Cell Organoid

Review

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Evolving from organoid to assembloid with enhanced cellular interactions

Authors

Jiarui Liu, Yitong Shi, Xianqin Shen, Wei Zhang, Xi Wang*, Kai Wang*

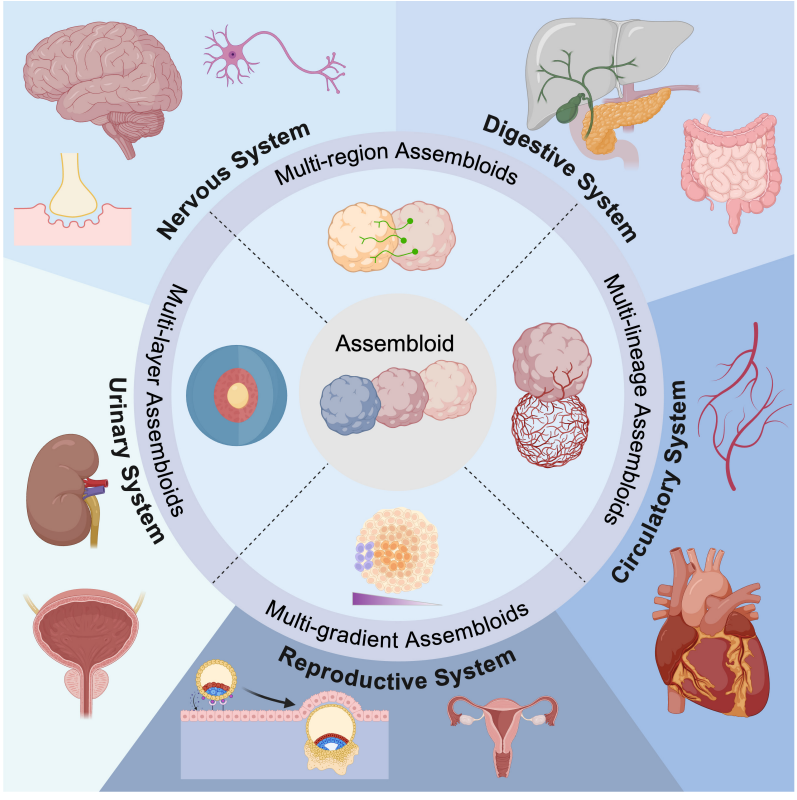
Correspondence

Xi Wang, xi_wang@bjmu.edu.cn; Kai Wang, kai.wang88@pku.edu.cn

In Brief

This review explores the emerging field of assembloid technology, which integrates multiple organoids or cell types into self-organizing three-dimensional (3D) systems to better model inter-tissue communication. It categorizes assembloids into four key assembly strategies and discusses their applications across various human systems, while also addressing challenges and future prospects.

Graphical abstract



Highlights

- Detailed analysis of the four key assembly strategies for building physiologically relevant assembloids: multi-region, multi-lineage, multi-gradient, and multi-layer.
- Application of assembloids in modeling complex interactions across human systems, including the nervous, digestive, urinary, reproductive, and circulatory systems.
- Identification of key challenges such as reproducibility and vascularization, with proposed solutions using bioengineering techniques and artificial intelligence to improve model accuracy and functionality.

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Evolving from organoid to assembloid with enhanced cellular interactions

Jiarui Liu[1], Yitong Shi[1], Xianqin Shen[1], Wei Zhang[2], Xi Wang[1,3,*], and Kai Wang[1,*]

[1] *Department of Physiology and Pathophysiology, School of Basic Medical Sciences, State Key Laboratory of Vascular Homeostasis and Remodeling, Clinical Stem Cell Research Center, Peking University Third Hospital, Peking University, Beijing 100191, China*

[2] *TianXinFu (Beijing) Medical Appliance Co., Ltd., Beijing 102200, China*

[3] *State Key Laboratory of Female Fertility Promotion, Department of Obstetrics and Gynecology, Peking University Third Hospital, Institute of Advanced Clinical Medicine, Peking University, Beijing 100191, China*

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ABSTRACT

In recent years, the study of complex cellular interactions has been hampered by the limitations of traditional two-dimensional (2D) and single-cell type culture systems, which fail to accurately mimic the intricate dynamics of human tissues. To bridge this gap, assembloid technology has emerged as a transformative approach. Assembloids are self-organizing three-dimensional (3D) systems formed by integrating multiple organoids or cell types, providing a more accurate model for studying inter-tissue and inter-organ communication. Here, we categorize current assembloids into four types based on assembly strategies—multi-region, multi-lineage, multi-gradient, and multi-layer—each designed to replicate specific biological phenomena with high fidelity. We also explore the diverse applications of assembloids across various human systems, demonstrating the broad application scope of assembloids. Finally, we highlight the challenges faced by assembloid technology and outline its future prospects. Overall, assembloids represent a powerful platform for advancing research in developmental biology, disease modeling, and drug discovery.

KEYWORDS

organoid, assembloid, cellular interaction, self-organizing

Introduction

A research model is crucial in shaping the complexity and biological relevance of the resulting scientific findings. Historically, due to technical and ethical limitations, studies on the functions of complex organs and tissues have often relied on simple cell culture models, while investigations into human disease mechanisms have frequently depended on non-human models. Traditional two-dimensional (2D) monolayer cultures and animal models have long been used in disease and drug development research. However, these models have significant limitations, including differences in cell arrangement and physiological and pathological functions, and suboptimal predictions of drug efficacy and toxicity. In contrast, three-dimensional (3D) cell culture allows cells to form spatial structures and interact with the surrounding cells as well as the extracellular framework in three dimensions. Many strategies have been developed to create a 3D biomimetic microenvironment for cells *in vitro* (Fig. 1).

Conventional scaffold-based strategies adopt a top-down approach to construct 3D tissue architectures by transplanting cells in combination with supportive scaffolds and biomolecules. These scaffolds and biomolecules provide support and create a spatial, chemical, and physical microenvironment for cells to some extent[1]. However, the cellular microenvironment constructed through such strategies is relatively simple[2]. Scaffold-free strategies rely on the self-organizing properties of cells to form tissues. Organoids, for instance, are defined as *in vitro*-generated cellular systems that emerge through self-organization, incorporating multiple cell types and displaying cytoarchitectural and functional features reminiscent of specific organs or organ regions[3]. Through cell self-assembly, organoids mimic natural tissue and organ formation processes. However, organoids typically contain only a limited number of cell types or a single tissue type, and they lack a defined topography[4–7], which are generated by gradients of signaling activity during the development *in vivo*[8,9]. Region-specific organoids attempt to overcome positional heterogeneity by restricting cellular identities to a single area. And assembloids, defined as self-organizing cellular systems resulting from the combination of a type of organoid with another type of organoid or with different specialized cell types, resulting in their integration[3], are able to reconstruct not only domain-specific but also tissue-

*Address correspondence to Kai Wang, kai.wang88@pku.edu.cn; Xi Wang, xi_wang@bjmu.edu.cn

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interconnected models for studying larger-scale processes, such as neural projection[10], gut–brain axis interactions[11], heart–brain axis interactions[12] and immune–tumor cell interactions[13], and results in emergent features of the system. Additionally, assembloids promote enhanced functional maturation and better spatial organization. These make assembloids a great tool for modeling developmental processes and diseases, offering a more holistic and sophisticated platform for research and drug development.

