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REVIEW

Prospects for 3D bioprinting of organoids

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Abstract
Three-dimensional (3D) organoids derived from pluripotent or adult tissue stem cells seem to possess excellent potential for studying development and disease mechanisms alongside having a myriad of applications in regenerative therapies. However, lack of precise architectures and large-scale tissue sizes are some of the key limitations of current organoid technologies. 3D bioprinting of organoids has recently emerged to address some of these impediments. In this review, we discuss 3D bioprinting with respect to the use of bioinks and bioprinting methods and highlight recent studies that have shown success in bioprinting of stem cells and organoids. We also summarize the use of several vascularization strategies for the bioprinted organoids, that are critical for a complex tissue organization. To fully realize the translational applications of organoids in disease modeling and regenerative medicine, these areas in 3D bioprinting need to be appropriately harnessed and channelized.

Keywords Three-dimensional bioprinting · Bioink · Organoid · Microfluidics · Extracellular matrix (ECM) · Biomaterial

Introduction

Organoids are defined as miniature organs that are derived from tissue-resident stem/progenitor cells or embryonic stem cells in the presence of organ-specific cues and matrices in culture dishes. Organoids resemble an organ in both structure and function—hence the term 'organoid,' where 'oid' stems from the Latin word 'oides' meaning resemblance. During organoid formation, cells undergo self-organization, proliferation and then differentiation into specific cell types similar to the processes in vivo (Fig. 1). The first organoid structures were derived from both normal and tumor murine mammary glands by Mina Bissell and colleagues, who showed that the mammary cells assembled themselves into a three-dimensional (3D) structure, when cultured in the presence of extracellular matrices (ECM) like, laminin and collagen IV [1]. After that, several pioneering studies by Clevers et al. have demonstrated derivation of intestinal organoids from intestinal adult stem cells and those by Sasai et al. [2, 3] have shown fabrication of cortical organoids and the retinal optic cup from the induced pluripotent stem cells. Subsequently, organoids have been generated using different cellular sources in several species. As the derivation of organoids employs basic principles of development biology, ideally, to generate an organoid, the entire process of organ development from stem cells should be faithfully mimicked. However, this is a feat which has been difficult to achieve in vitro. Nonetheless, the intrinsic ability of stem cells to self-organize into a near-native microanatomy along with some knowledge of the external signals required for the differentiation of stem cells to specific cell types has led to the successful production of organoids.

For organoid development, we usually start with a single stem cell, either induced or embryonic pluripotent stem cells (iPSCs or ESCs) or tissue-resident stem/progenitor cells isolated from adult tissues, that after cell expansion undergoes self-organization into a homogeneous cell population [4]. Self-organization is termed as the capacity of a cellular system to spatially rearrange under the guidance of cell-intrinsic mechanisms independent of the environmental signals. Many organoid derivation protocols also involve the co-culturing of the stem cells with one or more supporting cells to facilitate the self-organization and differentiation of the stem cell. Addition of supporting cells allows

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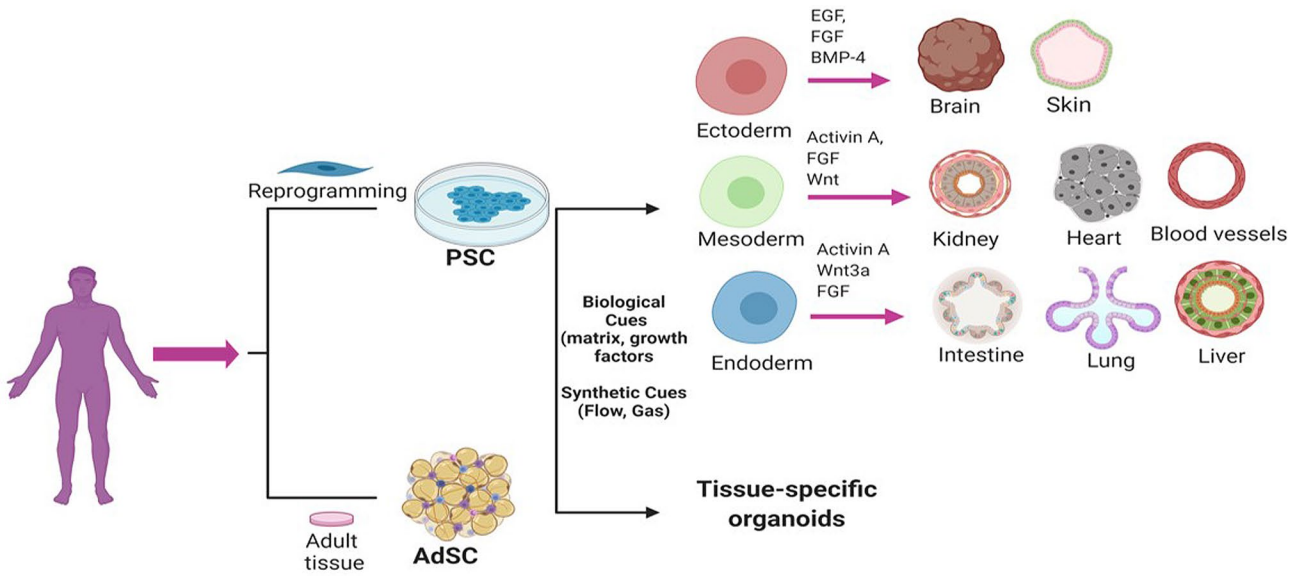

Fig. 1 Derivation of organoids from pluripotent stem cells (PSC) or adult stem cells (AdSC). After isolation and culture of single stem cells, both tissue-specific biological cues including matrix, growth factors and synthetic signals such as blood flow and oxygen guide the differentiation of cells into their specific lineages and organoids. (*EGF* epidermal growth factor, *FGF* fibroblast growth factor, *BMP* bone morphogenetic protein, *Wnt* wingless related integration sites)

paracrine interactions between all the cells where they also arrange themselves into precise organ-specific architectures. For example, culture of liver organoids from liver-specific stem cells (Lgr5+/EpCAM+) would require the support of endothelial cells, Kupffer cells and fibroblasts as hepatocyte function is largely regulated by its surroundings and the addition of supportive cells for heterotypic interactions helps in maintaining its viability, morphology and function [5, 6].

Challenges for biofabrication of organoids

The cells in organoids can exist in varying states where outermost cells are most prolific due to a good supply of nutrients and the innermost cells are mostly hypoxic with quiescent, apoptotic and/or necrotic states owing to accumulation of metabolites in the innermost regions [7]. This arrangement of cells also generates differential biochemical gradients between the inner and the outer cells. The organoids thus faithfully replicate the in vivo cell–cell interactions and cell–matrix interactions and possess immense potential for the generation of biofabricated organs. However, for developing 3D bioartificial organs, we need to scale up the organoids both in terms of size and complexity. With increasing complexity, several challenges with regard to the reproducibility of 3D structures, automation of organoid production and inclusion of perfusion networks will have to be addressed.

