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Systematic time-dependent visualization and quantitation of the neurogenic rate in brain organoids

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ABSTRACT

Organoids mimicking the formation of the brain cortex have been demonstrated to be powerful tools for developmental studies as well as pathological investigations of brain malformations. Here, we report an integrated approach for the quantification of temporal neural production (neurogenic rate) in organoids derived from embryonic brains. Spherical tissue fragments with polarized cytoarchitectures were incubated in multiple cavities arranged in a polymethylmethacrylate chip. The time-dependent neurogenic rate in the organoids was monitored by the level of EGFP under the promoter of *Tbr2*, a transcription factor that is transiently expressed in neural fate-committed progenitors during corticogenesis. Importantly, our monitoring system exhibited a quick response to DAPT, a drug that promotes neural differentiation. Furthermore, we successfully quantified the temporal neurogenic rate in a large number of organoids by applying image processing that semi-automatically recognized the positions of organoids and measured their signal intensities from sequential images. Taken together, we provide a strategy to quantitate the neurogenic rate in brain organoids in a time-dependent manner, which will also be a potent method for monitoring organoid formation and drug activity in other tissue types.

1. Introduction

Organoid culture systems that mimic brain formation derived from pluripotent stem cells have been demonstrated to be promising models for developmental [1–3], evolutionary [4,5], and pathological studies, especially in microcephaly resulting from mutations in MCPH genes [6] and the recently investigated Zika virus infections [7–9]. Although those organoids display the typical cytoarchitecture of the developing cortex, including apicobasal polarity and laminar formation, high heterogeneity among the self-assembled organoids and stochastic growth patterns are observed [10,11]. Therefore, for attempts to utilize organoids for experimental models to understand brain formation and for evaluations of drugs that act against brain dysfunctions, it will be essential to establish a method to monitor the cellular responses of a large number of organoids to overcome the heterogeneity of individual organoids. Along this line, semi-automated cellular activity recording systems based on quantitative time-lapse imaging strategies have been expected [12]. However, such quantitative temporal monitoring of cellular activities in a large number of organoids has been challenging due to the randomized position of each organoid in a gel culture or high mobility in floating cultures in conventional dishes or multi-well plates. Here, we present a novel method to visualize the temporal shift in neural production from fate-committed progenitors (hereafter referred to as the "neurogenic rate") in a large number of brain organoids using a multiple cavity chip prepared with microfabrication technology. Semi-automated quantification of the neurogenic rate was successfully demonstrated by applying image processing to the sequentially acquired time-lapse images.

2. Materials and methods

2.1. Animals

Transgenic mice expressing *Tbr2*-EGFP were obtained from the Mutant Mouse Regional Resource Center. The midday on which the virginal plug was detected occurred on embryonic day (E) 0.5. All

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mouse experiments were approved by the Institutional Animal Care and Use Committee of the RIKEN Center for Developmental Biology, and were conducted according to the guidelines for animal experiments at the RIKEN Center. On the day of the experiments, mice were euthanized through cervical dislocation and then used in the experiments. All efforts were made to minimize suffering.

2.2. Preparation of neural ball organoids

The preparation of the neural balls from the embryonic mouse brains was performed based on the previously described method [13] with some modifications. Briefly, the forebrains of E10.5 mice were harvested in ice-cold DMEM/F12 medium (Invitrogen) supplemented with 2.9 mg/ml D-(+) glucose (SIGMA). Dissected embryonic forebrains were digested in 0.25% trypsin in the same medium at 37 °C for 30 min followed by mechanical fragmentation by gentle pipetting. Tissue fragments were incubated for overnight in the culture medium (dissecting medium with 10% fetal bovine serum) to allow for self-formation of the neural balls. Subsequently, filtration of the neural ball size was performed using four types of cell strainers (40-, 70-, 100-, and 500-μm pores, Corning, USA). The sizes of the neural balls in each fraction were measured from microscopic images. Prism 4 (GraphPad Software, USA) was used for the statistical analyses.

2.3. Preparation of the multicavity chip

The multicavity chip used in this study was designed by authors and manufactured by STEM Biomethod Corporation (Japan) based on a previously described method [14] with some modifications. Multicavities (200-μm diameter and 200-μm in depth) in a triangular arrangement were bored into the polymethylmethacrylate (PMMA) chip using a programmable micromilling system (PMT Corp., Japan). Subsequently, another PMMA plate caved to have a medium chamber and liquid handling canal was placed on the chip and bonded by press heating. The surface of the chip was coated with platinum using an ion sputter unit, and the entire chip was immersed in 5 mM polyethylene glycol (PEG) carrying a thiol group in an ethanol solution to produce a non-adhesive surface. The chip was rinsed thoroughly in distilled water and 50% ethanol and then immersed in the culture medium prior to use.