In this review, we provide a comprehensive summary of the various strategies used to construct assembloids, covering almost all currently available strategies (Fig. 2). We also broaden the scope by exploring the application of assembloids in modeling a wide range of human systems and organs *in vitro* (Fig. 3). Additionally, we highlight the challenges facing assembloid development and discuss potential future directions, offering a more holistic view of their capabilities and impact in biomedical research (Fig. 4).

Assembly strategies

The assembly strategies are critical, as they determine how well these models can recapitulate *in vivo* environments. This section outlines the main strategies used to assemble the four primary types of assembloids: multi-region, multi-lineage, multi-gradient, and multi-layer.

Multi-region assembloids

When the objective is to study a specific function or region of an organ with fine functional segmentation, such as the brain, using a guided organoid approach is recommended. Through region-specific differentiation, you can generate an *in vitro* organoid model that most closely resembles the region of interest. However, if your focus is on the interactions between different brain regions, such as the migration of interneurons from the subpallium to the cortex, or if a single region cannot adequately reproduce the physiological process or disease phenotype you are interested in, assembling them into an assembloid could offer new perspectives and discoveries. Additionally, many organs and anatomical structures in the body develop from originally isolated groups of cells; for example, the partitioning of the heart into a four-chamber organ results from a series of fusion events of primordial cellular structures[14]. Therefore, the stepwise combination of organoids from different specific regions into a larger unit, capable of addressing cross-tissue cell interactions, offers a promising strategy to mimic complex developmental processes and study inter-regional communication *in vitro*.

The assembly of multi-region assembloids involves the fusion of organoids derived from different regions (Fig. 2a), allowing the study of inter-regional communication and connectivity. Obtaining multi-region assembloids is generally straightforward; assembly strategies often involve pre-patterned embryoid bodies (EBs) or young organoids being co-cultured either in a microwell or a tube[10,16,17] or embedded together in a single Matrigel droplet[17]. Eventually, the two regions fuse and exhibit complex cell interactions (Fig. 2a). For instance, the organoid fusion approach has been particularly insightful in the field of neuroscience, such as studying the migration of cortical interneurons[15–17]. The fusion of dorsal and ventral telencephalic organoids makes it possible to visualize the characteristic saltatory migration of cortical interneurons and to study the impact of genetic and chemical perturbations on their migratory properties. Additionally, this strategy is pivotal in studying processes such as long-range corticothalamic and thalamocortical projections by fusing region-specific cortical and thalamic organoids[10] to replicate the complex wiring of these brain regions.

Organoid fusion methods are powerful techniques to visualize and study complex inter-region interactions, and in the future, these fusion approaches will help build more complex brain circuitry and neuronal connections with non-neural organoids or primary tissues.

Multi-lineage assembloids

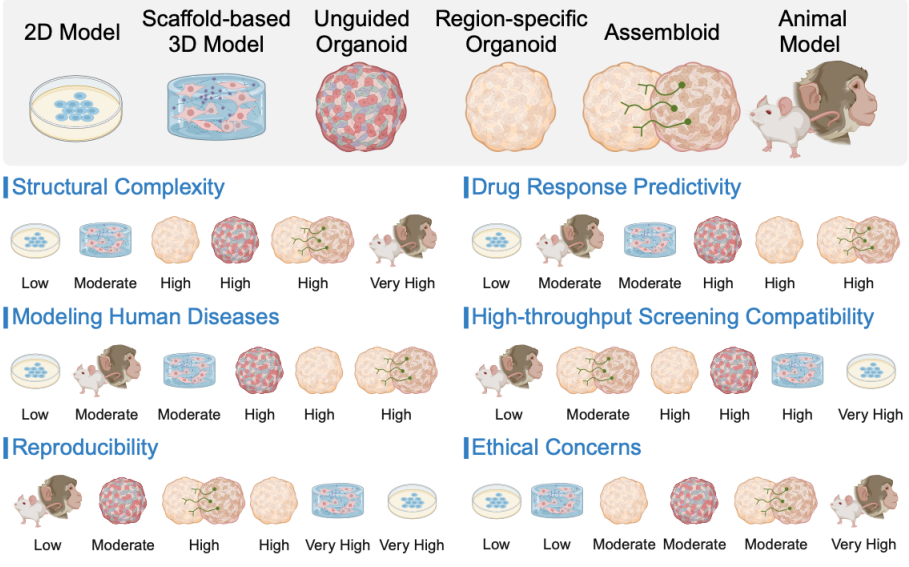

Figure 1. Comparison of commonly used models in biology. Multiple factors influence the choice of commonly used models in biology. This figure provides a comparison of the capabilities of commonly used models, including 2D models, conventional scaffold-based 3D models, unguided organoids, region-specific organoids, assembloids, and animal models, in terms of structural complexity, modeling human diseases, reproducibility, drug response predictivity, high-throughput screening compatibility, and ethical concerns. Created with BioRender.com.

In vivo organs contain multi-lineage cells, such as vascular and immune cells; however, most organoids often lack heterotypic cellular communication because they arise from tissue-specific committed stem cells and do not receive signals from tissues of a different origin. This is a clear distinction between organoids and their *in vivo* counterparts. This difference limits the maturation of organoids *in vitro* and their ability to model complex multi-system diseases. Currently, although rare, some organoids can achieve crosstalk between diverse lineages of cell types, either through the simultaneous or sequential application of patterning factors[18–20], or by inducible expression of fate-specifying transcription factors[21]. However, the former approach often restricts multi-lineage cellular communication to cell fates that are not mutually repressive, while the latter is not applicable for the introduction of all lineage-specific cells, as the differentiation protocols mediated by various transcription factors are still not fully developed. Constructing multi-lineage assembloids by directly assembling organoids with different lineages of cell types, or organoids containing these cell types, offers a potential solution to more directly enable interactions between a broader range of cell types across different lineages.

Multi-lineage assembloids are created by co-culturing organoids with different specialized cell types like microglia, immune cells, or endothelial cells (sometimes also with organoids, such as vascular organoids) to mimic the complex interactions across various lineages present in tissues and organs (Fig. 2b). A common example is the co-culture of brain organoids with microglia, the brain's resident immune cells, to study neuroinflammation and neurodegenerative diseases. This is typically done by introducing microglia precursors into brain organoid cultures at specific time points, allowing them to integrate into the organoid structure[22,23]. Another example involves incorporating endothelial cells into organoids, where the endothelial cells form capillary-like networks that interact with the surrounding tissue[24]. Overall, the multi-lineage assembloids strategy enhances the physiological relevance of the model by replicating tissue-tissue interactions that occur *in vivo*.

