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Microengineered Tools for Studying Human Brain Organoid Biology

THESIS

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MASTER OF SCIENCE

in Biomedical Engineering

by

Shijun Yan

Dissertation Committee:
Associate Professor Elliot Hui, Chair
Assistant Professor Medha M. Pathak
Professor William C. Tang

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DEDICATION

To my parents and friends,

for your support and patience.

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ABSTRACT OF THE THESIS

Microengineered Tools for Studying Brain Organoid Biology

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Associate Professor Elliot Hui, Chair

Human brain organoid, derived from pluripotent stem cells, is the organotypic multicellular construct which emerging as a promising tool for modeling human brain development and related disease. The developing organoids experience varied mechanical forces in vivo, these forces are essential factors for regulating organoids development and maturation. However, it is a great challenge to completely reproduce the in-vivo like complicated microenvironment in vitro, and the limited biomechanical culture system may result in immature organoids and unreliable physiologically models.

In current brain organoid 3D cultures, the neurulation-like development often comes with the uncontrolled generation of plentiful neuroepithelial tissues, a.k.a. neural rosettes. Each of them exists in indiscriminate sizes and shapes and develops as morphogenesis center individually. Thus, the presence of numerous rosettes confounds reproducible morphogenesis events and may limit the coordinated tissue development.

One of the purposes of the study was to develop the microfluidic system to probe the effects of fluidic shear stress (FSS) on the in vitro development of human brain organoids. In chapter 2 and chapter 3 different systems were designed and tested respectively, in order to manipulate the shear stress forces on organoids and study the biology of in vitro organoid models. In chapter 4, a culture platform for engineering induction single rosette generation was built. The geometric confinement of initial tissue using stencil micropatterning shows reliable results on generating iPSC-derived neural differentiation islands and singular neural rosette formation.

Chapter 1: Introduction

In vitro brain models

Traditionally, 2D neural cultures have been used to study mouse and human neural differentiation of pluripotent stem cells. This approach is able to recapitulate partial key mechanistic pathways and induce differentiation coordinated with relatively simple techniques. The numerous neurons provided by this simple culture method advanced the relevant insights into neural cell biology. However, the disadvantages of the 2D culture of stem cell neural differentiation are equally notable. The interactions with substrates may exceed cell-cell and cell-ECM interactions. The cells or tissues derived from organs often become flat in culture dishes, which are unable to represent their morphological characteristics completely and further affect their biological behaviors [1]. The brain, in particular, has extremely sophisticated 3D structures and extracellular matrices, making it impossible to recapitulate complete neural tissues in 2D culture systems. In addition, the interaction between cells and the extracellular matrix, the growth factors may be altered in the 2D setting [2].

Compared to 2D systems, 3D cell culture systems require a more elaborate experimental setup and costly fabrication process, but it also offers a more physiological relevant microenvironment. Neuroscientists have employed several 3D culture systems for neural spheres or brain organoids [3, 4].

Brain organoids

In recent years, exploiting the self-organizing and differentiation abilities of human pluripotent stem cells (hPSCs) to generate the 3D tissue structures, known as organoids or organ spheroids, has tremendously advanced our understanding of human brain organ development and function. The 3D brain organoids can recapitulate the brain's cytoarchitectural arrangement not only at the cellular level but in development trajectory. Therefore, it provides new opportunities to model human organogenesis, homeostasis, and disease [5, 6]. The self-assembly ability during hPSCs differentiation in brain organoids allows for the emergence of complex structures, including region-specific organoids[7].

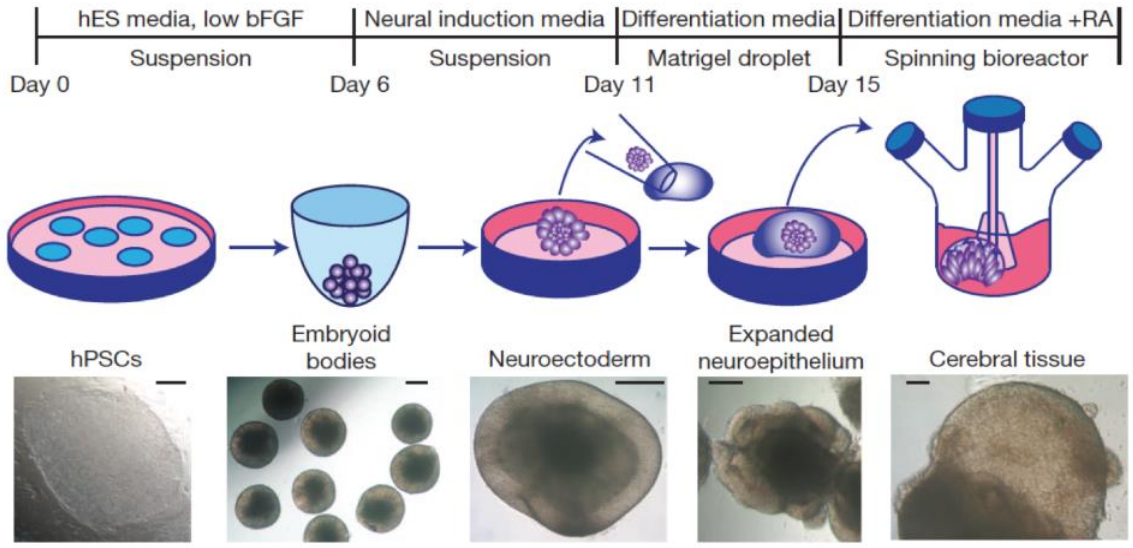


Fig.1.1. Schematic of a traditional 3D cerebral organoids culture system [8].

The most widely used protocol for generating brain organoids from hPSCs is the 3D suspension cultures maintained by spinning bioreactor with sequential growth factors. There are several advantages to this approach. Firstly, the 3D spinning culture improved the oxygen and nutrient delivery to support the growth of the tissue [1]. Moreover, it is a reliable approach for long-term culture (for almost two years) without genomic

alterations and enables the spontaneously in-vivo neurulation-like events occurring [9]. These 3D neural cell aggregates potentially have access to the great diversity of neural cell types and functional differentiation states.

Although this method has been widely employed due to its physiologically relevant and simplicity, it still has room for substantial improvement. Specifically, the simplicity of traditional 3D culture often comes at the expense of a lack of precise microenvironment control [10]. It is unable to model the tissue-tissue interactions at the organ level, and it has limited capacity to mimic the complex in vivo growth factors and biomechanical forces that a developing organ experience. The lack of biomechanical control raises challenges to generate more in-vivo like organoids and the reliability of in vitro human organ models. Further studies of organoid biology at the cellular and organismal levels are required to understand the influence of biomechanical forces on cell fate. One possible solution that has been undertaking is coordinating organoid culture with organ-on-a-chip technology [11].

Neural rosettes

Morphogenesis is a fundamental biological process during embryonic development that causes an organism to shape its body. Rosette formation is one of the critical morphogenetic processes that take part in various tissues and organs, which might reveal different functional cell stages [12]. Neural rosettes formation is a prominent feature during neural development, and the rosettes are morphologically identifiable structures containing neural stem cells to equipoise proliferation and differentiation. In the rosette structure, the cells surround a central lumen and undergo mitosis at the luminal side. The randomized generation of neuroepithelial rosettes was commonly

observed in current 3D hPSC-based culture systems. Even in a 2D culture system, the persistent existence of neural rosettes was reported, and they simulate early neurogenesis remarkably well [13, 14].

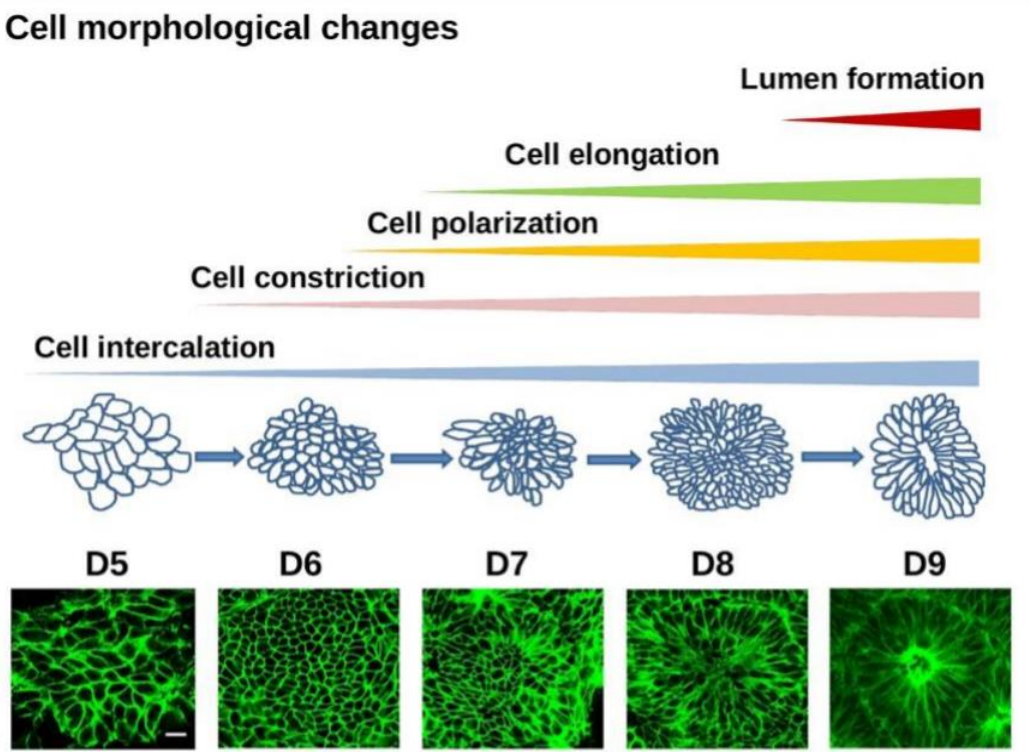


Fig.1.2 Cell morphology changes during neural formation [15].

Nevertheless, the randomized generation of numerous neural rosettes of varied shapes and sizes within the cultured tissues inevitably confounds the study of morphogenesis and further limits tissue maturation. It also provides large variability in cytoarchitecture even within the organoids produced in the same batch. Interestingly, the routinely single rosettes formation was found during the mouse embryonic stem cell-derived neuroepithelial cysts tissue culture [16, 17]. For hPSC-derived central nervous system tissues, manually isolation from culture tissues is the most widely used in vitro approach to get a single rosette [18, 19].

Recently, several studies showed that the geometric confinement of initial hPSC derived tissues on the 2D micropatterned substrate with a specific manner could be another solution for inducing single neural rosette [20-22].

Organoid-on-a-chip

Organ-on-a-chip is based on the technologies advanced in microfluidics, cell biology, bio-microelectromechanical systems, and biomaterials, mimicking the microenvironment of a physiological organ. This technology provides spatial and temporal control of the cellular environment and paves the way for rapid discoveries of human organ developmental biology and pharmaceutical sciences. It recapitulates the functional units of human organs in vitro by controlling the fluid flow, extracellular matrix properties, as well as biochemical and biomechanical cues [10, 23]. In addition to mimicking the biomechanical microenvironment, the OOC system permits the flow going through the tissue in a designated direction and controlled flow rates, which can be used to run the biological assessment of shear stress force at the single cell level. Beyond ensuring that the engineered tools can house a healthy population of cells, several parameters are vital for building a reliable, reproducible, and manufacturable platform.

The source of the tissue is one of the most significant components in an OOC system. The advanced understanding of pluripotent stem cell engineering has further improved the construction of 3D organ models. Organoids, as the frontier of stem cell research, is the ideal candidate for OOC [24, 25]. Many organoids-on-a-chip platforms are present in the literature and are widely used in clinical practice. For example, Wang et al. developed a perfumable OOC system that provides an in-vivo like brain environment allowing 3D brain organoids culture, in situ neural differentiation, organoid

regionalization, and organization in a controlled manner. Compared with 2D cultured organoids, mechanical fluid flow displays an essential role in brain organogenesis progress [11]. Another example of the microfluidic organoids-on-chip culture system was developed by Kasendra et al. They built a bioengineered and physiologically relevant microfluidic human small intestinal-on-a-chip, and this microfluidic chip has proved capable of replicating the essential intestinal villi-like structures and functions [26].

Fluid shear stress is a critical factor in advancing the understanding of cell biology, as an important mechanical force a developing cell would encounter. The flowing culture on OCC generates shear stress on cells; applying proper shear stress could improve organ maturation and induce polarity [27]. Although several studies have reported the effect of biomechanical forces on cell development using the OOC platform, to our knowledge, there was no relevant research on biological assessment of fluid shear stress on brain organoid biology.

