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Research paper

Polarization of organoids by bioengineered symmetry breaking

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ABSTRACT

Symmetry breaking leading to axis formation and spatial patterning is crucial for achieving more accurate recapitulation of human development in organoids. While these processes can occur spontaneously by self-organizing capabilities of pluripotent stem cells, they can often result in variation in structure and composition of cell types within organoids. To address this limitation, bioengineering techniques that utilize geometric, topological and stiffness factors are increasingly employed to enhance control and consistency. Here, we review how spontaneous manners and engineering tools such as micropattern, microfluidics, biomaterials, *etc.* can facilitate the process of symmetry breaking leading to germ layer patterning and the formation of anteroposterior and dorsoventral axes in blastoids, gastruloids, neuruloids and neural organoids. Furthermore, brain assembloids, which are composed of multiple brain regions through fusion processes are discussed. The overview of organoid polarization in terms of patterning tools can offer valuable insights for enhancing the physiological relevance of organoid system.

1. Introduction

Symmetry breaking in early mammalian development refers to the process by which the initially symmetric embryo acquires its asymmetrical features, leading to the establishment of the body plan and the formation of distinct structures (Zhang and Hiiragi, 2018). During embryo development, symmetry breaking often leads to the polarization of cells and tissues along the embryonic axes. For instance, in the formation of the body axes (anterior-posterior, dorsal-ventral, and left-right), the initial symmetry breaking event, such as the formation of the primitive streak in vertebrates, provides the directional cue for subsequent polarization along these axes. The establishment of cell polarity within cells of the developing embryo guides their behavior and differentiation, contributing to the proper formation of tissues and organs.

Polarization, driven by various molecular mechanisms, enables the organization and orientation of cells and tissues along specific axes, contributing to the formation of complex structures and body plans during embryonic development (Keller, 2002). Polarization involves the organization and orientation of cells and tissues along specific axes. It often relies on the establishment of cell polarity, which is the asymmetric distribution of cellular components and functions within individual cells. Cell polarity is essential for processes such as cell migration, tissue morphogenesis, and the formation of complex structures. Polarization is driven by various molecular and cellular mechanisms. These mechanisms include the activity of polarity proteins, such as Par proteins and planar cell polarity (PCP) proteins, which regulate the asymmetric distribution of cellular components and signaling pathways. Cytoskeletal dynamics, cell-cell interactions, and external cues also contribute to the establishment and maintenance of polarization (Wodarz, 2002). Thus, symmetry breaking and polarization are interconnected processes in embryo development.

Advent of revolutionizing 3-diemnsional (3D) cultures of neural tissues, called brain organoid or embryoid techniques have improved our understanding how human early development and organogenesis occur and provided hope for the personalized neuro-medicine and neuro-regeneration therapy (Xiang et al., 2020; Lee and Son, 2021; Lee and Sun, 2022; Susaimanickam et al., 2022). Although stem cells exhibit self-organizing property to establish *in vivo*-like complex structure and cell diversity in 3D culture, lack of sufficient signal for symmetry breaking often interferes with histogenesis. Thus, further driving the complex embryonic development often requires the control of symmetry breaking and subsequent polarization during the 3D culture of cells *in vitro*. There are several different approaches to induce symmetry breaking in cultured cells, which include the addition of polarizing

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factors for spontaneous symmetry breaking during the growth, the use of extracellular matrix proteins, and bioengineering approaches such as micropattern, microfluidics and biomaterials. The bioengineering approaches that utilize geometric, topological, and stiffness factors are increasingly being employed to induce symmetry breaking and polarization in 2D cultures as well as 3D organoids.

In this review, we first describe various engineering techniques for polarized cell patterning and then, focus on 2D cultures and organoids that recapitulate the developmental process from the blastocyst stage to brain formation, with emphasis on the occurrence of polarization, whether through autonomous mechanisms or bioengineering approaches.

2. Biophysical engineering affecting polarization

Biophysically engineering refers to the approach of directly applying physical forces to cells in both 2D and 3D environments. This engineering method involves limiting the space in which cells can grow, adjusting the stiffness of substrates that mimic the extracellular matrix (ECM), or directly applying forces such as compression and tension (Fig. 1a). These types of mechanical forces are linked to changes in cell signaling and ultimately influence cellular differentiation. However, these physical stimuli cannot be completely dissociable from biochemical signals, often the effects observed are combination of these factors. These are often caused by the use of biologically active ECM molecules, such as Matrigel, collagen, and decellularized brain ECM, which should affect both physical and biochemical status of the cells. However, recent discovery of the fact that biochemically inactive or least active hydrogels provide additional opportunity for segregating these factors (Cassel de Camps et al., 2022). For instance, it has been reported that alginate hydrogel can affect organoid growth, similarly to the Matrigel, suggesting that these effects are more likely owing to the physical constraint effect rather than ECM-derived signaling activation (Chooi et al., 2023).

2.1. Geometric confinement

Geometric confinement studies the alterations in the shape and function of cells when their available space for growth and movement is limited. An illustrative example of this is the application of micropatterning for geometric confinement. Geometric confinement via micropatterning has found extensive use in creating two-dimensional patterns for the formation of germ layers. The mere restriction of cell growth areas prompts a location-dependent receptor positioning within a colony due to a substantial increase in cell density in the inner region

