared by the conventional Millicell-CM[®] method.

Wistar/ST or Sprague Dawley rats (SLC, Hamamatsu, Shizuoka) at postnatal day 6 or 7 were deeply anesthetized

by hypothermia and decapitated. The brains were removed and horizontally cut into 300- μm -thick slices using a DTK-1500 vibratome (Dosaka, Kyoto) in aerated, ice-cold Gay's balanced salt solution. Entorhino-hippocampal stumps were maintained *ex vivo* by either of the two following methods: i) three or four slices were placed on a Millicell-CM[®] culture membrane (PICM030-50; Millipore, Bedford, MA, USA) (3), and ii) three or four slices were placed on an Omnipore[®] membrane filter (JHWP02500, Φ 25 mm; Millipore) that was laid on an O-shaped plastic plate, termed a "doughnut" plate (Fig. 1). The Omnipore[®] membrane filters are non-autoclavable because of the maximal operating temperature of 130°C. Instead, they were sterilized with ultraviolet light for 30 min and/or 70% ethanol for a few seconds in a clean bench. The doughnut plates were produced by custom order to an outside supplier (Hazai-Ya, Katsushika-ku, Tokyo). They are made of acylated resin and semipermanently reusable with sterilization in 70% ethanol for a few seconds (note neither an autoclave nor ultraviolet light can be used). The membrane was carefully placed onto a doughnut plate with forceps (Fig. 1C). Millicell-CM[®] wells and doughnut plates were inserted into six-well plates that were each filled with 1 ml of culture medium, out of which 0.9 ml of medium was changed every 3.5 days. The medium consisted of 50% minimal essential medium, 25% Hank's balanced salt solution, and 25% horse serum (Cell Culture Laboratory,

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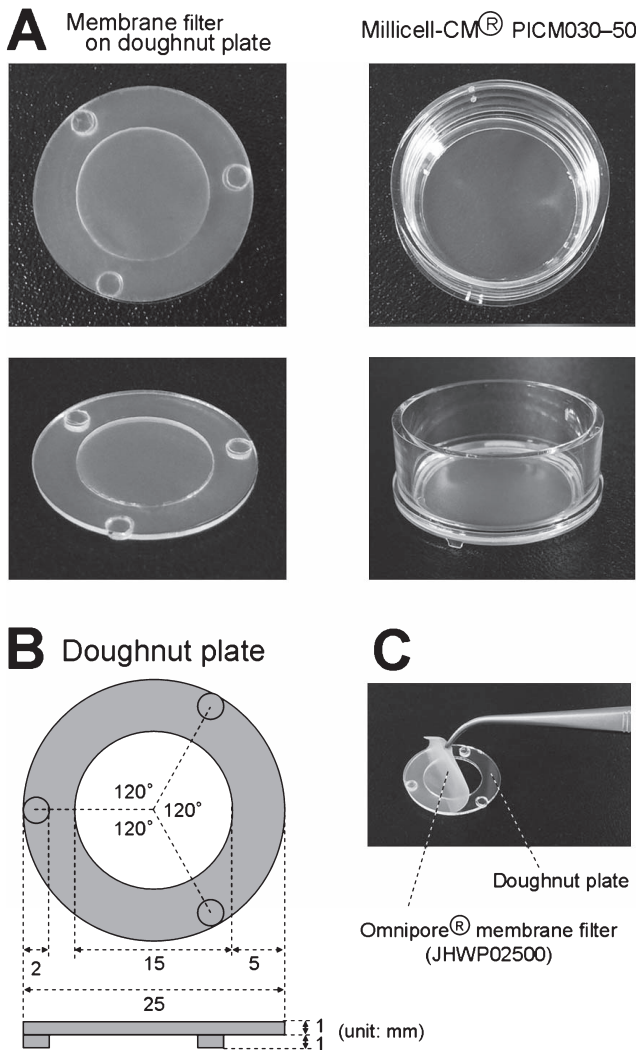


Fig. 1. Design for a low-cost method to organotypically cultivate brain slices. **A:** Photograph of Millicell-CM[®] (right) and a newly developed O-ring plastic plate, termed a “doughnut” plate, the top of which is covered with an Omnipore[®] filter (left). Top: top view, bottom: oblique view. **B:** Engineering drawing of a doughnut plate. Top: top view, bottom: side view. **C:** Omnipore[®] membrane was manually laid onto a doughnut plate.

