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DEVELOPMENT AT A GLANCE

Human assembloids

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ABSTRACT

Deconstructing and then reconstructing developmental processes *ex vivo* is crucial to understanding how organs assemble and how physiology can be disrupted in disease. Human 3D stem cell-derived systems, such as organoids, have facilitated this pursuit; however, they often do not capture inter-tissue or inter-lineage cellular interactions that give rise to emergent tissue properties during development. Assembloids are self-organizing 3D cellular systems that result from the integration of multiple organoids or the combination of organoids with missing cell types or primary tissue explants. Here, we outline the concept and types of assembloids and present their applications for studying the nervous system and other tissues. We describe tools that are used to probe and manipulate assembloids and delineate current challenges and the potential for this new approach to interrogate development and disease.

KEY WORDS: Organoids, Assembloids, Cell–cell interactions, Human development, Disease modeling, Pluripotent stem cells

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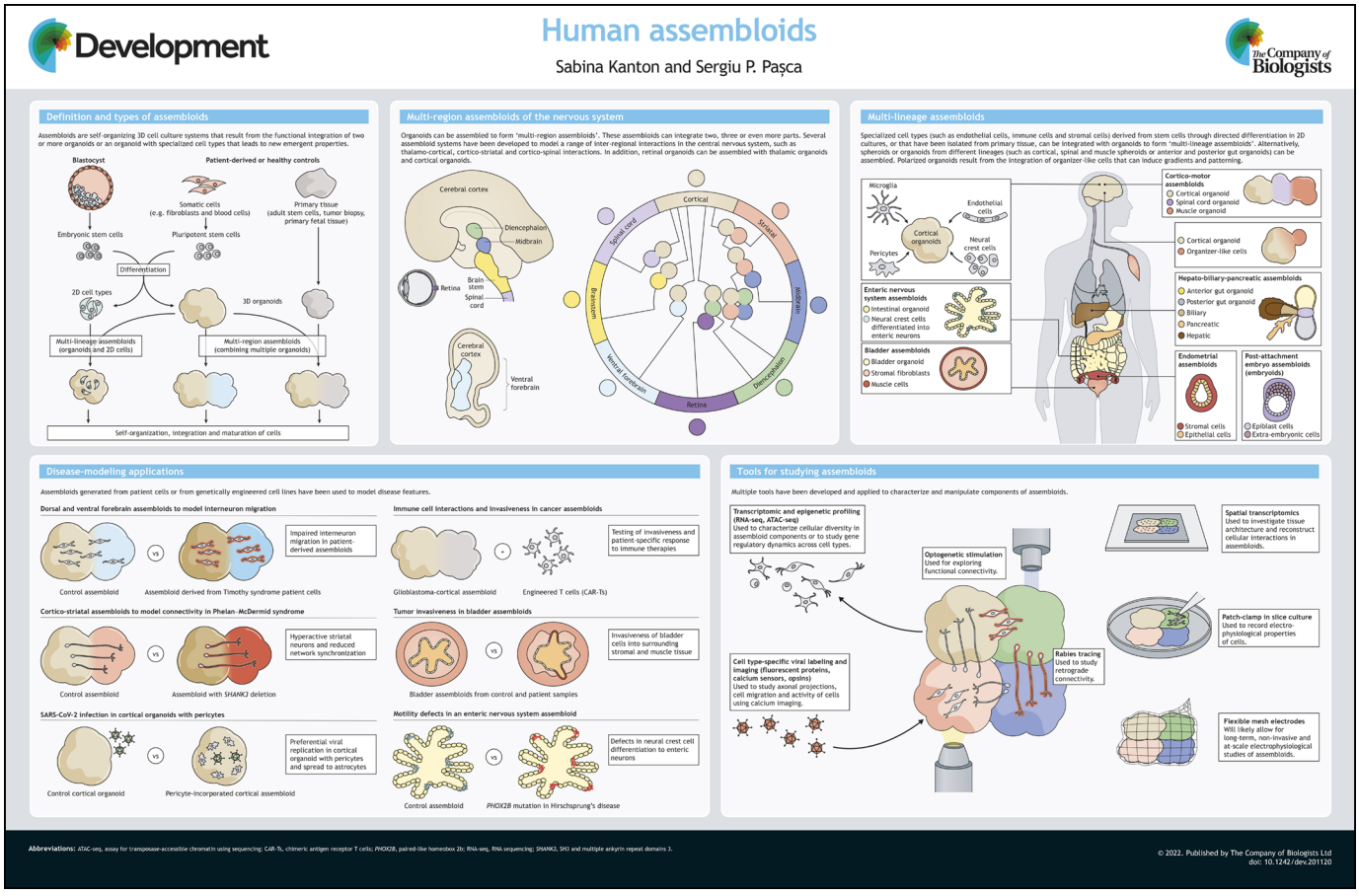
Introduction