In last few years, automated 3D bioprinting has evolved from 2 and 3D cultures as a promising technique that precisely places the cells in a specific environment, creating tissues that closely mimic the in vivo architectures (Fig. 2). 3D bioprinting can answer this problem of fabricating large tissue constructs by printing either stem cells or organoids. For successful bioprinting of organoids/stem cells, many parameters besides, cell-specific self-organization and exogenous chemical environment, need to be optimized including: the use of biomaterials, bioprinting method and finally efficient vascularization of the fabricated tissues. In the current review, we highlight these aspects of 3D bioprinting in terms of organoid derivation.

3D bioprinting for organoids

Started 30 years back as a mechanical process to create solid scaffolds by Charles Hull, 3D bioprinting technology is a promising technology to precisely position the biological materials, living cells and growth factors for the generation of bioengineered structures with the use of computer-aided transfer and build-up processes. It is a layer-by-layer integration of biomaterials and living cells, in a specific 3D architecture. This is accomplished by printing bioinks into acellular and cellular structures using automated 3D printers. 3D bioprinting for organoids or cellular aggregates has evolved recently and has immense potential to address the problem of obtaining large tissue constructs to fabricate biomimetic complex tissues and organs in vitro. Here, we highlight two major features of 3D bioprinting, bioinks and bioprinters.

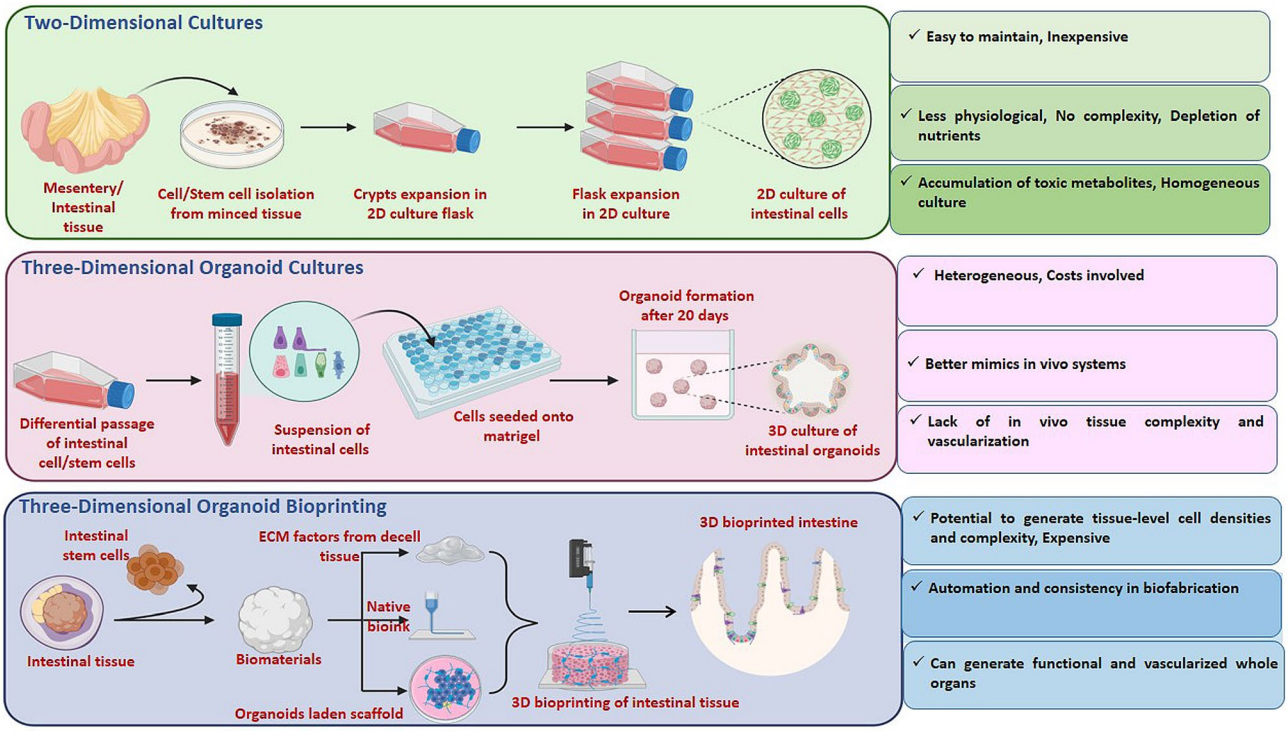

Fig. 2 Evolution of organoid printing from two-dimensional and three-dimensional organoid cultures. The characteristics of each are given. (*ECM* extracellular matrix)

Bioinks for organoids

Bioinks are printable biomaterials used in 3D printing. The bioinks used for cell or organoid printing should have properties requisite for conventional tissue engineering approaches, including biodegradability, bioactivity and non-toxicity to cells. In addition, two most crucial properties of bioinks for 3D printing applications are printability and mechanical properties. The printability of the bioink depends on parameters such as viscosity of the solution, surface tension of the bioink, the ability to cross-link on its own and surface properties of printer nozzle. Of these, viscosity is one of the crucial parameter that affects the printability of the bioink. If the viscosity is too high, a high pressure must be applied during the printing process, and this results in an unavoidable shear stress causing damage to the cells and organoids. On the other hand, low viscosity and slow gelation hamper structural stability and resolution of the printed model. Other conditions, such as nozzle gauge also determine the shear stress to which cells are exposed as well as the time required for the material to be deposited to form a 3D structure. Mechanical strength and integrity of the bioprinted constructs are other important criteria determining an optimal cell viability, gene expression and functionality post-printing (Fig. 3). A good mechanical strength of the biomaterial allows the constructs to be shaped into various micro- or nano-scale topographical geometries such as ridges, steps and grooves that play an important role in affecting cell shape, function, migration, differentiation and organization. A bioink with an optimum mechanical strength allows to generate tissue-like complex geometries.

Both natural and synthetic polymers are being used for 3D bioprinting of stem cells and organoids (Table 1). Hydrogel-based bioinks have been mostly used for both stem cell and organoid printing. The selection of the bioink depends on the specific application (e.g., target tissue), the type of cells and the printing method. Natural polymer-based bioinks such as agarose, alginate, collagen, hyaluronic acid are preferred due to their similarity to human ECM, and their inherent bioactivity. Matrigel, a natural hydrogel ECM purified from Engelbreth–Holm–Swarm mouse sarcoma is the most widely used matrix for culture of 3D organoids and also for 3D bioprinting in combination with other bioinks. For example, a hybrid 3D printable matrigel–agarose hydrogel system efficiently supports the growth of intestinal stem cells [8]. Other types of ECM proteins that have been used for derivation of mammary gland and intestinal organoids are collagen type I matrices. The collagen molecules self-assemble into interlocking fibers and fibrils. Alone, collagen-based bioinks are tough to handle when printing as the solution can undergo