2.4. Time-lapse image acquisition

Neural ball organoids with a size of 70–100 μm were harvested in the culture medium as described above and applied to each chamber in the multicavity chip for observation. Each neural ball was loaded to each cavity by the gravity flow. The medium was changed to remove excess neural balls from the chamber prior to start observation, and subsequently once per day during incubation period. Multi-area time-lapse images of the multicavity chip were acquired using a 10× objective lens on an inverted AS MDW microscope (Leica, Germany) equipped with a motorized XYZ stage and a temperature and CO_2 controlling chamber (Tokken Inc., Japan). The bright field (BF) and EGFP signals were acquired sequentially every 1 h for up to 72 h at different depths. Z-positions were set to acquire every 5-μm step to contain signals of 100-μm depths. The time- and z-series of the images were reconstructed using MetaMorph (Molecular Devices, USA).

2.5. Immunostaining

The immunostaining protocol for the cryosections of the fixed samples has been described elsewhere [15]. The primary antibodies were as follows: mouse anti-ZO-1 (1:250; Zymed, #33-9100), rabbit anti-Tuj1 (1:2000; Covance, #PRB-435P), mouse anti-Tuj1 (1:500; Covance, #MMS-435P), rabbit anti-Tbr2 (1:1000; Abcam, #ab23345), rabbit anti-phospho-histone H3 (1:2000; Upstate, #06-570), rabbit anti-Tbr1 (1:1000; Abcam, #ab31940) and mouse anti-PCNA (1:100; Oncogene Research, #NAO3). The secondary antibodies (1:500; Molecular Probes) were Alexa488, Alexa555 and Alexa647. DAPI (Molecular Probes) was used for the counterstaining of the nuclei. The samples were observed using a confocal laser microscope (TCS-SP2, Leica, Germany).

2.6. Quantitative image analyses

The center of each cavity for each time point was automatically detected in a BF image using the Hough transform for circle detection (Fig. 4D). Each BF image was first filtered with a Gaussian blur filter and then with a canny edge filter to enhance the edges of the cavities. By applying the circular Hough transform with the known radius of the cavities, the candidate positions of the centers of the cavities were detected. In the majority of cases, multiple positions were obtained for each cavity. To derive a single center position for each cavity, the candidate positions within the specified distance were clustered, and then their centroids were used as the center positions of the cavities.

The neural ball organoid in each cavity was detected using the images of the EGFP signals (Fig. 4Ea). To detect the part of an organoid with a low EGFP signal within an inhomogeneous background, the entropy filter was used to enhance the low intensity organoid-like regions. This method is simple but was sufficient for rough estimation. More sophisticated background extraction may improve the accuracy of the estimation. By combining the original images of the EGFP signal and their entropy-filtered versions (Fig. 4Eb), a rough mask was derived for each organoid. The sizes and EGFP intensities of organoids in each cavity were calculated by using the masks. All image processing was implemented with Matlab (MathWorks, USA).

3. Results and discussion

To establish a semi-automated quantitative imaging system for the neurogenic rate, we chose to utilize organoids derived not from pluripotent stems cells but from partially digested embryonic mouse brains because of the better homogeneity in the cytoarchitecture among organoids, which is required for efficient system optimization. Tomooka and his colleagues described the preparation method and named the brain organoids “neural balls” [13]. We confirmed that the tissue polarity of the developing brain is maintained based on the apical marker ZO-1 and the basal localization of Tuj1-positive neurons in the fragmented tissue as well as the neural balls cultured in collagen for 110 h (Fig. 1A). We noticed heterogeneity in the sizes of the neural balls prepared by the originally described method and improved this method to obtain homogenously sized neural balls by the combined use of cell strainers with different pore sizes (Fig. 1B).

To quantitate the neurogenic rate in each neural ball, the following conditions are required: 1) each neural ball should be cultured separately without adhering to others, 2) the positions of the neural balls should be maintained despite the horizontal and vertical movements of the microscope stage during multi-area time-lapse imaging, 3) the material for culture should be transparent for imaging, and 4) the material should not adhere to the neural ball so that the ball can maintain its spherical architecture. To fulfill all these requirements, we utilized a microfabricated PMMA chip with multiple cavities that was prepared by micro-electro-mechanical systems (MEMS) technology [14]. The surface of the multicavity chip was chemically modified with PEG to

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