Flow characteristics

The fluid shear stress (FSS,τ) is a mechanical force caused by friction between fluid particles, due to fluid viscosity. In the biology and microfluidics applications, shear stress is defined as the friction force of a biological fluid flow acting on cells or tissues. The FSS often presents in dyne/cm^2. The magnitude of the fluid shear stress depends on the radial position of the particle experiencing stress [28]. In order to calculate the FSS in a microfluidic platform, one has to get the velocity profile for the considered channels by solving the Naiver-Stokes equation, either numerically or analytically. The flow does not share the same velocity at every point in the microfluidic channel. Instead, the velocity profile can be approximated to be parabolic in most cases of laminar flow. The fluid

velocity reaches the maximum value at the center of the channel and is zero at the wall boundary. At the wall of the channel, cells usually experience the maximum value of fluid shear stress, whereas, at the axis of the channel, the cells fell the minimum FSS level. In biological experiments, the wall shear stress is commonly used when calculating the shear stress effects. [29].

In vivo shear stress

In vivo shear stress is mostly caused by the friction force of blood flow against the wall of vessels. Many cell types grow in a moving fluid environment, and it is especially important to culture cell types which occur in biofluidic systems under flow conditions, such as epithelial cells of the kidney, neural cells [30]. Many studies have been carried out to investigate the development of suspension-adapted cells to shear forces, and this mechanical stimulus has been proved to affect cell morphology significantly, cytoskeleton reorganization, gene expression, activation of ion channels [31, 32]. Moreover, there is evidence showing that shear forces can accelerate and improve retinal and kidney organoid maturation and differentiation [33,34].

The in vivo FSS values usually range from 0.1 to 120 dyn/cm², mostly depending on the tissue types and the size of the organism [35].

Shear stress in microfluidics

Microfluidics technology takes advantage of its physiological relevance, enables the biomimetic in vitro biology experiments, and recently, it has been employed in organ-on-a-chip to model the organ functional units in vitro. However, the new challenge that comes along to these applications is the lack of proper control of mechanical forces such

as shear stress. Therefore, it is crucial to study the flow shear stress theory before designing a microfluidic experiment.

Microfluidic transport equations are used to determine laminar flow regimes, the average velocity required for convection-dominated flow, and channel geometry and flow rates that ensure controlled shear stress on cultured cells. Average fluid velocity can be regulated by channel geometry, according to equation $v_{avg} = \frac{Q}{wh}$, where Q is the flow rate, w is channel width, and h is channel height. It is considered a flow is laminar if the Reynolds Number (Re) is less than 2300 and is turbulent if it is greater than 2300. In a rectangular channel, Re can be approximated by equation $Re = \frac{\rho v_{avg} D_H}{\mu}$, where ρ is fluid density, μ is fluid viscosity, and D_H is the hydraulic diameter, $\frac{2wh}{(w+h)}$ for a rectangular channel [36]. For most microfluidic devices, the flow is laminar due to the small dimension. To control the shear stress level in the microfluidic system, parameters such as channel dimensions and geometry, the flow rates and the flow delivery method should be considered [29]. There are several methods to measure the FSS in a microfluidic biology experiment. For laminar flow, one easiest and useful way is using fluid dynamics theoretical and numerical methods to evaluate the approximate shear stress. In some cases of simple geometries such as a wide rectangular channel or a cylinder channel, the analytic solution is straightforward. For instance, for a rectangular canal of dimensions, the wall shear stress can be approximated as $\tau = \frac{6\mu Q}{h^2 w}$, where τ is the maximum wall shear stress in dyn/cm² , Q is flow rate passing through, μ is the dynamic viscosity of the media (dyn·s/cm²), h is the height of the channel and w is the width [37]. For a cylindrical channel, the shear stress can be computed according to the equation $\tau = \frac{4\mu Q}{\pi R^3}$,

with R the radius of the cylinder [29]. As is evidenced by the equation, the magnitude of FSS is inversely proportional to h^2w of R^3. Thus, FSS is incredibly sensitive to changes in the dimensions of the channel. However, these simple calculations do not represent the shear stress for complex geometries, where it is generally better to run a computer simulation for the theoretical study to estimate the shear stress.

Summary

The invention of organoids has great potential to complement and improvement the study of human developmental biology and pathology. Microfluidic organoid-on-a-chip platforms have the unique advantage of precise control of microenvironment over other in vitro culture models. In the following chapter of this thesis, the focus will be on design, fabrication, and test of microfluidics devices for studies of brain organoid biology.

All these work was performed in collaborate with the Pathak lab. The design ideas came up based on their interests in understanding how mechanical forces modulate neural stem cell fate in development. Hui lab was mainly responsible for device design, fabrication, testing, and involved in some of the cell experiments. Still, most of the biological experiments were done by Dr. Jami Nourse and Dr. Mitradas Panicker in the Pathak lab.

For chapter 2 and 3, the aim is to develop a device that can manipulate the shear stress during brain organoid culture to study the brain organoid biology.

In chapter 2, a simple gravity-based culture system was developed, and it was demonstrated that the system could manipulate the shear stress by changing the media

level and porous membrane of the well-plate like device. Nevertheless, due to lack of precise control, and the frequent media change required, it was not suitable for long-term organoid experiments in the Pathak lab.

In chapter 3, the second-generation device: a peristaltic pump driven system was built. The system consisted of a homemade peristaltic pump and a PDMS chip for hosting organoids. It could provide a more precise and smooth flow control over the gravity-based system, and successfully engineered the shear stress on brain organoid. However, the evaporation of the culture media caused by the overheating problem of the pump dried the culture chamber after three days. Performance optimization of the system is required in order to run a long-term experiment.

The goal of chapter 4 is to develop an approach to generate single neural rosette within iPSC-derived tissues, the role of micropatterned morphologies in singular neural rosettes formation was investigated using stencil micropatterning technique. The method is based on Knight et al. paper in eLife [20]. The experiments found that the circular patterns of 250 μm diameter were capable of generating cell islands within iPSCs, and they did convert into neural cell types. However, we did not get the same efficiency with the previous studies, and further experiments are needed to develop a more method to engineer induce singular neural rosette emergence.

Chapter 2: Gravity Based Microfluidic Shear Stress Culture System

Background Information

Organoids are often grown in spinning flasks that offer 3D suspension culture, which improves diffusion of oxygen and provides efficient nutrients to support the growth of organoids. These culture flasks have the advantages of introducing a low-shear stress environment to these 3D tissues, which is significant because both hPSCs and organoids have been proved to be sensitive to FSS [38,39]. However, these commercially available spinner flasks are not specially designed for organoids; they limit the efficiency and production of organoids and also require a high volume of media for cultivation [7]. Despite lower reagent volumes, the organoids-on-a-chip technology can provide biomechanically controlled culture systems and simulate various mechanical forces such as shear force [40]. Compared with unfixed interaction rules in biological self-assembly systems, the strategic combination of micro-engineering principles can perform the biological assessment of mechanical forces at the single cell or organismal level.

There are several existing choices of the flow control system, such as pressure control, syringe pump, and peristaltic pump, these pump-based systems have the ability to generate an accurate flow control and have shown reliable results in system functionalities. However, the primary disadvantages of the pump system are the requirements of costly manufacture, complicated tubing connections, and elaborate manipulation. On the other hand, gravity-based flow microfluidic systems have the great

advantages of low cost, power-free, easy operation, and stability, and it has been successfully implemented to cell evaluation and cell culture application [41-43].

Evaluating the shear stress in microfluidic culture systems can be done in several ways. The wall shear stress calculation of simple geometries can be computed using a straightforward equation. For a cylindrical channel, the wall shear stress can be calculated as $\tau = \frac{4\mu Q}{\pi R^3}$, where Q is flow rate, μ is fluid viscosity, R is the radius of the cylinder. The formula is really useful to estimate the shear stress in simple geometries, even precisely, but it is generally better to run a computer simulation [29].

Specific Aim: The overall aim of this study is to develop a gravity-driven pumpless microfluidic device that can manipulate shear stress in the culture chamber for biological assessment of brain organoids.

The main goals are:

1. Design and build a microfluidic device using hydrostatic pressure to facilitate the slow, longer-lasting medium flow.
2. The tool should be able to hold multiple organoids at once for exposing organoids to shear stress ranges from 0.01~0.018 dyn/cm², and set up a control group for static culture.
3. Allow imaging access from the top and bottom, and have the external dimensions of a multi-well plate.

4. Analyze the fluid shear stress level and its effect on brain organoids.

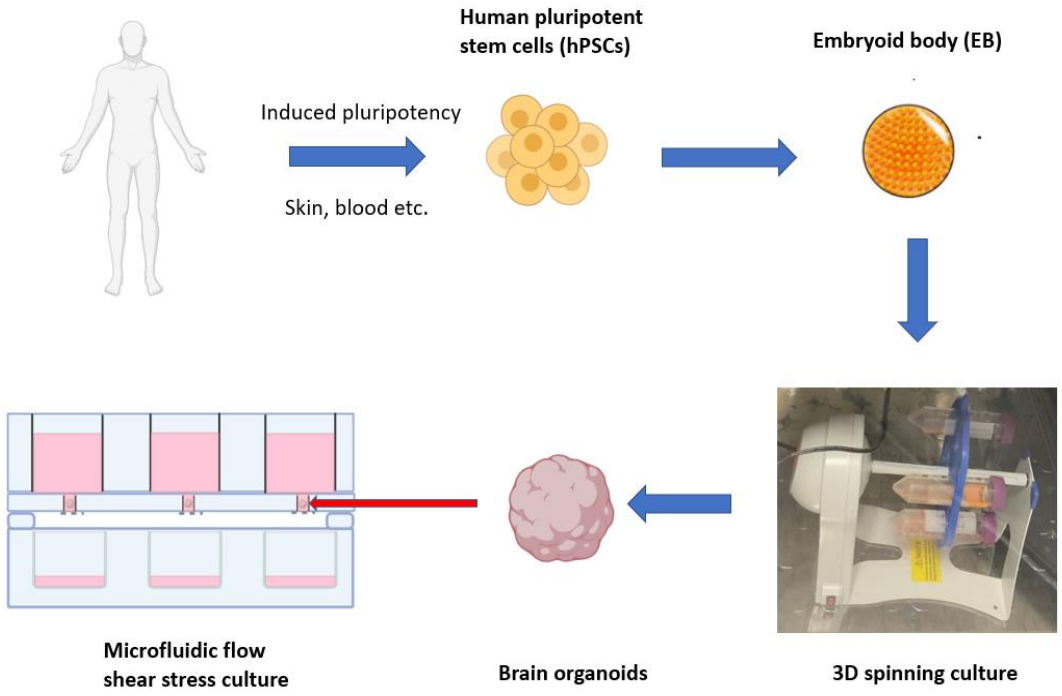


Fig.2.1. Schematic overview of the proposed human neural organoid-on-a-chip FSS culture platform.

In this study, an innovative power-free microfluidic system for analysis of the effect of shear stress on brain organoids was developed (Fig.2.1). It can control the flow rate by changing the volume of the culture media and different polycarbonate porous membranes. It was demonstrated that the platform allows for introducing required FSS to human brain organoids. The further biological assessment was not performed due to several limitations of the tool.

Materials and Methods

The device design and fabrication were done with the help of Erik Werner. The brain organoids and the detailed biological analysis in this experiment were provided by the Pathak lab.

Device Fabrication

Fig.2.2 presents the schematic diagram for the fabrication procedure of the microfluidic shear stress culture platform for studying brain organoid biology. The well plate structures and the organoid housing chamber were designed according to the technical data sheet of Falcon 6-well cell culture plate, to ensure that the device can be stacked on the top of a regular 6-well plate and fit in the lid. The platform was plotted and modeled using AutoCAD 2018, as shown in Fig.3A. The 6-well-plate like device consisted of two main layers made of acrylic, a ½ inch and a ⅛ inch transparent acrylic sheet were laser-cut by Universal VLS 4.60 laser cutter. The material for the laser cutting was Clear Scratch and UV-Resistant Cast Acrylic Sheets from McMaster-Carr.

The surfaces of the acrylic sheets were washed under deionized (DI) water. The fabricated acrylic sheets were put into the Ziploc bag, which was filled with a solution of ~1% Micsoap, then sonicated the bag for 20 minutes using Branson B2510 sonicator. After the cleaning process, the two acrylic pieces were bonded following a standard thermally solvent PMMA-PMMA bonding protocol [44].