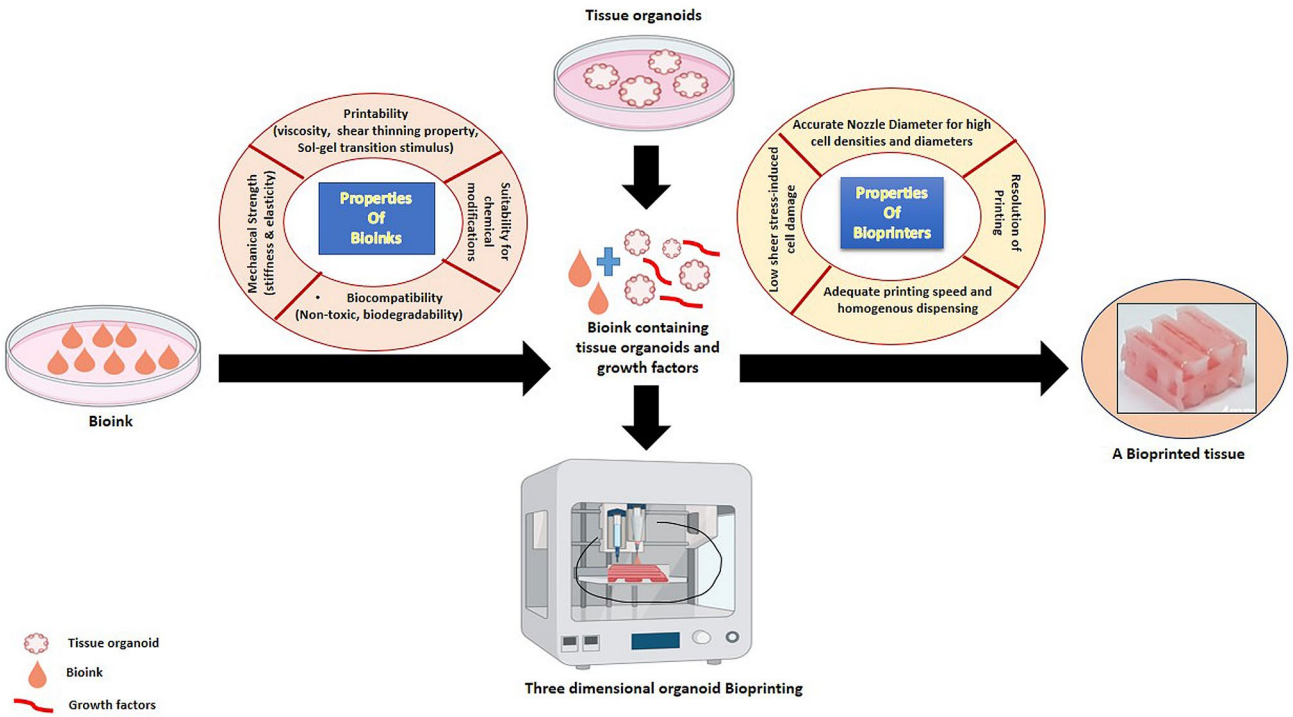

Fig. 3 Features of bioprinters and bioinks for organoid printing. Bioinks mixed with pre-formed tissue organoids and growth factors and printed using sophisticated bioprinters can fabricate intricate 3D tissue architectures. Characteristics of bioinks and bioprinters to be used for 3D bioprinting of organoids are listed (Bioprinted tissue image is reproduced from Lee et al. [62], copyright year 2017, with permission from Dr. Dong-Woo Cho)

premature setting. Hence, collagen is mostly used in hybrid bioinks with other bioinks such as alginate and hyaluronic acid. Alginate is one of the most frequently used natural biomaterials for microextrusion printing. It is a naturally occurring simple hydrogel and a polyanionic linear polysaccharide obtained from brown algae. Blends of alginate and gelatin are frequently used for extrusion-based bioprinting to combine the thermo-sensitive properties of gelatin with the chemical cross-linking capabilities of alginate. Gelatin is a denatured collagen, that forms a thermoreversible hydrogel with strength dependent on solution concentration. It can be modified suitably to enhance cross-linking and bioactivity. Methacrylated gelatin (GelMA) has been extensively used for bioprinting complex architectures. Other blended natural bioinks include fibrin and hyaluronic acid. Blending of two to three bioinks substantially improves the properties of different biopolymers. Cross-linking of the bioinks during or immediately after the bioprinting facilitates bioprinter deposition and stabilization of the printed tissue construct to create the final shapes of the tissue constructs. Among the natural bioinks, novel tissue-specific bioinks from decellularized tissues have been developed using different chemical cross-linkers to impart mechanical strength and structural fidelity to the otherwise weak tissue ECM. The addition of different molecular weight polyethylene glycol (PEG)-based cross-linkers as well as thiolated gelatin and hyaluronic acid into solubilized ECM have also been used to improve the viscosity of the bioinks and enable better control over the mechanical properties of the final printed tissues [9, 10]. Among the synthetic bioinks, various biomaterials such as PCL, PEG, pluronic have been used for 3D bioprinting. The advantage of synthetic polymers as bioinks is that they have strong mechanical properties. The natural bioinks are usually modified with the synthetic biomaterials to have suitable mechanical properties. Challenges in the use of synthetic polymers include poor biocompatibility and toxic degradation products. A combination of natural and synthetic bioinks has also been successfully employed in several studies. In one report, alginate has been mixed with a synthetic polymer PCL to create a hybrid material with enhanced mechanical properties. In this study, the PCL frame has been bioprinted first using inkjet bioprinting to form a framework and then subsequently gaps have been filled by bioprinting alginate laden with cells and growth factors. The study has shown that the hybrid bioink system supports cartilage regeneration both in vitro and in vivo [11].

Table 1 Bioinks used for printing for stem cells

S. no	Biomaterials	Type	Cells	Properties	References
1	Agarose	Natural	Pluripotent cells	Better gelling property, low melting point, provide high cell viability	Gu et al. [19]
2	Alginate	Natural	Pluripotent cells HepaRG cells	Fast gelation property under physiological conditions without forming harmful by-products	Gu et al. [19] Hiller et al. [50]
3	HA	Natural	iPSCs	Good rheological properties and high viscosity	Park et al. [51]
4	Gelatin	Natural	Embryonic stem cells	Biocompatibility, biodegradability, low antigenicity, accessible active groups, absence of harmful byproducts	Raof et al. [52]
5	Matrigel	Synthetic	Intestinal stem cells	Matrigel: natural biomimetic ECM, it undergoes thermal cross-linking at higher temperatures	Caiazzo et al. [53] Glorevski et al. [54]
6	PEG	Synthetic	Encapsulated MSC	Strong mechanical properties that facilitate the bioprinting processes and shape maintenance of the deposited constructs	Glorevski et al. [54]
7	Pluronic (Sacrificial ink)	Synthetic	Encapsulated chondrocytes	Good printability and temperature-responsive gelation	Kolesky et al. [55] Muller et al. [56]
8	Carboxymethyl-chitosan	Natural	Pluripotent cells	Biocompatible and biodegradable polysaccharide	Gu et al. [19]
9	NFC/A and NFC/HA	Synthetic	iPSCs with co-cultured with irradiated chondrocytes	Provides structural and mechanical support for forming the physiological mimetic environment, stem cell pluripotency, and supporting differentiation in 3D	Nguyen et al. [20]
10	Laminin	Natural	hESC-derived LESCs	Bioactive, promote cellular activity in 3D scaffolds and capable for regeneration	Sorkio et al. [22]
11	Alginate hydrogel matrix	Natural	hESCs and hiPSCs	Enhanced mechanical properties and high viscosity	Faulkner-Jones et al. [27]
12	GelMA and GMHA	Synthetic	hiPSC-derived hepatic progenitors (HPCs), (HUVECs) and (hASCs)	Biocompatible, supported the attachment and proliferation of the cells	Ma et al. [24]
13	GelMA	Synthetic	colonic crypt cells	Cross-linking density can be easily controlled during methacryloyl group activation or during photopolymerization which determines the physicochemical properties of the final construct.	Kolesky et al. [55]
14	PDMS	Synthetic	Keratinocytes	Enhances mechanical properties of fabricated samples, decreases porosity of bubble entrapment, facilitates adhesion of cells	Golden and Tien [42]
15	PLGA	Synthetic	Neural retina	Thermoplastic material, maintain scaffold structure and mechanical strength over long period and demonstrate better structural stability	Worthington et al. [57]
16	PGS	Synthetic	Bone marrow stromal cells	Elastomeric, degradable polymer, cytocompatible, display rapid degradation under physiological conditions	Visconti et al. [44] Zaky SH et al. [58]