Multi-gradient assembloids

During organogenesis, morphogen gradients play a key role in shaping organ structure, such as the diversity of neurons in the brain, which is driven by gradients along the anterior–posterior (AP) and dorsal–ventral (DV) axes[25]. Traditional organoid cultures, however, typically apply morphogens at uniform concentrations, limiting cellular diversity. By establishing *in vitro* gradients that mimic developmental processes, we can model a wider range of phenotypes in a single system. Multi-gradient assembloids offer a promising model, particularly for studying complex developmental diseases linked to regional specification during patterning.

The creation of multi-gradient assembloids leverages the concept of morphogen gradients to direct the spatial organization and differentiation of cells within the assembloid. These gradients are established by co-culturing organoids with cells that secrete signaling molecules, such as Sonic Hedgehog (SHH), which is crucial for patterning during embryonic development (Fig. 2c). To establish a gradient of morphogen activity and downstream patterning, Cederquist et al. established a novel way to generate axially patterned organoids using a two-step process of EB formation[4]. Initially, a small EB was generated from transgenic human pluripotent stem cells (hPSCs), capable of inducible expression of a morphogen. Subsequent seeding of additional hPSCs led to the formation of a larger EB harboring an inducible signaling center or organizer. Upon induction of morphogen expression, the secreted morphogen diffused in the tissue to create a gradient. Cederquist et al. used this method to create a gradient of the ventral-inducing morphogen SHH and successfully generated telencephalic organoids with a spatially arranged DV axis[4].

In the future, this new technology will pave the way to pattern longer developmental axes, such as the AP axis, and to combine different axes to mimic *in vivo* patterning. Axial patterning combined with organoid fusion will help recreate the complexity of the brain in a regulated manner.

Multi-layer assembloids

Multi-layer assembloids are particularly suited for modeling hollow organs with epithelial-mesenchymal structures, such as the gastrointestinal tract[26], bladder[27], and endometrium[28]. These assembloids are assembled by layering different cell types or organoids to recreate the distinct tissue layers found in these organs (Fig. 2d). For instance, gastrointestinal assembloids are created through a two-step process that starts with the isolation of murine colon crypts and stromal cells to generate three-dimensional epithelial organoids and primary stromal cell cultures. The first step involved mixing pre-cultured stromal cells into Matrigel droplets, followed by the addition of individual organoids to each droplet, thereby allowing for the self-organization of a stroma that closely mimics the *in vivo* cellular diversity and organization[26]. Similarly, endometrial assembloids are generated by combining gland-like organoids with stromal cells in Matrigel, with endometrial epithelial cells being inoculated on top of the Matrigel[28]. In addition to using primary cells[26], other cell sources such as cell lines[27] or PSC-derived cells of all three germ layers[29] can also be employed. In addition to epithelial and stromal cell layers, neural components[29] and muscle cell layer[27] can also be incorporated. These strategies enable the accurate modeling of complex epithelial-mesenchymal interactions, simulating the layered structure of mature organs, and more faithfully recapitulating the cellular microenvironment and organ macroscopic dimensions.

Given these assembly strategies, it is advisable to choose based on the balance between the complexity versus minimalism of the system required, and a combination of different strategies will help to unravel diverse phenotypes associated with the biological question under investigation. Only by carefully selecting the appropriate assembloid assembly strategy based on the developmental processes, cellular composition, and structural characteristics of the research target and area of interest can the *in vivo* state be best represented, followed by rigorous structural and functional validation, and interpreting the results with an awareness of limitations, ensuring the accuracy of the research findings.

Modeling applications of assembloids

Assembloids represent an advanced model in biomedical research, particularly due to their capability to mimic complex inter-organ and tissue interactions more accurately than traditional organoids. This section summarizes the applications and advantages of assembloids across various human body systems. We focus on the nervous, digestive, urinary, reproductive, and circulatory systems,

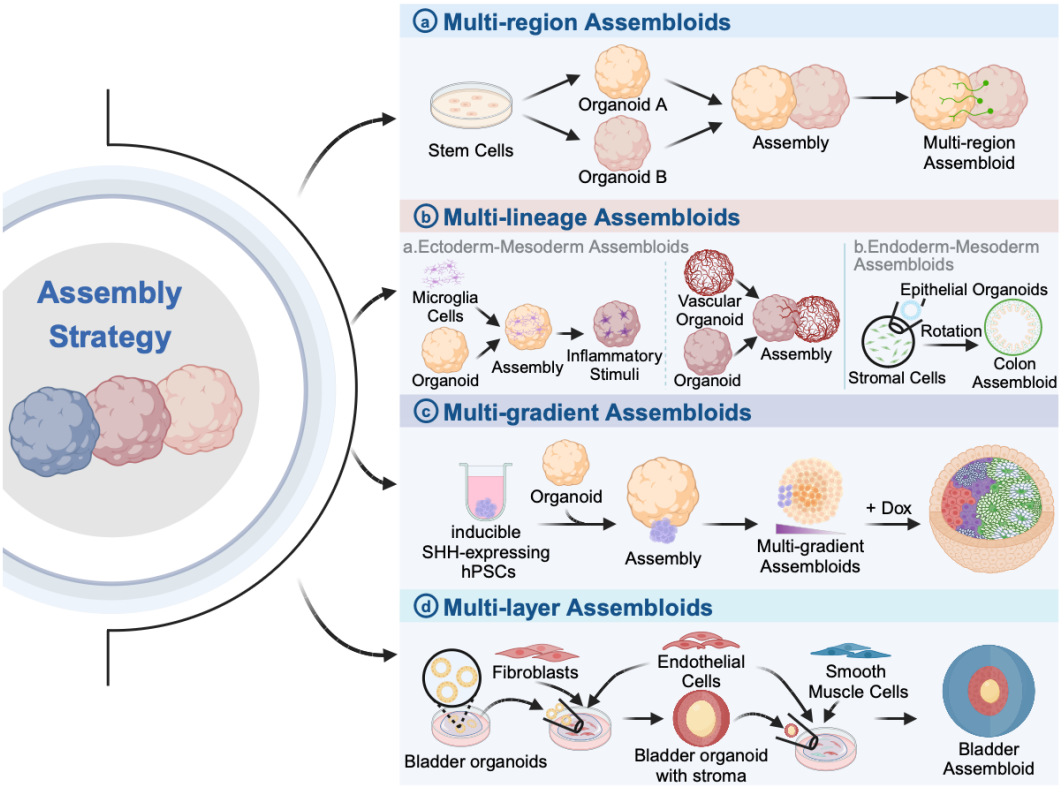

Figure 2. Assembly strategies for building the assembloids. Schematic overview of assembly strategies for the four primary types of assembloids: multi-region, multi-lineage, multi-gradient, and multi-layer. Created with BioRender.com.

as these are the areas where assembloid technologies have seen the most widespread application and significant progress (Fig. 3). These systems serve as representative examples, highlighting the potential and versatility of assembloids. Other systems, such as the immune, muscular, and endocrine systems, have already been explored in relation to the nervous and circulatory systems. While additional organs and systems may also benefit from assembloid modeling, research in these areas is still emerging and lacks sufficient studies to provide a comprehensive overview.