Next, the bottom surface of the device was cleaned by isopropanol, the biocompatible instant-bond adhesive (Loctite 4601) was coated around the edge of the small organoid chamber. Before allowing the glue to dry, a piece of porous polycarbonate membranes (Sterlitech Corporation, 0.1, 0.2, 0.4, 1.0, 3.0, 5.0, 8.0 μm in diameter, PVP-Free) was applied to each chamber. The device was then cured under UV light for 30 mins. PDMS (Dow Corning, Sylgard) was mixed at a ratio of 10:1 (silicone elastomer base: curing agent), then pour the mixed PDMS prepolymer into a petri dish to a depth of about 3mm. The dish was then placed into a vacuum chamber for 30 mins to completely

removed all the bubbles from the mixture. After degassing the PDMS, the dish was cured for 4h at 65°C in the oven. The PDMS layer was cut into pieces with the desired shape and size using a sharp blade and bonded to the corners of the device by the same biocompatible adhesive above.

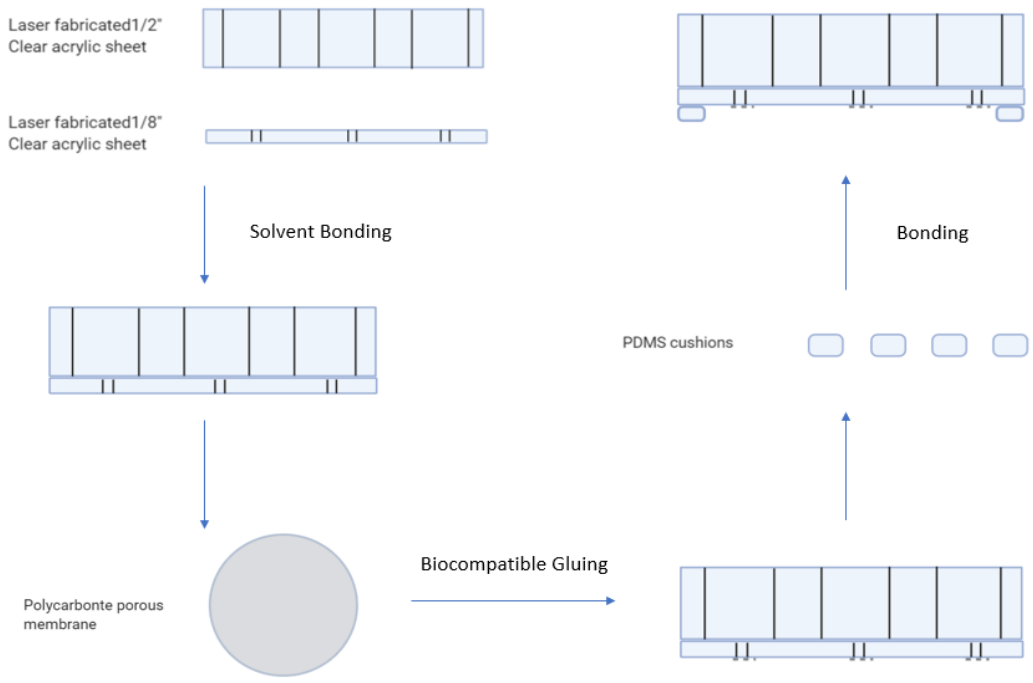


Fig.2.2. Fabrication procedure for the human neural organoid-on-a-chip platform.

The device consists of two main layers, a 6-well plate layer for holding culture reagent (Fig.2.3B) and an organoid culture layer for housing the organoids (Fig.2.3C). The outer dimension of the 6-well plate is 124.2 × 81.4 mm, each well is 35 mm in diameter. The organoid culture chamber has the same outer dimension as well plate, six holes of diameter 1.5 mm were drilled to serve as organoid culture chambers.

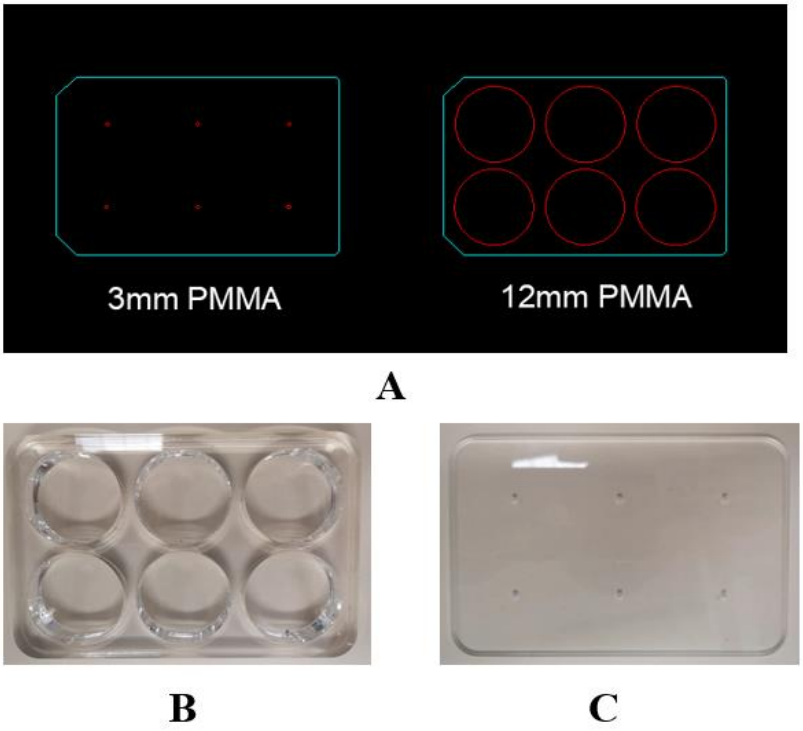


Fig.2.3. Fabrication procedure for acrylic “6 well-plate”. (A) Schematic design for the well plate. (B) Fabricated PMMA layer for holding culture media. (C) Fabricated PMMA layer for housing 1mm brain organoid.

Device testing

Fig.2.4 shows the image of the whole system setup, including the microfluidic device and a 6-well plate. To simulate and validate the design, two identical devices were made. Five chambers of each device were covered by different pores size semipermeable membranes (0.1, 0.2, 0.4, 1.0, 3.0, 5.0, 8.0 μm) for initial testing, and one chamber was completely sealed to provide a static culture and served as the control group. A 1/32 or 3/64 inch in diameter alloy steel ball (McMaster-Carr) was placed into each culture chamber to act as an “organoid” for simulation (Fig.2.5).

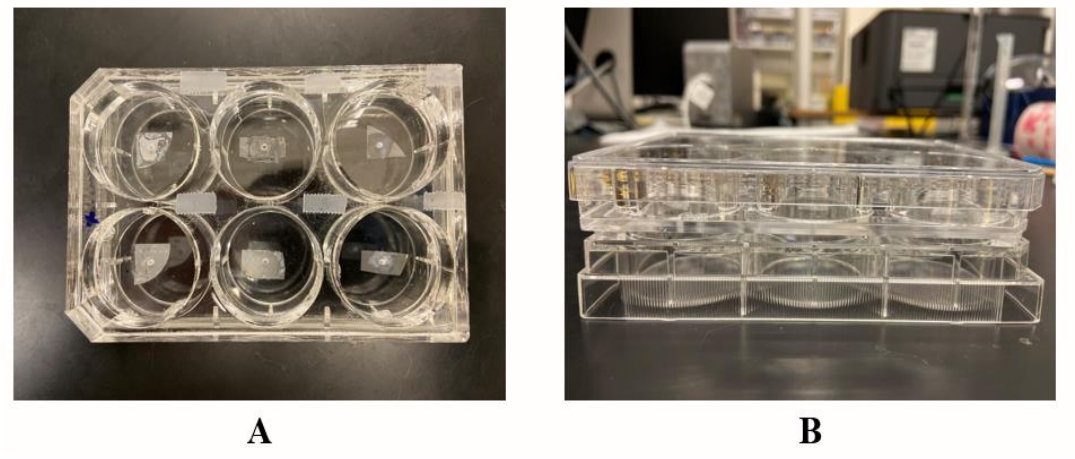


Fig.2.4. Human brain organoid-on-a-chip platform experiment setup. (A) Top view of the device. (B) Side view of the whole experimental setup.

The device was stacked on the top of the reservoir: a 6-well culture plate (Falcon ref #353046), and dispensed 10mL water into each well then covered the lid. The culture platform was immediately placed in the culture incubator. After 24 h stay in the incubator, the platform was transferred to a microscopy system for testing the seal of the semipermeable membrane and measuring the flow rate. To get the flow rate, the liquid in the corresponding reservoir was pipetted out to a measuring cup and recorded the liquid volume. After testing, calibrated the system again and repeated the testing steps for the new sample. The flow rate values were determined from multiple repeated experiments.

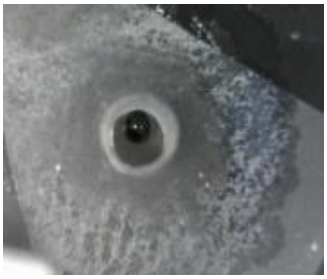


Fig.2.5. The simulated organoid (3/64″ in diameter) under microscope.

Analysis of the shear stress

The equations of shear stress were used to determine the initial experiment regimes to test and characterize the microfluidic culture device. The shear stress, for Newtonian fluids, can be calculated according to Newton's law with the following equation: $\tau = \mu \frac{\partial u}{\partial y}$, where μ is the dynamic viscosity of the fluid, u is the velocity of the fluid along the boundary, y is the height above the boundary, therefore, $\frac{\partial u}{\partial y}$ is the velocity gradient, in other words, the shear rate. In this experiment, the spheroidal organoid was placed in a cylindrical chamber, but the calculation for simple geometry cannot estimate the exact shear stress since the organoid occupied most of the channel. Therefore, considering the size of the organoid, the wall shear stress can be approximated by $\tau = \frac{4\mu Q}{\pi(R-r)(R^2-r^2)}$, where μ is the fluid viscosity, Q is the function of volumetric flow rate, R is the radius of the channel, and the r is the radius of the organoid sphere. Therefore, the shear stress can be controlled by changing the channel dimensions or the fluid flow rate.

In this model, the channel radius was 0.75 mm, and the organoid can be approximated as a sphere of radius 0.4~0.6mm. The maximum filling volume of the well is 12mL, and the media should be changed regularly to maintain the shear stress level. Ideally, under certain shear stress conditions, the lower flow rate can reduce the medium changing frequency, also can maintain the liquid level to keep a more stable flow. According to the equation, for 1 mm organoids, to successfully generate 0.01~0.018 dyn/cm² fluid shear stress in the culture chamber, the flow rate should be manipulated in the range of 3.3~5.8 mL/day.

FSS is also calculated using an analytical solution. A 2D computational model of the microfluidic device was computed in the COMSOL Multiphysics® (Version 5.1, COMSOL Inc.) software package to generate the flow velocity profile and simulate the shear stress along with the organoid culture chamber. In COMSOL, the shear stress module was defined as the following equation: τ = spf.sr*spf.mu where spf.sr is the shear rate and spf.mu solution viscosity.

Testing and Results

Flow Rate Testing

The relations between the flow rate and the pore size of porous membranes are shown in Table.2.1. The initial liquid volume was 10 mL; the average values were worked out by five repeated experiments. The groups in which the flow rate exceeded 10mL/ day provided over ranging shear stress to organoids and did not conform to the experimental requirements.

Flow Rate Of the System (mL/day)

Pore Size (μm) / Organoid Size (mm)	0.1	0.2	0.4	1	3	5	8	10
0.8	0.8	2.4	3.9	5.6	8.8	>10	>10	>10
1.2	1.4	1.8	2.4	3.7	5.5	8.2	>10	>10

Table.2.1. The flow rate testing results of varying sizes of porous membranes and organoids.

The wall shear stress in this model can be approximated by $\tau = \frac{4\mu Q}{\pi(R-r)(R^2-r^2)}$. According to the equation, for a 1.2 mm organoid, the flow rate should be manipulated in the range of 2.1~3.7 mL/ day to apply 0.01~0.018 dyn/cm² shear stress on the organoid. For a 0.8 mm organoid, a flow rate of 4.8~9.8 mL/ day is required to achieve the targeting shear stress range. Based on the flow rate chart, the porous membranes with 0.4 and 1.0 μm pore sizes were used. The culture media needed to be refilled every 24 hours to maintain the shear stress level.