Table 1 (continued)

S. no	Biomaterials	Type	Cells	Properties	References
17	ECM-based bioinks	Synthetic	hiPSC-derived oligodendrocytes	ECM-based bioinks possesses tissue-matching physicochemical properties, together with superior in vitro/in vivo biocompatibility	Cho et al. [59]
18	Hydroxyapatite	Synthetic	Osteoblasts and osteoprogenitor cell types	Calcium phosphate mineral, plays supportive functions for bone growth and osseointegration.	Catros et al. [60]
19	Fibrinogen-HA	Synthetic	ECFCs	Formation of a vascular-like network, increased the degradation time and improved the robustness of the constructs after cross-linking with thrombin / Ca2 + solution	Gruene et al. [61]

PEG poly-ethyl glycol, *NFC/A* nanofibrillated cellulose with alginate, *NFC/HA* nanofibrillated cellulose with hyaluronic acid, *GelMA* Gelatin methacrylate, *GMHA* glycidyl methacrylate-hyaluronic acid, *PDMS* polydimethylsiloxane, *PLGA* poly-lactic co-glycolic acid, *PGS* poly-glycerol sebacate, *HA* hyaluronic acid, *iPSCs* induced pluripotent stem cells, *ESCs* embryonic stem cells, *hESC* human embryonic stem cells, *HPCs* hepatic progenitors, *LESCs* liver endothelial sinusoidal cells, *hiPSCs* human-induced pluripotent stem cells, *HUVECs* human umbilical venules endothelial cells, *hASCs* human adipose tissue-derived stem cells, *hPSC* human pluripotent stem cells, *ECFCs* endothelial colony-forming cells, *SMC* smooth muscle cells, *EC* endothelial cells, *MSC* mesenchymal stem cells

Bioprinters for organoids

Automated 3D bioprinters are used for creating computer-aided designs and then creating scaffolds with geometries closely mimicking native tissues by precise positioning of different types of cells. Bioprinters include inkjet, micro-extrusion and laser-assisted printers (Table 2). In general, printing techniques can be sub-divided into nozzle-based and nozzle-free approaches. 'Inkjet bioprinting,' one of the first approaches to produce 3D biological constructs is a nozzle-based printing method [12]. The method uses inkjet cartridges and piezoelectric actuators, which exploit electricity for precise movement control, or thermal micro-heaters, based on temperature, to eject micrometric droplets of the bioink. Inkjet printing employs small (typically about 50-µm-diameter nozzles for printing low viscosity biomaterials and low cell densities about 1×10^6 cells/mL. The resolution of the printed patterns using the inkjet

Table 2 Advantages and disadvantages of different types of bioprinters

Types of bioprinters	Basic principle	Pros	Cons
Inkjet printing (nozzle-based)	Thermal and piezoelectric-based	Resolution of up to 50 µm High print speeds Low cost and a wide availability High cell viability	Restricted viscosities of bioink Inefficient cell encapsulation due to the low concentration of the ink Nozzle plugging
Extrusion bioprinting (nozzle-based)	Pressure-driven technology with pneumatic or mechanical dispensing system	Print with very high cell densities Uses highly concentrated and viscous hydrogel Resolution is 100 µm	Shear–stress during printing Low speed
Light-based bioprinting stereolithography and digital light processing (nozzle-free)	Ultraviolet (UV) or visible light to cure photosensitive polymers in a layer-by-layer fashion	No negative effects of shear pressure No limitation on bioink viscosity Resolution is 100 µm	Cytotoxic effects of the photoinitiators Damage caused by UV and near UV light to cell DNA Limited choice of photosensitive biomaterials Non-ideal density and uniformity of printed cells
Laser-assisted bioprinting nozzle-free)	Uses a laser as the energy source to deposit biomaterials on a substrate	High cell viabilities A resolution between 10 and 50 µm Ability to position multiple cell types with a high degree of accuracy	Expensive process to perform Low stability and scalability Fabrication of complex structures is not possible

printing method is about 20–100 mm. Although inkjet printing comes with several advantages such as low cost and high resolution, only bioinks with low viscosity can be printed to avoid clogging issues. This strongly limits its applicability of printing complex tissues and organoids with higher cell densities.

Another nozzle-based printing is 'extrusion bioprinting' that extrudes the bioink out of nozzles using pneumatic or piston-driven actuators, depositing cellularized filaments with micrometric resolution [13]. These bioprinters come with both small and large nozzle diameters. To avoid cell damage or cell clogging, nozzle diameters below 100 μm are used for printing lower cell densities ($< 5 \times 10^6$ cells/mL) and low viscosity cell suspension materials like pure cell medium or cell medium with low concentrated alginate. Higher cell densities comparable to native tissue ($> 1 \times 10^8$ per mL) and higher viscosity biomaterials for 3D printing are printed with larger nozzles. This method thus can use a multitude of bioink materials and the fabricated construct size is scalable. Scaffold-free dispensing by extrusion printing that utilizes the idea of biopaper and bioink is also a unique approach to print cell aggregates and organoids, where the bioink has the cells or cell aggregates being used and the biopaper forms the structural environment of the cells, mimicking tissue ECM. The bioink or biopaper can also be modified with bioactive molecules to improve cell viability and functions [14]. Extrusion bioprinting is one of the major methods that is currently being used for cell aggregates and organoids.

Laser bioprinting is a nozzle-free cell printing technique that avoids the high shear forces that might occur in nozzle-based approaches. This allows printing of high viscosity biomaterials and high cell densities. Laser-assisted bioprinting (LaBP) uses a planar donor slide loaded with the bioink and a laser-pen [15]. The bioink droplets from the donor slide are deposited layer-by-layer on an underlying collector glass slide by blade-coating, allowing the formation of arbitrary patterns and stratified 3D structures. High resolution and freedom in the bioink formulation are major advantages of laser bioprinting, while time-consuming slide preparation, high costs and low accessibility to the general user are key limitations. Being a nozzle-free approach, there are no clogging issues and printing can be achieved with a wide range of viscosities of bioink materials. However, the process requires rapid gelation of bioink materials to achieve a higher resolution of the printed constructs. This method is capable of precisely printing the cells and organoids with high cell viability and function.

