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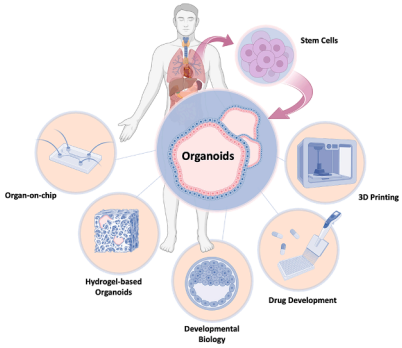
Engineered organoids for biomedical applications

Natan Roberto de Barros [a,1,*], Canran Wang [b,1], Surjendu Maity [a], Arne Peirsman [a,c], Rohollah Nasiri [d], Anna Herland [d], Menekse Ermis [a], Satoru Kawakita [a], Bruna Gregatti Carvalho [a,e], Negar Hosseinzadeh Kouchehbaghi [a,f], Rondinelli Donizetti Herculano [a,g,h], Zuzana Tirpáková [a,i], Seyed Mohammad Hossein Dabiri [j], Jean Lucas Tanaka [k,l], Natashya Falcone [a], Auveen Choroomi [a], RunRun Chen [a,g], Shuyi Huang [a,g], Elisheva Zisblatt [a], Yixuan Huang [a], Ahmad Rashad [a], Danial Khorsandi [a], Ankit Gangrade [a], Leon Voskanian [a], Yangzhi Zhu [a], Bingbing Li [a,g], Mohsen Akbari [j], Junmin Lee [m], Mehmet Remzi Dokmeci [a], Han-Jun Kim [a,n,*], Ali Khademhosseini [a,*]

[a] Terasaki Institute for Biomedical Innovation (TIBI), Los Angeles, CA 90064, USA
[b] Andrew and Peggy Cherng Department of Medical Engineering, Division of Engineering and Applied Science, California Institute of Technology, Pasadena, CA 91125, USA
[c] Plastic and Reconstructive Surgery, Ghent University Hospital, Ghent, Belgium
[d] Division of Nanobiotechnology, Department of Protein Science, Science for Life Laboratory, KTH Royal Institute of Technology, 17165 Solna, Sweden
[e] Department of Material and Bioprocess Engineering, School of Chemical Engineering, University of Campinas (UNICAMP), 13083-970 Campinas, Brazil
[f] Department of Textile Engineering, Amirkabir University of Technology (Tehran Polytechnic), Hafez Avenue, 1591634311 Tehran, Iran
[g] Autonomy Research Center for STEAHM (ARCS), California State University, Northridge, CA 91324, USA
[h] São Paulo State University (UNESP), Bioengineering and Biomaterials Group, School of Pharmaceutical Sciences, Araraquara, SP, Brazil
[i] Department of Biology and Physiology, University of Veterinary Medicine and Pharmacy in Kosice, Komenskeho 73, 04181 Kosice, Slovakia
[j] Laboratory for Innovations in Micro Engineering (LiME), Department of Mechanical Engineering, University of Victoria, Victoria, BC V8P 5C2, Canada
[k] Butantan Institute, Viral Biotechnology Laboratory, São Paulo, SP Brazil
[l] University of São Paulo (USP), São Paulo, SP Brazil
[m] Department of Materials Science and Engineering, Pohang University of Science and Technology (POSTECH), Pohang, Gyeongbuk 37673, Republic of Korea
[n] College of Pharmacy, Korea University, Sejong 30019, Republic of Korea

GRAPHICAL ABSTRACT



* Corresponding authors.
E-mail addresses: nbarros@terasaki.org (N. Roberto de Barros), hanjun@korea.ac.kr (H.-J. Kim), khademh@terasaki.org (A. Khademhosseini).
[1] Natan Roberto de Barros and Canran Wang contributed equally to this work.

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ABSTRACT

As miniaturized and simplified stem cell-derived 3D organ-like structures, organoids are rapidly emerging as powerful tools for biomedical applications. With their potential for personalized therapeutic interventions and high-throughput drug screening, organoids have gained significant attention recently. In this review, we discuss the latest developments in engineering organoids and using materials engineering, biochemical modifications, and advanced manufacturing technologies to improve organoid culture and replicate vital anatomical structures and functions of human tissues. We then explore the diverse biomedical applications of organoids, including drug development and disease modeling, and highlight the tools and analytical techniques used to investigate organoids and their microenvironments. We also examine the latest clinical trials and patents related to organoids that show promise for future clinical translation. Finally, we discuss the challenges and future perspectives of using organoids to advance biomedical research and potentially transform personalized medicine.

1. Introduction

Organoids are tiny, self-organized three-dimensional (3D) tissue cultures derived from stem cells that imitate organ structure and function [1,2]. To this end, organoids comprise multiple stem cell-derived organ-specific cell types that spatially self-assemble, similar to organs [3]. Additionally, these organized cell clusters exert specific functions of their *in vivo* counterparts (*e.g.,* filtration, neural activity, contraction, excretion) [4]. These diverse biological properties make organoids a suitable tool for developmental studies, drug testing, disease modeling, and regenerative medicine [5].

Organoid research has increased over the past decade, and the FDA's indication of the possibility of approving non-animal testing techniques has made organoids more viable as a drug testing platform than ever before [6]. Historically, the basic concepts that have led to organoid development have been taking shape since the early 1900 s [7]. In 1907, Wilson demonstrated the ability to dissociate spongy cells to reassemble, self-organize and form new functional spongy organisms [8]. Next, Tung and Kü [9], Weiss, and Taylor [10] performed dissociation-reaggregation experiments with amphibian and embryonic chick tissues. The first long-term human cell cultures were developed in 1975 [11]. The first isolations of embryonic stem cells (ESCs) began in 1981 (mice) [12] and 1998 (humans) [13]. Since the introduction of induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka in 2006, access to pluripotent stem cells (PSCs) has increased [5,14]. In addition, by adding four factors (Oct3/4, Sox2, Klf4, and c-Myc) to ESCs and adult fibroblast cell cultures, they reprogrammed these cells into PSCs.

Next to stem cell isolation and culture, differentiation plays an essential role in tissue recapitulation. In 1987, a milestone for organoid research, extracellular matrix (ECM) extracted from Engelbreth-Holm-Swarm sarcoma cells, was introduced into cell cultures [15]. Murine mammary epithelial cells cultured on this ECM extract formed 3D clusters and reorganized into functional ducts and ductules with milk production [16]. The introduction of ECM extract and the advances in stem cell technologies have fueled organoid research, resulting in the development of several organoid models: *e.g.,* brain [17], heart [18], gut [19], kidney [20], lung [21], and retina [22].

While the capability of culturing tissue-specific organoids offers great potential in various biomedical applications, their culturing methods must be optimized to ensure reproducibility and accuracy. In addition to ECM extracts for improving organoid culturing, engineered ECM-mimicking hydrogels [23], 3D bioprinting [24], and microfluidics [25] also play a significant role in advancements in organoid research, allowing the creation of microscale 3D tissue models that can be used for drug screening and personalized medicine.

The cornerstone of microfluidic and organ-on-a-chip technologies has revolutionized the field of organoid research. These technologies precisely control fluid flow and shear stress for creating *in vitro* models that better mimic the *in vivo* environment of organs—enabling the study of complex physiological processes such as immune cell interactions [26], tissue regeneration [27], and cancer metastasis [28]. Furthermore, microfluidic devices provide a platform for integrating multiple organ models and studying their interactions [29,30]. In this regard researchers introduced the InterOrgan tissue chip, noteworthy for its unique composition comprising four distinct tissues: heart, bone, liver, and skin. This design facilitated the integration of bioengineered tissues to closely replicate physiological conditions, with each tissue allocated a specialized environment that fostered tissue-to-tissue communication through vascular flow and maintained separation between the vascular and tissue compartments via a selectively permeable endothelial barrier. [30].

Analytical methods also play a crucial role in organoid research, enabling the visualization and analysis of their microenvironment and cellular responses. The image-based organoid analysis involves using microscopy to visualize organoid structure and function. At the same time, biosensors enable the measurement of cellular and molecular interactions within organoids. High-throughput approaches are used for drug screening, where large numbers of compounds can be tested for their efficacy and toxicity within organoids. Artificial intelligence is emerging as a promising tool for analyzing complex datasets generated from organoid experiments.

Herein, we aim to overview the generation of organoid technology and its biomedical applications (Fig. 1). We first summarize the recent progress in the engineering of organoids and the methods for improving their culturing. We then discuss the applications of organoids in regenerative medicine, drug screening, and drug development. We also present analytical methods for organoid research, recent clinical trials, and patents in organoids, along with a discussion of the challenges and perspectives of using organoids in biomedical applications.

2. Stem-cell-derived organoids and signaling pathways involved in organoid differentiation

Stem cells, in general, show a great potential to self-assemble into complex tissue-mimicking structures [31]. When cultured in a hydrogel (typically Matrigel, gelatin, or gelatin methacryloyl (GelMA)) containing various exogenous substances such as differentiation factors, differentiation of stem cells into complex tissue-like ordered clusters of cells that build structures can be induced [32]. Organoids can reproduce various biological features, such as the structural arrangement of diverse tissue-specific cells, cellular interactions with other neighboring cells or ECM, and specific physiological responses produced by the 3D organization within the organoids [33]. The ability to create a 3D structure from stem cells that can accurately replicate the intricacy of organs has shown several advantages, including *(i)* can form almost any tissue; *(ii)* can mimic embryological development, differentiation during growth, and even malignant transformation; and *(iii)* can be efficiently transplanted and maintained *in vivo* [34]. PSCs and ASCs are major sources of stem cells for organoid development [33]. Given a physiological or pathological microenvironment that mimics the target tissue or disease, these stem cells can develop into primitive target structures. The development of the human body is meticulously controlled by step-by-

step differentiation [35].

Creating PSC organoids combines guided differentiation, morphogenetic processes, and intrinsically directed cell self-assembly to simulate organogenesis in the developing embryo [36]. For instance, organoids can be created that mimic different tissues in the three layers that form the embryo: ectoderm, mesoderm, and endoderm. The first ESC line was generated in 1998 [13]. Still, there were a lot of controversies and ethical limitations to using human embryos as a cell source. Meanwhile, the discovery of the inductive potential of adult cells described as iPSCs in 2007 to the stem cell state created a novel source of PSCs and opened a new era of organoid research [37].

The mouse small intestine organoids were the first long-term ASC-derived organoid culture system without a feeder layer. Several steps and discoveries lead to these developments. First, Wnt signaling is necessary to preserve the stem cell compartment of the mouse small intestine, first published in 1998 [38]. Next, a Wnt target gene encoding G-protein coupled receptor 5 (Lgr 5) containing leucine-rich repeats was discovered [39]. It was a marker for small intestine and colon stem cells. Studies have shown that intestinal stem cells (ISCs) are proliferative and have an unlimited capacity to regenerate intestinal tissue *in vivo*. A follow-up finding is that the ectopic production of R-spondin, a Wnt agonist that acts as an Lgr5 ligand [40], causes crypt proliferation *in vivo* [41]. These breakthroughs paved the way for developing a method for *in vitro* production of ISCs. The earliest example of ASC-derived organoids comes from Sato *et al.*, who isolated intestinal crypt cells and cultured them under several differentiating factors, including Wnt3A [42]. They showed intestinal organoid formation and differentiation of mature enterocytes, goblet cells, and enteroendocrine cells (Fig. 2**. A**).

A similar Wnt-centered approach has been adopted for human fallopian tube organoids [43]. This study showed that single epithelial cells harvested from patients' fallopian tubes could generate secretory and ciliated cells. The organoids survived and proliferated for up to 4 months. Ectodermal organoids are also reported to be derived from ASCs [44,45]. The first is a mammary gland organoid, in which primary isolated mammary cells were cultured in compliant collagen gels and showed the development of a functional subunit of the mammary gland, 'terminal ductal-lobular units' [44] (Fig. 2. **B**).

Another example is even more interesting: the development of salivary gland organoids from ASCs. In one study, rare salivary gland ductal EpCAM^{+} cells exhibited nuclear catenins indicative of active Wnt signaling [45]. In addition, EpCAM^{++} (high) cells respond to Wnt signaling in cell culture, promoting self-renewal and the long-term development of salivary gland organoids, including all differentiated salivary gland cell types (Fig. 2**. C**). All these examples demonstrate the importance of Wnt signaling in ASC organoid generation.

The production of organoids using murine and human iPSCs (hiPSCs) avoids the shortage of high-quality human primary ASCs. Nevertheless, a comprehensive understanding of the factors involved in germ layer formation and differentiation into specific lineages requires a comprehensive understanding of these biological processes. Using iPSCs requires extra preparation, as somatic cells must first be transformed into iPSCs by expressing OCT4, KLF4, SOX2, and c-Myc [46]. After that, iPSCs are exposed to germ layer and tissue-specific patterning factors, which are then embedded in Matrigel to form 3D architecture and aid differentiation factor treatment to generate the desired organoids [47]. TGF signaling is stimulated in iPSCs to generate definitive endoderm and, depending on the culture conditions of the endoderm tissue, differentiate into relevant sections of the embryonic gut [5]. FGF-4 and WNT3A promoted hindgut and intestinal fate in definitive endoderm cells from human iPSCs [19]. Noguchi *et al.* showed that definitive endoderm differentiates into the foregut [48]. They established a method for differentiating stomach tissue from ESCs by inducing the expression of the essential gene Barx1, known for its role in stomach development. Under Barx1-inducing culture conditions, they generated spheroids resembling early stomach primordium, which later matured into stomach tissue cells in both the corpus and antrum when cultured in a 3D environment. This ESC-derived stomach tissue exhibited a gene expression profile similar to that of adult stomach tissue and demonstrated the secretion of pepsinogen and gastric acid. Additionally, overexpression of TGFA in led to hypertrophic mucus production and gastric anacidity, resembling Ménétrier disease *in vitro*.

