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Research paper

Patch method for culture of primary hippocampal neurons

Yadong Tang [a,1], Francesco Paolo Ulloa Severino [b,1], Federico Iseppon [b], Vincent Torre [b,*], Yong Chen [a,c,d,**]

[a] Ecole Normale Supérieure-PSL Research University, Département de Chimie, Sorbonne Universités - UPMC Univ Paris 06, CNRS UMR 8640 PASTEUR, 24, rue Lhomond, 75005 Paris, France
[b] Scuola Internazionale Superiore di Studi Avanzati, via Bonomea, 265, 34136 Trieste, Italy
[c] Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto 606-8507, Japan
[d] Institute for Interdisciplinary Research, Jianghan University, Wuhan, China

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ABSTRACT

Culture of primary neurons, and especially hippocampal neurons, is important for understanding cellular mechanisms in neurobiology. Actually, this is achieved by using culture dish or glass slide with surface coated proteins. Here, we proposed a patch method for culture of primary neurons on a monolayer of gelatin nanofibers electrospun and crosslinked on a honeycomb microframe of poly (ethylene glycol) diacrylate (PEGDA). This method allows us to minimize exogenous material contact of cells and largely increase the exposure area of cells to the culture medium. We found that neurons, and especially astrocytes, have a more *in vivo* like morphology comparing to that on culture dish or on glass slide. We also found that neurons were preferentially located in the suspended areas of the monolayer nanofibers. Finally, calcium imaging revealed that primary neurons have a higher degree of neural activity on the patch than on glass. These results suggest that crosslinked and monolayer gelatin nanofibers closely mimic the extracellular matrix structure and allow more effective culture of primary neurons than conventional methods, thus facilitating advanced studies of neural functions as well as cell-based assays.

1. Introduction

Primary neurons, especially the hippocampal neurons, are widely used for *in vitro* studies due to the relative simple nerve cell population and the expression of key neural phenotypic features as well as the involvement of the hippocampus in learning and memory [1–4]. In most of these studies, the primary neurons were cultured in culture dish or on glass slides with or without astrocytes [5]. It has been shown, however, that the neural growth and its functional performance are critically dependent on the culture conditions, especially the mechanical and biochemical properties of the substrate [6,7]. While the optimal culture should recapitulate the *in vivo* organization of extracellular matrix (ECM) of primary neurons [8,9], the conventional culture methods are not flexible to achieve this ultimate goal.

Electrospun nanofibers are *in vivo* ECM like, which are promising for advanced applications in tissue engineering and regenerative medicine [10,11]. Gertz et al. showed that electrospun nanofibers can significantly enhance neuritogenesis, maturation and the polarity formation of neurons compared to the conventional two-dimensional (2D) culture using glass substrates [12]. Our previous studies demonstrated that gelatin nanofibres could be used for long-term expansion of human induced pluripotent stem cells (hiPSCs) [13]. Although nanofibers made of different types of synthetic polymers can also be used for cell culture, natural bio-polymers such as gelatin, which is produced by hydrolyzing collagen, should be more relevant for cell based assays [14]. More recently, we used monolayer gelatin nanofibers in form of culture patch to differentiate hiPSCs towards mature motor neurons and cardiomyocytes [15,16]. Our results showed a number of advantages of this patch method over the conventional culture dish methods, including up-regulated expression of neural specific genes, accelerated neuron maturation as well as plug-and-play monitoring of neuron spikes by extracellular potential recording.

In this work, we extend the patch method to culture primary hippocampal neurons. We show that hippocampal cells, especially astrocytes, have *in vivo* like morphology on the culture patch. Our results also showed that most of neurons were found in the porous areas inside the honeycomb compartments and that primary neurons have a higher degree of neural activity on the patch than on glass.

2. Experimental

2.1. Fabrication of culture patch

The culture patch was fabricated as described previously [15,16]. Briefly, honeycomb microframe of 500 μm pitch size and 50 μm band

* Corresponding author.
** Correspondence to: Y. Chen, Ecole Normale Supérieure-PSL Research University, Département de Chimie, Sorbonne Universités - UPMC Univ Paris 06, CNRS UMR 8640 PASTEUR, 24, rue Lhomond, 75005 Paris, France.
E-mail addresses: torre@sissa.it (V. Torre), yong.chen@ens.fr (Y. Chen).
[1] Authors contributed equally to this work.