Stereolithography (SLA) is another nozzle-free 3D printing technique that uses ultraviolet (UV) or visible light to cure photosensitive polymers in a layer-by-layer fashion. It offers a fast and accurate fabrication, with resolutions ranging between 5 and 300 μm [16]. Polymerization occurs at the top where the biomaterial is exposed to the light energy. After each layer is polymerized, the platform supporting the structure is lowered in the vat, enabling a new layer to be photopolymerized on top. Stereolithography is a promising technique for printing cells and organoids as they permit guided self-organization and controlled differentiation. The absence of shear stress and no limitation on bioink viscosity makes it an appealing choice for incorporating cells within scaffolds, its only disadvantages being the damage caused by UV and near UV light to the bioprinted cells DNA and the limited choice of photosensitive biomaterials. A recent study has fabricated a novel photopolymerizable hydrogel using high-resolution SLA 3D printing that supported the growth and differentiation of intestinal cell lines for 3 weeks [17].

3D bioprinting of stem cells and organoids

Although printing of organoids is still in its infancy, two approaches have been reported with respect to printing of pluripotent stem cells, firstly, bioprinting undifferentiated pluripotent stem cells and the other bioprinting differentiated stem cells or organoids themselves [18]. In terms of methods, both extrusion and laser-based bioprinting have been reported for bioprinting of pluripotent cells. Gu et al. [19] performed extrusion-based printing of cells within a polysaccharide-based bioink made of alginate, carboxymethyl-chitosan and agarose with post-printing cross-linking in calcium chloride. They showed that bioprinted human iPSCs differentiate in situ to self-assembling 3D embryoid bodies that express markers of the three germ layers, thus demonstrating maintenance of pluripotency after printing. Nguyen et al. [20] have printed human iPSCs co-cultured with irradiated chondrocytes in a bioink composed of nanofibrillated cellulose with alginate (NFC/A) or hyaluronic acid (NFC/HA). In contrast to a loss in cell proliferation and pluripotency with NFC/HA, NFC/A bioink maintained pluripotency and cartilaginous tissue over five weeks. In stem cell printing, the composition of the bioink is important not only to ensure long-term viability and maintenance of stem cells in a 3D environment, but also to provide an appropriate physiological mimetic environment for cell differentiation. Hence, an appropriate combo of bioinks to support both stem cells self-renewal and differentiation is requisite. In a study, extensive testing of bioinks has revealed that laser printing with hyaluronic acid combined with pluripotency supporting cell medium on matrigel shows the best results, maintaining both their pluripotency and differentiation potential [21]. Human corneal tissues have been fabricated by laser-assisted bioprinting using limbal epithelial stem cells (LESCs) in laminin bioinks, which maintain not only their proliferation but also allow them differentiate to corneal keratocytes reproducing corneal stroma. In this work, hESC-derived 3D bioprinted LESCs have been shown to form a stratified

corneal epithelium, mimicking the cytoarchitecture of the uppermost part of the native human cornea [22].

Yu et al. [23] have presented another novel approach to fabricate microscale biomimetic tissue constructs with hiPSC-derived cardiomyocytes and hepatocytes using photocrosslinkable dECM bioinks. Photocrosslinking is one of the best methods for cross-linking and imparting mechanical strength to the bioprinted constructs. In DLP, UV light triggers photopolymerization of the hydrogel solutions. Tissue-matched dECM bioinks provide a conducive environment for maintaining high viability and maturation of the stem cells into differentiated cell types; however, they are inherently weak and hence need to be appropriately modified to shape them into tissue-like microarchitectures. This study has developed a novel direct method to produce highly tunable tissue-specific dECM-based constructs possessing biomimetic microarchitectures by using a custom digital light processing (DLP)-based scanningless and continuous 3D bioprinter and has shown that the printed cells maintain high viability and maturity. Ma et al. [24] have also used a DLP 3D printing method based on UV light-induced photopolymerization, where they have co-printed hiPSC-derived hepatic progenitors (HPCs), with supporting cells comprising of umbilical vein endothelial cells (HUVECs) and human adipose tissue-derived stem cells (hASCs). These cells have been resuspended in two different bioinks, specifically: GelMA, with stiffness similar to healthy liver tissues for hiPSC-derived hepatic progenitors, and a mix of glycidyl methacrylate-hyaluronic acid (GMHA)/GelMA to facilitate vascularization for the supporting cells. Hepatic progenitor cells (HPCs) and the supporting cells have been co-printed in a sequential manner in an architecture that resembled an array of liver lobules with a hexagonal architecture and physiological dimensions. The study has shown that the designed liver pattern is maintained, and also that the HPCs spontaneously reorganize in aggregates. Liver marker expression analysis has revealed enhanced maturation and functions of aggregated HPCs in the vascularized constructs, indicative of the fact that vascularization is one of the key attributes for maintaining the long-term survival and functionality of the bioprinted spheroids and organoids.

With respect to organoid printing with differentiated stem cells or organoids, only a few studies exist. This is mainly because unlike cells, organoids are cell aggregates and in order to bioprint organoids, they need to be made dispensable through a bioprinter nozzle, without clogging issue and structure destruction. Thus, an optimization of the dimension of tissue spheroid with respect to the bioprinter nozzle is a must for continuous and homogenous dispensing (Fig. 3). Cell aggregate-based bioinks can be homocellular, containing a single cell type or heterocellular, prepared by several cell types. In a recent study, mature colonoid cultures have been set up using colonic crypt cells and then bioprinted by extrusion bioprinting into multi-well plates using GelMA bioink into 96-well culture plates. The colonoids retain viability and display proliferation post-printing [25]. Yang et al. [26] have fabricated 3D bioprinted hepatorganoids using hepatic organoids differentiated from HepaRG cells with alginate as the bioink. The printed hepatorganoids possess liver functions, such as albumin secretion, drug metabolism and glycogen storage both in vitro and in vivo. In another study, Faulkner-Jones et al. [27] have used in situ differentiation of stem cells into hepatis organoids during 3D bioprinting. The study has printed hESCs and hiPSCs during the differentiation process by valve-based printing using alginate hydrogel matrix and an appropriate chemically defined media. Cells printed during the differentiation process did not show any differences in hepatocyte marker expression and similar morphology when compared to those differentiated in a non-printed mode highlighting the potential of in situ differentiation of stem cells into organoids during printing. Another recent study that has employed the unique approaches of in situ differentiation of stem cells has fabricated functional human heart muscle pumps. The study by amalgamating the technical advances in 3D bioprinting, and human organoid cultures have developed a photocrosslinkable formulation of native ECM proteins (bioink engaging several integrin heterodimers) in combination with hiPSCs and bioprinted them by extrusion-based printing to form a human chambered muscle pump. The iPSCs have been printed as such and differentiated during the process of bioprinting. They have shown an efficient differentiation of the bioprinted iPSCs toward human cardiomyocytes. Also, the human chambered muscle pumps have demonstrated macroscale beating and continuous action potential propagation with responsiveness to drugs and pacing. The connected heart chambers allow for perfusion and enabled replication of pressure/volume relationships fundamental to the study of heart function and remodeling with health and disease. This advance represents a critical step toward generating macroscale tissue geometries, akin to aggregate-based organoids. This implies that for an optimum function of the organoids, tissue-like geometries are essential, which can only be assembled using advanced techniques of bioprinting and suitable tissue-specific bioinks [28].