The holy grail of developmental biology has been deciphering how single cells are progressively programmed to form complex tissues, diverse organs, or end organisms with a high degree of resilience and spatiotemporal control [49]. On the other hand, synthetic biology attempts to design and construct biological systems [50]. So far, new

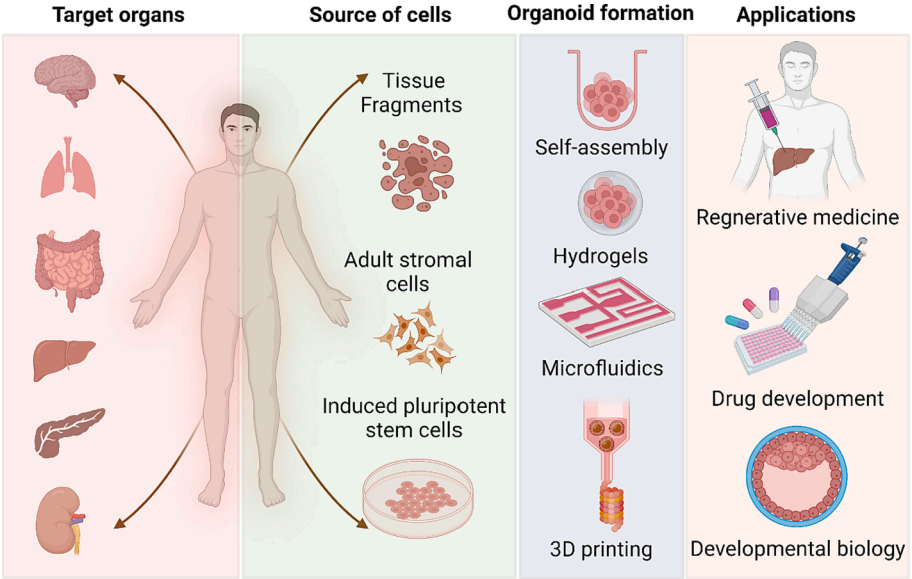

Fig. 1. Schematic diagram summarizing the generation and potential applications of organoid models in preclinical research and precision medicine. Organoids mimicking multiple essential patient tissues such as the brain, lung, and liver could be developed. Diverse sources of cells could be harvested from humans and cultured to form organoids. Organoid development could be facilitated by advanced engineering approaches such as induction of self-assembly, hydrogel scaffold formation, microfluidics fabrication, and additive manufacturing. These approaches can revolutionize current research and novel therapeutic strategies in regenerative medicine, drug development, and developmental biology.

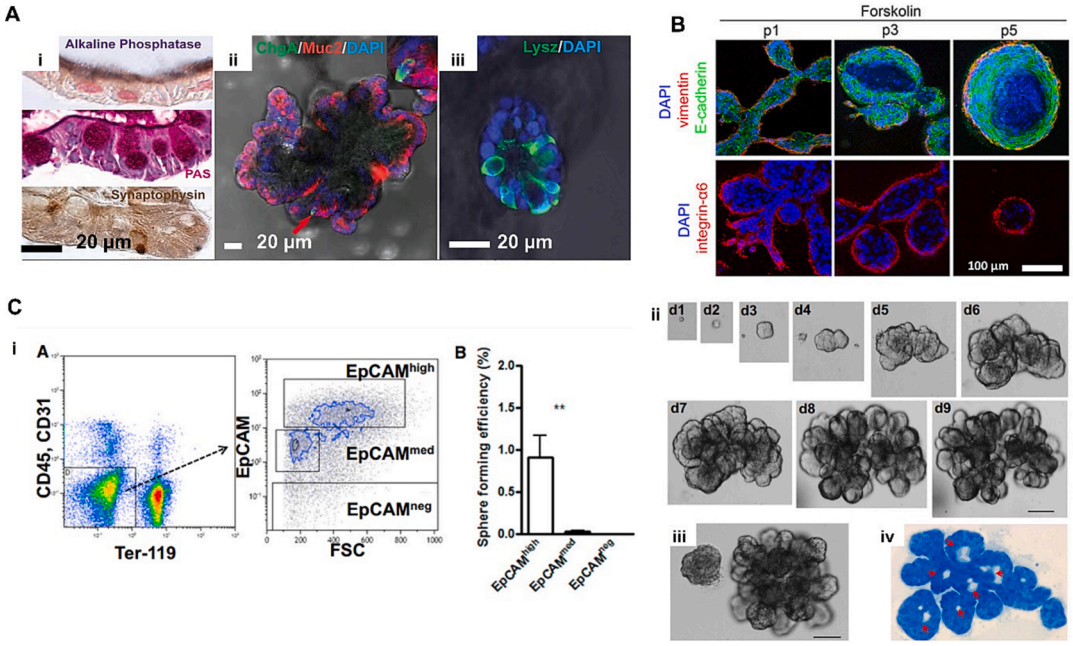

Fig. 2. Examples of stem-cell-derived organoids and organoid differentiation. Ai. Human crypt ISCs could differentiate into the different cell types of the intestine (top: Alkaline phosphatase for mature enterocytes, middle: Periodic acid–Schiff staining (PAS) for goblet cells, bottom: Synaptophysin for enteroendocrine cells). Aii-iii Immunofluorescence stains using Mucin2 (red) for goblet cells and Chromogranin A (green) for enteroendocrine cells (red arrow and inset) and Lysozyme (Lysz; green) for Paneth cells. Reproduced from [42] with permission from Elsevier 2011. B. Confocal laser scanning microscopy of terminal ductal-lobular unit resembling structures at different passages showing Vimentin (red), E-cadherin (green), integrin-α6 (red), and DAPI: blue, Scale bar: 100 μm). Reproduced from [44] with permission from The Company of Biologists 2015. C. Ci, (A) Representative FACS gating strategy for the analysis of ductal cells in the salivary gland. Left panel shows the exclusion of lineage marker-expressing cells. Right panel depicts the distribution of $EpCAM^{high}$, $EpCAM^{med}$, and $EpCAM^{neg}$ cells in dissociated adult mouse salivary gland. FSC, forward scatter. (B) Sphere-forming efficiency of $EpCAM^{high}$, $EpCAM^{med}$, and $EpCAM^{neg}$ populations (**$p < 0.005$). Data are expressed as the mean ± SEM of three independent experiments. Cii, Differential interference contrast image of a growing minigland until 9 days of culture. Ciii, Representative example of a salisphere and a minigland originating from single $EpCAM^{high}$ in 9-day-old culture. Civ, Toluidine blue staining shows uniform lumen formation throughout minigland (arrows). Scale bars represent 100 μm (ii, iii) and 10 μm (iv). Reproduced from [45] with permission from Cell Press, Elsevier 2016.

signaling pathways, artificial proteins, organelles, and even cells have been constructed or manufactured. Controlling multicellular systems and tissue generation is a new cutting-edge technology in regenerative medicine and basic biology. Tissue engineering, stem cell differentiation, and *in vitro* organoid formation are based on current multicellular system manipulations, which differ from the past. At the same time, it faces challenges in terms of functional integration, complexity, and precision [51]. During embryonic development, undifferentiated progenitor cells are guided to generate all of the diverse tissue types of the mature organism [52]. Many of the self-organizing control instructions are programmed at the genetic level. In addition, some networks regulate genes and cell signaling cascades, as indicated by regenerative, developmental, and stem cell biology.

In a recent study, researchers used synthetic pathways that rely on cell–cell contacts to create a synthetic patterning circuit in a naive epithelial cell line [53]. When the first ligand stimulates the receptor, it also triggers gene transcription of the second ligand. Next, neighboring cells detect second ligands that activate secondary reporter genes in the cascade. For example, primary hippocampal neurons were transfected with a synthetic green fluorescent protein (GFP)-Notch receptor, and sender cells (which transmit primary signals) were added to the culture. In the presence of primary ligands, neuron cells were activated, and GFP production was observed as early as 4 h. This is an example of synthetic, multicellular organoid triggering and development. The signaling pathway used in this example, Notch signaling, is contact-dependent and can be used for precise cell patterning.

A unique technique to generate and, subsequently, co-differentiate hiPSC-derived progenitors have been reported [54]. In this study, researchers use genetically engineered GATA-binding protein 6 (GATA6) to stimulate the rapid development of all three germ layers in a tissue context. As a result, they produced a complex tissue that attempted to simulate early embryonic processes, with a phenotype similar to liver bud, containing both stromal and hematopoietic cells. Moreover, they can generate hepatic neuronal niches within two weeks. With this approach, fetal hematopoiesis has been demonstrated in multicellular fetal organoids.

When mouse ESCs transiently overexpressed the NKX2-1 and PAX8 transcriptional factors, they were found to be sufficient to induce differentiation into follicular thyroid cells in the presence of thyrotropin [55]. They also assemble into 3D follicular structures. In addition, iodide-organizing activity was observed in these *in vitro*-derived thyroid follicles. Finally, Sozen *et al.* combined several different cell sources to mimic the gastrulation step of embryonic development [56]. ESCs can be implanted into the germline of a growing embryo. However, their ability to simulate embryogenesis is limited when cultivated alone. In the 3D ECM scaffolds they created, ESCs interacted with trophoblast stem (TS) cells *in vitro*. As a result, they can construct embryo-like structures that simulate an early post-implantation embryo.

Furthermore, crosstalk between all three stem cell types observed in the normal embryos could form complete embryo-like entities representing gastrulation cell rearrangements [56]. With this in mind, in the next step, they created culture conditions that allowed for the three stem cell lines that make up the mouse embryo: ES, TS, and extra-embryonic-endoderm (XEN) cells. These multicellular organoids showed that this interaction led to the spontaneous self-assembly of structures resembling actual mouse embryos containing all three layers and extra-embryonic compartments. These examples demonstrate that synthetic biology and cell bioengineering are major approaches to obtaining multicellular spheroids and controlled cell patterning.

3. Methods for improving culturing of organoids

Despite the complexity of organoids formed by the self-organization of cells, organoid cultures need to apply additional stimuli and establish specific cell niches to exhibit biological properties similar to actual organs. It is also necessary to develop more complicated tissue structures and mimic the dynamics (the mechanics of tissue tension and fluid flows and biocomponent concentrations) of the human body's internal environment. Recently, multiple methods have been developed to improve organoid culture models, such as optimizing ECM, integrating with bioprinting technologies, and implementing microfluidic technologies.

3.1. ECM for 3D culture

The ECM is a 3D fibrous network of macromolecules surrounding cells. It provides structural and physiological support and regulates cellular functions, including cell growth, migration, proliferation, and differentiation [57]. The composition of the ECM is tissue-specific and reflects the specific role of the tissue and its constituent cells. The basic building blocks are proteoglycans, fibrous proteins (collagens, elastins, fibronectins, and laminins), glycosaminoglycans, and water [58,59]. Cells attach to the ECM through cell surface receptors such as integrin, discoidin domain receptor, and glypicans. Cell-ligands mediate cell proliferation and migration through the ECM by linking the intracellular cytoskeleton to the ECM.

Additionally, the ECM plays an essential role in cell fate, as biochemical and biomechanical signals are transduced from the ECM to cells, activating various signaling cascades [60]. ECM components such as proteoglycans can bind to growth factors to store these growth factors in the ECM and modulate their bioavailability. Proteolytic enzymes, including matrix metalloproteinases (MMPs), cleave the bond between the ECM and growth factors, release growth factors, activate signaling pathways, regulate the morphological organization of the ECM, and eventually direct physiological functions [57,61,62].