During development, cells migrate and interact to form complex tissues and organs. For example, in the central nervous system, which is primarily of neuroectodermal origin, microglia migrate in from the yolk sac (Alliot et al., 1999), and mesoderm-derived endothelial cells invade and form blood vessels that support both growth and migration of other cells in the nervous system (Shen et al., 2004). In fact, essential to brain function are intricate interactions between different regions of the neural axis that involve extensive cell migration, such as that of inhibitory interneurons into the dorsal forebrain to assemble the circuits of the cerebral cortex, and the formation of axonal projections and synaptic connections at distance, such as thalamic projections bringing sensory input into the cerebral cortex and cortical neurons projecting back to the thalamus (Kelley and Paşca, 2022).



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In recent years, human cellular models derived from stem cells have been developed in an effort to offset the paucity of human primary tissue for studying many of these inaccessible interactions (Rossi et al., 2018). 3D *in vitro* systems, such as organoids, leverage self-organizing forces and can be coaxed to contain organ-specific cell types to recapitulate some tissue functions and cellular interactions (Lancaster and Knoblich, 2014; Paşca, 2018). However, to model the contribution of cell types derived from other germ layers or to model the interactions between different tissues, more complex models are needed. Assembloids mitigate this issue by combining different organoids or by introducing missing cell types into organoids to enable additional emerging properties of tissue development (Paşca, 2019).

Definition and types of assembloids

Assembloids are 3D cell culture systems that result from integrating a type of organoid with another organoid or that contain specialized cell types and display self-organizing features. An organoid is therefore defined as a self-organizing 3D *in vitro* culture system that contains a set of specialized cell types and recapitulates some function of an organ or region of an organ. There have been recent efforts to provide a framework for nomenclature of neural organoids and assembloids (Paşca et al., 2022).

Multiple organoids can be assembled to form 'multi-region assembloids'. These organoids can be derived from healthy, patient-derived or genetically engineered human induced pluripotent stem cells (hiPSCs), human embryonic stem cells (hESCs) or even primary tissue sources, such as isolated adult stem cells, excised tumors with growth potential or fetal tissue. These assembloids can integrate two, three or even more parts.

Alternatively, specialized cell types derived from stem cells through directed differentiation in 2D cultures or that have been isolated from primary tissue can be integrated with organoids to form 'multi-lineage assembloids'. Assembloids generated from organoids of different individuals that are used to study cell-autonomous effects are classified as 'inter-individual assembloids', and those obtained by combining organoids from different species (e.g. human and chimp) as 'inter-species assembloids' (Paşca et al., 2022).

Generally, the assembly of these components needs to be accompanied by cellular self-organization and new emergent properties, such as cell fate specification, changes in morphology, enhanced maturation or circuit formation.

Multi-region assembloids of the nervous system

Several two-part assembloid systems have been developed to date to model a range of inter-regional interactions. To model the migration and integration of ventral forebrain-generated interneurons into dorsal cortical circuits, dorsal and ventral forebrain organoids can be combined, which results in nucleokinesis-based migration of interneurons followed by morphological and synaptic integration with glutamatergic neurons (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017). This assembloid system revealed differences in the morphology and kinetics of migration between humans and mice (Birey et al., 2017).