Vascularization of organoids

In spite of making significant leaps in understanding the mechanisms of vascular development and function, recreating vascular structures in vitro is still a formidable task. A functional organoid vascularization has although been demonstrated in vivo after transplanting the organoids into host animals, where native vasculature integrates into the implanted organoid; however, ex vivo vascularization strategies for organoids to ensure a consistent supply of

oxygen, nutrients and removal of wastes are still lacking. Vascularization and nutrient supply do not pose a problem in small-scale 2D and 3D cultures as the cells need only static conditions and they are mostly perfused in enough media that is changed at regular intervals. Currently, 3D organoid culture is also majorly attempted in culture plates, where static media conditions are only utilized. In large-scale 3D cell/organoid cultures, however, where the cells are in a thick tissue-like arrangement, vascularization and a dynamic nutrient/waste flow throughout the entire construct are essential to avoid cell necrosis, especially at the center of the 3D tissues. Indeed, one of the biggest limitations of organoid cultures to generate functional tissues is that upon reaching a certain size, organoids cease to proliferate and develop a necrotic core. Hence, along with biofabrication of complex organoids through bioprinting, achieving adequate vascularization and perfusion of the bioprinted organoid is also requisite. This can be achieved through incorporation of endothelial cells along with the stem cells/organoids, by culturing the organoids onto 3D printed vascular structures or by using microfluidic devices. The use of endothelial cells to vascularize hPSC-derived organoids ensures oxygen and nutrient distribution in large organoids, thus contributing to the maturation of adult-like organoids through paracrine interactions. Extrusion bioprinting has been the most widely employed technique for printing both endothelial cells along with other cells using direct extrusion. In a study, the authors have combined mouse hepatic progenitor cells (HPC) with mouse liver sinusoidal endothelial cells (LSECs) in 1:1 ratio to generate hepatobiliary organoids with liver-specific vasculature. As compared to HPC organoids, HPC/LSEC organoids have demonstrated enhanced survival and hepatic functions. This study has presented a novel method to develop vascularized hepatobiliary organoids, with both in vitro and in vivo results confirming that incorporating LSECs with LPCs into organoids significantly increases the differentiation of hepatobiliary tissue within organoids and their survival post-transplantation [29]. A joint administration of vessel-forming cells [human mesenchymal stromal cells (MSCs) and endothelial colony-forming cells (ECFCs)] along with renal tubule-forming cells derived from human adult and fetal kidneys into the subcutaneous and subrenal capsular space has been attempted and resulted in self-organization of donor-derived vascular networks that connected to host vasculature, alongside renal tubules comprising tubular epithelia of different nephron segments [30]. A recent study has developed a tissue-engineered physiological tumor organoid model using endothelial cells in co-culture with mammary tumor cells. Imaging has revealed that tumor organoids integrate into the endothelial cell lining, resulting in mosaic vessels [31].

Vascularized brain organoids have been fabricated using several approaches. To model perineural vascular plexus (PNVP) in vitro, human embryonic stem cells (hESC)-derived endothelial cells (ECs), neural progenitor cells and microglia (MG) with primary pericytes (PCs) in synthetic hydrogels in a custom-designed microfluidics device have been co-cultured. The formed vascular plexus include networks of ECs, MG, and PCs and an overlying neuronal layer. PNVP also showed functional properties such as increased brain-derived neurotrophic factor secretion and differential metabolite secretion [32].

Another study has employed a multicellular 3D neurovascular unit organoid containing human brain microvascular endothelial cells, pericytes, astrocytes, microglia, oligodendrocytes and neurons to model the effects of hypoxia and neuroinflammation on blood–brain barrier function. Organoids cultured in this neurovascular unit under hypoxic conditions have shown increased permeability, pro-inflammatory cytokine production and increased oxidative stress, indicating immense potential in disease modeling and therapeutic development [33]. A recent study has profiled, for the first time, the electrophysiological properties of vascularized cerebral organoids consisting of various brain cells, such as neurons, astrocytes and vascular cells (endothelial cells and smooth muscle cells) at molecular and cellular levels. Intriguingly, two-month organoids have exhibited action potentials, multiple channel activities, and functional electrophysiological responses to the anesthetic agent, propofol [34]. In an elegant approach, a study has described a protocol to generate self-organizing 3D human blood vessel organoids from hPSCs that exhibit morphological, functional and molecular features of human microvasculature. These organoids have been differentiated via mesoderm induction of hPSC aggregates and subsequent differentiation into endothelial networks and pericytes in a 3D collagen I-Matrigel matrix. The in vitro-differentiated human blood vessel organoids when transplanted into immunocompromised mice have incorporated successfully in the mouse circulation [35]. Another study has used a methylcellulose-based hydrogel system, and generated spheroids consisting of EC/hPSC-SMC (vascular organoids), which has led to rapid generation of a complex functional vascular network both in vitro and in vivo [36]. These vascular organoids could be of immense use to vascularize large tissue-specific organoids.

Another method that has been in use for developing vascular and perfusable structures is the bioprinting of fugitive/sacrificial inks by extrusion bioprinting. Fugitive inks or sacrificial inks are extruded in the form of solid tubular structures, followed by extrusion of other hydrogels as the bulk adjacent layers, and the initial sacrificial ink is then removed by dissolution leaving behind a hollow conduit in the gel. Sacrificial bioinks are soluble in water, under specific temperatures, or rapidly degrade to allow their removal from the construct. Sacrificial bioinks include non-cross-linked

gelatins, pluronics and other materials that can be easily removed [37]. Perfusable microchannels lined with viable endothelial cell monolayers have been successfully demonstrated. Human MSC–alginate–gelatin hydrogels have been printed using dual-syringe layout with a thermal control, in which bioink at elevated temperatures was deposited onto a cooled (10 °C) substrate, and gelatin was used as a sacrificial bioink [38]. Recently, a method of using hydrogels as sacrificial scaffolds has allowed cells to form self-organized clusters or organoids, resulting in highly reproducible multicellular structures on a large scale. A dynamic change in the cross-linking state of alginate results in the release of cell-based structures from a surface without significantly disrupting the organoid structures or underlying cell function. The self-organization or organoid formation is done in growth-factor free medium, followed by providing a vasculogenic medium with growth factors to induce cell–cell interactions including sprouting of blood-vessel-like structures. The fabricated organoids have blood-vessel-like structures, and could be gently released by dissolving the alginate microwells [39].