Cleveland, OH, USA), supplemented with 33 mM glucose. Slices were maintained at 37°C in a humidified and CO₂-enriched atmosphere. Wet filters are tightly stuck to doughnut plates through surface tension, allowing stable cultivation for long time. Experiments were performed at 7 to 11 days *in vitro*.

For immunohistochemical staining, cultures were fixed in 4% paraformaldehyde and permeabilized with 0.4% Triton X-100 overnight at 4°C. Nonspecific antibody binding was blocked by 60-min incubation with 2% goat serum at room temperature. The cultures were stained with a primary mouse monoclonal antibody against NeuN (1:1000; Chemicon International,

Temecula, CA, USA) overnight at 4°C and with secondary anti-mouse IgG Alexa-594 (1:1000; Invitrogen, Gaithersburg, MD, USA) overnight at 4°C and imaged with a laser-scanning confocal system (MRC-1024; BioRad, Richmond, CA, USA).

Functional multineuron calcium imaging (fMCI), an optical recording technique that detects action potentials as somatic calcium transients, was carried out, as previously described (4). Briefly, slices were incubated with 0.0005% Oregon green 488 BAPTA-1 AM (Invitrogen) for 1 h at 37°C and then transferred to a 32°C recording chamber perfused with ACSF consisting of 127 mM NaCl, 26 mM NaHCO₃, 3.3 mM KCl, 1.24 mM KH₂PO₄, 1.0 mM MgSO₄, 1.0 mM CaCl₂, and 10 mM glucose. Calcium transients evoked by spontaneous spike activity were simultaneously measured from about 100 hippocampal CA3 neurons. Images were captured at 10 frames/s with a CSU10 Nipkow-disk confocal microscope (Yokogawa Electric, Musashino City, Tokyo) and a cooled CCD camera (Cascade 512B/F; Roper Scientific, Tucson, AZ, USA). Spike timings were determined as the onset of individual calcium transients by custom-written Visual Basic software.

Hippocampal slices cultured by the doughnut-plate and Millicell-CM[®] methods both developed tissue organizations that closely resembled those observed *in situ*, as assessed by immunostaining against NeuN, a neuron-specific marker. Confocal microscopic inspection did not reveal any difference in cell alignment or morphology between the two methods (Fig. 2). Similar results were obtained with Nissl staining (data not shown).

fMCI revealed that a portion of the CA3 neuron populations was spontaneously active (Fig. 3A). Neither the percentage of active neurons to the total (Fig. 3B) nor the mean activity frequency per neuron (Fig. 3C) was different between the doughnut-plate and Millicell-CM[®] methods. The distribution of inter-activity intervals was also not different (Fig. 3D) between the two methods, indicating that the network activity pattern was statistically indistinguishable.

In addition to the above-described studies, we have carried out a variety of experiments with the new culture method, including patch-clamp recordings, neurite tracing with carbocyanine fluorescent dyes, gene transfection, and neurodegeneration assay with propidium iodide or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), but we found no difference between the doughnut-plate and Millicell-CM[®] methods, with only one exception: Timm staining, a histochemical technique to detect mossy fiber terminals because of their high Zn²⁺ content, unexpectedly resulted in silver