Axonal projections between different brain regions are essential for circuit formation and maturation of the nervous system. The thalamus serves as a hub relaying information between the cortex and multiple other brain regions, and input from the thalamus into the cortex is crucial for cortical maturation. To model thalamo-cortical interactions, thalamic and cortical organoids can be assembled to form bi-lateral projections (Xiang et al., 2019). Cortico-striatal circuits control motivated behaviors and contribute to multiple diseases and syndromes, including autism spectrum disorder and schizophrenia (Shepherd, 2013). Reminiscent of the directionality of this circuitry, when striatal and cortical organoids are assembled only cortical neurons project into the striatum but not vice versa. This increases the intrinsic activity of medium spiny neurons, revealing disease-related defects in assembloids derived from patients (Miura et al., 2020). Cortico-spinal projections can also be studied in assembloids by integrating cortical, spinal and skeletal muscle organoids, in which contraction can be achieved upon electrical or optogenetic activation of cortical glutamatergic neurons (Andersen et al., 2020). Lastly, to model aspects of the ascending visual pathway, retinal organoids can be assembled with thalamic organoids and cortical organoids to build retino-thalamic and thalamo-cortical projections (Fligor et al., 2021).

Multi-lineage assembloids

A limitation of organoids and assembloids is the lack of functional vasculature to supply nutrients and other trophic factors. To achieve vascularization, brain organoids have been combined with endothelial organoids and mesenchymal cells (Song et al., 2019), derived from ETV2-induced endothelial differentiation (Cakir et al., 2019), or integrated with hESC-derived vascular organoids (Sun et al., 2022). Pericytes, which are neural crest derived, interact closely with endothelial cells and astrocytes. Therefore, when hiPSC-derived neural crest cells were integrated with cortical organoids, the differentiated pericytes secreted components of the basement membrane that led to more mature astrocytes (Wang et al., 2021). Microglia, the immune cells of the central nervous system, play a vital role in the phagocytosis of apoptotic cells, infection defense and neurodegeneration. To model neuro-immune interactions, microglia-like cells derived from hiPSCs can be introduced into midbrain (Sabate–Soler et al., 2022) and cortical (Xu et al., 2021) organoids. Oligodendrocytes, which are myelinating support cells in the brain, often have to migrate to reach their final destination, for example from the ventral to the dorsal forebrain (Jakovcevski, 2009). Oligodendrocytes can be generated in neural organoids (Madhavan et al., 2018; Marton et al., 2019; Shaker et al., 2021) and their migration could, in principle, be modeled by generating two-part assembloids. Lastly, assembloids of the peripheral (e.g. enteric) nervous system can also be derived by combining hiPSC-derived intestinal organoids with neural crest cells (Workman et al., 2017).

To study the specification of and interactions between endoderm-derived organs, anterior and posterior gut organoids can be juxtaposed to ultimately generate hepato–biliary–pancreatic domains (Koike et al., 2019). Moreover, multilayered bladder assembloids can be built by integrating long-term bladder organoids with stromal fibroblasts and muscle cells, which can lead to increased maturation of the bladder cells (Kim et al., 2020).

Studying very early stages of human development, including embryo implantation and gastrulation, is challenging owing to technical and ethical limitations. In fact, modeling interactions between the various cell lineages of the blastocyst and the uterus can help with understanding the early stages of embryogenesis and organogenesis. Assembloids derived by combining hESC-derived epiblasts and extra-embryonic cells allow investigation of post-implantation stages of embryonic development (Simunovic et al., 2022). Moreover, endometrial assembloids generated by reassembling stromal and epithelial cell fractions from endometrial biopsies revealed that senescent cells are important in preventing implantation failures (Rawlings et al., 2021).

Another type of assembloid aims to recapitulate spatiotemporal patterning by creating organizer-like cellular structures that can be

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embedded into organoids to generate 'polarized organoids'. For instance, to pattern across the dorsoventral and anterior and posterior axes of the forebrain, hPSCs can be induced to express sonic hedgehog (SHH) when being incorporated into early-stage forebrain organoids; this leads to local patterning and spatial organization within the organoid and the subsequent formation of dorsal and ventral forebrain, hypothalamic and diencephalic domains (Cederquist et al., 2019).

Disease-modeling applications of assembloids

Assembloids generated from patient cells or from genetically engineered cell lines have been used to model disease features. For instance, forebrain assembloids derived from patients with Timothy syndrome (TS), a genetic disorder associated with autism and epilepsy, have uncovered a defect in cortical interneuron migration and function. TS neurons display higher saltation frequency but shorter saltation length, which is related to changes in actomyosin signaling and GABAergic receptor sensitivity (Birey et al., 2017; 2022).