Nervous system

The human nervous system, comprising the central and peripheral nervous systems, is responsible for sensing, processing information, and responding to stimuli. Assembloids have proven particularly effective in modeling the nervous system, providing a platform that surpasses traditional organoids in complexity and functionality. This allows for more detailed studies of neurogenesis, brain development, and complex neuronal interactions (Figs. 3a–3d). Notably, traditional whole brain organoids based on self-patterning protocols have suffered from high variability[30], while assembloids offer a unique opportunity to generate organoids with improved reproducibility by assembling region-specific spheroids, which can be produced with lower heterogeneity.

Neurogenesis

The original purpose of generating assembloids was to model the interplay between region-specific organoids or spheroids, making them ideal for studying neurogenesis, particularly brain development. The human brain consists of various encephalic regions, and its developmental processes have many human-specific traits, such as the expansion and gyrification of the cerebral cortex[31], and the appearance of the outer subventricular zone and outer radial glia progenitor cells[32], which are largely absent in the developing cerebral cortex of lissencephalic species such as mice, rats, and rabbits. As a result, studying neuronal assembloids can provide valuable insights into human-specific neurogenesis.

For example, Birey et al. fused human cortical spheroids with human subpallium spheroids, successfully modeling the migration of interneurons from the subpallium to the cortex, a process previously observed in the fetal brain. This model also generated functional microcircuits to some extent[15,33]. Additionally, cortico-striatal and midbrain-striatal assembloids have demonstrated axon projections from cortical or midbrain regions into striatal parts, indicating the possibility of synaptic connection formation[34,35]. Assembloids have also been used to model the peripheral nervous system, such as in the functional cortico-spinal-motor model, which linked human cortical spheroids, hindbrain/cervical spinal cord spheroids, and skeletal myoblasts (Fig. 3a)[36]

Assembloids can also recapitulate interactions between neuronal organoids and mesodermal tissues. For instance, the neuro-mesodermal model replicates many aspects of neural crest cell (NCC) behavior during peripheral nervous system development and neuro-mesodermal interactions[37]. This model confirmed that in neuro-mesodermal assembloids, NCCs tend to be recruited to the neuro-mesodermal interface to infiltrate the mesodermal part, with peripheral ganglion-like cells found at the interface, indicating that neuro-mesodermal interaction is required for NCC induction. Observing these assembly processes in such models may provide novel insights into human neurogenesis, including cell migration, projection formation, and