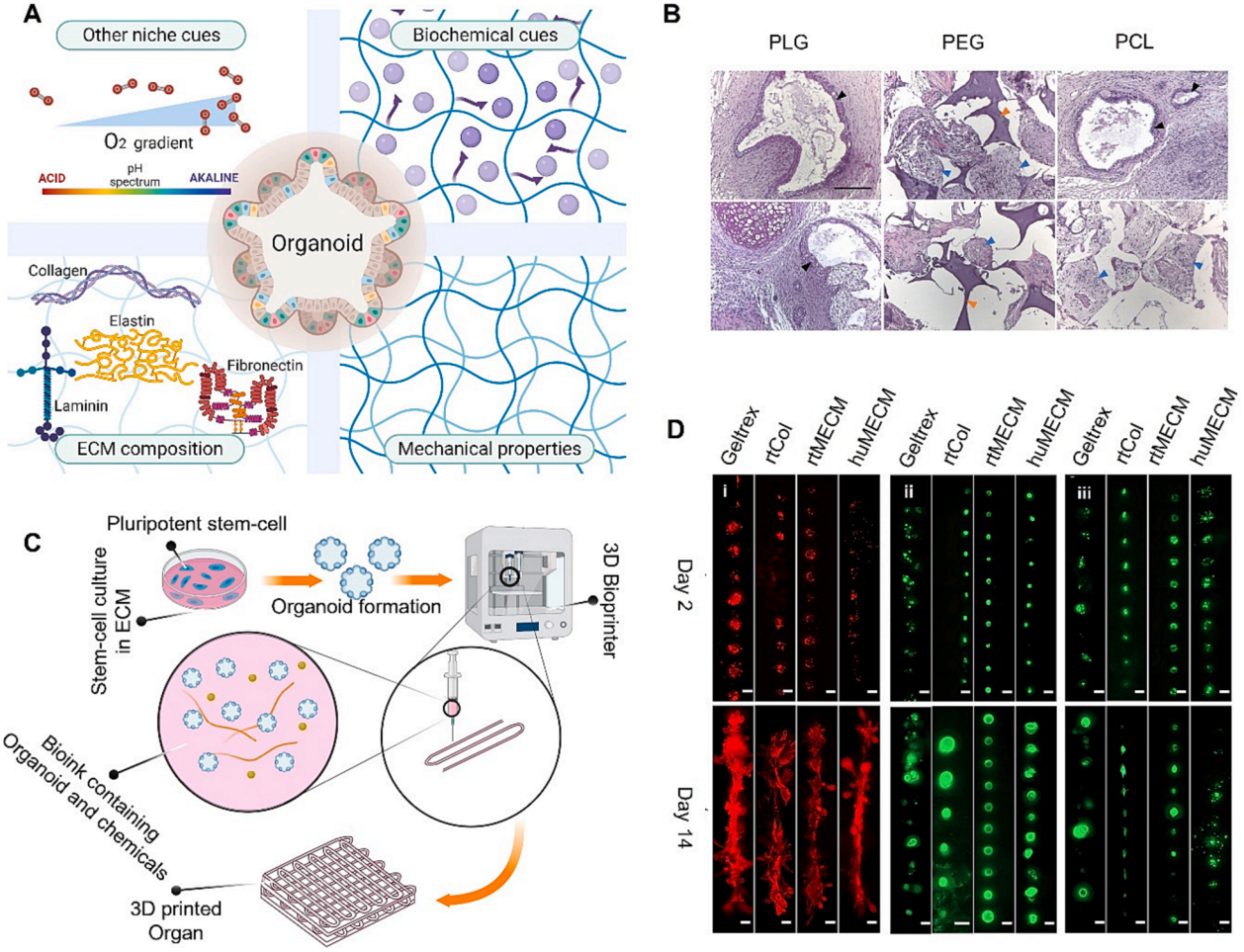

Fig. 3. Approaches to improving organoid development. A. Schematic representation of organoid culture in ECMs to recapitulate native tissue. B. Effect of the degradation rate of scaffolds on the hPSCs-derived lung organoid maturation into airway structures as cultured on poly (lactide-co-glycolic acid) (PLGA), PEG, and PCL scaffolds and transplanted into the epididymal fat pad of nonobese-diabetic-severe-combined-immunodeficient (NOD-SCID) IL2Rgnull (NSG) mice. The Black arrows show pseudostratified epithelium duplicating native airway epithelium (degradable scaffolds). Histological examination of organoids seeded on PEG and PCL scaffolds demonstrated intact scaffold (orange arrows) and clusters of cells (no-organized epithelium, blue arrows). Scale bar = 200 μm. Figure adapted with the permission of Elsevier from [83]. C. Schematic representation of 3D bioprinting approach to deposit bioink containing organoids into a specific architecture. D. Bioprinted epithelial organoids and tumoroid in Geltrex, rat-tail collagen (rtCOL), rat mammary ECM (rtMECM), and human mammary ECM (huMECM) hydrogels. Cellular arrays with ~ 50 cells at a time at 500 μm intervals were printed to recapitulate the architectural features of tumor organoids. Fluorescence image of (i) mammary epithelial cell line (MCF-12A), (ii) human breast cancer cell line (MCF-7), and (iii) MB-MDA-468 printed in a linear pattern within different ECM hydrogels. Large organoids of MCF-12A cells with duct-like luminal morphologies (exceeding 3 mm in length) were generated in all ECM substrates. Meanwhile, MCF-7 cells generated grape-like morphology in Geltrex and maintained a spherical shape in other ECM substrates after 14 days of culture. Also, MB-MDA-468 cells formed small clusters in Geltrex and single tumoroid in rtCOL, and rtMECM. In comparison, cells failed to grow appropriately in huMECM. Scale bars 200 μm. Figure reproduced from [119] with permission from Elsivier.

During organoid development, organoids are cultured within a 3D ECM composed primarily of hydrated proteins (*i.e.,* collagen, elastin, laminin, and fibronectin) and polysaccharides to recapitulate the properties of native tissues, including ECM composition, tissue mechanical properties, biochemical cues, and oxygen and pH gradients [23,63] (Fig. 3**. A**). Organoid formation in 3D culture requires precise control over the extracellular environment, ECM, and conditioned media that stimulate specific stem cell signaling pathways to control stem cell expansion, differentiation, and self-organization. To this end, it is expected to understand the role of the ECM, cell-ECM interactions, and intrinsic/extrinsic biochemical signals that determine the fate of stem cells. Factors such as mechanical properties, ligand presentation, matrix geometry, matrix degradation, and remodeling affect ECM-cell interactions and organoid formation [1,23,32,64].

The "gold standard" ECM material for expanding organoids is Matrigel, a solubilized basement membrane extracted and purified from Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, composed mostly of laminin, collagen IV, and entactin [65]. Despite having greater versatility than other options for organoid expansion, Matrigel is unsuitable for translational clinical applications, mainly due to the potential to induce immunogenicity by introducing the extractor and cancer-derived enzymes and growth factors into the body [65,66]. Proteomic analysis showed that Matrigel contains nearly 2000 unique proteins and peptides, making identifying niche components essential for the organoid structure and function impractical for clinical applications. Moreover, batch-to-batch inconsistencies observed in Matrigel formulations reduce organoid growth reproducibility and further limit Matrigel's application. Heterogenicity of mechanical properties, low stiffness, lack of tunable mechanical and physical properties, and impracticality of tailoring Matrigel to meet specific organoid niches are other factors hindering its application in translational settings. Therefore, organoid technology is increasing the demand for well-defined 3D biomaterial engineering that remodels the *in vivo* stem-cell niches and enables reproducible and precise control of organoid cultures [1,64,65].

Engineering chemically defined biomaterials as substitutes for animal-derived ECMs is a promising approach for controlling the environment for organoid expansion [65]. Various engineered ECMs made from natural or synthetic polymers have been suggested to overcome limitations such as a lack of physicomechanical tunability, lot-to-lot variability, and immunogenic potential observed in the animal-derived ECMs (Table 1) [65]. To make this section more comprehensible, the following ECM-like matrices were reviewed by dividing them into two main classes: hydrogel and non-hydrogel matrices.

3.1.1. Hydrogel matrices for organoid culture

Various ECM-like materials have been studied for organoid cultures. Natural polymers have been widely used for organoid cultures owing to their cytocompatibility and structural similarity to the secreted ECM of target organisms [67]. These polymers are classified as protein-based (*e.g.,* collagen and fibrin) and polysaccharide-based (*e.g.,* alginate, hyaluronic acid (HA), and chitosan). Additionally, the biophysical and biochemical properties of natural polymers can be easily tailored to recapitulate stem-cell niches [68,69]. Luo *et al.* developed a dual peptide-loaded hybrid system by mixing bone-forming peptide-1 (BFP-1)-containing mesoporous silica with alginate modified with cell adhesion peptides units (RGD) to facilitate osteogenic differentiation of human mesenchymal stem cells (hMSCs) [70]. The presence of cell adhesion peptides promoted the survival and migration of hMSCs within alginate-based matrices at an early stage, and the osteogenic differentiation of cells was mediated by mesoporous silica containing BFP-1 after cell proliferation. In another study, Drzeniek *et al.* contrived a hMSCs-laden hybrid hydrogel system based on collagen I and thiol-modified HA to study matrix-guided cell behavior and secretion of therapeutic proteins [71]. Their finding suggested that the engineered matrix provides essential biochemical and biomechanical cues for cell survival and migration. It also enhances the secretion of proangiogenic, neuroprotective, and immunomodulatory paracrine factors. In the context of tumor organoids, Ng *et al.* used an enzymatic crosslinking approach to tailor the biomechanical and biochemical properties of phenol-conjugated gelatin and HA hybrid hydrogels when culturing and expanding patient-derived colorectal tumor organoids [72]. Conjugation of phenol to the backbone of ECM components governed matrix crosslinking in the presence of horseradish peroxidase (HRP) and H_2O_2. The level of crosslinking determined the mechanical properties of the matrix. The results indicated that phenol-conjugated gelatin supports colorectal tumor organoid growth and that a higher level of mechanical properties improved organoid growth and sensitivity to other colorectal cancer tumor therapies. However, it should be noted that the limited processibility, high level of complexity, and variability of natural-derived polymers hinder their use for organoid cultures [68].

Chemically defined synthetic polymers are a versatile alternative for engineering ECMs in which biochemical and biomechanical cues can be incorporated to optimize the growth of organoids [65]. Reproducibility and batch-to-batch consistency of organoid cultures, which result from controlling the fabrication process, are advantages of synthetic ECM over naturally derived ECM. Polyethylene glycol (PEG) is the gold standard synthetic polymer used in various organoid growth research

Table 1
Materials for organoid culture systems.

Material Class	Material Type	Material	Organoid Tissue	Cell Origin	Ref
Hydrogel	Decellularize tissue	Matrigel	Cardiac organoid	hiPSCs	[86]
			Brain organoid	hiPSCs	[87]
			Prostate organoid	Murine prostate epithelial cells	[88]
			Pancreatic organoid	hESCs and hiPSCs	[89]
			Lung	hiPSCs	[90]
		Decellularized porcine brain ECM	Brain organoid	hESCs	[91,92]
		Decellularized-Skeletal ECM	Cardiac Organoids	hESCs	[93]
		Decellularized- porcine intestinal ECM	Intestinal organoid	hESCs and hiPSCs	[94]
	Natural Polymers	Collagen	Intestinal organoid	Human intestinal epithelium	[95]
				Human adult intestinal epithelial stem cells	[96]
		HA	Kidney organoid	Murine kidney epithelial cells	[97]
		Alginate	Intestinal organoid	hESCs and hiPSCs	[98]
		Fibrin-laminin	Epithelial organoids	adult islet-depleted pancreatic tissue	[99]
		Collagen-Laminin-Fibronectin-Hyaluronan	Breast Organoid	Mammary stem cells	[100]
	Synthetic polymers	PEG	Intestinal organoid	ISCs	[101]
			hepatic organoids	Mouse and human ductal cells	[102]
		PEG-4-MAL	Intestinal organoid	hESCs and hiPSCs	[76]
		PNIPAM	Intestinal organoid	small ISCs	[80]
Non-hydrogel	Natural Polymers	silk	kidney organoids	hiPSCs	[84]
	Synthetic polymers	PLGA	Cerebral organoid	hESCs and hiPSCs	[78]
		PCL	breast tumor-derived organoid	mammary breast cancer	[79]

due to its unique chemical structure [65,73]. PEG can be specifically tailored to meet the desired properties of organoid culture. Tomaszewski *et al.* aimed to develop a fertility preservation option by functionalizing PEG with several peptides, such as heparin-binding (HBP and AG73) and basement membrane binder (BMB), to mimic the native ECM for expansion and maturation of ovarian follicular organoids [74]. They demonstrated that the functionalization of PEG-ECM with peptides facilitates native ECM secretion by ovarian follicles and stimulates follicle maturation. In another work, Kloxin *et al.* used a photodegradable crosslinking approach to control the function and morphology of encapsulated hMSCs. To this end, they focused on spatiotemporal control of chemical properties, mechanical elasticities, and distribution of adhesive peptides within a PEG-based ECM-like matrix [75]. Four-armed, maleimide-terminated PEG (PEG-4-MAL) has also been used to engineer synthetic ECM-like matrices that support organoid formation [76,77]. This ECM-like matrix supports human intestinal organoid growth from PSCs with organoid growth similar to Matrigel's. Other synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA) [78], Polycaprolactone (PCL) [79], poly(N-isopropylacrylamide) (PNIPAM) [80], and polyvinyl alcohol (PVA) [81] have been used as scaffolds for organotypic and organoid formation.