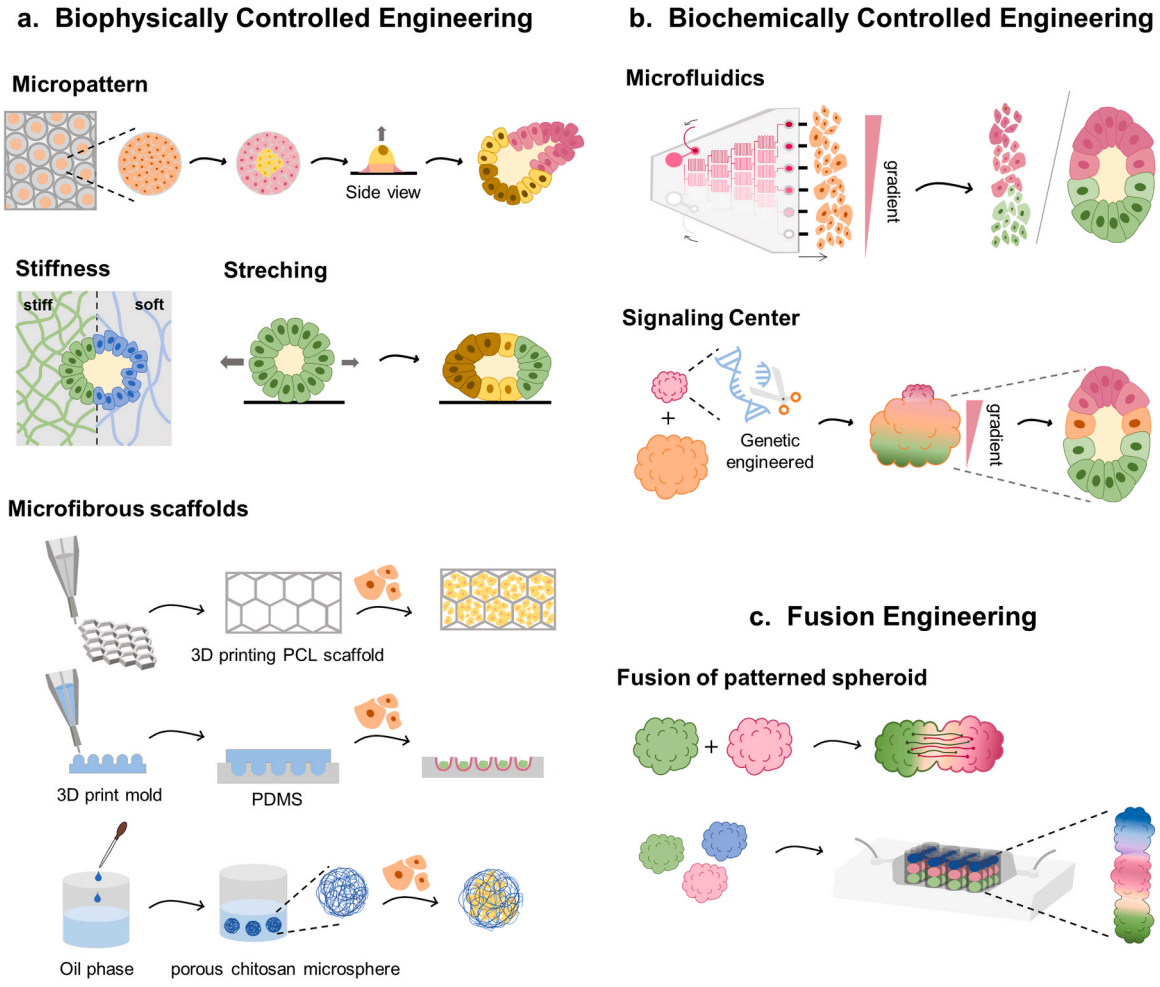

Fig. 1. Bioengineering tools for symmetry breaking in polarized embryoids and organoids. Recapitulating human development *in vitro* can be achieved by utilization of bioengineering tools to induce symmetry breaking and guide differentiation of human pluripotent stem cells (PSCs). These tools can generate germ layer patterning, body axis, and cell patterning and structured orders of brain subregions. a) Biophysically controlled engineering tools including micropattern, modulation of substrate stiffness, mechanical stretching and the use of microfibrous scaffolds exert direct physical forces on cells in 2D and 3D environments to induce symmetry breaking. b) Biochemically controlled engineering like microfluidics and establishment of signaling center generates concentration gradients within a single well or a single spheroid to guide and direct cell differentiation. c) Fusion engineering involves the artificial merging of differentiated or patterned cells and tissues to generate more intricate organization.

compared to the periphery, along with a dynamic signaling wavefront. As a result, cell polarity is established within a single colony. The polarity structure of human pluripotent stem cells (hPSCs) is derived from characteristics similar to epithelial cells, and it exhibits variances in cell shape, secretion of morphogens (Etoc et al., 2016), and the cell cytoskeleton (Xue et al., 2018) depending on the cellular position within the colony(Simunovic and Brivanlou, 2017) (Krtolica et al., 2009). These varying biological responses lead to complex patterning of germ layers in pluripotent stem cells (Bauwens et al., 2008; Warmflash et al., 2014). More recently, micropatterned hPSC colonies, in response to caudalized neural induction, have given rise to three-dimensional structures that ultimately develop into the spinal cord, exhibiting a dorsal-ventral axis (Seo et al., 2023). However, the precise mechanisms of how cell position and polarity impact fate determination remain largely unknown. To elucidate these mechanisms, various studies are ongoing. As an example, a recent development involves the observation of distinct protein distribution in cells located at the colony's edge. This observation led to the development of a technique utilizing dyes to separate edge cells from center cells (Kim et al., 2022). Using this technique, cells can be categorized into two groups based on their initial positions within the colony, allowing for an exploration of location-dependent gene expression profiles. The results of the Differential Expressed Genes (DEG) analysis showed that several gene classes related to cell movement and motility were significantly upregulated in the edge cells. Comparing these findings with previous research (Rosowski et al., 2015) highlighted differences in actin dynamics. Through an examination of the kinematics and mechanical dynamics of human pluripotent stem cell (hPSC) colonies and an analysis of cell trajectories, it was determined that central cells with lower mobility are strongly connected to neighboring cells, while edge cells, which exhibit active individual mobility, have weaker connections to neighboring cells. These differences in traction force lead to variations in the expression of mechanotransducer proteins and play a pivotal role in inducing symmetry breaking. Such research shows promise in uncovering the influence of cell positioning on patterning in stem cells.