COMSOL Simulation

A 2D computational model of the microfluidic device was computed in the COMSOL Multiphysics to run simulation for theoretical study of the FSS. Fig.2.6 is the 2D simulation of the FSS at a flow rate of 5 mL/ day with a 1.2 mm organoid in the culture chamber. As it can be observed in the figure, the culture chamber increases in FSS as the liquid flows down to the narrow space, and the organoid experiences approximately 0.014 dyn/cm² shear stress, which meets the requirements of the study.

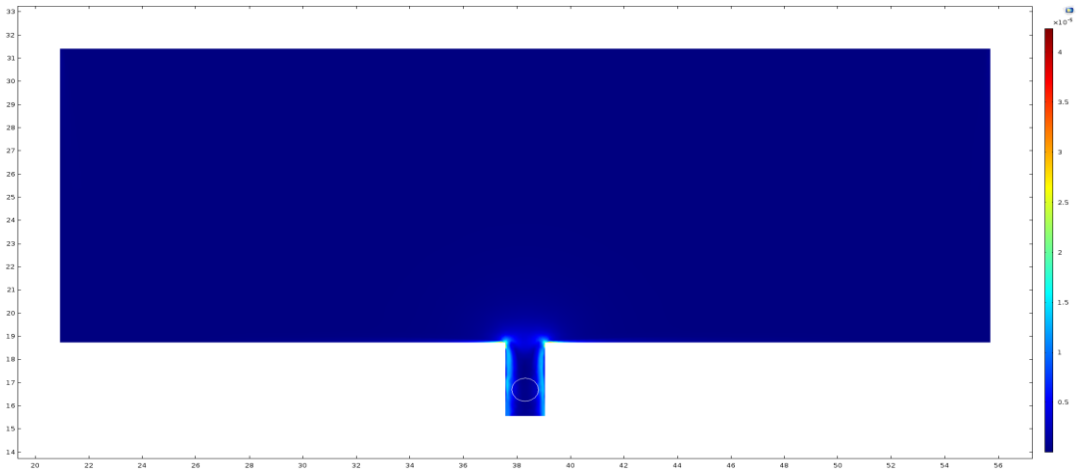


Fig.2.6. Fluid shear stress simulation in COMSOL.

Discussion

The purpose of this study was to develop a microfluidic device to culture organoids under a tiny FSS environment and run the biological assessment of FSS. A gravity-driven microfluidic device was built, considering the costly manufacture, complicated tubing connections, and elaborate manipulation of the pump flow control system. To ensure the device is capable of generating continuous shear stress, it was crucial to get a precise and smooth flow control. However, the flow rates of the system were not constant due to the dropping liquid level. The average flow rate in 24h cannot fully represent the dynamic flow the organoid experienced, and the shear stress might be out of the targeting range. Also, in order to maintain the flow rate and shear stress, the media need to be refilled at least once a day.

In this study, a novel gravity-driven approach for testing the shear stress on a single brain organoid was introduced. This method has the potential to be extended to culture other types of organoids or even cells. The main advantages of the gravity-based FSS culture system are as follows. (1)This system is a pumpless microfluidic system that avoids costly manufacturing, complicated experiment setup, and elaborate manipulation. (2) By changing the polycarbonate porous membrane or the initial medium volume, this experiment can be easily regulated to corresponding conditions and expose cells or tissues to certain physiological levels of fluid shear stress. (3) The device has been designed to fit in the microscope holder and has the imaging access from above, from bottom or both. Thus, it is capable of integrating mechanical force measurements and imaging cellular structures formation. (4) The 6 well-plate like device enables researchers

to test a series of experiments at the same time. It greatly saves time and effort over operating multiple devices simultaneously.

Although this system was able to generate and test the FSS in brain organoid culture, there were still several critical limitations and challenges. First of all, the flow rates of the system strongly depend on the size of the organoid and the liquid level in the well, and they are both changing over time. Therefore, the organoid experiences an unstable flow and dynamic shear stress force, both the numerical and computational methods calculate the shear stress using the approximated values; neither of them can predict the precise shear stress. In other words, the real shear stress on the organoid may be outside of the allowed range. Second, the effective permeable area of the porous membrane significantly affects the testing results. This is an inherent error of the system due to manual fabrication. As it is hard to unify the amount of the liquid adhesive and the bonding quality, the results obtained from the same experimental setup groups sometimes have an obvious difference. Finally, the chambers may be slightly tight for the mature neural organoids and had limited space for long-term culture. The organoids the Pathak lab has for this experiment ranged from 1.2 mm to 2.0 mm in diameter and will grow up to 3.5 mm in months, where the chamber is only 1.5 mm. However, the requirement of low shear stress limits the size enlargement of the culture chamber. The larger culture area requires a higher flow rate to achieve the targeting shear stress, which also requires more often media change. The fairly frequent media change would be a problem for researchers to run a long-term assessment.

These limitations caused the device not to meet the requirements of the experiments. The result of this initial testing prevents this design from a powerful tool for

study brain organoid biology, but it can still be used in other tissues that are not sensitive to shear stress or other cases that allows frequent media change.

Therefore, after the discussion with Pathak lab, we moved to a different design: a device that can provide more precise and smooth flow control and allow sufficient space for a long-term experiment.

Chapter 3: Peristaltic Pump Based Microfluidic Shear Stress Culture System

Background Information

By integrating the physiological microenvironments with human organoids, the OOC platform can replicate complex in-vivo physiological responses at the organ level. The controlling fluid enables better nutrient supply and offers the possibility to integrate with various sensors and actuators to simulate multi-organ interaction. Furthermore, the OOC system can manipulate the biomechanical forces with temporal and spatial precision, showing the feasibility of real-time monitoring biomechanical microenvironment by changing the flow direction and flow rates. Therefore, the selection of flow control systems is significant for developing OOC, especially when it comes to controlling fluid shear stress. As mentioned in the previous chapter, one disadvantage of the gravity-driven flow system was unable to provide precise and stable flow. To solve this problem, there are several existing options, such as syringe pumps, peristaltic pumps, pressure control systems. There are some things to avoid when dealing with shear stress, such as low response time and flow rate oscillations, and these drawbacks may result in a significant difference between the simulated shear stress and the real one [29,45]. In consideration of the advantages of low-price, ease of use and ability to recirculate the fluid, the peristaltic pump may be one of the best solutions for the long-term organoid culture application, although it has a major disadvantage of providing pulsatile flow due to the compression of the tubing [46]. A representative peristaltic pump control system example can be found in a microfluidic system developed for in vitro modeling of stomach organoids development. Lee et al. developed a stomach-on-a-chip platform that

allows long-term, 3D growth of human gastric organoids. With the use of the peristaltic pump, the system can mimic and apply the in-vivo like rhythmic stretch and contraction, and also deliver nutrients to organoids [47]. Besides, Homan et al. built a kidney organoids-on-a-chip system that was engineered in a 3D printed gasket. They utilized the culture system demonstrated that the flow-generated microenvironment enhanced the structural and functional development of human kidney organoids in vitro [34]. Although several works have reported the culture of hiPSCs-based brain organogenesis on the organ-on-a-chip, to our knowledge, no work has been devoted to the research of the role of fluid shear stress in brain organogenesis within these perfused 3D culture systems.

In fabricating a microfluidic perfusion culture device, several properties that need to be considered, including the selection of biocompatible materials, channel geometry, and fabrication techniques [48]. PDMS has been the most employed material to mold microfluidic devices because of its inexpensiveness, biocompatibility, deformability, gas permeability, and optical transparency [49]. Moreover, PDMS can be easy to mold structures at high resolution using soft lithography, and it can be bonded tightly to glass or another PDMS layer with a simple plasma treatment to avoid fluid leakage [50]. These characteristics made it an ideal material to offer compartmentalized microenvironments for culture cells and tissues under dynamic flow conditions.

In addition to the flow rates and the materials, microfluidic perfusion culture systems can be designed and operated to maintain shear stress level by manipulating the channel geometry. For rectangular channels with laminar flow, shear stress at the wall, τ, can be approximated according to equation $\tau = \frac{6\mu Q}{h^2 w}$, where μ is fluid viscosity, Q is fluid flow rate, h the channel height, and w is channel width. The shear stress can thereby be

controlled by changing the channel dimensions or by adjusting the pump flow rate. In the model, the medium (serum-free) viscosity is treated as water in room temperature and assumed to be 0.01 dyn·s/cm². The channels are designed 6 or 8 mm in width, 0.635 mm in height. Therefore, for a 6 mm width channel, to reach the desired FSS, the flow rate should range from 1.5~2.6 mL/h.

The primary aim of this study is to develop a peristaltic pump control microfluidic closed-loop system for culturing brain organoids under FSS. The main goals are similar to the requirements in Chapter 2, a bit of optimization explored to facilitate a more reliable tool for study brain organoid biology. More specifically, a system consisting of a PDMS fluidic culture channel and a peristaltic pump was developed (Fig. 3.1). Compared with the previous design, this platform has a larger culture area for brain organoids and the flow provided by the peristaltic pump allows more precise control of the shear stress.

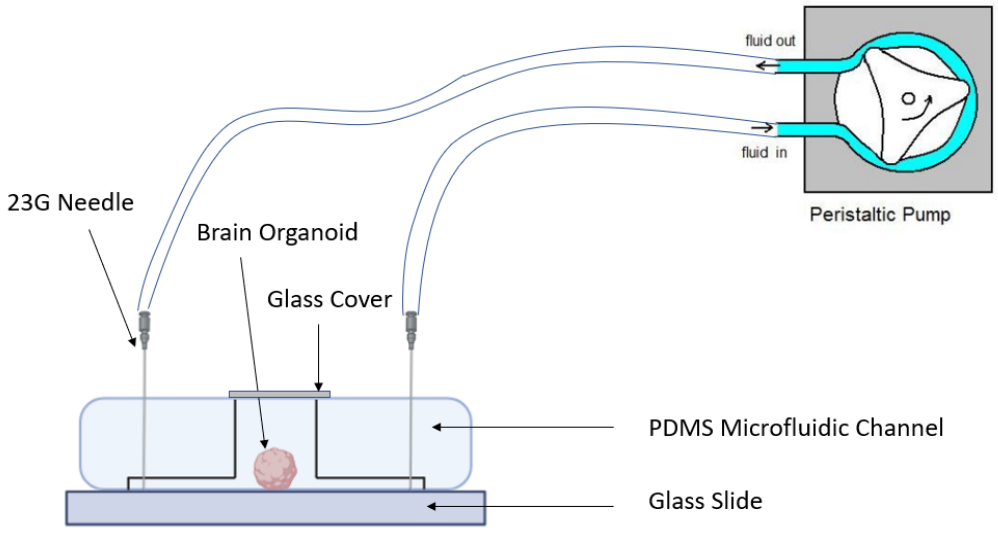


Fig.3.1. The schematic diagram of the experimental setup for generation of fluid shear stress in peristaltic brain-organoid-on-a-chip.

Materials and Methods

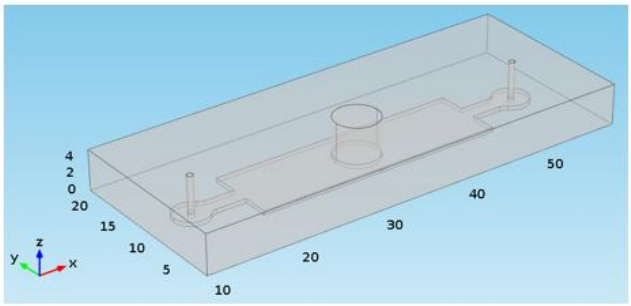
The homemade pump used in this experiment is designed and fabricated by Erik Werner, reconstruction and optimization on the tubing and connection were made to apply the pump into the experiment. The brain organoids culture and detailed biological analysis in this experiment were done collaborated with Pathak lab.