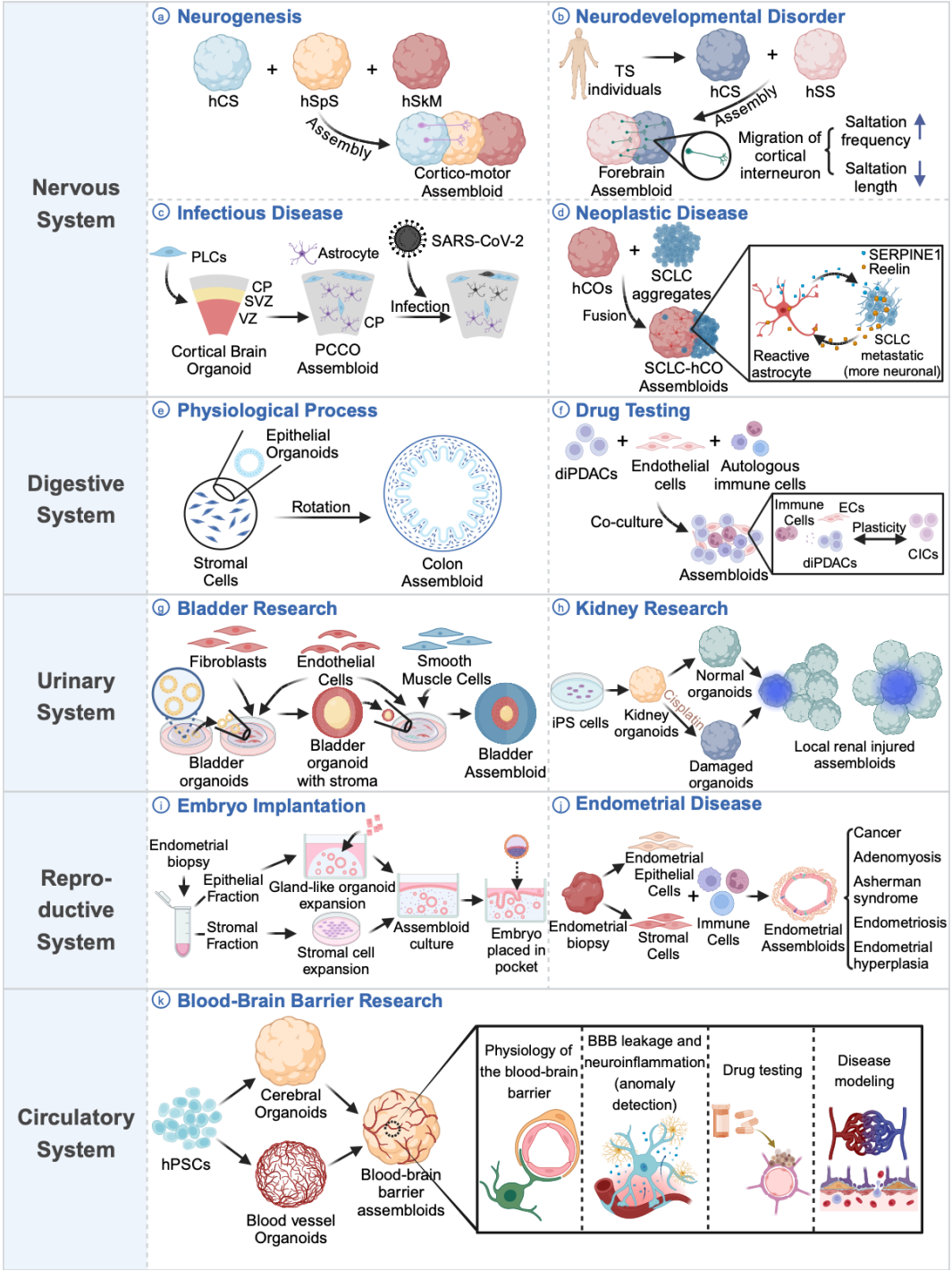

Figure 3. Assembloids for recapitulating organ features and disease modelling. (a) Cortico-motor assembloid integrating human cortical spheroids (hCS), human spinal spheroids (hSpS) and human skeletal muscle spheroids (hSkM)[36]. (b) Forebrain assembloid consisting of hCS and human subpallial spheroids (hSS) derived from Timothy Syndrome (TS) individuals[39]. (c) Human neural-perivascular assembloid with pericyte-like cells (PLCs) can be infected with SARS-CoV-2[45]. (d) Small cell lung cancer (SCLC)-human cortical organoid (hCO) assembloids mimicked SCLC brain metastasis[72]. (e) Murine colon assembloid modelled the interplay between epithelial crypts and stroma[26]. (f) Pancreatic ductal adenocarcinoma (PDAC) assembloids can be used in investigating drug resistance mechanisms[51]. (g) Bladder assembloids obtained by reorganizing bladder organoids with other components of the microenvironment[29]. (h) Locally injured renal assembloids constructed using the multifunctional acoustic tweezer[54]. (i) Co-culturing endometrial assembloids containing stromal cells with embryos[56]. (j) Human endometrial assembloids with luminal structures are used for modeling endometrial diseases[73,74]. (k) Combining brain organoids and vascular organoids derived from human pluripotent stem cells to simulate the physiology of the blood–brain barrier and related diseases[63]. Created with BioRender.com. CP, cortical plate. SVZ, subventricular zone. VZ, ventricular zone.SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2. diPDAC. differentiated pancreatic ductal adenocarcinoma. CICs, cancer-initiating cells. iPS cell, induced pluripotent stem cell. ECs, endothelial cells. hPSCs, human pluripotent stem cells. BBB, blood–brain barrier.

synapse formation.

Notably, the assembly strategy of assembloids is not without its challenges. Since most assembloids are constructed based on our current understanding of nervous system development and connectivity, there is a risk of omitting significant regions or cell types that could be crucial to their function, such as stromal cells. Addressing these gaps could improve the overall utility of the assembloid models. For instance, the retinal-thalamic-cortical assembloids developed by Fligor et al. demonstrated both the extension of projections from retinal to thalamic regions and from thalamic to cortical regions. They also showed improved survival of retinal ganglion cells, supporting the hypothesis that retinal ganglion cells require survival signals from thalamic regions[38]. To further investigate such interactions, assembloids that integrate different tissue types may be a suitable approach. This underscores the importance of carefully selecting the components included in the assembly system based on the research objectives.

Neurological disorders

Assembloids offer an unprecedented opportunity for studying neurodevelopmental disorders due to their capacity to model developmental processes of *in vivo* tissues. Combined with genetic manipulation techniques, such as CRISPR-Cas, mutations related to genetic disorders can be introduced to establish disease models that may yield new discoveries beyond those possible with animal experiments and traditional organoids.

For example, recent studies on Timothy syndrome (TS) have made considerable progress by using human subpallium spheroids (hSS) and human cortical spheroids (hCS) assembloids to model the migration of interneurons from the ventral forebrain to the dorsal forebrain[15,39,40]. TS is a severe multi-system disorder primarily caused by mutations in CACNA1C, which encodes a subunit of the L-type calcium channel Cav1.2. By generating hSS-hCS assembloids from patients with TS, researchers observed aberrant interneuron migration, including reduced saltation length and increased saltation frequency (Fig. 3b)[8]. Realizing the potential of their assembloid platform, the team further explored the mechanisms underlying these abnormalities, demonstrating that reduced saltation length is associated with aberrant actomyosin and myosin light chain phosphorylation, while increased saltation frequency is linked to enhanced GABA sensitivity[39]. They also tested the effect of antisense oligonucleotide therapies on restoring interneuron migration in their assembloids[40]. hSS-hCS assembloid model is compatible with many commonly used neuroscience tools, such as calcium imaging, long-term live cell imaging, pharmacology, whole-cell patch clamping and RNA sequencing[39], which greatly facilitated the study of a complicated process. Additionally, due to the involvement of multiple cell lines, assembloids are more likely to generate functional neural circuits and projections, improving cellular interactions and maturity. It is also worth mentioning brain assembloids can be studied with optogenetics and retrograde tracing[35,36], further validating their usefulness not only to interrogate molecular but also functional aspects of human neural circuits.

Although it remains unclear to what extent assembloids can recapitulate diseases that often manifest in later life stages, they are likely better suited for modeling neurodegenerative diseases, such as Huntington's disease (HD)[41] and Alzheimer's disease (AD)[42] compared to other *in vitro* models, due to their enhanced functionality and maturity.

For instance, the human striato-nigral assembloids constructed by Wu et al. successfully reproduced key pathological features of HD, including mutated huntingtin gene (mHTT) aggregation, decreased progenitor proliferation, and striatal neuron loss[41], demonstrating the potential of assembloids in modeling neurodegenerative diseases.

Infectious diseases of nervous system

Traditional brain organoids were instrumental in uncovering the teratogenic effects of the Zika virus during the outbreak, revealing its impact on early brain development by inducing structural abnormalities and inhibiting normal growth in infected progenitor cells[43,44]. Moving forward, assembloids are expected to have broader applications due to their ability to integrate specific cell types, creating a more authentic microenvironment. For instance, while traditional cortical organoids showed little evidence of SARS-CoV-2 infection, Glesson and colleagues successfully generated pericyte-containing cortical organoids (PCCOs) by integrating pericyte-like cells (PLCs) into cortical organoids. These PCCOs were susceptible to SARS-CoV-2 infection, with both pericytes and astrocytes showing viral susceptibility[45]. Moreover, PLCs in PCCOs promoted astrocytic maturation and the production of basement membrane components and appeared to serve as virus replication centers[45]. Therefore, PCCOs provide a more effective model for studying COVID-19, with improved cell components and viral susceptibility (Fig. 3c). Furthermore, the study by Kong et al. advanced this model by directly fusing cortical organoids with blood vessel organoids containing pericytes and endothelial cells, which allowed for the exploration of interactions between SARS-CoV-2 and Alzheimer's disease while promoting the activity of astrocytes (Fig. 3d), glial cells, and endothelial cells[46]. In conclusion, with advancements in controlling the components of assembloids, the modeling of infectious diseases will become more precise, offering greater opportunities to discover key mechanisms and potential therapies.