2.2. Stiffness and stretching

The physical resistance imposed on cells affects their shape, signal transduction, and differentiation. The forces generated when cells interact with their surrounding extracellular matrix (ECM) or other cell surfaces during movement are referred to as traction forces. The stiffness of the ECM is one of the physical properties of the cellular microenvironment, and ECMs of varying rigidity exert traction forces on cells, influencing their shape, differentiation, movement, and signal transduction. For example, naive mesenchymal stem cells (MSCs) form neural lineages on soft matrices mimicking brain-like conditions (Flanagan et al., 2002), muscle lineages on stiffer matrices resembling muscle tissue (Engler et al., 2004), and skeletal lineages on relatively rigid matrices (Engler et al., 2006). This underscores the significance of substrate rigidity in the field of pattern formation. Various studies have explored how the stiffness of the extracellular matrix (ECM) is biochemically translated, and a general model for this phenomenon has been elucidated (Klapholz and Brown, 2017). Integrins on the cell membrane, when binding to the ECM, form different types of integrin clusters depending on whether the ECM is relatively soft or stiff (Oria et al., 2017). These integrin clusters are connected to a protein called Talin within the cell membrane, serving as a molecular tension sensor that detects mechanical signals in the cell's surrounding environment. Particularly on stiff ECM, Talin links integrin clusters and detecting mechanical signals in the cell's surrounding environment. This molecular tension sensing, facilitated by Talin, activates two transcriptional co-activator proteins, Yes-associated protein (YAP), and Transcriptional co-activator with PDZ-binding motif (TAZ) (Elosegui-Artola et al., 2016). YAP and TAZ initially present in the cytoplasm, translocate to the nucleus upon receiving signals and regulate the expression and modulation of genes about cell differentiation and growth (Hansen et al., 2015). Based on these studies, researchers are exploring a variety of substrates with different rigidity levels in vitro, and they are also employing these substrates for differentiation purposes. Specific substrates have been observed to induce the self-organization of PSCs into 3D tissue structures, further mimicking developmental processes like the formation of luminal neuroepithelial (NE) cysts. In recent studies, there has been evidence of more sophisticated ridge-and-groove patterns emerging when researchers replicate the microenvironment found in neural generation sites within the human body or the extracellular microenvironment surrounding cells (Zheng et al., 2019).

Another form of physical resistance involves traction forces applied to cells or tissues by their surroundings, a process known as stretching. For example, during embryonic neurogenesis, mechanical forces exerted by surface epithelium adjacent to neural folds contribute to the bending of neural plates (Colas and Schoenwolf, 2001). These events within the body highlight the importance of providing active mechanical forces to tissues or cells, not only in mimicking physiological processes but also in pattern formation. To quantify these traction forces, analysis methods typically involve obtaining images before and after stretching, followed by image analysis using tools such as Matlab programs for traction force calculations. Recent research has provided evidence that increasing the stretching of three-dimensional environments promotes pattern formation and morphogenesis, further emphasizing the crucial role of mechanical forces in shaping both form and pattern (Abdel Fattah et al., 2021). Nevertheless, the mechanisms underlying these traction forces and cellular responses are still under investigation, and further research in this area is warranted.

2.3. Microfibrous scaffold (3D neural organoid)

Patterning can also be induced by controlling three-dimensional space, employing engineering techniques such as the microfibrous scaffold. The microfibrous scaffold offers diverse methods for its implementation. Using a 3D printer, it is feasible to fabricate structured scaffolds of desired (Chen et al., 2021; Rothenbucher et al., 2021). Additionally, techniques like emulsification and chemical reactions can be harnessed to create irregular scaffolds(Xue et al., 2023). Similar to micropatterns, the microfibrous scaffold constrains cellular space, exerting physical forces and enabling the transmission of signals within cells, leading to pattern formation. The differentiating factor lies in that while micropatterns confine spaces in 2D, scaffolds restrict spaces in 3D. Consequently, this allows for more intricate control, albeit necessitating more intricate processes.

3. Biochemical engineering affecting polarization

Biochemical engineering refers to the methods of enabling the creation of concentration gradients within a single well or a single spheroid, which leads to symmetry breaking and patterning (Fig. 1b). For instance, microfluidics technology involves the manipulation of small-scale fluid flows using channels and manipulation devices. This technology finds various applications, including its utilization as a gradient generator. Different media delivered from distinct inlets traverse microfluidic channels, creating concentration gradients through mixing (Qian et al., 2016; Vatine et al., 2019; Rifes et al., 2020). Design parameters such as the number of channels, fluid flow rates, device size, etc., can be designed and mathematically analyzed for diffusion coefficients, enabling the quantitative measurement of gradient profiles and facilitating concentration modulation.

Another strategy for generating concentration gradients involves the establishment of signaling center. A signaling center is a region that secretes morphogens or signaling molecules into the surrounding tissues, regulating the process of development. This concept can be found in the formation of the Sonic Hedgehog (Shh) signaling center in the early stages of embryo development. Mimicking the developmental

processes observed in vivo, there exist engineering techniques that enable the creation of artificial signaling centers even in vitro. Through genetic engineering, small clusters of cells can be engineered to secrete specific morphogens or signaling molecules. When these engineered clusters are attached to one side of embryonic stem cell (ESC) spheroids, an artificial signaling center is established within the ESC spheroid. Consequently, this prompts the occurrence of concentration gradients and facilitates processes like symmetry breaking and patterning. For instance, an artificial signaling center secreting WNT3A can be attached to EBs to induce symmetry breaking (Glykofrydis et al., 2021). Similarly, in forebrain organoids, a signaling center secreting Sonic Hedgehog (SHH) can be attached to induce patterning within the forebrain organoid (Cederquist et al., 2019) as demonstrated by various studies.

4. Embryoids mimicking early development

Embryoids refer to a structure or clusters of cells derived from PSCs that exhibit some characteristics of early embryonic development, such as blastulation, gastrulation, and neurulation through some degree of self-organization and differentiation. In this review, this term encompasses both 2D and 3D structures, including blastoids, epiblastoids, gastruloids and neuruloids.