3.1.2. Non-hydrogel matrices for organoid culture

Besides hydrogel matrices, non-hydrogel matrices have guided cells in 3D organ-like architectures. Combining the self-organizing capacity of organoids with bioengineered scaffolds with tunable configurations can increase reproducibility and enable guided self-organization into tissue-like structures [68]. Lancaster *et al.* engineered elongated organoids using fibrous microfilaments made of PLGA as floating scaffolds [78]. They demonstrated that microfilament scaffolds and organoids allow forebrain formation and neural migration with characteristic cortical tissue architecture such as polarized cortical plates and radial units. Non-hydrogel matrices enable precise control over the surface chemistry, porosity, and spatial confinement. Pores facilitate the diffusion of nutrition through the scaffold in the conditioned media to deliver nutrition and growth factors to organoids physically entrapped in the scaffold [82]. Dye *et al.* studied the role of physicochemical properties such as size and interconnectivity of pores and degradation rate from developing ESC-derived lung organoids to adult airway-like structures [83]. The lung organoid seeded on the porous PLGA scaffold or PCL scaffold produced a tube-like structure that recapitulated structural and cellular features similar to the adult airway. On the other hand, lung organoids seeded on the non-degradable hydrogel scaffolds made of PEG did not grow and mature. They demonstrated that lung tissue maturation is enhanced by the scaffold's interconnected porous structure and degradability (Fig. 3**. B**). *In vitro* organoid culture can also be supported using natural polymers with the desired shape and mechanical properties. Silk is an example of a natural polymer for organoid culture because of its high cell adhesiveness arising from its fibroin component. Gupta *et al.* fabricated porous silk as a sponge. They used this scaffold to study the growth and differentiation of hiPSCs-derived kidney progenitor cells and primary cells [84]. They showed that hiPSCs differentiated to epithelia corresponding to WNT/β-catenin signaling. However, their fabrication method did not allow precise control of the pore size and shape of matrices, resulting in poorly organized iPSCs-derived tubules. In addition, porous silk scaffolds have been successfully used to generate a 3D osteosarcoma model with physiological features such as the expression of proliferative angiogenic markers similar to those of *in vivo* tumors [85].

3.2. 3D printing and organoids

Advances in 3D biofabrication and bioassembly technologies have enabled organoids to be involved in the bioassembly of complex biomimetic tissues for regenerative medicine purposes [65] (Fig. 3**. C**). Microfabrication-based technologies, such as 3D printing and microwells, allow precise control over the structure and composition of organoids, as well as batch-to-batch consistency, and produce organoids with complex structures. Similarly, modifications of ECM-like biomaterials, such as the introduction of signaling proteins and peptides (*e.g.,* RGD peptides), adjustment of matrix stiffness, degradation, and remodeling, can be easily integrated into these biofabrication methods [24,103,104]. 3D bioprinting can fabricate vasculature within tissue structures to deliver oxygen and nutrients and remove waste. From a regenerative medicine perspective, combining the precision of 3D bioprinting with the innate self-organization of organoids enables a better recapitulation of macroscale tissues in the body [1,68,105].

The 3D bioprinting method enables researchers to control 3D culture parameters, including cell density, chemical formulation, deposition volume of bioink, and ECM components, including growth factors (Fig. 3**. D**). This allows a high degree of control and customization over cell types (homocellular or heterocellular bioinks). In addition to the typical properties expected from biomaterials, such as biocompatibility, biodegradability, printability, and mechanical properties, there are critical features for selecting bioinks for cells and organoid printing [24,106,107]. For instance, low-viscosity bioinks have limited printability due to slow gelation, reducing structural integrity and print resolution [108]. While printing high-viscosity inks, cells are exposed to high shear stress, damaging the entrapped cells and organoids.

Additionally, the mechanical properties of bioinks contribute to the shape, function, viability, migration, and differentiation of cells after printing. Efficient mechanical properties enable bioprinting of cells in various structures and geometries, including grooves, ridges, and stepwise [109,110]. Natural hydrogel-based bioinks such as Matrigel, alginate, agarose, collagen, GelMA, and HA and synthetic polymers such as PEG, Pluronic, and PCL have been used for 3D bioprinting of organoids [111]. For instance, Yang *et al.* developed an *in vitro* 3D printed liver tissue model (liver organoids) using HepaRG cells and alginate bioink [112]. The authors demonstrated that the liver organoids obtained liver functions, including albumin secretion, glycogen storage, and drug metabolism. This model further matured when implanted into a mice model. In another work, Bernal *et al.* engineered a rapid light-driven method for volumetric 3D bioprinting organoid-laden constructs to capture critical features of the human liver [113]. To this end, they printed liver organoids using a layerless, nozzle-free technique by printing a GelMA bioink in a lattice with varying degrees of porosity. The printed organoids acquire liver functions (albumin secretion and liver-specific enzyme activity) through hepatocyte differentiation and demonstrate the ability to modulate liver-specific ammonia detoxification.

Unlike 2D culture and small-scale 3D culture in static conditions, where nutrition is supplied through regular media exchanges, significant challenges associated with scale-up organoid cultures and generation of functional tissue are perfusion of nutrition/oxygen and waste removal [114]. Advances in organoid bioprinting technology allow the production of vascularized structures that facilitate nutrient transport to promote organoid expansion [115]. One approach to introducing vascular channels into organoids is to combine stem cells and endothelial cells (ECs). This approach allows the perfusion of oxygen and nutrients along large organoids that contribute to organoid maturation and growth [116]. Yap *et al.* generated vascularized hepatobiliary organoids by combining mouse liver progenitor cells and sinusoidal ECs [117]. The results showed a significantly positive impact of vascularization on hepatobiliary tissue differentiation and cell survival inside organoids after transplantation. Nevertheless, this method has limited scalability and cannot make organoids with complex geometries.

Another approach to creating a vascularized channel is using a sacrificial bioink. Skylar-Scott *et al.* reported a biomanufacturing approach to creating vascularized channels via sacrificial writings into organ-building block matrices composed of patient-specific iPSCs-derived organoids [118]. After forming a living organ budling block matrix containing approximately 500 million cells, sacrificial ink

(gelatin) was patterned within the matrix using an embedded 3D printer. As a result, a perfusable vascularized network was formed upon the sacrificial ink's removal. This biomanufacturing strategy helps create personalized, organ-specific tissues with embedded vascular channels at the therapeutic level.

3.3. Microfluidics and organoids

Microfluidics is the science of manipulating small volumes of fluids. Microfluidics for biological fluids can be used for a variety of applications, including point-of-care diagnostics [120], droplet generation [121], cell separation [122], microphysiological systems [123], organ-on-a-chip [124], and organoid-on-chip [125] platforms.

Organ-on-chip technology represents engineered microfluidic chips containing cells that recapitulate organ-level functions [123,126]. It provides fluid flow that supplies nutrients to cells, exchanges metabolic wastes, applies physiological shear stress, and regenerates the tissue-tissue interface. These platforms can offer relevant mechanical cues as well as continuous fluid sampling. Moreover, the transparent materials of the chips enable high-resolution imaging [127]. Due to the high control of laminar flow in microfluidics platforms, they can provide a controlled gradient of drugs/chemicals using gradient-generating devices [128]. These unique features show great promise for the advancement of personalized medicine.

In several recently reported studies, organoid-on-a-chip models have shown reproducibility and improved the potential of efficient organoid cultures [123,126,129]. In the conventional growth process of organoids, after reaching a limited size, the inner core of organoids can lose viability and undergo necrosis due to the restricted diffusion of oxygen, nutrients, and metabolites [130]. The challenge of restricted diffusion can be addressed with microfluidics, through which organoids can be perfused and thus can efficiently supply nutrients to support long-term organoid culture [131]. Hence, enhanced circulation of conditioned media in organoids through a laminar flow of microfluidics can reduce the necrotic core size and increase organoid size.

One of the most important examples of merging organoids and microfluidics is the brain chip system using hiPSC-generated organoids. Their self-organization constitutes a novel *in vitro* micro-organ system to mimic brain tissue development and disorders. Brain organoids are expected to be crucial for understanding brain development, but constructing brain biomimetic environments remains challenging. Wang *et al.* presented an organoid-on-a-chip platform for generating hiPSCs-derived brain organoids by a dynamic cell culture approach [132]. The platform uses fluid flow, multicellular tissue, and a 3D environment with Matrigel to create a biomimetic brain microenvironment. The authors developed an in situ neural differentiation, scaled 3D culture, and brain organoid on-chip platform with this multifaceted approach. Furthermore, brain organoids in perfusion culture express higher levels of cortical layer markers (CTIP2 and TBR1) than in static culture, demonstrating the importance of fluid flow in encouraging brain organogenesis (Fig. 4. **A**).

Investigating nicotine exposure to fetal brain development is challenging because of the limitations of animal models. To address this issue, Wang *et al.* developed a brain organoid-on-a-chip model using hiPSCs to study neurodevelopmental problems caused by prenatal nicotine exposure [133]. The organoids showed structured regionalization, neural differentiation, and cortical architecture, recapitulating crucial aspects of human brain development at an early stage. Brain organoids exposed to nicotine showed increased TUJ1 expression (the neuron marker) and early neuronal development. Additionally, these organoids exhibited poor cortical development and brain regionalization, as evidenced by the expression of hindbrain (PAX2 and KROX20), cortical neural layer (deep-layer CTIP2 and preplate TBR1), and forebrain (FOXG1 and PAX6) markers. Furthermore, neurite outgrowth from organoids treated with nicotine demonstrated impaired neuronal differentiation and migration (Fig. 4 **B**).

Multiple organ-on-chip platforms can be connected to provide a body-on-a-chip platform (multi-organ on-a-chip) to consider the tissue-tissue or organ-organ interactions and to achieve more accurate and physiological models [134]. These models could be employed to study every dimension of the drug metabolism process, including absorption, distribution, metabolism, and elimination (ADME) for drug screening and disease modeling applications. In a study, liver, bone marrow, and brain cortex micro-organoid-on-a-chip were connected in a systemic microenvironment [135]. In a more advanced multi-organoid-on-chip platform, Zhang *et al.* proposed a platform for connecting the liver organoid-on-a-chip to the heart organoid-on-a-chip with multiple sensor integration for biophysical and biochemical parameters detection in a real-time manner [136].

Although the organoid-on-a-chip shows great promise in organoid culture and for disease modeling and drug screening applications, standardization and scale-up are among the common challenges in

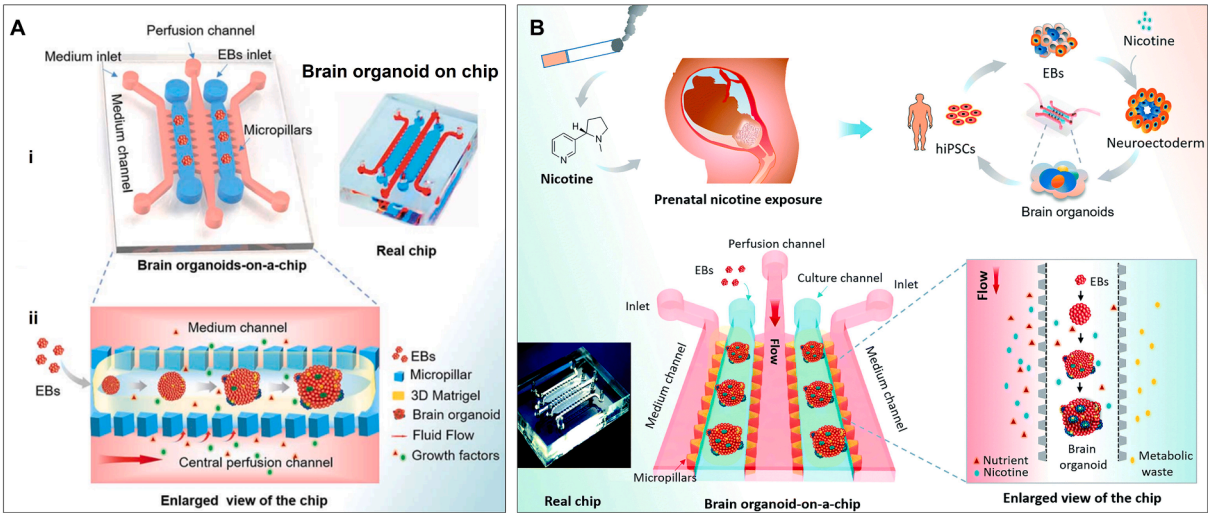

Fig. 4. Engineering microfluidics for organoid development. Ai. Schematic representation of brain organoids-on-a-chip. Brain organoids are formed from hiPSCs. Aii, Neurogenesis and tissue morphology of the organoids showing TUJ1 and SOX2 markers in 33-day organoids (indicated by arrows) on a chip. Reproduced from [148] with permission from The Royal Society of Chemistry 2018. B. The brain organoid-on-a-chip model proposed by Wang *et al.* (Reproduced from [133] by Royal Society of Chemistry 2018) to study neurodevelopment problems caused by prenatal nicotine exposure.

developing organoid-on-a-chip models [125]. Moreover, needing tubing, pumps, and connectors for operation hinder their widespread applications due to the limited accessibility and high-cost perfusion systems. Moreover, the long-term process (about 1 month) of making and culturing organoids from stem cells is extensive for a clinician to achieve personalized therapy options. More advanced technologies, such as 3D bioprinting and integration of screening with organoid-on-chip platforms and connecting multiple organoid-on-chip platforms can facilitate studying the absorption, distribution, metabolism, and elimination process [137].