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width was patterned on a Chrome mask with a micro pattern generator (μPG 101, Heidelberg Instruments). A 50 μm thick photoresist layer (AZ 40XT, MicroChem) was then spun coated on the mask and backside exposed with UV light. After development, the mask with resist pattern was treated in trimethylchlorosilane (TMCS) vapor for *anti*-sticking treatment. Polydimethylsiloxane (PDMS) pre-polymer and cross-linker (GE RTV 615) at weight ratio of 10:1 was mixed and then poured on the resist layer. After curing at 80 °C for 2 h, the PDMS layer was peeled off and placed on a glass slide. The PDMS-glass assembly was then degassed for 10 min in a desiccator. Afterwards, a PEGDA (average Mn = 250, Sigma) solution containing 1 v/v% Irgacure 2959 as photo-initiator was dropped on the edge of PDMS mould to fill the cavity between PDMS and glass with the help of degassing based micro-aspiration. After UV curing for 30 s at 9.1 mW/cm^2, the PDMS mould was release. In parallel, a 100 μm thick PEGDA ring with outer and inner diameter of 13 mm and 9 mm respectively was prepared with the similar method. This PEGDA ring was then mounted on the honeycomb microframe using PEGDA solution as binder and UV curing.

Gelatin nanofibers were prepared by electrospinning using the same protocol as described previously [8]. For easy collection of the nanofibers, 10 nm thick Au layer was sputtered on the PEGDA frame and the PEGDA frame was fixed on a silicon wafer (collector). 10 wt% gelatin was dissolved in a solvent mixture containing distilled water, ethylacetate acid and acetic acid at a volume ratio of 10:14:21. The gelatin solution was then ejected from a syringe to the collector at a distance of 10 cm and a pumping speed of 0.2 ml/h through a stainless steel needle (23-gauge) under a bias voltage of 11 kV. After electrospinning for 15 min, the sample was removed in a desiccator to remove the residual solvent overnight. An ethanol solution containing 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.2 M N-hydroxysuccinimide (NHS) was used for crosslinking the gelatin nanofibers during 4 h. Finally, the samples was rinsed with ethanol three times and dried completely in a desiccator overnight to eliminate the remaining solvent.

2.2. Cell culture

Human glioblastoma cell line U-87 was cultured in Dulbecco's Modified Eagle Medium (DMEM) completed with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2 supplementation for 3– 4 days to reach 80% confluence. Then cells were dissociated by Trypsin at 37 °C for 3 min. Then without any additional coating, 10^5 cells in 50 μl medium were seeded on the patch in the center of the ring. After incubation at 37 °C for 1 h for cell attachment, more medium was added gently around the patch, which was then transferred back to the incubator for continued culture.

Hippocampal neurons from Wistar rats (P2-P3) were prepared in accordance with the guidelines of the Italian Animal Welfare Act, and their use was approved by the Local Veterinary Service, the SISSA Ethics Committee board and the National Ministry of Health (Permit Number: 630-III/14) in accordance with the European Union guidelines for animal care (d.1.116/92; 86/609/C.E.). The animals were anaesthetized with CO_2 and sacrificed by decapitation, and all efforts were made to minimize suffering. All substrates (Glass and patch nanofibers) were sterilized under UV light (15 min each side), soaked for 2 days in PBS and other 2 days in 100 units/ml Penicillin and 100 μg/ml Streptomycine. Finally, they were coated with 20 μg/ml Laminin (Sigma-Aldrich, St. Louis, MO, USA) overnight and washed with water before Matrigel coating (1:50 diluted in the Culture Medium) (Corning, Tewksbury MA, USA) and cell seeding. Dissociated cells were resuspended in Culture Medium: minimum essential medium (MEM) with GlutaMAX™ supplemented with 10% dialyzed fetal bovine serum (FBS, all from Thermo Fisher Scientific, Waltham, MA, USA), 0.6% D-glucose, 15 mM Hepes, 0.1 mg/ml apo-transferrin, 30 μg/ml insulin, 0.1 μg/ml D-biotin, 1 μM vitamin B12 and 2.5 μg/ml gentamycin (all from Sigma-Aldrich). A drop containing 200.000 cells was deposited per each substrate and incubated for 30 min at 37 °C. The cells were then resuspended in Astrocyte Conditioned Medium (ACM) in 1:1 ratio with Neurobasal/B27 medium. After 48 h, 2 μM cytosine-β-D-arabinofuranoside (Ara-C; Sigma-Aldrich) was added to the culture medium to block glial cell proliferation. Half of the medium was changed every 2–3 days. The neuronal cultures were maintained in an incubator at 37 °C, 5% CO_2 and 95% relative humidity.