4.1. Blastoid and epiblastoid

Recapitulating the initial stages of human development can be achieved by utilization of bioengineering tools, like hydrogels, to guide differentiation of human pluripotent stem cells (PSCs) to form blastocyst and epiblast. Blastocysts contain an inner cell mass that gives rise to the embryo and a trophoblast, which forms extraembryonic tissues such as the placenta. Epiblast, which emerges during gastrulation, gives rise to the three germ layers including ectoderm, mesoderm, and endoderm. Models like blastoids and epiblastoids that are employed to replicate these developmental stages, can reproduce cellular determination, structured organization of this early stage, and the function like implantation (Table 1).

In blastoids, embryonic and abembryonic axis spontaneously forms according to the temporal and morphological development of *in vivo* embryo (Kagawa et al., 2022). Inhibition of Hippo, TGFβ and ERK signaling pathways in naïve hPSC aggregates in non-adherent hydrogel microwells efficiently produces trophectoderm, epiblast and primitive endoderm with spatial patterning. Furthermore, the interaction between epiblast and trophectoderm triggers the local maturation of polar trophectoderm, subsequently enabling the blastoid to acquire the ability to attach onto stimulated endometrial cells. Compared with the formation of this first axis in blastoids, when dissociated ESCs are dispersed as single cells into a mix of liquid hydrogel/Matrigel, they form spherical epithelia that spontaneously break AP symmetry with BMP addition to the medium (Simunovic et al., 2019). Initially, SOX2+ and/or Brachyury/T+ (Bra/T+) populations are spatially separated and later, markers of the primitive streak and EMT are induced, suggesting that WNT-DKK1 pair plays a crucial role in symmetry breaking in this epiblast model. This model demonstrates the ability of the epiblast itself to self-organize and break AP symmetry with minimal cellular and molecular components without the involvement of extraembryonic tissues or asymmetric ligand presentation.

Table 1
Polarized protocol mimicking early developmental stage.

Types	Dimensions	Methods	Patterning	References
Blastoid/ Epiblastoid	3D	Hydrogel microwell, Chemicals (PALLY)	Embryonic-abembryonic axis	(Kagawa et al., 2022)
		Hydrogel, BMP4	AP axis	(Simunovic et al., 2019)
Gastruloid	2D	Micropattern, BMP4	Germ layer patterning	(Warmflash et al., 2014)
		Micropattern, BMP4	Germ layer patterning	(Etoc et al., 2016)
		Micropattern, BMP, Wnt, Nodal	Primitive streak and organizer	(Martyn et al., 2018)
		Micropattern, BMP, Wnt, Nodal	Germ layer patterning	(Chhabra et al., 2019)
		Microfluidics, BMP4	Germ layer patterning	(Manfrin et al., 2019)
		Micropattern, BMP, Wnt	ML axis of ectoderm	(Britton et al., 2019)
	3D	Chiron (mESC)	AP	(Turner et al., 2017)
		Chiron (mESC)	AP, DV, ML	(Beccari et al., 2018)
		Chiron	AP	(Moris et al., 2020)
		Hydrogel microwell (mESC)	AP (+anterior neural tissue)	(Girgin et al., 2021)
		Genetically engineered HEK cells (mESC)	mesoderm	(Glykofrydis et al., 2021)
Neuruloid	2D	Micropattern	Neuroectoderm (neural plate-neural plate border)	(Xue et al., 2018)
		Micropattern	Neuroectoderm (neural progenitor-neural crest-sensory placode-epidermis)	(Haremaki et al., 2019)
		Microfluidics	AP	(Rifes et al., 2020)
	2.5D	Micropattern + Matrigel	Neural tube folding	(Karzbrun et al., 2021)
	3D (neural cyst)	Matrigel (mESC)	DV	(Meinhardt et al., 2014)
		Matrigel + Stiffness	DV	(Zheng et al., 2019)
		Stretching	DV	(Abdel Fattah et al., 2021)
		Matrigel + anisotropic PDMS	DV	(Tang et al., 2022)
		Matrigel (mESC)	AP	(Park et al., 2022)
	3D	Wnt activator	AP	(Libby et al., 2021)
	3D	Microfluidics	AP and DV	(Xue et al., 2024)

4.2. Gastruloid

Gastrulation is a critical stage of embryonic development in which a single-layered embryo transforms into a multi-layered structure characterized by distinct germ layers. Gastruloids, which serves as models of gastrulation derived from PSCs, can be developed to generate the primitive streak and the three germ layers with spatial patterning by employing bioengineering tools. While 2D gastruloids generated on micropattern typically exhibit the formation of concentric ring like-domains representing different germ layers, 3D gastruloids display tissue elongation and axial patterning which more closely mimic the dynamic process during gastrulation in vivo (Table 1).

To recapitulate gastrulation in 2D culture, micropattern which controls self-organization of PSCs with minimal geometric cues has been widely used. Upon exposure to BMP4, hPSCs that are geometrically confined on circular micropatterns, are differentiated into spatially organized germ layers including trophectoderm, ectoderm and mesendoderm, along the radial axis of colony (Warmflash et al., 2014). They