4. Biomedical applications of organoids

4.1. Transplantation and modeling of organ-specific tissues

The human body is a complex system composed of multiple organs with unique characteristics and functions. Modeling organ-specific tissues is a field of study that seeks to understand and replicate the behavior of these organs and their constituent cells *in vitro*. This is particularly important in medicine and biomedical engineering, where the ability to study and manipulate organ tissues outside of the body can lead to significant advances in diagnosis, treatment, and regenerative medicine.

Organ-specific tissue modeling involves various techniques, including cell culture, tissue engineering, and microfluidic devices. These approaches can recreate the cellular and ECM components of specific organs, allowing researchers to study their behavior under different conditions. For example, liver tissue models can investigate drug metabolism and toxicity [25]. In contrast, lung tissue models can be used to study respiratory diseases such as asthma [83].

Developing organ-specific tissue models also holds promise for regenerative medicine, which aims to create functional replacement tissues and organs. By understanding the processes involved in organ development and function, researchers can work towards creating tissue-engineered replacements for transplantation or other therapeutic applications [138,139].

Modeling organ-specific tissues is a rapidly advancing field with important implications for basic research and clinical applications. As technologies continue to improve, these models are becoming increasingly sophisticated, enabling researchers to gain more detailed insights into tissue development, disease development, and progression, especially in tissue regeneration. This section describes organ-specific organoid models for various applications, including implantation for regenerative approaches.

Although organoids can be grown *in vitro* to resemble organ-like structures, the long-term study of organoids can be constrained due to their transient longevity, nutrient supply, and low rate of cell differentiation and proliferation [140]. These issues inhibit organoids from maturing to the desired structure and procuring certain functionalities of organs, hindering therapeutic development progress. *In vivo* transplantation is imperative for the functional maturation of organoids. Through this, scientists may build animal models to investigate regenerative therapies, drug screening, and the mechanism of rare diseases and enhance the applicability of organoids in the clinical phase. Here, we elucidate recent studies on developing animal models, *in vivo* organoid transplantation, and microfluidic-based organoid models for tissue-specific modeling. Additionally, Table 2 summarizes the transplantation and modeling of organ-specific tissues subdivided by organ type and key findings and techniques.

Gastrointestinal organoids: In 2009, the concept of organoids was coined when the geometrically stable organoid was fabricated for the first time after discovering adult ISC proliferation and self-organization [141]. Many investigations reported discoveries of organoids derived/differentiated from fetal/adult rat/mouse intestinal or PSCs [76,139,142,143]. However, the first hPSC-derived intestinal organoids were transplanted under kidney capsules [143]. Interestingly, the transplantation showed successful engraftment followed by maturation. Later, transplantation of PEG-Maleimide encapsulated intestinal organoids showed efficient engraftment, similar to Matrigel encapsulated organoids [76]. In a separate investigation, authors have successfully created gastrointestinal organoids suitable for modeling gastrointestinal diseases, advancing drug development, and supporting tissue regeneration applications by utilizing extracellular matrix hydrogels derived from decellularized gastrointestinal tissues. They also found that the development and function of gastric or intestinal organoids grown in tissue ECM hydrogels are comparable to, and often surpass, those in Matrigel [144]. Furthermore, the mesentery transplantation of the organoids of intestinal cells displayed 85% engraftment [142].

Radiation proctitis is one of the dominant complications of radiotherapy in curing pelvic tumors [145]. Currently, there is no direct and consummate therapy to heal the damaged colonic epithelial caused by radiation proctitis. Jee *et al.* [138] developed a mouse model for investigating the ability of colon organoid-based treatment by applying a local irradiation technique to the recta of the mice to induce radiation proctitis, continued with transplantation of colon-organoid by injecting them into the rectum without surgery. Replanted colon organoids using fibrin glue successfully engrafted onto the damaged rectal mucosa and reassembled the epithelial structure. Crypts were isolated from mouse intestinal specimens and suspended in the Matrigel to produce colon

Table 2
Transplantation and modeling of organ-specific tissues.

Organ Type	Key Findings/Techniques	Reference
Gastrointestinal	- Organoid fabrication, Transplantation under kidney capsules, Mesentery transplantation, ECM hydrogels, Successful engraftment, Modeling gastrointestinal diseases, Tissue regeneration applications, Efficiency in engraftment	[76,139,142-144]
Liver	- Co-culturing (hiPSCs, hMSCs, HUVECs), Alginate-based biomaterial encapsulation, Microfluidic device, High maturation levels, Metabolism activity of drugs, Immune response studies, Hepatic functions in organoids	[151-155]
Heart	- 3D self-organization, Electrophysiological activity detection, Transplantation into 3D-printed basket, Heart beating, Cardiomyocyte maturation markers, Electrophysiological activity post-transplantation	[156]
Brain	- 3D in vitro culture system, Transplantation into retrosplenial cortex, Microfluidic system, Complete vascularization, Engraftment and vascularization studies, Interaction between micro brain and organoids	[17,92,159-164]
Lung	- 3D self-assembly of LEPCs, Transplantation with microporous PLGA scaffolds, Engraftment and maturation studies, Interaction with host cells, Vascularization	[165-167]
Pancreatic	- Transplantation into cystic fibrosis model, Orthotopic transplantation, Expression of pancreatic markers, Validation of cystic fibrosis, Engraftment and viability studies	[169,170]
Retinal	- 3D retinal sheet development, Transplantation into stationary night-blinded mice, Shear stress-free bioreactor, Integration within degenerate retinas, Maturation of photoreceptor sections, Enhanced retinal organoid preservation	[171-176,178,179]
Kidney	- 3D self-assembly, Transplantation into resected location of LNs, Integration into endogenetic lymph vasculature, Rehabilitation of lymphatic drainage and perfusion	[131,180-183]
Lymphoid	- Lymphoid-organoids composed of LN stromal progenitor and dECM-based scaffolds, Integration into endogenetic lymph vasculature, Rehabilitation of lymphatic drainage and perfusion	[185,186]

organoids; a small lump of organoids was injected near the irradiated area of mice using Mainz COLOVIEW endoscopic system [138]. ISCs are responsible for the repair of the colonic epithelial tissues [146], and the depletion of ISCs in patients from radiotherapy treatment impedes the process of epithelial regeneration [147]. As the Lgr5^{+} acts as a marker of ISCs, the influence of Lgr5^{+} in the regenerating process was examined. Colon organoids were cultured in the medium without or with Wnt-3a to reduce or increase the amount of Lgr5^{+} stem cells, respectively (Fig. 5). The organoid-transplanted area with a lower number of Lgr5^{+} stem cells was noticeably smaller than those with a more significant number of Lgr5^{+} stem cells. This emphasizes the significance of Lgr5^{+} stem cells in the regenerating process. EGFP^{+} crypt formed after transplanting organoids on the irradiated area of the mouse, manifesting the sign of cell proliferation and differentiation. The result indicates that the colon-organoid can differentiate into multiple types of intestinal epithelial cells. Notably, the timing of organoid transplantation is crucial for effective regeneration on injured sites. In this study, it shows the success rate is optimal between 6 and 10 days after the radiation. The transplanting mouse and donor mouse have a correspondent genetic background which exhibits the potential of organoid transplantation in humans [138].

Current animal models and *in vitro* strategies suffer critical shortcomings in modeling human gastrointestinal diseases [148]. Importantly they cannot closely mimic the multicellular human primary tissues. The production of human gastric organoids (hGOs) through guided differentiation of hPSCs was one of the significant milestones toward this aim. The luminal epithelium is critical to stomach functions and gastric disease mechanisms. However, getting into the epithelium within the organoids is challenging. Kug Lee *et al.* developed a bio-engineered human stomach-on-a-chip system to provide luminal flow over hGOs and closely recapitulated *in vivo* gastric functions [149]. The stomach-on-a-chip enabled stable, long-term hGO cultivation and luminal administration using a peristaltic pump. Furthermore, their platform enabled the contraction and stretching of the organoids in a rhythmic manner, similar to gastric motion.

Although mimicking several functional and structural features of the *in vivo* tissues, most organoid models suffer from inadequate vascularization. Schulla *et al.* used a microfluidic device to cultivate human small intestine organoids (hSIO) with vasculature in a triple co-culture system with ECs and fibroblasts [150]. They revealed that organoids in mono-culture in microfluidic devices have a low survival rate. In contrast, cultivating hSIO with ECs and fibroblasts in a co-culture system improves the viability and stemness of hSIO.

Liver organoids: The first-ever liver organoids were created by co-culturing the hiPSCs, hMSCs, and human umbilical vein ECs (HUVECs) [151]. The cells were self-organized into 3D structures to transform into liver buds at the primitive stage. The organoids were transplanted into nude mice to study vessel formations and donor-host tissue connection, showing high maturation levels. In addition, the metabolism activity of drugs was tested on the organoid model. Despite the positive results from the transplant, there was a need for biomaterials to transplant the organoids. Using organoids formed with

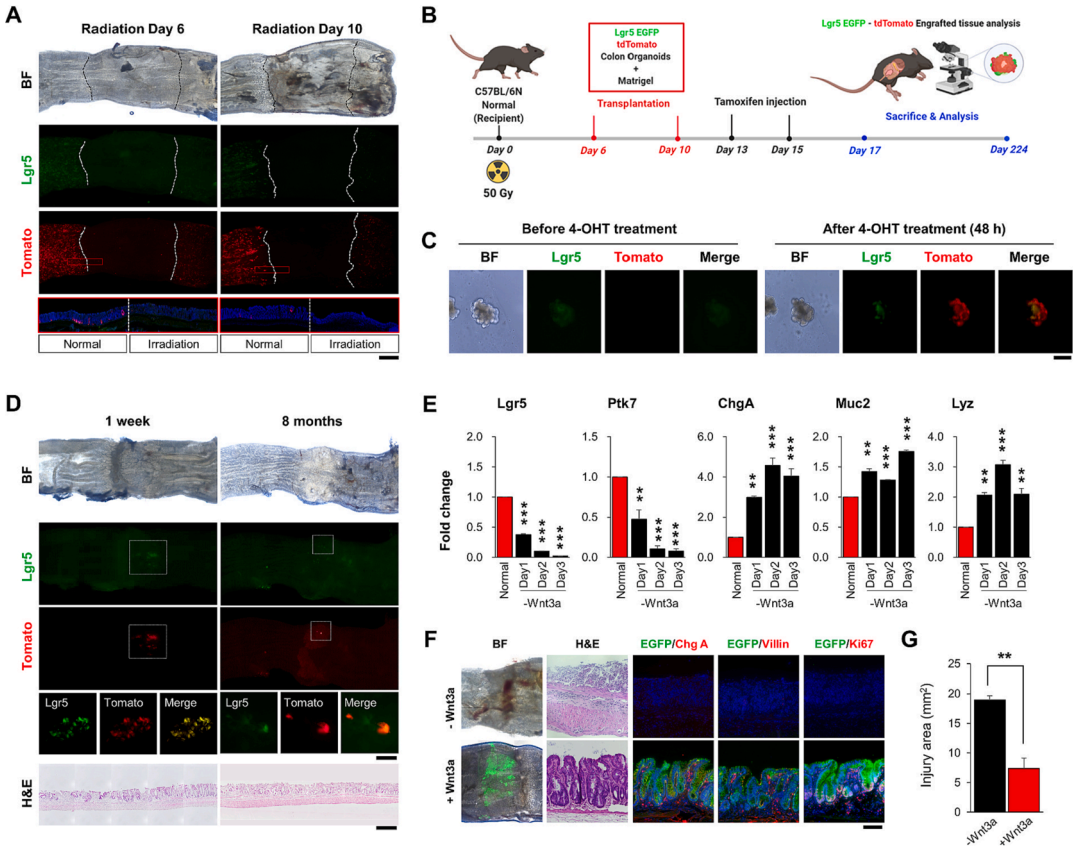

Fig. 5. Engineering and characterization of Lgr5-EGFP-tdTomato colon organoids. A. Lgr5^{+} cell depletion in colon tissue after radiation exposure. The black and white dotted lines indicate irradiated areas. Scale bar: 200 μm. B. Strategy for colon organoid transplantation in the mouse model of radiation proctitis. C. Evaluation of colon organoids *in vitro* treated with 4-hydroxytamoxifen (4-OHT) before and after transplantation. Scale bar: 200 μm. D. Lgr5 + cell expression in colon organoid-engrafted tissue at 1 week and 8 months after transplantation. Scale bars: 200 μm (organoid engrafted tissue), 500 μm (HandE-stained tissue). E. Lgr5, PTK7, Chg A, Muc2, and lysozyme mRNAs were expressed in colon organoids grown in medium with or without Wnt3a for 3 days. F. Representative images of tissues engrafted by colon organoids cultured in medium with and without Wnt3a (bright field and HandE, GFP/Chg A, villin, and Ki67 staining). Scale bars: 100 μm (HandE, EGFP/Chg A, villin, Ki67 staining). G. Areas of proctitis injury 2 weeks after transplantation of colon organoids cultured in medium with Wnt3a or without Wnt3a. Reproduced from [138] with permission from Elsevier 2021.

different protocols, Song *et al.* encapsulated liver organoids into alginate-based biomaterial to transplant into an immunocompromised animal [152]. The biomaterial-encapsulated spheroids were studied regarding the immune response. As a result, the presence of IL-2 directed the immune response; however, the immune system did not reject the capsules. Nie *et al.* transplanted single-donor functional multicellular liver organoids into mice to investigate the treatment of acute liver failure [153]. The multicellular liver organoids were transplanted into the renal subcapsules of acute liver failure mice model. The organoids rapidly assumed hepatic functions, presenting an improved survival rate.