Neoplastic diseases

The interplay between cancer cells and the tumor microenvironment (TME) has garnered increasing attention due to its significant impact on tumor behavior. Assembloids, compared to traditional models using animal-derived culture media, offer a more precise replication of the human TME by integrating critical elements such as astrocytes[47] and blood vessels[48]. For instance, Qu et al. successfully mimicked small-cell lung cancer (SCLC) brain metastasis by assembling SCLC cells with human cortical organoids. Their study revealed that the crosstalk between astrocytes and SCLC cells promotes tumor growth through the recruitment of astrocytes. Besides, tumor in situ can also be modelled. Kong et al. fused blood vessel organoids with glioblastoma spheroids, effectively recreating the perivascular niche, including CD19-positive pericytes. This model contributes to the further exploration of CAR-iNK therapy for glioblastoma. These studies show the potential of assembloid model systems as effective platforms for exploring tumor biology and testing new therapies due to their ability to recreate the TME *in vitro* with high fidelity.

In conclusion, assembloids have emerged as a powerful tool for advancing our understanding of the nervous system. The ability to replicate human-specific traits and interactions within a controlled environment gives assembloids a significant edge over traditional

organoid models. As the technology continues to evolve, assembloids are poised to play a critical role in unraveling the complexities of the nervous system, offering unprecedented opportunities for innovation.

Digestive system

Consisting mainly of digestive tract and glands, the human digestive system can digest, absorb and excrete the food we intake. Organoid technology was initially introduced to the study of the digestive system earlier than for other biological systems. As early as 2009, Sato et al. developed small-intestinal crypt-villus structures from single Lgr5 stem cells[49]. With the advent of assembloids in neuroscience, the idea of integrating different organoids or organoids with various cell types has also been applied to studying digestive system physiology and disease models. The ability of assembloids to co-culture different cell types has enabled researchers to investigate bidirectional interactions between these components. For example, the development of a human hepato-biliary-pancreatic (HBP) assembloid through the adjacency of foregut and midgut tissues has resulted in a structurally and functionally integrated model, paving the way for studying the intricate interactions at the foregut-midgut boundary[50]. And Lin and colleagues developed a murine colon assembloid co-culture system by adding individual epithelial organoids into Matrigel droplets containing pre-cultured stromal cells (Fig. 3e). Their study revealed that in the murine colon, the stroma provides key growth factors for crypt formation, while the diversification and positioning of stromal cell subsets require signals from the epithelium[26].

Assembloids have also been applied to research digestive system neoplastic diseases. For instance, Choi et al. co-cultured pancreatic ductal adenocarcinoma (PDAC) organoids with human umbilical vein endothelial cells (HUVECs) and peripheral blood mononuclear cells. They observed that endothelial cells and immune cells could reprogram differentiated epithelial ductal carcinomas into cancer-initiating cells. Using their assembloid model, they identified the NOTCH2/JAG1 pathway between endothelial cells and PDAC as a pivotal factor in cancer cell plasticity, demonstrating the effectiveness of assembloids in investigating tumor drug resistance mechanisms (Fig. 3f)[51]. Xu et al. took this further by encapsulating patient-derived xenograft organoids and human cancer-associated fibroblasts in microgels via droplet microfluidic technology, resulting in assembloids that exhibited high consistency with primary tumors at both the transcriptomic and histological levels[52]. Moreover, Lv et al. employed a mold-replacement strategy to develop a colorectal cancer peritoneal metastasis model with pre-formed vessels. This model exhibited similar heterogeneity and drug penetration patterns to parental xenografts, and by implanting their assembloid into animal peritoneal cavities, they constructed a peritoneal metastasis animal model with controllable tumor burden, underscoring its potential in preclinical drug evaluation[53].

While assembloids have demonstrated their efficacy as *in vitro* models, research using assembloids in the digestive system is still relatively limited. It is believed that there is plenty of scope for the leveraging of assembloids in digestive system, when combined with frontier researches like metabolic syndrome and precision medicine, etc.

Urinary system

The urinary system, comprising the kidneys, ureters, bladder, and urethra, is critical for waste filtration and substance regulation via urine production. The bladder, a hollow organ lined with urothelium—a specialized epithelium—sits atop the lamina propria, beneath which lies the muscular detrusor layer. Assembloids provide a superior platform for physiological modeling of such multilayered organs by more accurately replicating their complex architecture and function. For instance, Kim et al. developed bladder assembloids by integrating bladder organoids with layers of fibroblasts, endothelial cells, and smooth muscle cells (Fig. 3g). These assembloids demonstrated high fidelity with primary bladder tissue, exhibiting functional responsiveness that closely mirrors *in vivo* conditions[27]. Additionally, Kim's team developed bladder tumor assembloids derived from aggressive urothelial carcinomas, observing that the HUVECs within the assembloids formed interconnected vascular networks that facilitated tumor growth, effectively replicating *in vivo* tumor progression[27].

In the kidney, epithelial cell types—such as nephrons and collecting ducts—are embedded within the renal interstitium, which contains a vascular network. In renal diseases, nephron damage often occurs sequentially within this functional unit. However, traditional organoid or cell models typically suffer from global damage *in vitro*, failing to mimic the progressive nature of kidney failure. To address this, Gao et al. utilized acoustic tweezers to structurally assemble heterogeneous kidney organoids, creating specific regional renal injury assembloids that more closely imitate the physiological and pathophysiological conditions observed *in vivo* (Fig. 3h)[54]. This advancement is expected to significantly contribute to the study of kidney injury and the progression of chronic kidney disease.

In conclusion, assembloids represent a transformative tool in the modeling of the urinary system. By offering a more accurate and detailed replication of the intricate architecture and function of organs like the bladder and kidneys, they pave the way for deeper insights into both physiological processes and disease mechanisms.

Reproductive system

The human reproductive system is responsible for producing sex cells, facilitating fertilization, and supporting the development of offspring, with key biological processes including gametogenesis, fertilization, implantation, and embryonic development. Studying these processes often requires interactions between multiple cell types, making the use of assembloids especially valuable for *in vitro* modeling. For example, endometrial assembloids have been developed to model embryo implantation[55–58]. Thomas M. Rawlings and colleagues established human endometrial assembloids by co-culturing gland-like organoids with primary stromal cells in Matrigel, which enabled them to model the impact of decidual senescence on embryo implantation[56]. Furthermore, by creating pockets within these endometrial assembloids and implanting human embryos, researchers have been able to study embryo–endometrial interactions (Fig. 3i)[57].

In the context of embryogenesis, embryo-like assembloids have been constructed by assembling naive hESCs and extraembryonic cells. These models are valuable for elucidating the mechanisms and interactions of germ layer development and exploring the cellular behaviors and signaling interactions that drive human embryogenesis[59]. Research on gametogenesis, which focuses on human primordial germ cells (hPGCs)—the precursors of sperm and eggs—has also advanced. These cells are specified during weeks 2–3 after fertilization *in vivo*. A recent study has shown that

resetting human primordial germ cell-like cells (rhPGCLCs) co-cultured with human hindgut organoids progress at a pace reminiscent of *in vivo* hPGC development, offering a promising tool for studying human gametogenesis *in vitro*[60].