and others show that hPSC colonies on micropattern prior to exposure to morphogen are prepatterned (Warmflash et al., 2014; Etoc et al., 2016; Kim et al., 2022). Due to the high cell density at the center on micropatterned colony, receptor lateralization occurs at the center of colonies while receptor maintains the apical localization. Furthermore, apical structures and functions as well as transcriptomes at the center cells are different from those at edge cells. These differences generate spatial patterning in response to BMP4, although BMP4 are presented homogenously in media. Additionally, diffusible inhibitors such as Noggin induced by BMP4 are also necessary for the spatial pattern formation. Chabra *et al.* show that the dynamic events in the BMP, WNT and NODAL signaling cascade is important for the gastruloid patterning on micropattern, not a spatial pattern in signaling (Chhabra et al., 2019). As well, Wnt3a is sufficient on micropatterned colonies to induce primitive streak, producing patterned epiblast, mesoderm and endoderm (Martyn et al., 2018). Furthermore, micropatterned colonies that are stimulated with Wnt3a and activin express the organizer-specific marker, and acts as a secondary axis when transplanted into chick embryo. These studies show the strength and reliability of the gastruloid model established in a confined micropattern geometry. Geometric confinement through micropatterning offers several advantages over other cell patterning strategies. It allows for easy manipulation, exhibits high reproducibility, and facilitates quantitative measurements. However, these micropattern-based germ layer patterning does little control over self-organization beyond adding morphogens uniformly to the entire colonies. To control intrinsic capacity of PSCs for self-organization extrinsically, microfluidic approach is combined with micropattern (Manfrin et al., 2019). Microfluidics enables micropatterned colonies to be exposed to graded BMP4, which mimics the behavior of morphogens diffusing from signaling centers *in vivo*. This system makes axial arrangement of cell fates with breaking the radial symmetry of the micropatterned colonies, which have a more posterior cellular identity localized close to the BMP4 source.

While these studies based on micropattern generate 2D gastruloids with spatial patterning, polarized 3D gastruloids systems are established from mESCs (Turner et al., 2017; Beccari et al., 2018). Unlike the 2D system that utilizes BMP4 as a stimulus, the 3D system employs Wnt signaling activator for gastruloid formation. Turner *et al.* show that gastruloids undergo an intrinsic symmetry breaking process with WNT activation, resulting in axial elongation with expression of ~~BraT~~Bra/T in one end (Turner et al., 2017). With an extended culture period, these gastruloids develop three axial organization including AP, DV, and ML axes, spontaneously (Beccari et al., 2018). Specifically, collinear distribution of HOX gene are established spatially, demonstrating the existence of AP axis. Nevertheless, self-organizing capacity of ESCs leads to low level tissue organization due to absence of extraembryonic tissues, localized sources of signals, mechanical interactions and constrains that characterize the developing embryonic text. Human 3D gastruloids are generated by making subtle adjustments to the treatment time of Chiron (CHIR99021) (Moris et al., 2020). Three germ layers are organized spatiotemporally and elongation along the AP axis occurs although they curl or retract after 72 hr incubation. In common, these mouse and human gastruloid models are not able to develop brain or head structure, which is implicated with the absence of SOX1-positive region. The limitation of lack of anterior neural tissues is overcome by hydrogel microwell array (Girgin et al., 2021). When Wnt inhibitor is added to epiblast-like aggregates which are derived from stimulation with FGF2/ActinvinA, they become gastruloids with the presence of SOX1+ and SOX2+ cells in front of the extended ~~BraT~~Bra/T+ domain. The occurrence of symmetry breaking through the use of an artificial signaling center is also reported (Glykofrydis et al., 2021). When HEK cell clusters that are engineered to overexpress P-cadherin and produce functional mouse Wnt3a, are attach to the outer surface of EBs, symmetry breaking occurs. Regions of EBs in close proximity to the HEK clusters exhibited a predominant induction of ~~BraT~~Bra/T+ mesoderm. This indicates that these engineered HEK clusters serve as spatially localized signaling centers that mimic the function of in vivo organizers.

4.3. Neuruloid

Neurulation represents a critical stage in early embryonic development marked by the formation of the neural tube, starting from neural plate and later establishing regional identity within nervous system. In neuruloids, which serve as models of neurulation, the neuroepithelium initially exhibits shared characteristics across all regions and later differentiates into specific brain regions based on AP and DV axis. The transition from nonpolarized cells to polarized neuroepithelial cells in this stage can be facilitated in neurulation models by various bioengineering tools. Micropattern and microfluidics are employed to guide the differentiation and organization of neural cells in 2D culture system, and Matrigel and biophysical engineering tools are used to generate DV or AP organization in 3D neural cyst culture system (Table 1).

Micropattern is widely used in symmetry breaking in germ layer patterning in gastrulation, and this approach can be extended to modeling neurulation stage. BMP stimulus following nodal inhibition in micropatterned hESCs establishes medial-lateral patterning of ectoderm, with producing neural, neural crest, placodal and epidermal progenitors with the same organization as found *in vivo* (Britton et al., 2019). hESCs at the center of micropatterned colonies differentiate into neural cells. Moving outward from the center region, concentric rings of cells emerge, consisting of neural crest and placodal progenitors in order. Close to the outer edge of the colonies, non-neural ectoderm cells are located. Dual SMAD inhibition followed by BMP4 in micropatterned hESCs also generates neuruloids containing neural progenitors, neural crest, sensory placode and epidermis (Haremaki et al., 2019). Unlike the previous ectoderm model, epidermis component in this neuruloid covers the spatially organized neural ectoderm, neural crest and placode. This arrangement closely resembles the organization of the ectoderm observed *in vivo*, in terms of spatial morphology and structure. These neuruloids was utilized for modeling Huntington's disease, which is a dominant autosomal disease caused by an expansion of CAG repeats, leading to an increase of polyglutamine expansion at the N terminus of the huntington (HTT) protein. Neuruloids derived from both isogenic HD hESCs and homozygote null HTT-/- mutants exhibited highly reproducible phenotypes (Haremaki et al., 2019). In addition, micropattern has been used for neuroectoderm patterning (Xue et al., 2018). Dual SMAD inhibition results in neural induction at the center of micropatterned colony and brief treatment of Chiron induces neural plate border in the periphery. Similar to previous micropattern-based studies (Etoc et al., 2016), this study also demonstrate that fate patterning in hPSC colonies depends on colony geometry and cell density. And this micropattern system reveals that neuroectoderm patterning occurs in the absence of non-neural ectoderm or mesenchymal tissues, indicating the autonomous patterning and regionalization of neuroectoderm tissues. Based on the result that the increasing concentration of WNT activator induces differentiation of hPSCs into progressively caudalized neural fates (Kirkeby et al., 2012), Rifes *et al.* establish microfluidics system mimicking single gradient of WNT signaling to drive specific regional differentiation within the neural lineage (Rifes et al., 2020). hPSCs exposed to a linear gradient of GSK3 inhibitor in a microfluidic device are differentiated into a single tissue that exhibits the entire cranial rostral to caudal neural axis containing forebrain to midbrain to hindbrain.