Cortico-striatal assembloids created from patients with Phelan–McDermid syndrome, in whom the striatal-enriched *SHANK3* gene is often lost, show increased calcium activity and reduced network synchronization (Miura et al., 2020). Importantly, this defect is not present in non-assembled striatal organoids, highlighting the important role of the interaction with cortical glutamatergic neurons in revealing this functional phenotype, which would be challenging to dissect in an animal model.

Assembloids incorporating immune components and vascular cells have already revealed some of the cellular mechanisms of central nervous system infection with Zika virus and SARS-CoV-2 viruses. For example, the infection of assembloids generated by integrating cortical organoids and pericytes with SARS-CoV-2 has highlighted pericytes as replication hubs for the spread of the virus to astrocytes (Wang et al., 2021). Neuro-immune assembloids (cortical organoids and microglia) infected with Zika virus have shown pruning of synapses during infection (Xu et al., 2021).

To study neuro-intestinal interactions in Hirschsprung's disease, enteric nervous system neurons can be assembled with intestinal organoids, revealing that a mutation in *PHOX2B* leads to defects in neural crest cell differentiation and functional integration with smooth muscle cells (Workman et al., 2017).

Assembloids combined from tumor organoids and stem cell-derived organoids hold potential for investigating invasion into healthy tissues and screening for targeted therapies (da Silva et al., 2018; Linkous et al., 2019), for example combining glioblastoma tumor organoids (GBOs) or assembloids of GBOs and neural organoids with engineered T cells [chimeric antigen receptor T cells (CAR-Ts)] to probe patient-specific responses to cell therapies or autoimmune reactions (Jacob et al., 2020). The etiology and progression of tumors often depends on microenvironment remodeling. To investigate this, bladder assembloids have been used to model different types (basal and luminal) of urothelial carcinomas by combining tumor organoids with endothelial cells and with matched fibroblasts, or they have been applied to study invasiveness into the surrounding stromal and muscle tissue and responses to T cells (Kim et al., 2020).

Tools for studying and manipulating assembloids

Multiple tools have been developed and applied to characterize and manipulate components of assembloids. Single-cell RNA sequencing and chromatin accessibility (ATAC-seq, CUT&RUN) assays can be used to characterize cellular diversity in assembloid components or to study gene regulatory dynamics across cell types. These approaches are also helpful in revealing more subtle transcriptional changes that occur upon assembly of different organoids following migration or axonal projection. Moving forward, high-resolution spatial transcriptomics offers an opportunity to investigate tissue architecture in organoids and assembloids and to computationally reconstruct cellular crosstalk of interacting cells in assembloids.

Cell type-specific viral labeling, including retrograde rabies tracing, and imaging of projections in assembloids has been employed to study axonal projections (Andersen et al., 2020; Miura et al., 2022). Functional connectivity can be probed by recording light-induced activity on one part of the assembloid following delivery of opsins and genetically encoded calcium indicators. Alternatively, electrophysiological properties of cells can be recorded using patch-clamping in slice culture (Birey et al., 2017). Recent developments in extracellular recordings, as well as flexible electrodes, will likely allow for long-term, non-invasive and at-scale electrophysiological studies of assembloids.

Challenges and future trends

Assembloids have already proved useful for modeling complex cell-cell integrations within and across tissues and to reveal disease pathophysiology. Moving forward, there are still a number of limitations to address and features to incorporate. Compared with the complex inter-regional interactions in the brain and the inter-organ crosstalk in the body, current assembloid models are still limited in their complexity. However, with recent advances in specifying more regions of the nervous system in 3D cultures and improving culture conditions, three-, four- or even five-part assembloids will be built. Recent successes in automating large-scale production and phenotyping of organoids (Narazaki et al., 2022 preprint; Renner et al., 2020) will also facilitate screening for circuit features and inter-cellular defects. Benchmarking of assembloids to *in vivo* tissues and circuits remains a challenge owing to the inaccessibility of primary tissue. To explore the maturation potential of assembloids, transplantation into rodents and subsequent vascularization of the grafts presents a promising approach (Mansour et al., 2018; Revah et al., 2022). Assembloids and organoids, combined with emerging genomic and functional technologies, will continue to shed light on human biology and disease processes.

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Competing interests

Stanford University holds multiple patents on organoids and assembloids with S.P.P. listed as an inventor.

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