To enhance angiogenesis and blood vessel growth, natural or synthetic scaffolds can also be functionalized with a combination of proangiogenic biomolecules, such as vascular endothelial growth factors (VEGFs), platelet-derived growth factors (PDGFs) and basic fibroblast growth factors (bFGFs) that promote neo-angiogenesis for formation of mature vascular networks [40, 41]. In addition to a bioprinting of capillary networks or using angiogenic growth factors, microfluidic approaches have recently been proposed to spontaneously vascularize engineered tissues. A microfluidic system, consists of many microchannel networks integrated into a single chip 3D cell culture. It is the most appropriate approach to recreate tissue- and organ-specific vascular architecture. Microfluidic devices that allow uniform distribution of flow and mass transfer can be produced using soft lithographic technique, with polymers such as PDMS, poly-lactic (co-glycolic acid) (PLGA) and poly-glycerol sebacate (PGS). Successful spontaneous vascularization of human lung fibroblast spheroids grown in microfluidic chip has been illustrated in a chip with parallel fluidic channels allowing cell migration and proliferation between the channels [42–45]. The study has used human lung fibroblasts and HUVECs together to generate composite spheroids of approximately 600 µm in diameter and then have fabricated the microfluidic chip where the middle channel consisted of the lung spheroids while the adjacent side channels were seeded with HUVECs. This has allowed the HUVECs to form a network around and within the lung spheroids. A combination of bioprinting of

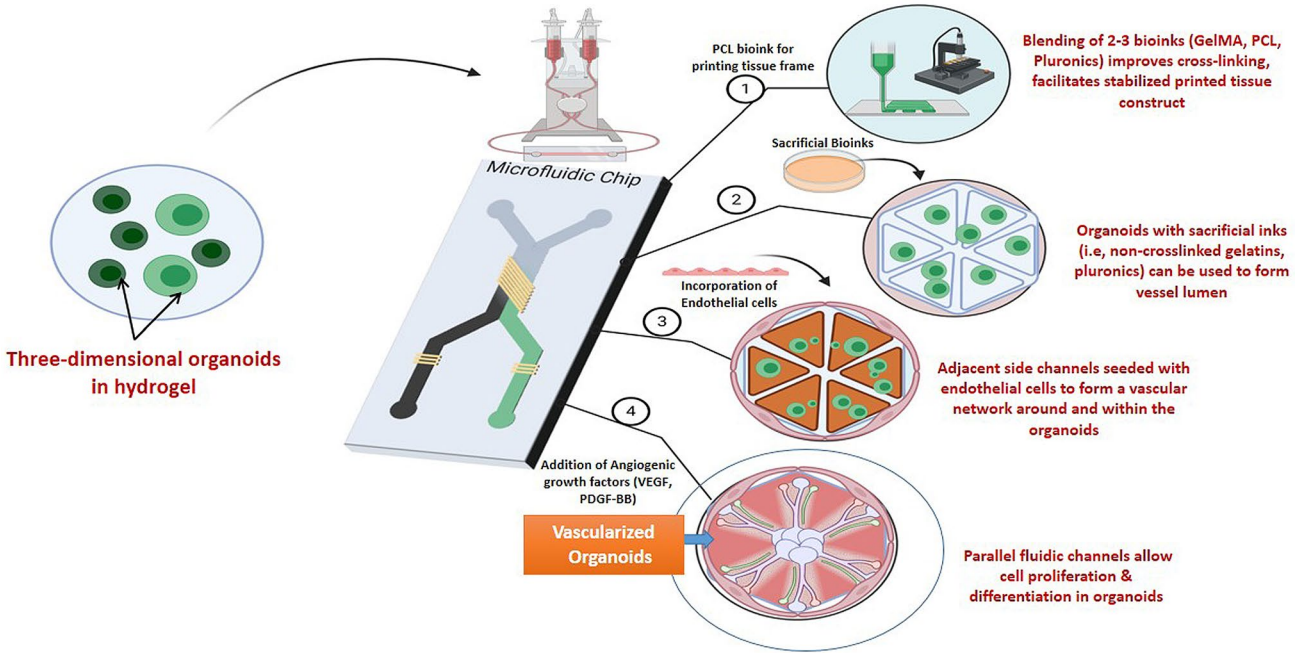

Fig. 4 Vascularization strategies of Organoids. The organoids can be vascularized using different strategies (1) Blending of bioinks to form frame work for channel-like structures (2) Using sacrificial bioinks that can be removed leaving the lumens behind (3) Bioprinting of endothelial cells along with the stem cells or organoids (4) Use of angiogeneic growth factors in the media. The vascularized organoid can be connected to a media perfusion device which mimics its in-vivo microcirculatory patterns. (PCL- Polycaprolactone, GelMA-Gelatin methacrylate, VEGF-Vascular endothelial growth factor, PDGF-BB-Platelet derived growth factor-2 beta subunits)

organoids with native endothelial cells and tissue-specific ECM on microvascular patterned and bioprinted organoid-on-chip microfluidic channels will be the next step forward for ensuring precise positioning of the right cells at the right place and establishing a fully perfusable organoid systems ex vivo (Fig. 4).

Applications of bioprinted organoids

The structural and physiological relevance of the currently available organoids is very limited, thereby reducing their relevance for functional studies, disease modeling and regenerative therapy. 3D Bioprinting and vascularization strategies can enhance this relevance by not only increasing the complexity and size of organoids but also by providing tissue-specific geometries and architectures with anatomical and functional similarity to human tissues and organs. The bioprinted tissue organoid structures find major applications in: (1) development and disease modeling (2) drug screening and discovery (3) regenerative therapy. The long-term in vitro expansion of organoids allows modeling of both development and disease. Several complicated anatomical structures of brain, kidney tubules, liver tubules and microvillus structures of intestines have been fabricated in 3D organoid models so far but their functional applications can only be enhanced by techniques like 3D bioprinting. Although the generation of specific organoids relies on the core knowledge of developmental biology, they offer numerous possibilities of further insights into early human development, enable evolutionary studies by comparing species-specific mechanisms of key developmental events.

For disease modeling, human stem cell-derived organoids provide a lucrative platform for unraveling human disease biology. Any disease model developed in cell lines in 2D cultures cannot be used to faithfully replicate the biology of the disease or accurately predict efficacy of any investigational drug for treatment. 3D bioprinted organoids that reproduce both cell-to-cell and cell–matrix interactions would address the limitations of cell lines and animal models. The organoids can be treated with various inflammatory stimuli for disease modeling and mechanistic studies. Various bacteria and virus-induced intestinal organoid models have been reported [46]. As compared to animal models, organoids represent only an approximation of the human biology as they lack key in vivo features such as a defined body axis, a functional immune or nervous system and organ to organ interactions. Hence, disease modeling in organoids can be taken up as a complementary approach to existing animal disease models.