2.3. Scanning electron microscopy (SEM) observation

For the samples with cells, they were firstly fixed with 4% formaldehyde for 30 min, and rinsed three times with PBS. Then the samples were immersed in 30% ethanol solution (in distilled water) for 30 min. Afterward, the samples were dehydrated using graded ethanol solutions with concentrations of 50%, 70%, 80%, 90%, 95%, and 100%, respectively, each for 10 min and dried by nitrogen gas flow. Before observation, both dehydrated cell samples and samples without cells were deposited with a 2 nm thick Au layer by sputtering. Finally the samples was observed with a scanning electron microscope (Hitachi S-800) operated at 10 kV.

2.4. Calcium imaging

The cells were loaded with 4 μM of a cell-permeable calcium dye Fluo4-AM (Life Technologies) dissolved in anhydrous DMSO (Sigma-Aldrich), stock solution 4 mM, and Pluronic F-127 20% solution in DMSO (Life Technologies) at a ratio of 1:1 in Ringer's solution (145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes, pH 7.4) at 37 °C for 1 h. After incubation, the cultures were washed and then transferred to the stage of a Nikon Eclipse Ti-U inverted microscope equipped with a piezoelectric table (Nano-ZI Series 500 μm range, Mad City Labs), an HBO 103 W/2 mercury short arc lamp (Osram, Munich, Germany), a mirror unit (exciter filter BP 465-495 nm, dichroic 505 nm, emission filter BP 515-555) and an Electron Multiplier CCD Camera C9100-13 (Hamamatsu Photonics, Japan). The experiments were performed at RT, and images were acquired using the NIS Element software (Nikon, Japan) with an S-Fluor 20×/0.75 NA objective at a sampling rate of 3–10 Hz with a spatial resolution of 256 × 256 pixels for 10 min. To avoid saturation of the signals, excitation light intensity was attenuated by ND4 and ND8 neutral density filters (Nikon).

2.4.1. Ca^{2+} imaging processing and analysis

The initial video was processed with the ImageJ (U. S. National Institutes of Health, Bethesda, MA) software. The image sequences were then analysed as described previously [17]. Appropriate ROIs around the cells bodies were then selected. The time course of the fluorescence intensity, $I_f(t)$, in this ROI was displayed, and any decay, which is a consequence of dye bleaching, was evaluated. The Ca^{2+} transients of each cell signal were extracted in a semi-automatic manner by selecting a threshold for the smallest detectable peak that was equal to three times the standard deviation of the baseline. $I_f(t)$ was then fitted to the original optical signal to compensate for dye bleaching, and the fractional optical signal was calculated as follows: $DF/F = (Y(t) + I_f(t))/I_f(0)$, where $I_f(0)$ is the fluorescence intensity at the beginning of the recording.

2.4.2. Computation of the correlation coefficient of Ca^{2+} transient occurrence

The times, t_i, at which transient peaks occurred were used to calculate the rate of activity. The correlation coefficient of the calcium transients for neuron i and neuron j (σ_{CTij}) was computed as follows: The total recording time, T_{tot}, was divided into N intervals (1,..,n, …,N) of a