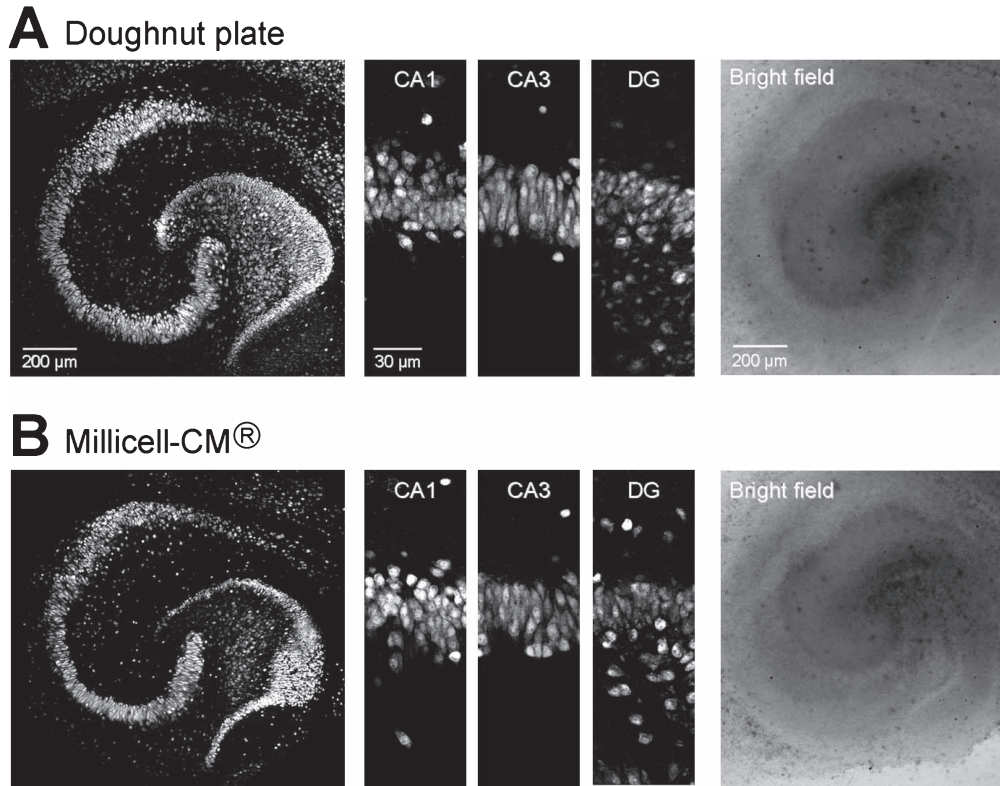


Fig. 2. No histological difference in hippocampal slices cultured by doughnut-plate (A) and Millicell-CM[®] methods (B). Hippocampal slices prepared from P6 rats were cultured for 7 days and immunostained with anti-NeuN antibody. Confocal fluorescence images were obtained with 10× objectives for the whole slices (left) and 20× objectives for high magnification of the subareas CA1, CA3, and dentate gyrus (DG) (middle panels). Bright field images (right) were obtained with 10× objectives. Images to be compared were taken under the same conditions such as illumination intensity and exposure time.