Liver organoids have great promise in understanding liver development and contributing to drug testing and toxicological assessment [148]. Due to the lack of controllability of cellular microenvironmental inputs, traditional approaches for generating organoids provide limited liver functions and lower repeatability. To produce advanced liver organoids that play a pivotal role in drug discovery and toxicity testing, Wu *et al.* established a microfluidic device that allows for applying environmental cues and pressure stimuli by heart beating, which was challenging in conventional organoid fabrication [154]. In another work for liver organoids, Schepers *et al.* created a liver organoid-on-a-chip platform by culturing iPSCs from a patient and growing them as perfusable 3D organoids, which performed robustly even when the perfusion flow rate was changed [155]. They created hepatocyte aggregates from iPSC-derived and primary cells in monoculture and co-culture with support cells. To include these aggregates on a perfusable platform, they embedded the cells in a PEG hydrogel to avoid aggregation and overgrowth while on-chip. Their chip architecture allowed organoids' stable loading in a wide flow rate range. The final liver organoid chip showed a lifetime of at least 4 weeks.

Heart organoids: Heart/Cardiac organoids are still a poorly explored area in the field of disease modeling and treatment. Primarily, organ physiology and tissue dynamics could be investigated in detail. Varzideh *et al.* studied the development of hiPSCs-derived organoids [156]. The organoids contained three types of cells, including ECs, MSCs, and cardiac progenitor cells (CPCs). The cardiac organoids were self-organized into a 3D stable structure. The electrophysiological activity, such as heart beating and cardiomyocyte (CM) maturation markers, were detected before transplantation. Organoids were placed into a 3D-printed basket and transplanted into the internal abdominal muscle of nude mice to study the maturation. After four weeks of transplantation of cardiac organoids enclosed in collagen type I, the electrophysiological activity, sarcomeric structures, extensive neovascularization, and cardiac markers were found in the transplanted system.

Brain organoids: The brain is the most complex organ with numerous complex functionalities [157]. The standard cell culture could not recreate all the functionalities. Therefore, the fabrication of brain organoids has shown the potential to model and treat brain-related diseases [17,158]. In 2013, a 3D *in vitro* culture system mimicking brain tissues was developed to investigate microcephaly [17]. Later on, GFP hESC-derived organoids were transplanted into the cavity of the retrosplenial cortex of NOD-SCID mice to study the complete vascularization of the organoids [17,159,160]. Daviaud *et al.* transplanted hESC-derived neuron progenitor cells and cerebral organoids into the frontoparietal of P8-P10 mice to investigate engraftment and vascularization [161]. The transplanted organoids showed better graft survival, neuronal differentiation, and vascularization compared to the transplantation of hESC-derived neuron progenitor cells [161].

In a different work, Wang *et al.* transplanted organoids into the middle-cerebral-artery-occlusion of rats to study the reverse of the damage from stroke [162]. After 6 to 24 hrs of the transplantation, the organoids were found to reduce the stroke damage volume, synaptic reconstruction, and the recovery of neurological motor function by cell multilineage differentiation, proper vascularization, and cell survival. Recently, Dong *et al.* developed small human brain organoids and transplanted into the medial prefrontal cortex of mice to investigate the interaction between the micro brain and the organoids [163]. The transplantation of brain organoids showed maturation, survival of the organoids, and extension in length during engraftment. In addition, the connection between the host and the organoids was well-established, along with the proper synaptic connections and the electrophysiological activity.

Employing microfluidics, Cho *et al.* engineered brain organoids using a human brain ECM. The microfluidic system enables brain-specific stimulation, increased lifespan, and reduced organoid variability [92]. As a result, neurogenesis was significantly enhanced in a brain organoid model made from brain ECM-enhanced hiPSCs. Dynamic culture in microfluidic chamber devices further enhanced volumetric augmentation, cortical layer development, and human brain organoid electrophysiological function. Qian *et al.* designed and presented a spinning microchip for generating forebrain-specific human iPSCs-derived organoids [164]. Their organoids recreate critical features of human cortex development, including gene expression, neurogenesis, progenitor zone structure, and a separate layer of outer radial glial cells. They created methods for organoids from the midbrain and hypothalamus. Finally, Zika virus (ZIKV) infection was simulated using the organoid platform. Quantitative investigations revealed that either Asian or African ZIKV strains selectively and effectively infect brain progenitors.

Lung organoids: Lung organoids are one of the promising tools in transplantation to model and treat pulmonary diseases [165]. The lung organoids are commonly generated by the 3D self-assembly of lung epithelial progenitor cells (LEPCs) with stem cells, PSCs, and with/without the MSCs [165,166]. In 2016, lung organoids were first transplanted with microporous PLGA scaffolds to test the different conditions of the transplantation [167]. The PLGA scaffold showed the best result in the maturation of human airway epithelial and the generation of secretory lineages after one day of *in vivo* engraftment. Tan *et al.* used adult bronchial epithelial cells, lung fibroblasts, and lung ECs to help fabricate human airway organoids [165]. The developed organoids were transplanted into kidney capsules. The host's cells were proliferating, and proper vascularization was observed. Weiner *et al.* performed alveolar type 2 (AT2) organoids transplantation into influenza-infected mice to study the elevation of oxygen exchange in the infected mice [168]. After thirteen days, the AT2 organoids transplanted into influenza-infected mice showed good engraftment and retained the AT2 fate. Although, the oxygen exchange capability could not be elevated even after transplantation of the spheroids.

Pancreatic organoids: Developing pancreatic organoids could potentially treat autoimmune diseases like type I diabetes mellitus, where β-cells are destroyed without insulin. In this respect, Hohwieler *et al.* transplanted pancreatic ß-cells spheroids to a cystic fibrosis model [169]. These PSCs-derived pancreatic organoids containing acinar and ductal cells expressed pancreatic markers and structurally and functionally resembled the pancreas. The organoids were transplanted orthotopically to trace the expression of cell markers and validate cystic fibrosis. In a study by Lebreton *et al.*, islet cells with human amniotic epithelial cells (hAECs) created pancreatic organoids that were transplanted into an immunocompromised type 1 diabetes mouse model to investigate the extent of vascularizations [170]. Incorporating hAECs into islet organoids enhanced engraftment, viability, and graft function, providing great potential in developing cell-based therapies for type 1 diabetes.

Retinal organoids: Impairment and vision loss are primarily due to the loss or damage to the photoreceptors. In recent years, multiple studies have reported macular degeneration and retinitis pigmentosa [171]. However, no cure has been discovered or identified to treat this disease. Therefore, the transplantation of retinal derivatives is the need of the hour. With the advancement of 3D and iPSC-derived cells, different human and mouse retina parts were significantly developed [172–175]. Gonzalez-Cordero *et al.* developed embryoid bodies (EB) differentiated retinal organoids and transplanted them in stationary night-blinded $Gnat1^{-/-}$ mice to study the maturation [176]. The transplanted

organoids were shown to integrate within the degenerate retinas of adult mice and mature into outer segment-bearing photoreceptors.

Similarly, in a study by Assawachananont *et al.*, mESC and mouse iPSC derived a 3D retinal sheet in rd1 mice, which showed rapid and progressive RP [175]. Post-transplantation, retinal structure maturation, new synaptic connections, formation of new photoreceptors, and incorporation with the outer nuclear layer (ONL) were observed. Santos-Ferreira *et al.* developed mESC-derived retinal organoids and transplanted them in the subretinal region of mild or severe rod-con degenerated mice [177]. For Prom1$^{-/-}$mice, the transplanted organoids showed the synaptic connection, maturation, and expression of the protein of the phototransduction pathway. In contrast, tg (Cpfl1; Rho$^{-/-}$) seemed immature, with no synaptic markers but photoreceptor-expressed rod markers.

Retinal organoids generated from stem cells (RtOgs) are a new type of tissue replacement therapy [171]. A shear stress-free bioreactor was proposed by Xue *et al.* for practically labor-free and long-term retinal organoid preservation [178]. They improved the chip architecture by utilizing *in-silico* simulations and *in vitro* investigations to ensure effective mass transfer and concentration homogeneity in each cultivation chamber. RtOgs were cultivated on an optimized chip for over a month at different differentiation phases. They applied various quantitative and qualitative methodologies to describe the RtOgs produced by organoid culture in perfused bioreactor and static dish culture methods. They discovered that RtOgs cultivated in the bioreactor had cell types and morphologies similar to RtOgs in static culture conditions and identical retinal gene expression levels. Furthermore, the outer surface region of RtOgs grown within the bioreactor exhibited a similar free/bound nicotinamide adenine dinucleotide (NADH) ratio and a lesser long lifetime species (LLS) ratio compared to static cultivated RtOgs.

In another study for retina organoids, Achberger *et al.* proposed the retina-on-a-chip (RoC), incorporating about seven vital retinal hiPSCs-derived cell types [179]. The microfluidic system enables vasculature-like perfusion and recapitulates the interaction of mature photoreceptor sections with retinal pigment epithelium (RPE) *in vitro*. They demonstrated that this interaction improves the creation of outer segment-like configurations and the formation of physiological processes similar to *in vivo* conditions, including calcium dynamics and outer segment phagocytosis.

Kidney organoids: Many people suffering from kidney disorders worldwide need kidney transplantation and hemodialysis [180]. The kidney has the most significant complexity among all organs that comprise different cell types [181]; therefore, the transplantation of organoids becomes a good investment for the scientific community. Moreover, the lack of an efficient animal model to exhibit the rare disease mechanism hinders the development of potential therapies. For instance, it was challenging that during the *in vivo* modeling of some rare genetic diseases, the inactivation of TSC1 or TSC2 can cause embryonic destructiveness [182]. Therefore, Hernandez and colleagues strived to assemble a mouse model by generating a kidney organoid [183]. The model could recapitulate the mechanism of a rare kidney tumor-angiomyolipoma (AML) associated with Tuberous Sclerosis Complex (TSC). They discovered the presence of myomelanocytic cells in TSC2-/- hiPSCs-derived organoids, which also obtained the common essential characteristics of kidney AML and other TSC-associated kidney diseases. It was remarkable that the formation of renal epithelial cyst is likely because of the loss of TSC2. In a different work, Hernandez *et al.* transplanted TSC2-/- derived AML organoids into immunodeficient rats [183]. They noticed the intensification of phenotype in organoids and the shrinkage in the size of organoids after the injection of rapamycin-loaded nanoparticles under kidney capsules. Besides drug screening and disease mechanisms, organoid transplantation can be an appropriate method to seek potential regeneration therapies.

Homan *et al.* presented an *in vitro* approach for cultivating kidney organoid-on-a-chip in the presence of flow, which increases the endogenous group of endothelial progenitor cells and produces vascular networks [184]. Compared to static controls, vascularized kidney organoids cultivated in flow showed more tubular compartments and matured podocytes and improved adult gene expression and cellular polarity. The capacity to promote significant morphological maturation and vascularization of kidney organoids *in vitro* in the presence of flow brings up new possibilities for investigating diseases, regeneration, and kidney development.

Lymphoid organoids: Surgical abscission of lymph nodes (LNs), irradiation, and infections can injure vasculature, which impairs the function of lymphatic drainage and perfusion, leading to a diminishment of the immune response [185]. Elisa Lenti *et al.* developed a mouse model to advance the regenerative method by employing lymphoid-organoids (LOs) composed of two major components-lymph node stromal progenitor and dECM-based scaffolds to structure the secondary lymphoid tissues [186]. Immortalized stromal cells were used for dECM production, and primary stromal progenitors (pSP) were acquired from neonatal mesenteric LNs, combinedly to generate the LOs by seeding pSP cells on the top of dECM scaffolds and then transplanted into mice. The observation and data collected in this research displayed that after transplanting LOs to the resected location of LNs, LOs merged into endogenetic lymph vasculature and rehabilitated the lymphatic drainage and perfusion. Correspondingly, 60% of LOs contained T and B immune cells that aid antigen-specific immune responses around the transplanted area [186]. The authors demonstrated the possibility of injured lymphatics therapy and that LOs could stay in the transplanted area for at least two months.

4.2. Tumor organoids and drug screening

Tumor organoids mimic the architecture and functions of the original tumor tissue and have emerged as a promising tool for studying tumor biology, drug discovery, and personalized medicine. Tumor organoids have several advantages over traditional 2D cell cultures, such as maintaining the genetic heterogeneity and cellular complexity of tumors, better recapitulation of the tumor microenvironment, and improved predictive value for drug responses. The use of tumor organoids in biomedicine has revolutionized our understanding of cancer biology and provided new avenues for developing targeted therapies. In this context, Shirure *et al.* developed a tumor-on-a-chip microfluidic system replicating mass transfer in the tumor environment near a capillary's arterial [187]. A quiescent perfused 3D microvascular network was established before loading tumor cells or patient-derived tumor organoids in an adjacent compartment as a major feature. The vascular network thus facilitates the physiological supply of nutrients and medicines to the tumor. They studied the growth, cultivation, and treatment of tumor cell lines and patient-derived breast cancer organoids. Their proposed microfluidic system enables real-time investigation of tumor growth characteristics such as cell migration, angiogenesis, cell proliferation, and tumor cell intravasation. Primary breast tumor organoids were also alive for several weeks in the device and induced substantial sprouting angiogenesis.