Device Fabrication

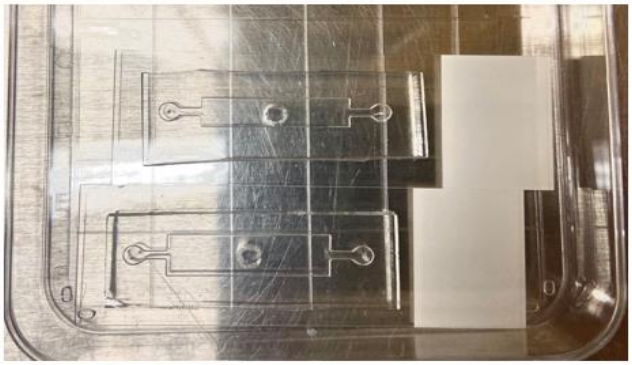
A widely used technique that is utilized to develop microfluidic perfusion culture chips is incorporating soft lithography with either laser cut or 3-D printed molds. Fluidic layer with positive features was designed using AutoCAD 2018 and was cut into molds from acetal resin strip (McMaster Carr, Derlin, White; dimensions: 5 ft × 4 ″, 0.025 ″ thickness) using an automated laser cutter (Universal VLS 4.60). Before device fabrication, molds were cleaned using sonication, DI water washes, and blown dry with compressed N_2. After the cleaning process, the molds were glued to a petri dish. PDMS (10:1 w/w; Sylgard 184, Dow Corning) was used as the polymer for the device. After mixing, the PDMS was degassed using a vacuum desiccator. Then PDMS replicas were created by pouring degassed PDMS into the petri dish to a thickness of 5 mm, and the dish was placed in an oven at a temperature of 65°C and cured overnight.

Once released from the mold, the PDMS was cut into the desired shape using a razor blade. Inlet and outlet ports were made by coring through the entire PDMS layer using a 1 mm biopsy punch. The organoid chamber was cut in the middle of the PDMS channel using a hole punch for 3/16 ″ holes in diameter (McMaster-Carr). The cut PDMS channel thereby contained inlet and outlet, the main fluid channel, and a hole for housing

organoid. The surfaces of PDMS were cleaned with sonication, IPA, DI water, followed by compressed N_2 and 3M magic tape to dry and remove debris. The clean PDMS layer and glass slide were placed in the plasma cleaner (PDC-002, Harrick Plasma) for about three minutes with the surfaces to be bonded facing up. After removal from the plasma cleaner, the PDMS layer was immediately put in contact with the glass slide firmly. To increase bond strength, the device was placed at 65°C for 20 minutes. The materials should be permanently and irreversibly bonded. Fig.3.2 presents a 3D rendering of the microfluidic channel for organoid culture and the fabricated PDMS channel.



A



B

Fig.3.2. The PDMS channel for organoid culture. (A) 3D rendering of the 8mm PDMS channel in COMSOL. (B) Fabricated PDMS channels (top: 6 mm in width, bottom: 8 mm in width).

Establishment of stable FSS culture microenvironment

Prior to settling down the organoids, the tubing and the microfluidic channel needed to be filled with the culture medium. The air bubbles could be introduced into the channel, which can greatly affect the performance of the system and have a detrimental effect on organoid viability. Thus, the tubing was filled using a low pumping speed (2 RPM) to minimize the effect of air bubbles. After a few months after culturing in the spinning bioreactor, a human brain organoid was collected and relocated into the central chamber of the brain organoid-on-a-chip platform. A round coverslip was placed at the top of the chamber and gently pressed down to seal. Blunt 23G needles were inserted to the inlet and outlet ports, and the needles were directly connected to the Tygon tubing (McMaster Carr 1/16 inch inner diameter, 1/8 inch outer diameter), the culture medium was run through a homemade peristaltic pump (Fig.3.3, designed and fabricated by Erik Werner) for generating the FSS culture microenvironment. Then the chip and the pump were immediately transferred to the culture incubator. The pump consisted of a box housing the Arduino chip for controlling the motor and two peristaltic pumps. The FSS protocol can be followed by running the pump at a desired speed.

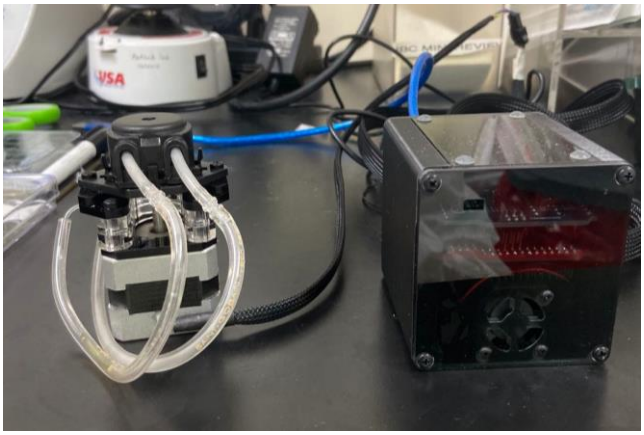


Fig.3.3. The homemade peristaltic pump.

Testing and Results

Pump testing

Prior to the experiment, a testing experiment was performed to assess the pumping performance of the homemade peristaltic pump. The following formula can be used to calculate the flow rate of the peristaltic pumps. Theoretical flow rate (ml/min) = A * L * N * RPM, where A is the flow area of occluded tubing (cm^2), L is the tubing length that will be occluded by pump rollers (cm), N is the number of rollers on the rotor, RPM is the pump revolution per minute [51]. For this model, A roughly equals 0.02 cm^2, L is 4 cm, and N is 3. However, the flow rates may vary depending on tube material, suction height, outlet pressure, and viscosity of the media pumped. It is not a surprise that there is a difference between the prediction value and the real one. Therefore, the flow rates of the homemade pumps (pump#0 and pump#1) were measured, and the relationship between flow rate and RPM (using Tygon tubing, internal diameter 1/16 inch) was shown in Table.3.1. According to the table, pump#0 allows smoother flow control and fits the experiment requirements (flow rate in the range of 1.5~2.6mL/ h) better. Therefore, it was employed in the experiment. Fig.3.4 showed the flow rate delivered by the pump#0 at 0.4 RPM over time, using the tubing's internal diameter of 1/16 inch, the flow varied between 0-35μL/min over a period of 270 seconds. Although the average flow rate was around the set value (25μL/min), it sometimes reached negative values. Nevertheless, the periodicity and the pattern of the oscillation are reproducible over time.

Flow rate of the peristaltic pump (mL/h)

RPM	Pump #0	Pump #1
0.1	/	0.6
0.2	0.7	1.2
0.3	0.9	1.8
0.4	1.2	2.0
0.5	1.3	2.6
0.6	1.4	3.0
0.7	1.6	3.2
0.8	2.0	3.7
0.9	2.2	4.3
1.0	2.5	5.0
1.1	2.8	6.1

Table.3.1 The flow rate of the pumps correspond to different RPM.

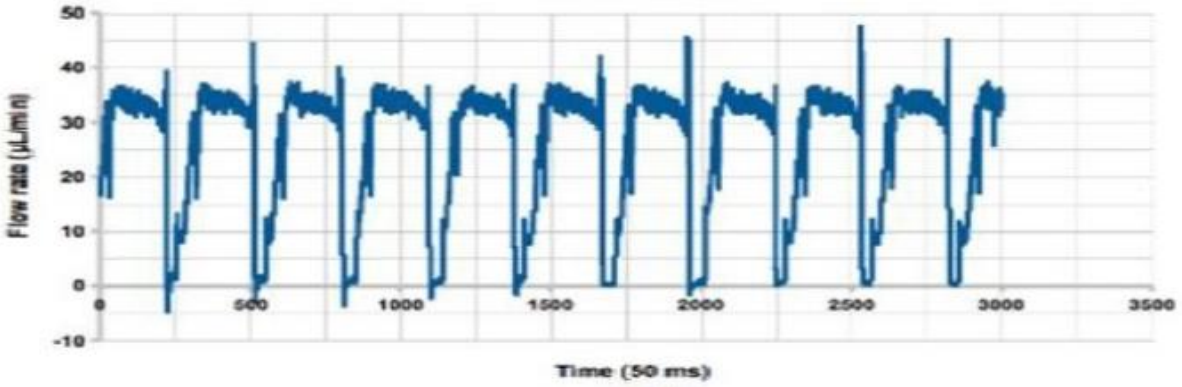


Fig.3.4 The flow rate delivered by a peristaltic pump over time. [46]

Brain Organoid Microfluidic Culture

Experimental setup for the long-term flow of brain organoid-on-a-chip was described in Fig.3.5. The chip had a central chamber for culturing the organoid. Two small brain organoids were collected and placed into the chamber, and images were acquired under light microscopy for the initial experiments (Fig.3.6). The performance of

the platform and the viability of organoids were checked every 24 h. It was observed that the pump was able to generate a stable and smooth flow through the microfluidic chip, and there was no leakage in the system. However, a few bubbles were found in the system, probably due to the compression of rollers on the tubing. Besides, it was reported that the long-time operation caused the rising temperature of the pump, and the heat from the pump increased the media evaporation. At day 3, the media almost dried out (Fig.3.7), but the organoids remained alive well.

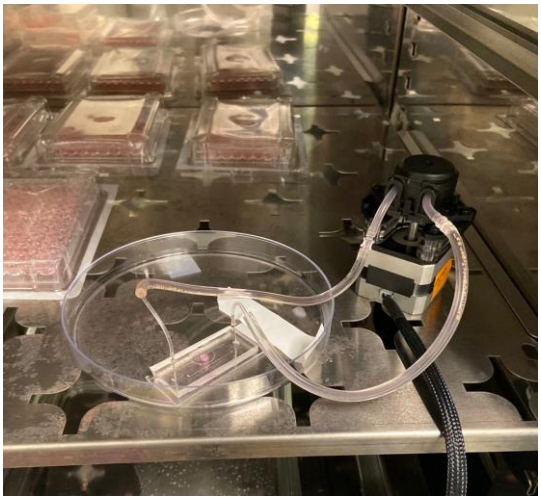


Fig.3.5. The human brain organoid shear stress controlled culture platform

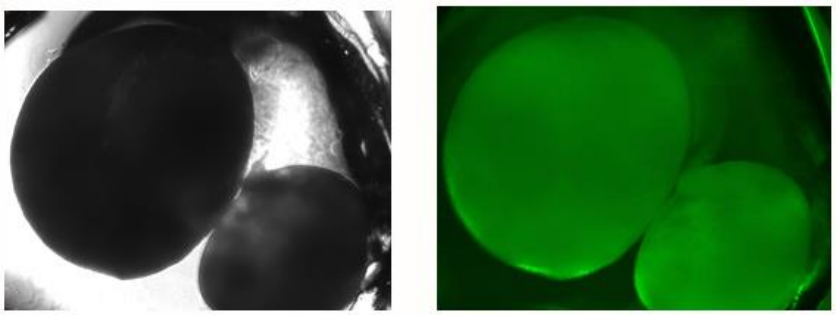


Fig.3.6. The images of two organoids co-located in the culture chamber under light microscopy. (A) Transmission image of organoids. (B) GFP fluorescence image

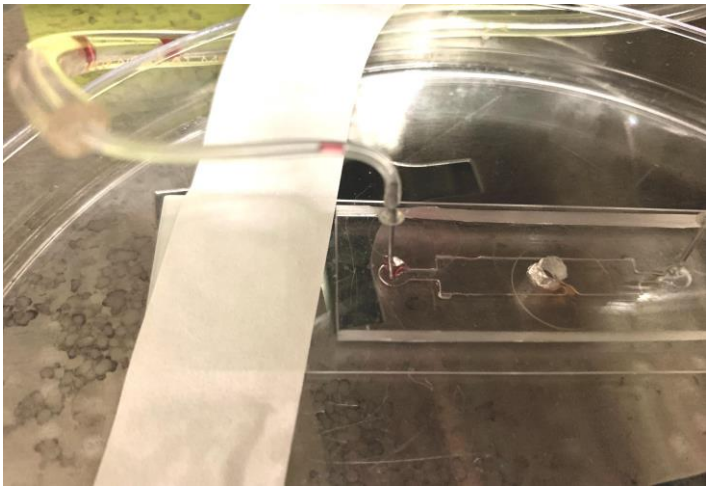


Fig.3.7. The dry channel at day 3.

Discussion

There are several advantages to employing the pump-controlled culture system over the gravity-driven system. The second generation culture platform remedied the failure of unstable flow rate by utilizing a peristaltic pump flow control system. The pump eliminates the time-dependent change of shear stress caused by the dropping liquid level of culture media. In addition to the more precise flow control, the microscale channel required lower culture media, and the automatic circulation closed-loop system reduced the media change frequency. Furthermore, geometric manipulation of microfluidic devices enabled tunable laminar flow that can be used to adjust shear stress on organoids. The second-generation design also offered a larger culture space for the organoids, and it can hold two early-stage brain organoids simultaneously or a fully matured organoid where the previous culture chamber was designed for a single organoid.