However, these 2D approaches do not recapitulate three dimensional cell-fate patterns and neural tissue morphogenesis. Using chip-based culture system that combines surface micropatterning and transition 2D culture to 3D structure induced by Matrigel, neural tube-like structure covered with ectoderm is generated, with mimicking human neural tube folding (Karzbrun et al., 2021). As neural plate width and neural fold morphology varies along the AP axis, by varying micropattern size, they show that neural tube shape depends on neural tissue width. However, clear DV patterning cannot be achieved in this neural tube

model. The suitability of this organoid as a modeling system for neural tube defects was assessed by testing its response to small molecule inhibitors, such as a ROCK inhibitor and an HSP-90 inhibitor (Karzbrun et al., 2021). Application of these inhibitors resulted in morphological defects reminiscent of neural tube defects. Furthermore, exposure to valproic acid, a well-known teratogenic agent that causes neural tube defects in humans and also in spinal cord organoid model (Lee et al., 2023b), led to an inversely bent neural plate. These results strongly suggest the pathophysiological relevance of this neural tube model for studying neural tube defects. Recently, Xue et al. reported the establishment of AP and DV axes within neural tube-like structures using microfluidics (Xue et al., 2024). Moreover, they demonstrated that the pre-patterning of axial identities in neural crest progenitors within this system.

Neural tube with DV patterning that contains floor plate cells is successfully reproduced with neural cyst models of mESCs (Meinhardt et al., 2014) and hESCs (Zheng et al., 2019). When single mESC is embedded in Matrigel or synthetic matrix, neuroepithelial cyst with single lumen is generated, and the following posteriorization by RA treatment results in DV pattering (Meinhardt et al., 2014). Using similar 3D ECM but additional soft tissue bed, human NE cyst with default dorsal identity is produced and the following RA and SHH activation lead to DV patterned NE cysts (Zheng et al., 2019). Although these two NE cyst systems display DV axis, they do not have all progenitor domains along the DV axis *in vivo*.

Neural cyst that is derived from single PSCs and contains a sin178gle lumen is a good model to study axis formation in neural tissues. When single hiPSCs seeded onto geltrex-coated coverslip are differentiated under neural induction medium further supplemented with soluble geltrex, they become 3D neural cyst with floor plate patterning (Tang et al., 2022). Actomycin contractility, Yap signaling and interfacial cues such as anisotropic surface and confining microniches, influence on cyst formation and patterning. On the other hand, neural cysts derived from mESCs in Matrigel establish AP axis from midbrain and spinal cord and generate neural crest cells (Park et al., 2022). However, they do not have DV axis, with default dorsal fate. It is notable that in this system, the relatively low initial cell seeding density is used and AP patterning occurs without Chiron or RA which are generally used for AP pattering in other systems. To mimic mechanical force that plays an important role in neurulation during which an initially flat neural plate is changed into a pseudostratified epithelial neural tube, Abdel Fattah et al. add matrix actuation to gel-embedded neural cysts (Abdel Fattah et al., 2021). This mechanical force increases organoid growth and patterning through actomyosin contractility, cytoskeletal remodeling and planar cell polarity machineries.

5. Neural organoids

Neural organoids are PSC-derived 3D structures that exhibits self-organization and differentiation into diverse cell types found in the central nervous system, mimicking certain aspects of the structure and function of the developing human brain. Region-specific neural organoids can be generated by utilizing small molecules and recombinant growth factors with precise timing of exposure to guide PSC differentiation. However, simply adding molecules to culture media often falls short in establishing distinct regional compartment and axial patterning (Lancaster et al., 2013; Jo et al., 2016; Lee et al., 2022). Bioengineering tools such as micropattern, scaffold and genetic engineering can enhance the spatial organization and structural integrity of region-specific cells in neural organoids. These approaches allow for the replication of features such as wrinkled cortical surface, neuromuscular organization as well as axis formation within specific brain regions (Table 2).

5.1. Cerebral/brain organoid

Cerebral organoids are one of the most widely used organoid system, but most of them in earlier days lack significant patterning induced by externally added morphogens or other factors. However, careful analysis of the cerebral organoid finds that forebrain signaling center, cortical hem and pallial-subpallial boundary (PSPB) are present discretely in cerebral organoids, indicating endogenous patterning is self-organized, in spite of significant variability from organoid to organoid and between experiments (Renner et al., 2017). To recapitulate *in vivo* spatial patterning, an inducible SHH expressing hPSCs are embedded at one pole of a developing forebrain organoids (Cederquist et al., 2019). This cell cluster function as a signaling center with making SHH protein gradient, which enables forebrain organoid to establish DV and AP axis. Interestingly, depending on the distance from SHH source, tissue cytoarchitecture differs. The region distal to SHH source displays circular, rosette-like morphologies, but the proximal region contains thin and highly extended neuroepithelia. In addition, extensive cortical

Table 2
Polarized protocol mimicking neural development.