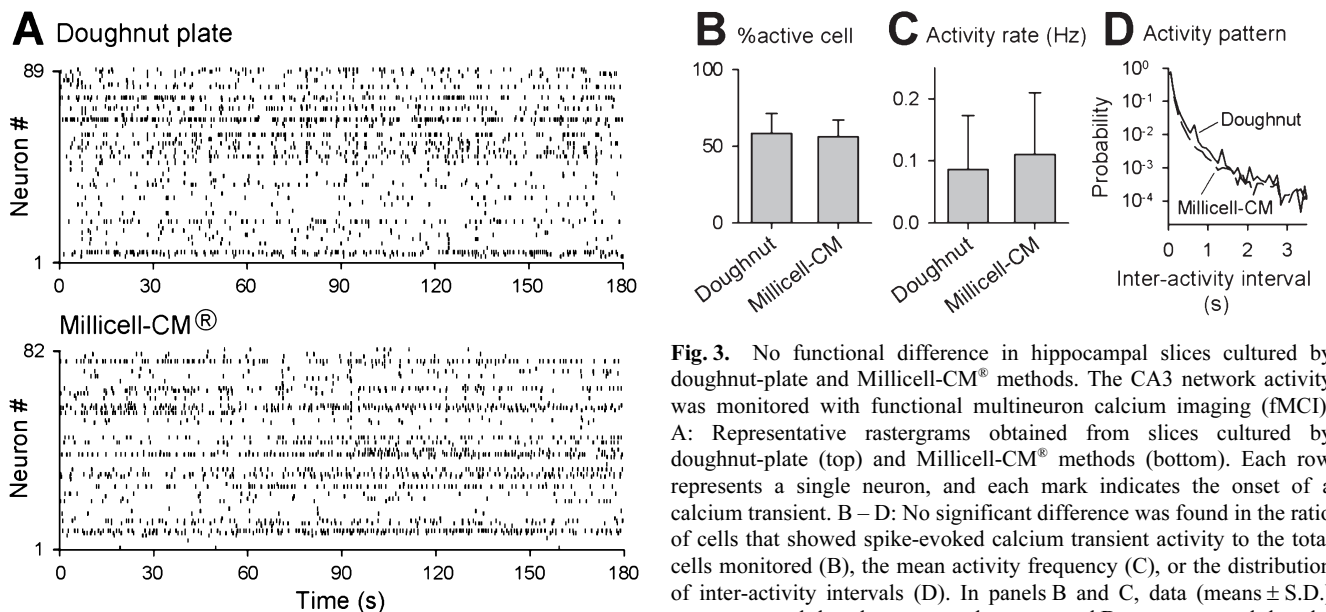


Fig. 3. No functional difference in hippocampal slices cultured by doughnut-plate and Millicell-CM[®] methods. The CA3 network activity was monitored with functional multineuron calcium imaging (fMCI). A: Representative rastergrams obtained from slices cultured by doughnut-plate (top) and Millicell-CM[®] methods (bottom). Each row represents a single neuron, and each mark indicates the onset of a calcium transient. B – D: No significant difference was found in the ratio of cells that showed spike-evoked calcium transient activity to the total cells monitored (B), the mean activity frequency (C), or the distribution of inter-activity intervals (D). In panels B and C, data (means ± S.D.) were assessed by the *t*-test, whereas panel D was assessed by the Kolmogorov-Smirnov test. *n* = each 4 slices.

deposition on the surface of Omnipore[®] membrane for unknown reasons.

A critical difference, however, is membrane transparency. The thickness of the Millicell-CM[®] and Omnipore[®] membrane is approximately 30 and 60 μm , respectively, and thus, Omnipore[®] is more opaque and may blur bright field or fluorescence images across the membrane (Fig. 2).

The important advantage of our method is its inexpensiveness. As doughnut plates are repeatedly usable, our method is one order of magnitude more economical as a whole (see appendix). Moreover, doughnut plates are not absolutely necessary for short-term cultivation, because the Omnipore[®] membrane floats on the surface of culture medium. Without doughnut plates, the total cost will be reduced (5). Thus, the Omnipore[®] method provides a promising tool for genomic, proteomic, and pharmacologic approaches for functional screening in the central nervous system.

Appendix

The suggested list prices of Millicell-CM[®] and Omnipore[®] are ¥880 (approx. US\$7.50) and ¥70 (approx. US\$0.60) per unit, respectively. The unit price of a doughnut plate depends on the amount ordered: ¥1,050 for less than 50 pieces, ¥735 for less than 100 pieces, ¥525 for less than 200 pieces, ¥420 for less than 300 pieces, and ¥350 for 300 and more pieces (Hazai-

Ya, URL: <http://www.hazaiya.co.jp>).

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