In another study for drug screening in patient-derived organoids, a low-cost microfluidic device for growing and expanding organoids was presented by Pinho *et al.*, called OrganoidChip [188]. OrganoidChip was used to develop patient-derived colorectal cancer organoids. Their survival and proliferative activity increased dramatically after cultivation within the chip. There were no significant changes in the organoids' responses to 5-fluorouracil (5-FU) therapy on-chip and on-plate. Compared to traditional culture on a 24-well plate, the OrganoidChip culture considerably increased colorectal cancer organoid-forming efficiency and total size.

Haque *et al.* presented a tumor organoid-on-a-chip platform to recapitulate the pancreatic ductal adenocarcinoma TME using patient-derived tumor organoids and stromal cells, particularly pancreatic stellate cells and macrophages [189]. patient-derived tumor organoids generated in a microfluidic device technology extend cellular function

and survival while establishing an organotypic tumor microenvironment with immune cells and desmoplastic stromal cells. In monoculture conditions, there was no influence on cell viability when initial cancer cells were exposed to stroma-depleting chemicals. Targeting stroma in their tumor-chip model, on the other hand, led to a considerable enhancement in the chemotherapeutic effect on cancer cells, proving the tumor-chip platform's utility for drug screening.

Sachs *et al.* used patient-derived breast cancer organoids to recreate the reaction of metastatic breast cancer to tamoxifen [190]. *In vitro* experiments with organoids indicated therapeutic response or resistance, reflecting clinical outcomes in 13 patients treated with tamoxifen in a standard care situation. Broutier *et al.* presented an innovative, more physiological liver organoid cultivation technique in which human primary liver cells create organoids with preserved tissue genetic stability and function by growing long-term [191]. They also used their culture system to propagate primary liver cancer (PLC) organoids using the most prevalent subtypes of PLC: cholangiocarcinoma (CC), hepatocellular carcinoma (HCC), and combination HCC/CC (CHC) tumors. PLC-derived organoid cultures retain the gene expression, histological architecture, and genomic landscape of the original tumor even with long-term cell growth in identical medium conditions, allowing for distinction among various tumor tissues and subtypes. According to xenograft experiments, PLC-derived organoids' tumorigenic ability, metastatic properties, and histological characteristics were retained *in vivo*. PLC-derived organoids were used to identify biomarkers and test drugs, which led to the finding of the ERK inhibitor SCH772984 as a potential treatment for PLC.

Hou *et al.* proposed a high-throughput screening method that combines a cell-repellent surface with a 3D-bioprinting technique and a magnetic force to enable the consistent creation of primary pancreatic tumor organoids in flat-bottom 1536- and 384-well plates [192]. They tested the effects of well-known drugs on four patient-derived pancreatic cancer KRAS mutant-associated primary cells to validate this homogenous mechanism. A cytotoxicity pilot screen of 3300 certified medicines was used to test this technology's compatibility with high-throughput screening automation.

Besides tumor organoids, various other types of organoids can also be utilized for drug screening applications. Using cardiac organoids, Mills *et al.* performed functional screening of over 100 small compounds with pro-regenerative potential [193]. Their results found a significant discrepancy between the human cardiac organoid method and typical 2D assays. Furthermore, functional investigations revealed that several compounds had adverse implications. The pro-proliferative chemicals activated a cell-cycle network and the mevalonate pathway synergistically, according to high-throughput proteomics in human cardiac organoids. Their findings indicate the importance of human cardiac organoids in developing pro-regenerative drugs, such as understanding critical biological pathways and reducing adverse side effects.

In summary, organoids have shown great promise in drug testing for different diseases, especially in cancer, using conventional organoid culture and organoid-on-chip methods. Further research in this field will enable the thriving culture and maintenance of organoids and high-throughput drug screening applications. Moreover, optimizing and standardizing organoid culture protocols to achieve improved reproducibility and generate uniform organoids will result in more accurate drug testing results [194]. Additionally, organoid-based therapeutic efficacy will be determined by their cellular composition, engraftment, vascularization into the host, and proof of functional activity [195].

It is well known that organoids may mimic the original tissue architecture better *in vitro* than spheroids or 2D culture systems [131]. In this sense, in the last years, according to the databank ClinicalTrials.gov (https://clinicaltrials.gov/), clinical evaluation of human organoids has been used to select individual patients for novel targeted therapy trials (46 interventional and 71 observational studies). It is noteworthy that 96 are related to cancer research among them. Most of the ongoing investigation involves using patient-derived organoids in basic research, biobanking, disease modeling, and precision medicine. For instance, a phase III clinical evaluation of the human tumor organoids approach is ongoing to treat pancreatic cancer (Clinical Trial #NCT04931381 and #NCT04931394). By comparing the organoid drug sensitivity and patients' treatment response, the results can optimize therapy strategy for an individual patient by predicting drug effects.

Some advanced technical approaches have also been applied for observational studies in colorectal cancer to achieve precise tumor treatment without side effects. For instance, biofabrication has been used to validate if 3D tumor models can foresee the colorectal cancer organoid response to chemotherapy (Clinical Trial #NCT04755907). This clinical trial compared the response of chemotherapy drugs in 3D bioprinted models to corresponding patients in treatment. In another study, researchers tested the accuracy and sensitivity of organoids-on-chip for anticancer drug screening in human-derived tumor organoids (Clinical Trial #NCT04996355). In addition, recent studies showed that personalized medicine using patient-derived organoids in clinical trials could be improved by using organoids-on-a-chip technology and high-throughput drug screening [1]. Although organoids have different potential applications, they still lack essential native cellular components such as immune cells. Most recent studies show that preclinical and clinical use of organoid-derived products can expedite their bench-to-bedside translation in regenerative medicine and precision medicine [196].

5. Analytical methods in organoid research

In recent years, the convergence of cutting-edge technologies in image analysis, biosensors, and artificial intelligence has revolutionized our ability to decipher the intricacies of organoids. This section explores how the synergy between image-based techniques, biosensors, and AI has opened new frontiers in analyzing organoids and accelerated innovative therapies' development. A summary of the advantages, disadvantages, and characteristics of image analysis, biosensors, and artificial intelligence on organoid analysis is presented in Table 3.

5.1. Image-based organoid analysis

With significant progress in organoid technology, characterization methods for 3D cultures have become more imperative. Image-based analysis has been one of the most widely used and prominent ways to analyze organoids. Traditional imaging techniques capture cross-sectional images of samples with limited resolution, in which organoid samples are sliced into thin sections to facilitate imaging [197]. More recently, however, volumetric imaging has emerged, enabling the visualization of the 3D architecture of an entire organoid [198]. Moreover, with advanced microscopic technologies such as confocal [199] and light-sheet microscopes [200], acquiring images at a considerably higher resolution is now possible than traditional microscopes.

In contrast to 2D imaging, 3D imaging can provide more information regarding organoid structures, such as intercellular interactions, cellular composition, and shape. Immunofluorescent labeling represents a crucial step in sample preparation to yield high-resolution fluorescent images. The staining process involves multiple steps that require a degree of optimization, such as incubation time and antibody concentration. For 3D imaging, samples are often "cleared" rather than sliced before imaging to make them transparent [201]. For clarification, different solvents are used, such as organic solvents, high-refractive-index aqueous solutions, and hyperhydrating solutions.

Recently, well-developed protocols for high-resolution volumetric imaging were described by Dekkers *et al.* [202] and later refined by the same group [203]. The authors obtained high-quality 3D images for different types of organoids from various imaging modalities such as confocal, multiphoton, and light-sheet microscopes. Another exciting study presented a novel imaging method using positron-emission microscopy, which tracks tracer molecules such as ^{8}F-fluorodeoxyglucose

Table 3
Advantages, disadvantages, and characteristics of image analysis, biosensors, and AI in analyzing organoids.

	Advantages	Disadvantages	Characteristics	References
Image-based organoid analysis	- High-resolution imaging - Visualization of 3D architecture	- Requires sample preparation - May need specialized equipment	- Uses confocal and light-sheet microscopes - Can visualize live cells	[157,197-207]
Biosensors for organoid analysis	- Direct sample readout - Label-free detection	- 2D sensors may not be suitable for 3D mapping - Longevity challenges	- Monitors physiological signals - Can detect microenvironment parameters	[1,217-226]
Organoids and Artificial Intelligence	- Can handle complex data - Less manual feature engineering	- Requires training and validation - May need specialized software	- High-resolution imaging - Visualization of 3D architecture	[227-234]

in organoids [204]. The glycolytic activity within patient-derived tumor organoids was successfully visualized and subsequently quantified. The findings demonstrated the potential clinical translation of the proposed technology for personalized therapies. Apart from the fluorescently-labeled images, imaging of live cells has garnered interest as it allows the visualization of cells without sacrificing samples, enabling spatio-temporal monitoring of organoids [205,206]. Scholler *et al.* recently proposed full-field optical coherence tomography for live imaging of organoids with outstanding spatial and temporal resolution [207]. Such live imaging techniques are instrumental in ensuring the proper formation of organoids generated in a high-throughput manner, as they keep the samples intact while significantly speeding up the screening process [208].

Once organoid images are collected, there are multiple options to analyze the data. One typical analysis performed is counting cells [2,209,210] and specific cellular components of interest (*e.g.,* mitochondria and nuclei) [211,212]. For counting, images typically require post-processing steps such as denoising and masking, followed by another filtering [210,211]. However, depending on the counted object, the approach can vary significantly between studies. Another metric often used for organoid characterization is the intensity of cell markers [202,213]. When measuring intensity, it is critical to normalize against the appropriate background intensity to obtain the "true" intensity. In addition to these quantitative assessments, 2D and 3D morphological characteristics are often examined, including the diameter, area, shape, volume, and sphericity [201]. Simple analyses, such as distance measurements, can be carried out using imaging software that comes with the microscope. However, to perform more advanced and customized analyses, multiple software programs are available today, such as ImageJ [213,214], CellProfiler [215,216], and MATLAB [215,217]. While imaging is undoubtedly an invaluable tool for the characterization of organoids, there are other means to study organoids, one of which is biosensors.

5.2. Biosensors for organoid analysis

Electrochemical biosensors were also developed for organoid analysis in recent years. Electrochemical sensors enable a direct sample readout compared to image-based analysis without requiring dedicated imaging equipment such as microscopes, additional data processing software, and real-time calibration to avoid environmental optical interferences [1,218]. In addition, the label-free detection feature of electrochemical sensors minimizes the influence of cell behaviors. It broadens its application in 3D cell culture systems [219]. Flexible, miniaturized, integrated platforms for multiplexed biosensing have been applied to investigate and monitor the physiological signals of organoids.

In **Fi.g 6 A**, an organ-on-chip platform integrated with multiple biosensors to continuously monitor cardiac and hepatic organoids, is represented [220]. By functionalizing electrodes with antibodies, the platform could detect a panel of microenvironment parameters, such as temperature, pH, and oxygen, and protein biomarkers, such as albumin and glutathione S-transferase α (GST-α) for liver organoids assessment and creatine kinase MB (CK-MB) as cardiac biomarkers. Excellent sensitivities of the three biosensors were realized as 1.607, 1.105, and 1.483 $\log(ng{\cdot}mL^{-1})^{-1}$, respectively. The as-designed platform was contacted with data processing modules for direct readouts and incubators to maintain organoid culturing conditions. These designs endowed the system with automated and in situ biosensing features, realizing stable organoid culturing and monitoring for up to 5 days. Drug-induced organ toxicity was evaluated on the platform, showing the promising potential for its future application in drug screening. Similar designs were incorporated into organ-on-a-chip systems for monitoring multiple biosignals, including oxygen [221], ions [222], metabolites [223], and macromolecules [224,225].

Despite various biosensors developed for organoid monitoring, 2D biosensors may not be suitable for mapping organoid conditions at different locations. To address this issue, technologies for 3D bioelectrical interfacing could benefit multisite organoid monitoring. Kalmykov *et al.* developed a self-rolled biosensor array around the cardiac spheroid to perform electrophysical tissue scale measurement of cell–cell communications (Fig. 6 **B**) [226]. The device was first patterned on a planar surface with a sacrificial layer. The elimination of the sacrificial layer generated stress between different constituent layers, transforming 2D electrodes into a 3D shape. The curvature was tunable by controlling the deposition process and film thickness. The fabricated microelectrode arrays were evaluated on a cardiomyocyte organoid model derived from hESCs. Direct and stable interfacing of microelectrode arrays with the spheroid was achieved to record field potentials at multiple locations with desired signal fidelity. Coordination of frequency between calcium ion transient and field potential spikes were observed. The spheroid sensing platform provides a promising approach to multisite and simultaneous measurement of 3D organoids with high spatial resolution.