Assembloid models of the human reproductive system hold significant potential for advancing reproductive medicine and infertility research, offering insights that were previously unattainable with individual organoid models (Fig. 3j).

Circulatory system

The circulatory system, including the cardiovascular and lymphatic systems, is essential for maintaining normal bodily functions by transporting nutrients and removing metabolic waste. In this section, we summarize the development and applications of vascularized assembloids and cardiac assembloids, which are key advancements in circulatory system modeling.

Vascularized assembloids

Vascularization is critical for maintaining organ function in the body. In organoid research, the lack of vascularization often leads to hypoxia, accumulation of metabolic waste, and functional decline[6,61]. To overcome this, researchers have proposed two main vascularization strategies: pre-constructed vascularization and post-engraftment vascularization[62]. Vascularized assembloids, as a form of pre-constructed vascularization, successfully form vascular networks by assembling organoids with vascular organoids or endothelial cells.

For example, a study fused brain organoids with vascular spheroids to create vascularized brain organoids. The formation of blood vessels improved the proliferation of neural progenitors and promoted the establishment of blood-brain barrier-like structures, providing a new platform for studying neurological diseases (Fig. 3k)[63,64]. Similarly, other organoids, such as kidney[65] and pancreas[66], have shown promising vascularization outcomes when combined with vascular structures. These findings demonstrate that vascularized assembloids can effectively address hypoxia in organoids, improve cell growth and survival, and promote the maturation of organ functions. Beyond resolving nutrient supply, vascularization is crucial for reproducing physiological cell interactions and constructing more accurate disease models, thereby enhancing drug screening accuracy[63–65].

In contrast, *in vivo* vascularization, achieved by transplanting organoids into animals (e.g., under the kidney capsule), can result in more physiologically relevant vascularization by utilizing the host's vascular system. However, this method faces challenges like immune rejection. On the other hand, vascularized assembloids have the advantage of a fully controlled *in vitro* environment, where researchers can precisely regulate cell types, culture conditions, and the addition of vascularization factors, making the process more reproducible and efficient. However, this also means vascularized assembloids may differ from *in vivo* environments, particularly in long-term culture, where the scale, functionality, and stability of the vascular network still require further validation.

Cardiac assembloids

Cardiac assembloids have emerged as a transformative tool in heart modeling, offering an advanced platform for studying heart function, disease, and development. Unlike traditional *in vitro* models, which struggle to replicate the heart's complex electrical properties, cardiac assembloids combine multiple cell types to create a more physiologically relevant system.

A significant advantage of cardiac assembloids is their ability to simulate multi-chamber heart development. By generating multi-chambered cardioids—representing the right and left ventricles, atria, and atrioventricular canal—researchers can study the interactions between different heart compartments. These models recapitulate the gene expression profiles, morphologies, and functional dynamics of the human heart during development. They provide a valuable system for investigating congenital heart defects, drug responses, and the effects of genetic mutations[67].

Another key application of cardiac assembloids is in modeling the atrioventricular (AV) conduction axis, which coordinates the contraction of the heart's chambers by facilitating the sequential propagation of electrical impulses. By assembling atrial, atrioventricular canal and ventricular spheroids/organoids, researchers have successfully recreated the natural "fast–slow–fast" conduction pattern. This model is ideal for studying atrioventricular conduction disorders, such as those caused by LMNA gene mutations, where intracellular calcium mishandling has been identified as a key factor in conduction block[68].

The primary advantage of cardiac assembloids lies in their ability to replicate both the structural and functional complexity of the heart. They offer a controlled environment for studying heart function and dysfunction, overcoming the limitations of traditional animal models in terms of reproducibility and ethical concerns. Furthermore, their scalability makes them ideal for high-throughput screening of potential therapeutic candidates.

To summarize, the utilization of assembloids represents a paradigm shift in biological research, transcending disciplinary boundaries and offering unprecedented insights. These applications of assembloid not only deepen our understanding of complex biological systems but also hold immense promise for revolutionizing approaches to drug discovery, toxicology, and personalized medicine.

Challenges and prospects

Despite significant advances in assembloid technology, several challenges remain unresolved (Fig. 4). One of the primary obstacles in organoid research is the reproducibility of results, particularly in models where self-organization is critical. The process of culturing single organoids and assembly would bring additional unccontrollable issues to assembloid systems compared with conventional organoids. Besides, differences in the differentiation status and rate of various regions in assembloid might cause more problems which are hard to predict. Basic contributing factors include genetic variability among stem cell lines, differences in differentiation potential[69–71], inconsistencies in differentiation protocols, and the presence of undefined components in cell culture reagents[6,49]. In assembloids, the formation of complex, multicellular, mature, and functional structures often compromises reproducibility due to the challenges in coordinating the proliferation and differentiation of multiple cell types. The intrinsic heterogeneity of assembloids, characterized by diverse and unevenly distributed cell types, hinders high-throughput screening applications that require consistent and predictable results while also making it challenging to scale up assembloid production with consistency across batches. Moreover, this heterogeneity complicates high spatiotemporal resolution imaging, as it obstructs the precise observation of cellular and tissue dynamics over time. Addressing

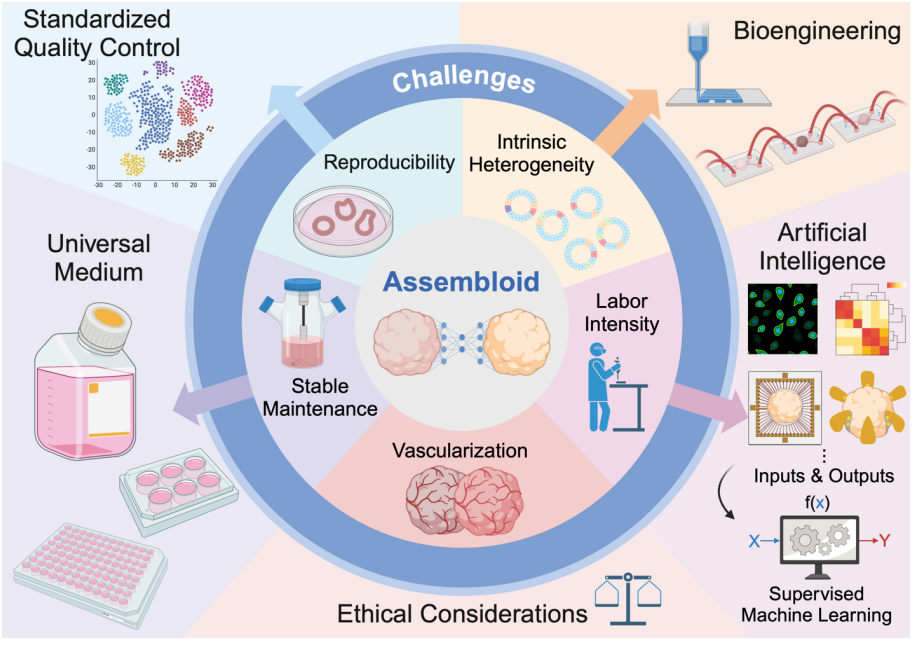

Figure 4. Key challenges and future directions in assembloid technology. Assembloid research faces several critical challenges, including low reproducibility, intrinsic heterogeneity, high labor intensity, difficulty in maintaining stability, and lack of vascularization. To address these, standardized quality control and bioengineering approaches can improve reproducibility and reduce heterogeneity; artificial intelligence may alleviate labor demands by minimizing manual intervention; and the development of universal media could enhance the long-term stability of assembloids. Additionally, as assembloid technologies progress, ethical considerations must not be overlooked.