The second-generation design is more reliable than the previous one, but it still has its flaws. First of all, the flow delivered by the peristaltic pump was pulsatile due to the squeeze of the tubing. The brain organoids are sensitive to shear stress, and the acute pulsation can activate an unexpected response. Secondly, the compression of tubing may introduce bubbles into the system, which can affect both the shear stress on organoids and the viability of the tissues [52]. To alleviate potential bubble formation, there are several methods, including flushing the system with ethanol before the experiments, avoiding sharp corners in the design, or incorporating a bubble trap [53]. Finally, the critical problem of the system is the heat from the pump. It significantly increased the evaporation of the media, also raised the temperature of the culture environment. Fortunately, several options exist to address this problem. The homemade pump can be redesigned and lengthen the tubing, in this way, the pump can be located outside of the incubator, and the culture environment would not be affected. A better solution is using a commercial peristaltic pump instead of the homemade one, the commercial pump may handle overheating problems better, and can provide a more accurate and constant flow.

Although the peristaltic pump met the unexpected overheating issue, it has been proven capable of providing brain organoid a suitable shear stress microenvironment, and the organoids can survive in the system for at least three days.

Chapter 4: Micropatterning for Engineering Induction of Single Neural Rosette

Background

The micropatterning technology allows a better understanding of cell behaviors in vitro by fixing cells on specific substrates in a controlled manner. Thanks to the advances in bio-microfabrication techniques, the in vitro cell assays are able to carry out more efficient and reliable results [54]. In fabricating a micropatterning platform, several characteristics of the substrates should take into consideration, such as conductivity, environmental factor, cost, and accessibility. Given these properties, PDMS, polymethylmethacrylate (PMMA), glass, cyclic olefin copolymer (COC) are the most commonly used biomaterials for substrates and platforms. Besides, other low-cost materials like graphene are increasingly popular [55].

Numerous cell patterning techniques have been carried out [56], but only a few of them have been successfully employed in hPSCs cell arrays, such as microcontact printing [57-59] and microstencils [20, 60]. The biggest challenge of hPSCs micropatterning is that hPSCs are extremely vulnerable and have strict requirements for ECM and growth factors. The microcontact printing is the most widely used approach for 2D hPSCs patterning on glass substrates. However, this method requires a two-folds coating process and specific control of atmospheric and humidity conditions to ensure the ECM micropatterns [20,59]. The primary concern of the micropatterning method is whether the pre-treated substrate is able to generate hPSC-adhesive ECM micropatterns with desired shapes and sizes while minimizing the unspecific attachments.

Stencil micropatterning is one of the easiest ways to generate hPSC micropatterns, and it is a method that is highly biocompatible with hPSC. The desired geometry of the cells correspond to the perforated patterns of the thin stencil membrane, which physically restrain the locations where the cells can access to the underlying substrate. It can determine the hPSC differentiation by regulating the cell-cell polarization and cell-ECM attachment. The advantage of this approach is that it can generate cell patterns without any additional substrate modification and has great flexibility to apply to hPSC-compatible substrates. In addition, a variety of materials can be used to fabricate the stencils, including metals [61, 62], PMMA [59], and most commonly, PDMS [63-67]. The PDMS stencils allow patterning on a curved surface due to its flexibility [68] and can be made using different methods depending on the desired features, such as CO_2 laser-cutting and photolithography.

Neural rosettes formation is a common feature found in both 2D and 3D hPSCs-based cultures. However, the random generation of neural rosettes inevitably affects the further studies of organogenesis. Recently, the geometric confinement of initial patterns has shown the feasibility of inducing singular neural rosette emergence within hiPSC tissue differentiation. Based on previously discussed studies, the influence of micropatterned morphologies of neural differentiating hiPSCs on singular neural rosette emergence was investigated in this chapter. A methodology for fabricating PDMS stencils with varied patterns by laser cutting and the generation of singular neural rosette within hiPSC patterns was developed.

Specific Aim: Develop an approach that can effectively induce singular rosette formation within iPSC-derived tissues.

The main goals are:

1. Develop a micropatterning method and protocol that can generate desired hPSC-adhesive ECM patterns.
2. Induce reproducible singular neural rosette emergence within hPSCs tissue by stencil micropatterning.

Materials and Methods

The stencil design was based on the Knight et al. paper [20]. The detailed biological analysis and optimization of the formation of neural rosettes were performed by the Pathak lab.

Design and fabricating PDMS stencils

The stencil gasket (18 mm circle) and stenciling sheet with through-holes of desired geometry and size (250 μm and 500 um circles) were designed in AutoCAD 2018. A modified version of stencils with only 28 holes (250 μm diameter) was also made. The gasket was designed to fit in the MatTek culture dishes (with 20mm diameter glass coverslip at the bottom, Fig.4.1).

The stenciling sheet and the gasket were cut on 250 μm PDMS mylar (Gleicher Manufacturing Corp.) and pre-casted 2 mm thick PDMS sheet respectively (10:1 w/w; Sylgard 184, Dow Corning) using the automated CO_2 laser cutter (Universal VLS 4.60). Before the bonding process, molds were cleaned using sonication, DI water washes, and blown dry with compressed N_2. The gasket and the stencil sheet were bonded using liquid uncured PDMS and curing at 65°C for 4 hours. After the fabrication process, the stencils

were sterilized using either 70% EtOH or autoclaving at 120°C for 30 min, and drying overnight.

Fig.4.1. The 35 mm MatTek culture dish (with 20 mm coverslip)

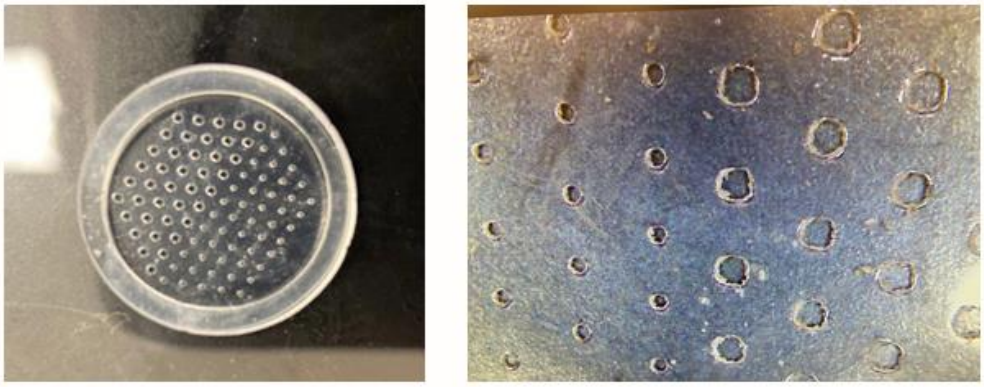


Fig.4.2. The fabricated PDMS stencil. (A) The PDMS stencil with 250 μm and 500 μm circular patterns on each half. (B) The patterns under microscopy.

Stencil micropatterned cell culture

Two different protocols were used to generate hPSC islands and neural differentiation. They are the same in the stencil setup and cells but different in the ECM coating and cell culture process. The detailed methods were as follows.

Early protocol

Cells used: a variant of WTC-11 i.e., ZO1-GFP from the Allen Cell Science collection – AICS-23 – (MONO-ALLELIC mEGFP-TAGGED TJP1 WTC iPSC LINE (TAG AT N-TERM)) i.e., Gene: TJP1, Protein: Tight junction protein ZO-1, These cells are routinely cultured on Vitronectin-XF coated dishes in NutriStem-XF media (from Biological Industries, USA). Cells were passaged by mechanical dissociation or using Accutase. The cell suspension was in NutriStem-XF with 10μM ROCK inhibitor. The media contained Pen-Strep usually, and more lately, Primocin was used– an antibiotic and antimycotic.

The cultural aspect of the method is based on Knight et al. paper in eLife [20]. Prior to cell patterning, a PDMS stencil was sealed at 20 mm glass coverslip of a 35 mm MatTek culture dish by dispensing 70% EtOH into the glass bottom. The PDMS insert bottom surface was cleaned using Scotch Tape. The alcohol was removed either by overnight drying or by removing the excess alcohol and then leaving the dish at 65°C for 15 mins. Check no bubbles exists underneath the stencil to ensure that the stencil well with the glass.

Vitronectin-XF (Primorigen Biosciences, Inc, USA) was diluted to 10ug/ml in DPBS, and around 250 ul solution was pipetted onto the PDMS stencil. The dish was placed in a vacuum desiccator and lab vacuum applied for about 20 mins or till most of the air was removed and tapped to release air bubbles. If the air bubbles still remained and trapped in the wells, then the desiccator with the dishes under the vacuum was placed at 4 °C overnight. The dish was then transferred to the incubator for 8 hours at 37°C and 5% CO_2 before use. After the vitronectin settled down, the solution on the PDMS stencil

was aspirated away, and the stencil was removed gently using a pair of autoclaved forceps. The dish was examined under a microscope to check if the ECM attached to the glass substrate, and the glass bottom was washed twice with DPBS then used immediately for seeding the cells onto it.

The cell suspension was diluted and the cell density was determined using a hemocytometer, then the required cell seeding density was calculated, and the stock cell suspension was diluted to the desired number with the culture medium supplemented with ROCK incubator. To get a confluent monolayer of single cells, the cell seeding density was about 5 x 10^5 cells/ml to a 1 x 10^6 cells/ml, determined based on a few experiments. 250µL cell suspension containing the desired number of cells was added into the glass coverslip area of the dish, and the dish was transferred into the incubator for 3h for cell seeding.

E6 media is a media available from Invitrogen, USA. It is E8 without the growth factors. E8 is also known as Essential 8 [69]. The next day, prior to adding the culture medium, the culture dish was checked under the microscope for cell attachment. The cell suspension was then removed away from the dish and washed gently with 2mL E6 medium without ROCK inhibitor. The media was then replaced with 2 mL E6 media. Media (E6) changes were performed daily thereafter, and the GFP patterns were examined under microscope

Latest protocol

The method of hPSC-derived cultures to generate a single rosette was performed using a modified version of Haremak et al. protocol [70]. The preparation prior to cell

seeding and the cell types (ZO1-GFP from Allen Institute for Cell Science, USA. Cell Line ID: AICS-0023 cl.20) were roughly the same as the early protocol.

For ~250 ml of media for Dual SMAD inhibition, it combined 125 ml of DMEM-F12, 125 ml of Neurobasal medium, 1.25 ml of N2 supplement, 62.5 µl of insulin, 2.5 ml of GlutaMAX supplement, 1.25 ml of MEM-NEAA and 2.5 ml of penicillin-streptomycin (Thermo-Fisher USA.). Also, 87.5 µl of a 1:100 dilution of 2-mercaptoethanol in DMEM-F12, 2.5 ml of B27 supplement, 10 µM of SB 431542 (Biological Industries, USA), and 0.2 µM LDN193189 (Biological Industries, USA) were added to the medium.

Instead of vitronectin, the dish was coated with LaminStem (Laminin 521 from Biological Industries, USA). LaminStem was diluted to 10ug/ml in PBS with Ca++ and Mg++. Next, 200 ul of the coating solution was added to the dried 250 µm PDMS stencil. The dish was placed in a vacuum desiccator and applied house vacuum for 15 minutes with some tapping to release air bubbles. The dish was then moved under the microscope to check for air bubbles. If the air bubbles were found, repeated the cycle of vacuum treatment, and then placed the dishes in the incubator overnight.

After overnight treatment, the coating solution was aspirated away, and the stencil was peeled off with autoclaved forceps. The dish was washed using PBS with Ca+ and Mg+ twice. The bead of PBS seen indicated the spots where the Laminin attached to the glass substrate. The dish was then kept with PBS solution till use.

The cells were washed with DPBS w/o Ca and Mg, and the cell dissociation buffer (Thermo-Fisher Scientific, USA) was added, then the dish was placed in the

incubator for around 8 mins. After cells detached, they were triturated using a 1 ml pipette to ensure single cell suspension, and accutase was diluted out with NS-XF medium supplemented with 20 ng/ml bFGF and 10uM ROCK inhibitor Y-27632. Then the cells were spun at 1000 RPM for 4 mins using the centrifuge (Eppendorf Centrifuge), and the cell survival rate was approximately 75%. The cells were resuspended in the same medium, and 2 x 10^5 cells in 500 ul of the above media was placed over the Laminin micropatterns on the MatTek dish after the PBS was aspirated off. The dish was then transferred to the incubator at 37°C and 5% CO_2. After 3h in the incubator, the cell suspension was aspirated away, and the micropatterns on the dish were washed with NS-XF media. Then the medium was replaced by 2 mL of media for Dual SMAD inhibition, and the dish was placed back in the incubator. The medium was replaced by the same fresh medium every two days.