Recent advances in bioelectronics have revealed the potential of monitoring multiple signals of organoids for downstream analysis and research [227]. However, there are still several limitations that need to be addressed. One major issue is to assess the organoids from acquired signals quantifiably. Physiological signals of organoids vary significantly due to different cell lines, cell culture conditions, and device designs. Further investigations of converting recorded physical and chemical signals from various organoids into meaningful and general information are highly demanded. In addition, the longevity challenge is another issue for biosensing [227]. On the one hand, the aqueous microenvironment for organoid culturing contains reactive oxygen species (ROS) and plenty of metabolites and enzymes, which can lead to the mechanical or electrical failure of the biosensors. Antifouling property should be taken into account when designing the devices. On the other hand, the interface between organoids and biosensors may interfere with cell behaviors. Also, it is required to employ materials with specific permeability of nutrients and oxygen to maintain biocompatibility and durable signal detections.

5.3. Organoids and artificial intelligence

Over the last decade, artificial intelligence (AI) has revolutionized many sectors, including healthcare and biomedical research [228,229].

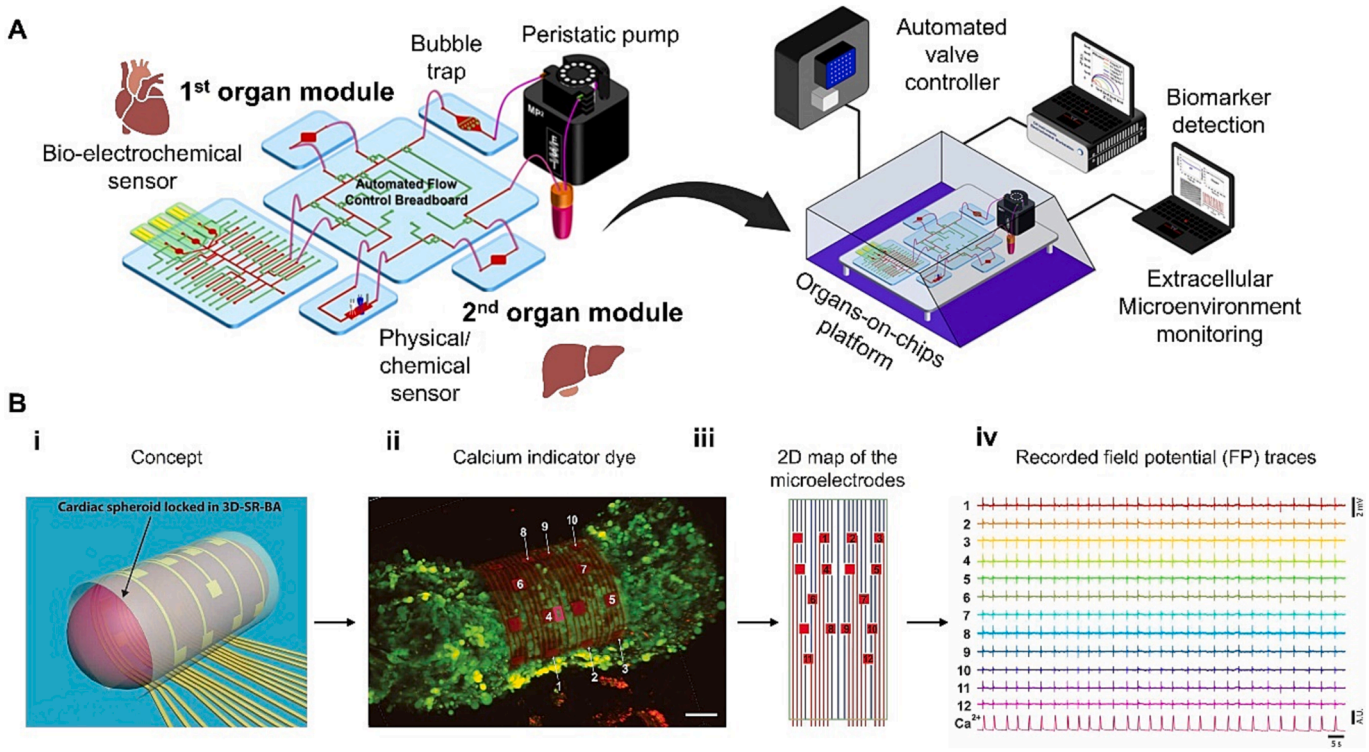

Fig. 6. Organoid system integrated with biosensors and high throughput functionality. A. Schematic representation of integrated multiorgan-on-a-chip platform consisting of micro bioreactors, breadboard, reservoir, bubble trap, physical sensors, and electrochemical biosensors for automated sensing of various biomarkers, data processing, and optimized cell culture condition maintenance. Reproduced from [246] with permission from the National Academy of Sciences 2017. **B.** Organ-on-an-electronic-chip for electrical interrogations of human electrogenic spheroids. **Bi,** Cardiac spheroids encapsulated a 3D self-rolled biosensor array (3D-SR-BA), allowing electrical measurements in 3D. **Bii,** A confocal microscopy 3D image of an encapsulated cardiac spheroid labeled with Ca^{2+} indicator dye (green fluorescence) in a 3D-SR-BA. Scale bar = 50 μm. **Bii$_i$,** 2D map of the microelectrodes in the biosensor array. **Biv,** Representative field potential (FP) traces measured from the channels of the array. Reproduced from [252] with permission from the American Association for the Advancement of Science 2019.

In particular, there has been a surge of interest in applying machine learning (ML) to various scientific tasks such as image analyses [230] and omics studies [231], in which the excessively large and complex data render and data analyses overwhelmingly challenging to be performed by humans. This is attributed, at least partly, to the recent rise of deep learning (DL), which is more versatile than traditional ML algorithms in handling highly complex data [258]. Moreover, DL requires far less manual feature engineering, thereby minimizing the loss of information encoded by the raw data [232]. Concurrent with the growing interest in DL coupled with the developments of high-throughput systems described above, more studies have shown the potential use of ML for organoid-based research and high-throughput screening applications. MOrgAna is a recently-developed image analysis software that utilizes ML for image segmentation and measurements of morphological features and fluorescent intensities [233]. Furthermore, the Python-based program has a user-friendly visual user interface, enabling the end users to conduct analyses without much programming. In contrast, advanced users have the option to customize the codes. Another analysis tool named 3DeeCellTracker was proposed by Wen *et al.,* which performs data processing, cell segmentation, and tracking [234]. Using the software, one can train a 3D U-Net to achieve state-of-the-art performance on 3D image segmentation of organoids. On the other hand, a spheroid monitoring and AI-based recognition technique, or SMART for short, is geared toward predicting tumor spheroids' boundaries via a modified CNN model called the pyramid scene parsing (PSP) U-Net [235]. The expected limits enable the calculation of the excess perimeter index and the multiscale entropy index for the quantification of tumor invasiveness.

Organoid research has greatly benefited from innovative image analysis tools and machine learning techniques developed by several research groups. Borten, Michael A., *et al.* made a significant contribution by creating OrganoSeg, open-source software tailored for the analysis of brightfield images of 3D cultures, including organoids. Their software simplifies the categorization of complex morphologies in cancer spheroids and organoids, providing an essential tool for researchers in this field [215]. Kassis, Timothy, et al. approached the challenge of image analysis by developing OrgaQuant, a deep convolutional neural network designed to automate the detection and quantification of human intestinal organoids in brightfield images. Their method involves the creation of an annotated dataset and training an object detection pipeline using TensorFlow. OrgaQuant's ability to fully automate image analysis without user intervention significantly expedites research by providing a high-throughput solution [236]. Kok, Rutger NU, *et al.* focused on tracking individual cells within organoids, a crucial aspect of organoid research. They developed a semi-automated cell tracker that utilizes convolutional neural networks for nuclei detection. This tracker efficiently connects detections at different time points to form cell trajectories, aided by a min-cost flow solver. By incorporating warnings to identify potential errors, Kok and colleagues enhanced the precision and efficiency of cell tracking, making it a valuable tool for organoid studies [237].

Furthermore, Arganda-Carreras, Ignacio, *et al.* introduced the Trainable Weka Segmentation (TWS), a versatile machine learning tool for pixel classification in image analysis. TWS is equipped with a user-friendly graphical user interface (GUI) and has a wide range of applications, from boundary detection to object localization. It streamlines the development of segmentation algorithms, empowering researchers to prototype methods efficiently within Fiji. The adaptability and accessibility of TWS make it an essential resource for organoid image analysis [238]. Guan, Yuan, *et al.* exemplified the practical use of TWS to

quantify immunostained and trichrome-stained images. Their approach involved manual labeling of positive spots in specific regions to train classifiers, enabling the identification of specific image features. These trained classifiers then generated probability maps, highlighting significant areas for further analysis. Guan and colleagues demonstrated how TWS streamlines image analysis in organoid research, particularly in identifying and quantifying specific features within complex images [239]. Together, these tools significantly advance organoid research by providing enhanced efficiency and accuracy of image analysis.

6. Challenges and perspectives

Organoids are endowed with the capacity to mimic physiological and pathological structures and critical functions of real organs. It enables establishing a simplified, scalable, and accessible approach to address the human tissue accessibility constraints and the gap between the animal model and patients. These advantages make organoids promising in basic biomedical research and promote the path to preclinical and clinical translation. However, bottlenecks remain in expanding organoids' translational relevance and applicability.

One main issue is the difference between large-scale tissues and micro-scale organoids. The *in vitro* miniaturized organoids and culturing microenvironment differ from the human body in multiple aspects, especially mechanical parameters, such as strains, interstitial fluid, blood flows, and forces from cell-tissue interaction. Additionally, recapitulating the intricate interactions among various cell types in organoid models presents challenges in tissue engineering. Organoids, aiming to mimic native tissue, have issues such as cell heterogeneity, spatial organization, vascularization, and replicating the complex ECM composition. Addressing these hurdles involves employing a diverse array of cell types, utilizing 3D bioprinting and microfluidics for spatial control, introducing vascular networks through co-culture and microfluidics, incorporating ECM components, promoting functional integration via co-culture and fusion techniques, and addressing immunological concerns through immune-privileged sites or immunosuppression. Also, complicated cell signaling and biochemistry interactions are involved in real organs for development and disease progression. In comparison, the current development of organoids mainly relies on the self-organization of cells with limited stimuli. Further fundamental and quantifiable research is required to understand the basic process in patient bodies to precisely establish more advanced models to mimic organoid culture conditions precisely.

In human bodies, the crosstalk context with other organs (*e.g.,* the fluctuation of multiple cytokines and hormones and cell migrations) should not be ignored. For example, to develop a diabetic wound model, an artificial pancreas system could be integrated as a source of insulin variation for the wound. Also, reservoirs for immune cells could be added to resemble the process of the prolonged inflammation stage of diabetic wound healing. There are some reports on fabricating multi-organs-on-a-chip, which could be a potential approach to address this issue. However, monitoring multiple organs and controlling their function simultaneously to maintain system stability is still challenging. Integrating a more complicated system using bioengineering strategies, electromechanical operations, and optimizing protocol to recapitulate multiorgan interactions and real-life cell niches are needed. In addition, defining the level of complexity is necessary. For all physiological and pathological models, a trade-off between maintaining the key features and keeping the simplistic and accessible procedure should be considered. By integrating multiple cell lines and stimuli, organoids reproduce and provide more information than traditional cell cultures. Advanced technologies such as microfluidics and bioprinting enable the establishment of more complex structures of organoids. However, organoid models are impossible to provide system-level complexity as animal models, and the increase in complexity may reduce the robustness and reproducibility of the organoid system. More importantly, the lifespan of organoids is generally short (approximately several weeks on average), and the functional organoid can reach homeostasis at a certain point, which means it is difficult to increase the complexity without limitation. Thus, it is essential to choose specific characteristics when developing organoid development protocols. Also, for future development, scalability and quality control are critical. It is highly demanded to have standardization and a universal methodology for organoid model establishment.

One critical issue for the preclinical and clinical translation of organoids is the involvement of animal components such as Matrigel, gelatin, and proteins from bovine serum. These non-human source biochemicals/hydrogels may cause undesired immune responses such as foreign body reactions and functional variations from the original organs. Also, the implantation of these materials may not be safe in the long term due to the potential for cytotoxicity and tumorigenicity. The replacement of these components for organoid development is required in the future. This challenge could be addressed by developing approaches for synthesizing pure chemicals and extracting, purifying, and propagating patient-derived cells or biocomponents. These could also be beneficial to improve the resemblance of an organoid model with patient real organ conditions.

Using biosensors and AI for organoid analysis holds great promise in advancing our understanding of complex biological systems and accelerating drug development. However, several challenges and limitations must be addressed. Developing standardized protocols for organoid analysis is crucial to ensure reproducibility in experiments. Also, enhancing the sensitivity of biosensors through innovative technologies is necessary to detect subtle changes accurately. Managing and analyzing the vast amount of data generated requires AI-driven automation and integration with other omics data sources. The complexity of organoid models can be dissected using advanced imaging techniques and AI-based segmentation algorithms. Furthermore, these tools can help identify relevant drug targets, streamline drug screening processes, and model disease variability, particularly through patient-specific organoids derived from iPSCs.

In summary, addressing all these challenges demand integrating multidisciplinary approaches and technologies. It may usher into a new era of organoid development and clinical translation from the bench to the real world.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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