these challenges requires the development of standardized quality control methods that encompass the entire differentiation process. Advanced characterization techniques, such as single-cell RNA sequencing, are crucial for ensuring the reliability and biological fidelity of assembloids. These methods enable the identification of off-target cells, refinement of differentiation protocols, and comparison of organoids/assembloids with *in vivo* tissues. Recent advancements in assessment technologies highlight the importance of integrating multi-omics analysis and functional property evaluations to better understand heterogeneity and functionality at both macro and micro levels[75].

Maintaining the long-term stability of multiple tissue types within a single assembloid platform presents another significant challenge, as most culture media are not optimized to support tissues from different germ layers simultaneously. Although the use of a common medium has been proposed as a solution, this approach may compromise the maturation and stability of assembloids over extended culture periods[19,36,76]. For instance, in some organ-on-a-chip (OoC) devices, mixed media have caused tissues to revert to more immature states after prolonged culture. In such cases, maintaining tissue-specific media while allowing inter-tissue communication through selectively permeable barriers has shown promise in preserving tissue function. However, in assembloids, where shared interfaces are essential for certain developmental processes, the challenge of developing a universal medium that supports multiple tissue types remains unresolved. Continued research in this area is critical for the further advancement of assembloid technology.

Another challenge in assembloid development is achieving sufficient vascularization, particularly as the constructs increase in size. An increase in size, structural complexity and metabolic level means more demand for energy, oxygen, signal transduction and other important factors, but it could also lead to more difficulty in diffusion. Without adequate vascularization, assembloids are prone to necrosis and the formation of hypoxic cores. Besides, different parts of the assembloid may have distinct demands for vascularization, which brings a greater challenge for the construction of functional vascular networks. Strategies such as co-culturing with endothelial cells to create perfusable channels have been explored, but these approaches often fail to replicate the complex vascular networks found *in vivo*. Additionally, the endothelial cells used in these models may not be tissue-specific, leading to discrepancies in the organization of vascular structures compared to their natural counterparts.

Advancing assembloid technology through bioengineering techniques offers significant improvements in reproducibility and reduction of heterogeneity. Bioprinting enables precise construction of 3D tissue structures using "bio-ink," which integrates living organoids with biomaterials to enhance structural complexity and functionality[77], addressing variability issues. Microfluidic platforms, or OoC systems[78], further this progress by replicating key aspects of organ physiology, including controlled fluid flow and shear stress[79], supporting vascular network development[80] and providing more relevant conditions for long-term stability. However, it is essential to balance these engineered approaches with the need to capture the complexity of cellular self-organization found in natural tissues. Ensuring that these models accurately reflect *in vivo* interactions and functions remains a critical challenge, necessitating careful integration of bioengineering techniques to maintain the fidelity and applicability of assembloid models.

The establishment of organoid systems is highly dependent on human intervention, making the process of optimizing strategies, analyzing data, and verifying efficacy time-consuming and labor-intensive. The integration of artificial intelligence (AI) offers a promising approach for efficiently optimizing culture protocols and even identifying a universal medium. AI can analyze large datasets from multi-omics analyses, imaging, and biosensor signals, predict outcomes, and refine differentiation strategies,

potentially leading to more efficient and sophisticated assembloid models[81–84]. For instance, Kanda et al. developed a robotic AI platform that utilized a batch Bayesian optimization algorithm to autonomously guide stem cells into differentiating into retinal pigment epithelial cells by testing 143 conditions from 200 million possible parameter combinations, achieving an 88% differentiation rate based on pigmentation scores, demonstrating the immense potential of autonomous robotic AI in dramatically accelerating systematic and unbiased exploration of experimental search spaces[85].

As these technologies advance, ethical considerations must also be addressed, particularly as assembloids increasingly resemble human tissues. Establishing robust ethical guidelines and ensuring responsible research practices will be crucial for the continued development and application of assembloids in both academic and commercial settings. Despite these challenges, the prospects for assembloid technology are promising, with the potential to revolutionize biomedical research, drug development, and personalized medicine, provided that these obstacles are addressed through innovative approaches and interdisciplinary collaboration.

Conclusions

Assembloid technology represents a significant leap forward in the quest to replicate the complexity of human tissues and organ systems *in vitro*. By enabling the co-culture and integration of diverse cell types into organized, multicellular structures, assembloids provide a powerful platform for exploring intricate biological processes and disease mechanisms with a level of detail previously unattainable. These models have already demonstrated immense potential in advancing our understanding of human development, disease pathology, and therapeutic responses, particularly in systems where traditional organoids fall short.

However, the path to fully realizing the potential of assembloids is fraught with challenges. Issues such as reproducibility, cellular heterogeneity, and the integration of functional vascular networks remain significant obstacles. Overcoming these challenges will require a concerted effort to refine and standardize assembly protocols, enhance bioengineering methods, and leverage emerging technologies such as artificial intelligence for predictive modeling and optimization (Fig 4).

Looking ahead, the continued evolution of assembloid technology will likely play a critical role in the future of biomedical research and clinical applications. The ability to create increasingly complex and functionally accurate models will not only accelerate drug discovery and personalized medicine but also offer new avenues for studying the fundamental aspects of human biology. Yet, as we push the boundaries of what is possible with these models, we must also navigate the ethical implications of creating systems that so closely mimic human tissues. Ensuring that research is conducted responsibly and with careful consideration of these ethical concerns will be crucial as assembloids become an integral part of the biomedical landscape.

Research ethics and patient consent

Not applicable

Availability of data and material

Not applicable

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Declaration of conflicting interests

K.W. serves as the Associate EiC of the journal, and Q.W. serves as a member of the Editorial Board. They were not involved in the reviewing process of, or decisions related to, this manuscript.

Author contributions

Jiarui Liu conceived the structure of the review and led the writing and visualization process, with assistance from Yitong Shi and Xianqin Shen in drafting and figure creation. All authors participated in the discussion of the results, contributed to the revision of the manuscript, and approved the final version.

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