Results

The previous studies [20, 71] demonstrated that circular neuroepithelial could effectively reduce the number of neural rosette formation. The optimal morphology for singular neural rosette emergence in forebrain neuroepithelial tissues is 200-250 mm diameter circular micropatterns. The paper also indicated the approximately 5-day persistence of neural emergence in E6 media. Based on the results observed, time-course analysis over micropatterned culture experiments using iPSCs was designed. The initial experiment started with seeding iPSCs (WTC-11 ZO1-GFP) on PDMS stencils with 250 μm and 500 μm circular patterns on each half of the stencil (Fig.4.2). When the cells were allowed to differentiate towards the neural lineage, they formed different types of cells,

and the ZO-1 protein tended to aggregate in tight junctions between cell types as increased GFP fluorescence got localized to various structures.

However, it was observed that 2-6 neural rosettes routinely formed within 500 μm cell micropatterns, while usually 0-2 rosettes found within 250 μm cell islands. Therefore, the cell patterning was switched to another type of stencils, which only have 250 μm diameter sparser through holes (Fig.4.3).

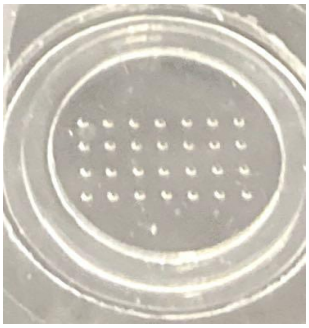


Fig.4.3. The PDMS stencil with 250 μm circular patterns.

Results from the early protocol

The attachment of vitronectin and cells was successful in that no air bubbles were trapped underneath, and the cells were restrained in circular patterns (Fig.4.4). Fig.4.5 shows the progression of iPSCs developing towards neural differentiation. At the early stage, the ZO-1 GFP protein established the cell boundaries, and the cells in the middle of the island were getting bigger and reorganizing to form a rosette. At day 4, some of the cell islands started forming the rosette-like structure, which appeared as the aggregation of GFP fluorescence. It was also observed that the cells obtained the same phenotype with near uniformity. However, only a few cell islands had a clear circular distribution of ZO-1 GFP (Fig.4.6A) at day 6. Most of the islands had a large aggregation of ZO-1 GFP

with some sporadic smaller aggregations (Fig.4.6B), which ideally should be observed as a tight circular junction. These were similar to what was found in neural organoids but better to some degree since there was only one large aggregation.

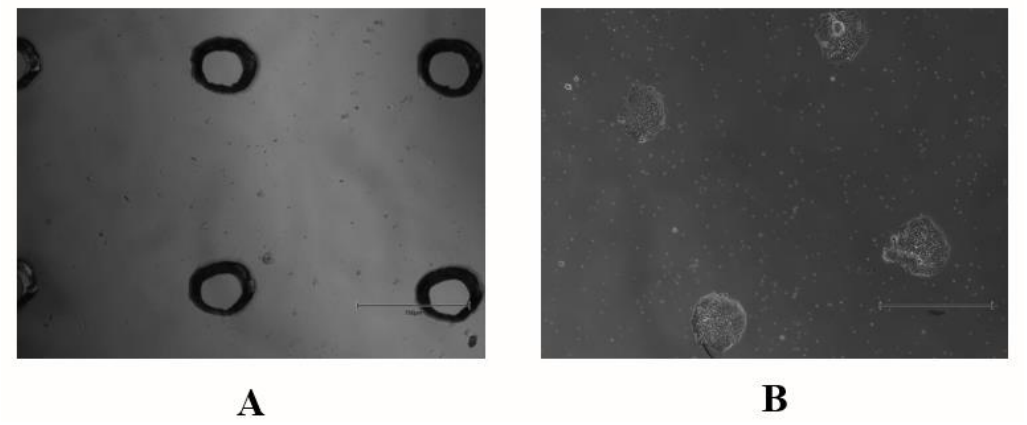


Fig.4.4. The patterns on the culture dish from the early protocol. (A) The PDMS stencil with vitronectin at day 0. (B) ZO1-GFP cell islands 24 hours after incubation.

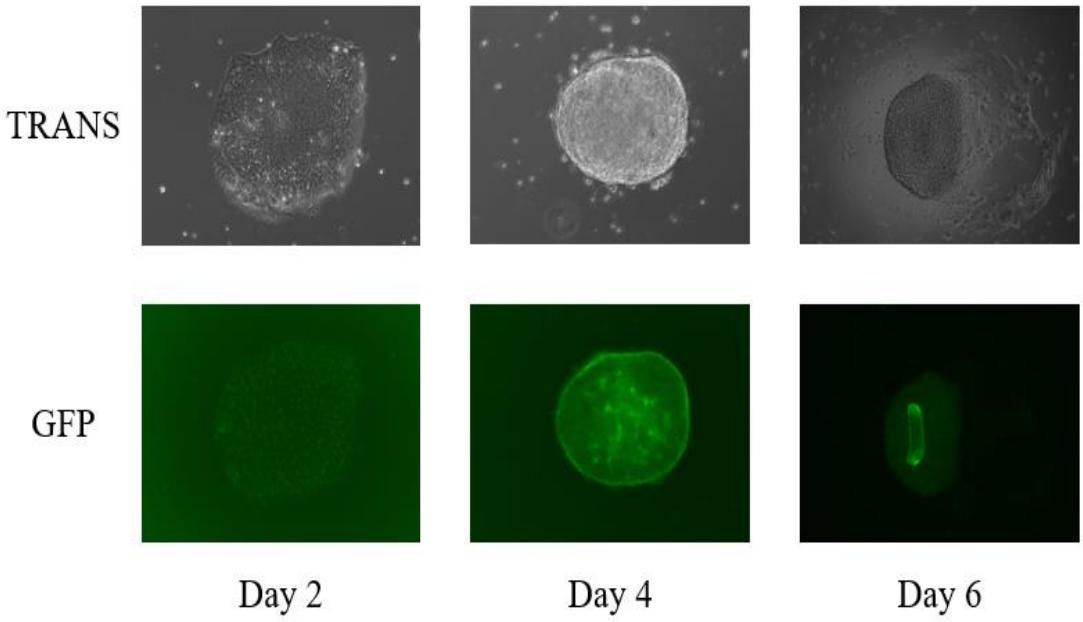


Fig.4.5. Progression of iPSCs (WTC-11 ZO1-GFP) developing towards neural rosette from the early protocol. (20X transmission and GFP fluorescence image of a cell island).

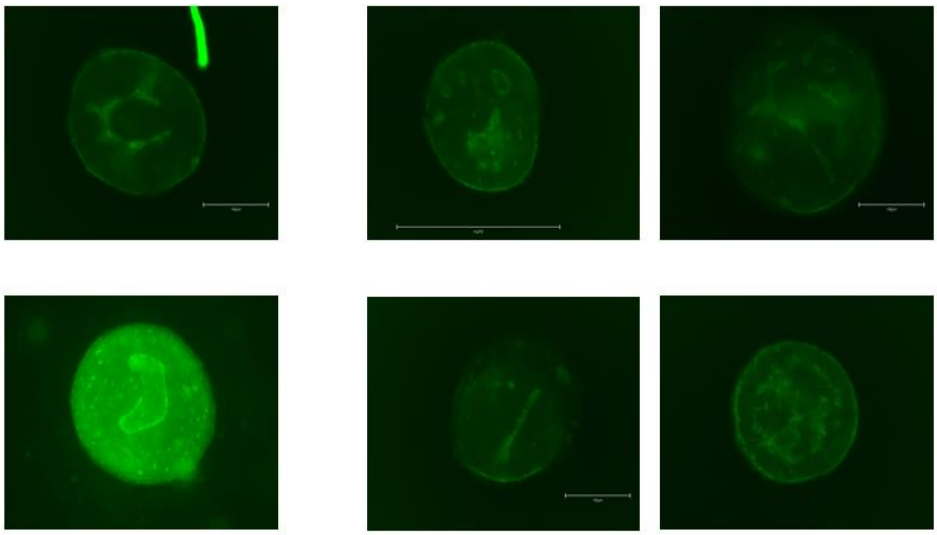


Fig.4.6. Example 20X GFP fluorescence image of cell island at day 6 from the early protocol. (A) Examples of cell islands with a clear circular distribution of ZO1-GFP. (B) Examples of cell islands which have large ZO1-GFP aggregation.

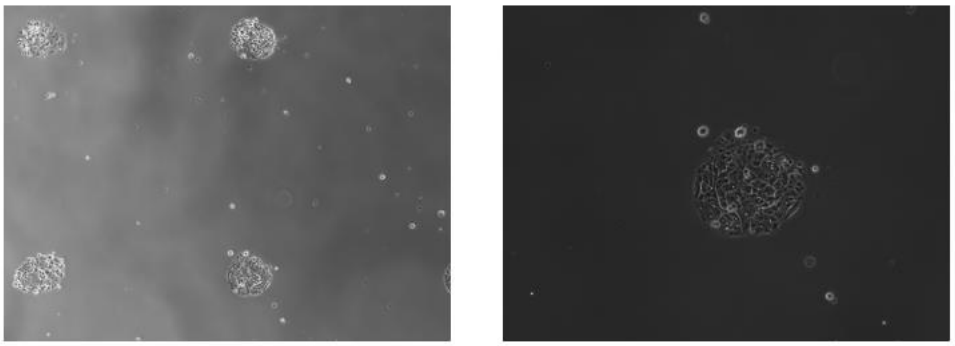
The results indicated that the stencil micropatterning could be used in generating iPSCs islands, and they did differentiate into desired neural cell types. The 250 μm circular geometric confinement of the E6 culture of iPSCs has the potential for reproducible induction of single neural rosette emergence, though with a low efficiency compared with the results (80% efficiency while seeding $D^{-}1$ hESCs) from Knight et al. [20].

Results from the latest protocol

The hPSCs (ZO1-GFP) were seeded onto the 250 μm diameter circular micropatterned subtract and treated with SB and LDN to induce neural differentiation. So far, the experiment has been carried out for 9 days, and is continuing. The Laminin and

cells showed a successful adhesion to the glass substrate. The cell islands were seen nearly uniform, and no cells attached out of the micropatterned area (Fig.4.7).

Regardless of the size and shape of the islands, all the cells had converted to certain neural cell types. The cell polarization was seen towards a central lumen within some of the cell islands, as delineated by expression of GFP fluorescence. These islands displayed complete closure of a single lumen, and a radial pattern was formed surrounding the lumen (Fig.4.8). Interestingly, the formation of a single rosette was not constant. Among 28 cell islands in this experiment, clear rosette structures were observed as early as day 7 within micropatterned cell islands but sometimes disappeared on day 9. Some rosettes were seen till day 9, while no rosette structures were observed in several cell islands so far (12 of 28). The results showed similar dynamics with the cell fate occurred in regular 2D cultures. It still remained a challenge to engineer reproducible hPSCs morphogenesis effectively, thereby controlled the formation of single neural rosette cytoarchitecture.



A B

Fig.4.7. The examples of micropatterned cell islands (ZO1-GFP) on the dish. (A) 10X transmission image of the micropattern. (B) 20X transmission image of a cell island.

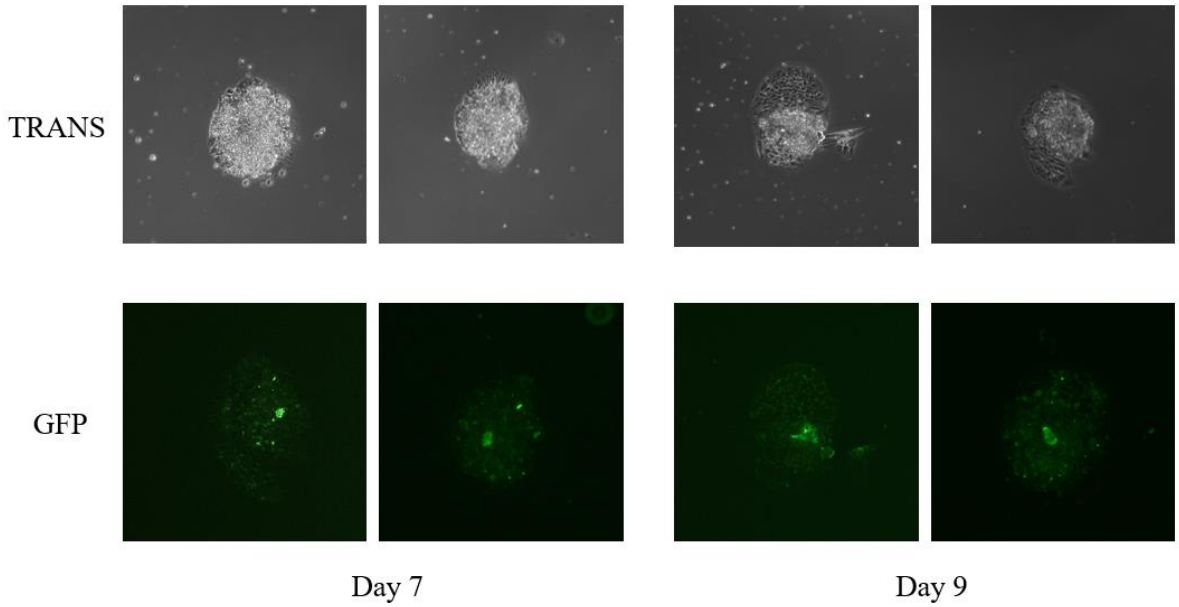


Fig.4.8. Examples of cell islands with a clear single rosette structure on day 7 and day 9. (Top: example 20X transmission image of a cell island, bottom: example 20X GFP fluorescence image of a cell island).