Types	Dimensions	Methods	Patterning	References
Neural organoids	3D (Cerebrum/Brain)	Genetic engineering (PTEN deletion)	Wrinkle	(Li et al., 2017)
		Autonomous	Forebrain regions	(Renner et al., 2017)
		Microfabricated compartment	wrinkle	(Karzbrun et al., 2018)
		Genetic engineering-signaling center (SHH)	DV	(Cederquist et al., 2019)
		Microwell fabrication	Wrinkle	(Chen et al., 2021)
		PCL scaffold	Wrinkle	(Rothenbucher et al., 2021)
	2D (Midbrain/ Hindbrain)	Micropattern	AP	(Xie et al., 2022)
	3D (Cerebellum)	SDF1α	Laminar layering (granule cell layer/ Purkinje cell layer)	(Atamian et al., 2024)
	3D (Spinal cord)	BMP, SHH	DV	(Ogura et al., 2018)
		BMP4	Dorsal pattern	(Duval et al., 2019)
		Autonomous	Neuromuscular junction (spinal cord- skeletal muscle)	(Faustino Martins et al., 2020)
		Composite scaffold	DV	(Xue et al., 2023)
		Micropattern	DV	(Seo et al., 2023)
	3D	Assembloid	DV (forebrain)	(Birey et al., 2017) (Bagley et al., 2017) (Xiang et al., 2017)
		Assembloid	Multiregion (cortex-thalamus)	(Xiang et al., 2019)
		Assembloid	Multiregion (cortex-striatum)	(Miura et al., 2020)
		Assembloid	Multiregion (cortex-spinal cord-muscle)	(Andersen et al., 2020)
		Assembloid-Microfluidics	Multiregion (cortex-hippocampus-thalamus)	(Zhu et al., 2023)

folding due to massive increase of neural progenitor population that result in expansion of the human cortex, is replicated in several cerebral organoid systems. PTEN deletion in human cerebral organoids expands radial glial and intermediate progenitor population which result from cell cycle re-entry and transient delayed neuron differentiation. These lead to VZ/SVZ expansion, overall size increase and larger surface area, and finally surface folding (Li et al., 2017). In contrast, PTEN deletion contributes size increase, but not surface folding in mouse cerebral organoids. To evaluate the efficacy of this genetically modified cerebral organoid system in modeling complex structural malformation of the developing human brain, Zika virus (ZKV), a pathogen known to affect fetuses and newborns, was applied to organoids (Li et al., 2017). Infection of ZKV, which leads to microcephaly, lissencephaly, pachygyria, and cortical atrophy in human, significantly disrupted the formation of expanded and folded cerebral organoids, indicating that this organoid system can recapitulate key aspects of the structural abnormalities observed in ZKV infection. Microfabricated compartment culture system using hESCs shows that mechanical instability drives cortical wrinkling (Karzbrun et al., 2018). Active contraction at the core and cell-cycle-dependent nuclear expansion at the outer regions induce differential growth in organoids, leading to cerebral folding. The organoid wrinkling was notably reduced in this on-chip organoids derived from LIS1 heterozygous (+/-) mutant hESCs (Karzbrun et al., 2018). This mutation is closely associated with lissencephaly, a severe smooth brain malformation. These results underscore the potential of this organoid system to faithfully model the interwined physics and biology underlying early human brain development. Microwell culture platform based on 3D printing also generates human cerebral organoids with wrinkling. (Chen et al., 2021). Engineered flat brain organoid system using polycaprolactone scaffold together with Matrigel develops cerebral folding (Rothenbucher et al., 2021).

5.2. Spinal cord organoid

Many efforts to generate spatially patterned spinal cord organoids have been made, and successful but incomplete organoid systems have been reported. Ogura *et al.* develop spinal cord organoids with dorsal patterning (Ogura et al., 2018). Several dorsal domains including roof plate are simultaneously induced and maintained in a 3D structure. Differentiated cells can be more dorsalized or ventralized by activation of BMP or SHH signaling pathway. Duval *et al.* also generate dorsal spinal cord organoids that are patterned with spatial dynamics of BMP4 signaling (Duval et al., 2019). Upon exposure to BMP4, 3D embryoid bodies (EBs) show stereotyped concentric patterns of expression organized along the outer-inner axis. By controlling BMP concentration, exposure time and duration, spatial patterning of organoids are controlled. However, these two systems do not contain all domains from dorsal to ventral regions. DV axis formation using engineered ventral organizer is successfully achieved (Xue et al., 2023). When differentiated hESCs are integrated with SAG loaded into a porous chitosan microsphere followed by thermosensitive Matrigel coating, sustained-release of SAG produces patterned spinal cord organoids. Ventral Nkx6.1+ and Olig2+ cells are close to SAG organizer and dorsal cells are mainly located outer layer of organoids. Recently, we reported polarized spinal cord organoids with DV organization (Seo et al., 2023). Initial symmetry breaking in 2D micropatterned cultures leads to DV patterning in 3D elongated structures. As micropattern size determines neural fold shape in neural tube folding model system (Karzbrun et al., 2021), micropattern size controls protrusion morphology and dorsal/-ventral proportion in pSCOs. However, while axial elongation is more associated with AP axis formation in other organoid models, elongation is associated with DV axis in our pSCOs. On the other hand, AP patterning in spinal cord organoids is achieved in hPSC aggregates treated with Wnt (Libby et al., 2021). Wnt activation induces axial elongation, and elongated organoids consist of neuroepithelial and neuromesodermal compartments. Progressive activation of HOX gene clusters demonstrates AP axis in this organoid. TBXT+ mesoderm is necessary for proper axial elongation, singular extension and HOX gene expression. Furthermore, neuromuscular organoids (NMOs) that simultaneously generate spinal cord neurons and skeletal muscle cells are produced from hPSC-derived neuromesodermal progenitors (Faustino Martins et al., 2020). Functional neuromuscular junctions (NMJs) supported by terminal Schwann cells shows CPG-like circuits and muscle contraction. These organoids have an important advantages in that motor neurons and muscle fibers develop in parallel and timely interact during development, in comparison to other systems that depend on the use of skeletal muscle from human biopsies or immature muscle s generated separately from hPSCs. Notably, these NMOs has been shown to recapitulate key aspects of myasthemia gravis (MG), a common autoimmune disease affecting the NMJ, suggesting their potential as a disease modeling platform (Faustino Martins et al., 2020).

5.3. Organoids for other brain regions

Micropattern has been also applied to anteroposterior regionalization of early midbrain and hindbrain (Xie et al., 2022). AP patterning was achieved by activating WNT, RA, BMP, and SHH signaling in micropatterned hPSCs, resulting in the formation of two distinct zones, OTX2+ midbrain and HOXB4+ hindbrain zones.