Organoids generated from patient stem cells are quite valuable for the study of hereditary diseases and even to explore the mutational steps underlying tumor initiation and progression. Patient-derived liver organoids have been used to model and investigate the disease pathology of two genetic liver disorders, α1-antitrypsin (A1AT) deficiency and Alagille syndrome [47]. Patient-derived organoids offer the best models for dissecting the complex disease mechanisms in the dish. These models are especially valuable for studying hereditary diseases. An exciting new development in the field of patient organoids is the creation of 'living biobanks' (Fig. 5). Biobanks of patient-derived organoids have the potential to be accessible worldwide to advance research and treatment. These depositories have been currently proposed/created for tumors of various origins, and the day is not far when we would have organoid banks for all diseases. Organoids derived from patient-derived iPSCs have immense potential to be developed as accurate pre-clinical models for testing pharmacological and/or biological interventions. More than 90% of the drugs fail when they go to human clinical trials from preclinical models. In this regard, patient organoids offer a valuable screening platform after preclinical testing. Drugs which work successfully in human organoids would certainly be safe in clinical trials. Organoid-based drug testing is thus a rational approach for investigating repurposed drugs and also new drug discovery after preclinical trials. Patient-derived organoid repositories also encompass the genetic diversity of different patients which can be used to identify specific drug–genetics interactions.

Organoids can serve a plethora of applications in regenerative biology. Vascularized organoids can be transplanted in vivo similar to an organ transplantation and thus replacing the need for donor organs. Sometimes, the organoids transplanted in vivo demonstrate better structural features that are usually not seen in vitro. In vivo transplantation of hIPSC-derived intestinal organoids, comprising both endoderm and mesoderm have shown to differentiate into fully vascularized guts when implanted into immune-compromised mice. A recent study has illustrated the therapeutic potential of transplanting hepato-organoids in mice models of acute liver failure [48]. Other tissues which have been evaluated for regenerative potential are the lung, skin and hair [49]. A major challenge in organoid transplantation studies is standardizing the number of organoids (and thus cells) transplanted, because most organoid methods result in variably sized organoids embedded within a hydrogel, making quantification difficult. Without this standardization it is difficult to compare experiments from different batches under different conditions. Also, for transplantation, vascularized organoids have better chances of long-term viability and functions under in vivo conditions. Kiryu et al. [29] have developed an efficient organoid method to combine multiple cell types, enabling the derivation of relatively uniform organoids per single well with a consistent number of cells, and

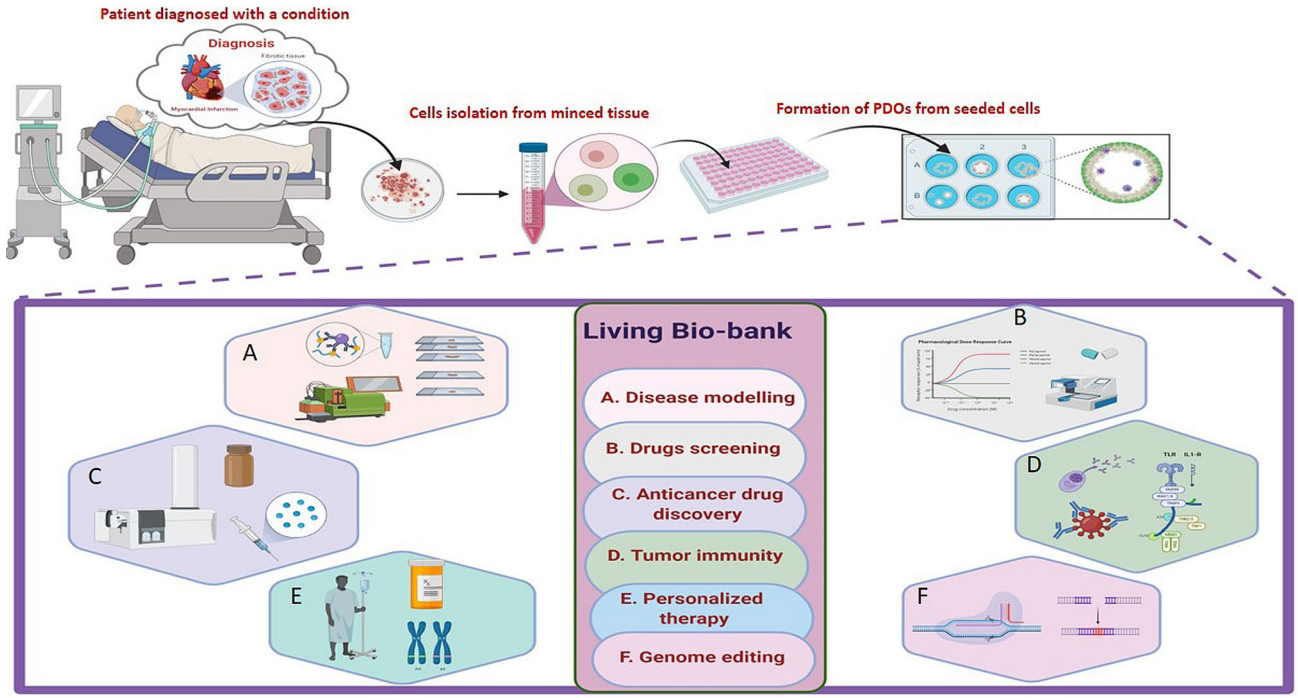

Fig. 5 Living Bio-bank formed by patient-derived organoids (PDOs). A patient's biopsy are used to isolate stem cells and derive organoids. A collection of a vast number of PDOs can result in the formation of a living biobank. (**a**-**f**) Applications of bio-bank i,e. Disease modelling, Discovery and screening of drugs (for various anomalies), Screening of immunity against tumor, generating personalized treatments and genome editing

is hydrogel-free facilitating transfer for transplantation. An 'ultimate' goal or organoid regenerative therapy would be to combine organoid technology with gene therapy/editing to correct genetic disorders.

Summary and future perspectives

Automated 3D bioprinting has prospects in scaling up the production of organoids and tissue constructs. Currently, 3D bioprinting is mainly used for pluripotent stem cells before organoid development to achieve high cell densities before differentiation, which can then be facilitated using defined growth factors and small molecules. The next challenge is of creating intricate 3D bioprinted organoids to encompass a spectrum of physiological and physiological tissues. For post-organoid bioprinting, many hurdles including improvement of the resolution of bioprinting process, shear stress-induced cell damage due to high cell densities, development of newer bioinks for depositing cell aggregates with fidelity, effective vascularization techniques, etc. need to be crossed. The trick lies in finding a balance between the intricate structural patterning of the printing process and the self-organizing nature of the organoid supported by the vascular network perfusing this whole structure. As these techniques and biomaterials are being refined daily to obtain an ideal balance, the bioprinted tissue organoid structures are undoubtedly here to stay and play crucial roles in comprehending disease mechanisms, and developing novel regenerative therapies over the next decade. The day is not far when we would be able to fabricate all the organs and connect them to a vascularized flow entity to mimic a working model of human to target any disease modelling and/or for a comprehensive drug testing to avoid burden on human trials.

Author contributions SK and DMT were involved in conceptualization of the study. PR wrote the manuscript and designed the figures. DMT supervised the figure designing. SR and SK reviewed and edited the final draft. All authors approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

Ethical approval This study does not contain any studies with human or animal subjects performed by any of the authors.

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