Another experimental group with larger cell seeding density was also carried out for 5 days. The occurrences of rosette-like structure on day 5 (Fig.4.9) further confirmed the dynamic cell fate of neural rosettes. However, the cell islands were not as uniform as the other group, probably because of some variations in the cell micropatterning process. Despite the accelerated formation of rosette structures, this group of cell islands had a clearer single rosette cytoarchitecture and was reported more effective of formation of single rosette. It proved that the cell seeding density is significant in the induction of neural rosette. Although the study did not get the same efficiency as Knight et al. reported (>80%), it showed that the circular patterns of 250 μm diameter were capable of inducing singular rosette emergence with hPSC tissues as it reached the levels of 60-65% efficiency.

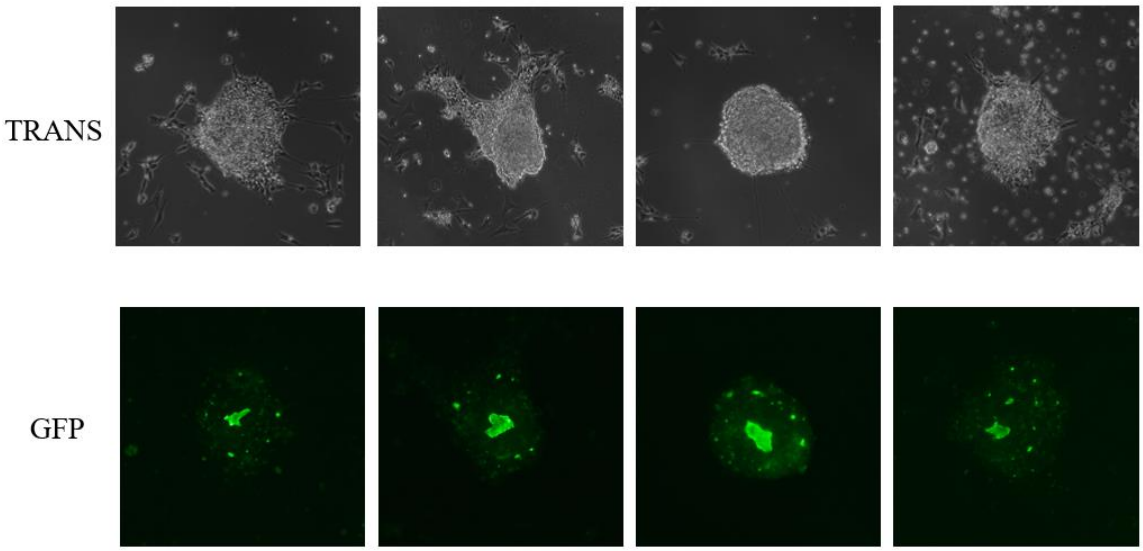


Fig.4.9. Examples of cell islands with a circular single rosette structure on day 5. (Top: example 20X transmission image of a cell island, bottom: example 20X GFP fluorescence image of a cell island).

Discussion

A consistent limitation in current neural organoids studies is the spontaneously yielding random generation of numerous neuroepithelial tissues, a.k.a. Neural rosettes, which present in indiscriminate sizes and shapes, and each act as a morphogenesis center independently. The dynamic formation of neural rosettes not only confounds the coordinated morphogenesis and further limits the organoid reproducibility. The study aims to engineer reproducible, singular rosette formation within hPSC-derived tissues using micropatterning technology. In this study, a protocol of making neural differentiation cell micropatterned using iPSCs (WTC-11 ZO1-GFP) was developed. The proposed method using PDMS stencil, laser cutting, and a glass-bottom culture dish to apply a localized pattern array of iPSCs.

The stencil technique is an ideal method to generate hPSC micropatterns for investigating neural differentiation patterning. The main advantage of stencil patterning is that it does not require complex fabrication, and the stencils can be reused [72-74], and it also provides great cell adhesion to the glass surface without additional processes.

There are several key steps during the stencil fabrication that may affect micropattern quality. The fidelity of generating the desired geometries within the hPSCs micropatterns relies on the clarity and consistency of perforating fabrication on the thin stencil sheet. The stencils in this study were fabricated by laser cutting on a thin PDMS membrane. The resolution of laser cutting depends on the spot size of the laser beam and the accuracy of the laser beam path mechanical controller. The resolution of the CO_2 laser cutter used in this experiment is 25 μm, which is capable of making 250 μm through holes ideally. However, due to the high temperature of the laser and the small patterning feature (250 μm circles in diameter), the periphery of holes on thin PDMS membrane melted during cutting. The diameter of the holes varies from 250 to 350 μm, and the cell islands lack uniformity because of the error in stencil sheet fabrication (Fig.4.10).

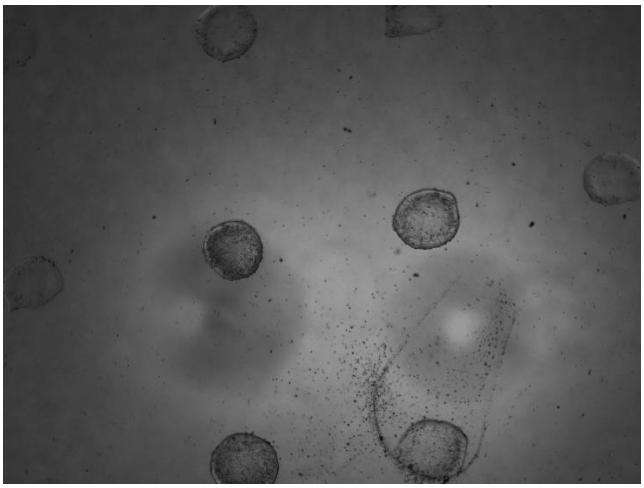


Fig.4.10. Non-uniform cell islands.

This degree of variation may not be acceptable for the experiment, and it might be the reason for inconsistent outcomes of the rosette formation. To address this problem, the through-holes can be fabricated by molding PDMS on a photolithographic silicone template with desired features instead of laser-cutting. Alternatively, there are micropatterning and non-fouling surface coating techniques that can generate more uniform patterns over stencil patterning.

The early culture protocol of the approach is based on Knight et al. paper [20], but slightly different in the cell types and cell density. However, there is a considerable difference in efficiency. In this study, the 250 μm circular micropatterned was shown to induce ZO1-GFP aggregate in the middle of the rosette, which is similar but better than what was found during organoid cultures. Although this protocol enables the distorted singular rosette emergence, it has limited capacity to generate real singular neural rosette so far, while Knight et al. reported effectively (>80%) induction of single rosette-forming using the same geometry confinement.

The latest protocol was inspired by Haremaki et al.[70]. While using the same micropatterns, it displayed better results in the induction of single rosette over the previous experiments. The emergence of single rosette was accelerated by the geometrical confinement (250 μm diameter circular micropatterns) of initial tissues. Moreover, the micropatterning technique provided a powerful tool to investigate the early-stage processes of human neural rosettes induction and morphogenesis in vitro.

A bit of optimization and several critical steps during cell patterning were discussed as follows. Firstly, the confluency of hPSC is important for cell differentiation. Cells at 70%~85% confluence were considered high-quality resources for seeding. Over-

confluent hPSCs patterning often causes spontaneous differentiation, and if these differentiating areas are not removed before cell seeding, it will affect the subsequent normal differentiation.

Second, the cell seeding density plays a pivotal role in generating a confluent monolayer of cells and cell adhesion. Different cell seeding densities were tested in the study, and it was reported that cell density is a vital factor that directly affected the rosette formation, and further experiments are required to determine the optimal cell density. Considering the hPSCs tend to grow as colonies, inadequate cells will cause the bald spot within the micropattern, which means a low fidelity of desired pattern geometry. On the contrary, excessive cell density will lead to overcrowded cells within micropatterns. Multi-layers of cells will form within the pattern, especially at the edge of the shapes. The biomechanical properties have a great influence on the rosette emergence behavior. It is critical to modulate the mechanical factors by the uniform cell micropattern. If multi-layers of cells form, mediated mechanical forces of cell adhesion will not only exist in the x-y plane of the substrate. Thus, the mechanical factors would be difficult to maintain, and the resultant differentiation would be more random. In fact, in our experiments, it was observed that the topmost z plane was not flat, and the lumen in the middle may be surrounded by raised cell membranes in the experiments, this further confounded the post-deposition cell growth.

Finally, the size and geometry of the pattern determine the tissue morphology, and this directly regulates the formation of neural rosette. The spatial cell pattern culture generates mechanical forces to push, expand, and reshape tissues towards specific differentiation. Specifically, studies on micropatterned cultures have found that the

mechanical forces originating from the contraction of cells lead to certain morphological growth [75]. In our experiments when the rosettes were small, they were usually seen circular and uniform, but once started growing, most of them became distorted in varying degrees. Besides the fabrication quality, one likely cause is that it is difficult for multicellular tissues to maintain even mechanical forces within a symmetrical pattern. However, only 250 μm and 500 μm circular patterns were tested, the optimal geometry of the patterns was still unclear. Therefore, the reproducibility of single rosette formation may be further optimized by varying the size and shape of the micropatterned tissues — for example, triangle, square, rectangle, and semi-circle of different sizes.

Overall, it was proved that the stencil micropatterning is a low-cost, easy-operate method to engineer induction of single human neural rosette structure emergence and to study morphogenesis within hPSCs-derived tissues.

Chapter 5: Conclusion

The frontier neural organoid technology has enabled the in vitro recapitulation of biomimetic and cytoarchitectural human brain models. It has been widely employed in the human brain organogenesis studies and neurological disease platforms. However, neural organoid biology needs to be further studied in order to develop engineered tools for reproducible organoid formation. Besides, while the impressive self-assembled ability of their in vitro morphogenesis, the spontaneous neurulation-like events occurrence enables the organoid development also limits the mass production due to the short of reproducibility in their cytoarchitectural arrangement and development trajectory.

In chapter 2 and 3, two different engineered tools for assessing the impact of shear force on brain organoid development were designed and fabricated. Both of them can generate targeting shear stress to brain organoids. In chapter 2, a rough shear stress simulation was performed, and it was seen that the gravity-driven system could provide dynamic flow. Therefore, this system is more suitable for tissues that are not particularly sensitive to shear stress. Although it still needs to be improved and optimized, the gravity-driven approach can be a low-cost, easy-operate solution for studying the effect of shear stress on cells or tissues.

The peristaltic pump system can manipulate the shear stress smoothly and precisely. The pump flow control platform is a more widely used approach to facilitate the OOC platform for organoids and also conforms to the requirements of the study. It was shown that the brain organoid can survive in the fabricated microfluidic system for

three days and is a useful tool to perform brain organoid biological analysis under the FSS microenvironment. It is overall a more complete and more reliable tool than the gravity flow system. However, this system requires further improvement on the pump to run a longer experiment. Also, detailed assessments of the shear stress should be included in the experiment to enhance the understanding of the role of shear stress in brain organoid biology.

In chapter 4, a different study of engineer tools for inducing single neural rosette emergence within hiPSCs-derived tissues was carried out. The previous studies have proved that geometric confinements of initial hiPSCs can induce the emergence of single neural rosette. Here, we use the stencil micropatterning technique to investigate the neural rosette morphogenesis and to engineer singular rosette emergence. The stencil micropatterning was proved reliable to generate hPSC islands and convert them to neural differentiation. Furthermore, our protocol effectively improved the reproducibility of the single rosette formation.

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