Although bioengineering tools were not employed, an external cue facilitated the establishment of organized laminar layering in cerebellar organoids. During development, SDF1α guides the tangential migration of granule cell precursors (GCPs) along the pial surface by activating the corresponding CXCR4 receptor, which is expressed in GCPs. This results in the formation of external granule layer (EGL), with Purkinje cells settling below the EGL. In 2-month-old cerebellar organoids, this organization resembling the *in vivo* cerebellum was not initially observed. However, upon the addition of SDF1α to the 2-month-old cerebellar organoids, GCPs aligned adjacent to the organoid edge, and Purkinje cell precursors were observed beneath this layer (Atamian et al., 2024). This indicates that the application of external cues inspired by developmental processes at the appropriate timing and concentration can enhance the anatomical organization in organoid systems.

6. Fusion engineering

As explained previously, the challenge lies in generating organoid systems that encompass multiple subdomains or major axis in a single structure because these subdomains require specific morphogens or gradients of morphogens to properly develop. In contrast to autonomous pattern formation, fusion engineering involves the artificial amalgamation of differentiated or patterned cells and tissues to generate more intricate artificial patterns (Fig. 1c). Although this fusion engineering does not involve the process of symmetry breaking as explained earlier, this review addresses it for comparison because the fusion strategy can replace symmetry breaking for the induction of polarization of the organoids. An example of this is the assembly of two or more different organoids, termed "assembloid." Unlike signaling center, which are utilized as auxiliary components to induce patterning in ESC spheroid, assembloids feature the distinctive aspect that all fused spheroids or organoids contribute to the patterning process. These assembloids can replicate an axis observed within a single organ, or they can involve attaching organoids that replicate various regions within a single organoid to craft more sophisticated, higher-order organ-mimicking assembloids (Table 2). Furthermore, assembloids can extend to the fusion of different organ organoids, simulating the connectivity and circuitry within the human body (Andersen et al., 2020).

In the fusion neural organoid, neurons from one spheroid project into the counterpart spheroid, which represents the directionality of neural circuits. Although detailed protocols for organoid generation and fusion are different, three groups develop dorsal-ventral forebrain assembloids (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017). All of these

organoids show robust interneuron migration from ventral to dorsal region. These migrated interneurons functionally integrates into dorsal side to form functional microcircuits (Birey et al., 2017; Xiang et al., 2017). The broad applicability of these modular systems would benefit if brain region-specific spheroids could be generated. When thalamus organoids are fused with cortical organoids, reciprocal thalamocortical projections establish with mimicking thalamocortical targeting and synaptogenesis (Xiang et al., 2019). Cortico-striatal pathways are produced in assembloids of striatum-cortex (Miura et al., 2020). Cortical neurons send axonal projections into striatal organoids with making synaptic connections. To assess the suitability of this cortico-striatal assembloid as a model system for neurodevelopmental disorder, assembloids were derived from patients with 22q13.3DS, a severe disorder characterized by global developmental delay, profound intellectual impairment, delayed speech and autism spectrum disorder (Miura et al., 2020). The mutant assembloids exhibited aberration in calcium activity, indicating their utility in recapitulating the altered neural activity observed in human disease states. The concept of modularity has been expanded, leading to the generation of three part-assembloids (Andersen et al., 2020). First, human cortical and spinal cord organoids are separately generated and assembled to establish corticofugal projections. Second, spinal spheroids and 3D human skeletal muscle derived either from adult muscle biopsies or iPSC are combined together to enable functional connections between spinal cholinergic projections and muscles. Finally, cortical spheroids are fused with spinal spheroids and 3D skeletal muscle. These 3 component assembloids successfully replicate cortico-motor circuit, where cortical neural activation controls muscle contraction through motor neuron activation. Zhu *et al.* reported brain assembloids based on microfluidic technology (Zhu et al., 2023). Brain region-specific spheroids which are made in microcapsules are introduced into multilayered microfluidic chip and fused together, resulting in the formation of brain assembloids. Cortical-hippocampal-thalamic assembloids generated by this system exhibit active neural migration and interaction, demonstrating the functionality of the system.

7. Conclusions and Perspectives

Increasing engineering efforts are required to provide sufficient environmental cues for the proper morphogenesis of later developmental processes. Thus, along with continuous efforts for mimicking later developmental processes, these engineering techniques are in a state of constant evolution. More recently, there has been an increasing effort to amalgamate various engineering methods to craft more sophisticated patterning. For instance, a recent publication showcased the utilization of microfluidics technology to achieve the fusion of assembloids in desired configurations. Through these combined techniques, the ability to control the linear connection or clustering of three organoids has been achieved (Zhu et al., 2023). While certain forms of combined controlled engineering are yet to be developed, their emergence is anticipated. Especially, current engineering technology have been focused on the spatial regulation of the developmental cues, but temporal controllability of these techniques is relatively poor. Since developmental cues for the polarization are precisely controlled at both spatial and temporal levels, improvement and combining of temporal controlling system will greatly provide technological improvements. Optogenetic or chemicogenetic protein engineering, context-controllable assembly of hydrogel molecules, and other brilliant concepts are good candidates for driving improvements (Lee et al., 2023a; Legnini et al., 2023; Qazi et al., 2022).

Considering that these 3D culture systems aim to mimic *in vivo* process of embryonic development, limitation of the current technology often stems from the shortage of our knowledge how the corresponding developmental control occurs *in vivo*. For instance, measurement of physical forces applied to the mammalian embryos *in vivo* is very difficult, and the current physical parameters for symmetry breaking have been mostly determined empirically. Thus, technical improvement on protein force sensors and their *in vivo* measurement are necessary for better understanding of the physiological relevance of the *in vitro* physical force-related symmetry breaking technologies. With these improvements, we will better understand how physical forces are converted to the biologically important signals during the development, and the thorough understanding of the biological process will help designing engineering for the *in vitro* production of embryoids/organoids.

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