Bioprinting with Live Cells

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1 Introduction

The loss or failure of an organ or tissue is one of the most devastating, and costly problems in health care. The current treatment methods for organ/tissue loss or failure include transplantation of organs, surgical reconstruction, use of mechanical devices, or supplementation of metabolic products. Due to the growing need for organ transplantation and a lack of donor organs, tissue or organ engineering has progressed as a multidisciplinary field combining life sciences and engineering principles to restore, maintain, or improve function of tissues or organs [1, 2].

Traditionally, tissue engineering strategies are based on the cell seeding into synthetic, biological or composite scaffolds providing a suitable environment for cell attachment, proliferation and differentiation. A scaffold is highly porous complex structure providing an interconnected network that is designed to act as an artificial extracellular matrix (ECM) until the cells form their own ECM. In scaffold-based tissue engineering, three steps must be followed including finding a source of precursor cells from the patient, seeding these cells in vitro into the desired places of scaffold and surgically implanting the scaffold into the patient [3]. Scaffolds have been used to fabricate various tissue grafts including skin, cartilage, bone, blood vessels, bladder and myocardium [4–12]. Bioengineered tissue scaffolds attempt to mimic both the external shape and internal architecture of the replaced tissues.

The modeling of scaffolds has a great impact on the growth and proliferation of cells and a spatially and temporally controlled scaffold design could improve cell growth and differentiation [13]. Although many different scaffold manufacturing techniques such as salt-leaching, porogen melting, gas foaming, electrospinning,

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fiber deposition, molding and freeze-drying have been investigated in the past, it is challenging to control pore size, porosity, pore interconnectivity and external geometries of scaffolds. In recent years, various additive manufacturing based methods such as bioplotting, bioprinting, ink-jet printing and stereolithography have been used for biomanufacturing of complex three-dimensional (3D) tissue scaffolds to overcome the limitations of conventional tissue engineering methods [14]. These additive manufacturing based techniques allow to fabricate scaffolds layer-by-layer with controlled external and internal geometries based on computer-aided models of targeted tissues [15]. Several researchers have investigated designing functionally gradient porous scaffolds with controllable variational pore size and heterogeneous porous architecture [16, 17].

In scaffold-based tissue engineering, different biomaterials are used for scaffold fabrication such as porous materials composed of biodegradable polymers (polylactic acid, polyglycolic acid, hyaluronic acid and several copolymers), hydroxyapatite or calcium phosphate-based materials and soft materials like collagens, fibrin, and various hydrogels. Although there is a plenty of choice for scaffold materials, an ideal biomaterial for scaffold fabrication should be nontoxic, nonimmunogenic, capable of maintaining mechanical integrity for tissue growth and differentiation with controlled degradation [3].

After implantation of a scaffold, it should degrade in a controlled manner and the seeded cells should proliferate and migrate into scaffold to replace the scaffold biomaterial. Newly-formed extracellular matrix (ECM) fills the places which were previously occupied by the biomaterial of scaffolds. However, there are some drawbacks to create tissues with biodegradable scaffolds. Mostly, oxygen/nutrient delivery and removal of metabolic waste are insufficient through the micro-channels of a scaffold. Additionally, biodegradation of the scaffold induces inflammation. Even though the biomaterials used may not be directly toxic, they can be metabolized to toxic byproducts [18].

Because of the above mentioned drawbacks, the recent tissue engineering studies tend towards ‘scaffold-free’ techniques. During the embryonic maturation, tissues and organs are formed without the need for any scaffolds [19, 20]. The self-assembly and self-organizing capabilities of cells and tissues are main driver for the field of scaffold-free tissue engineering. Self-assembly based tissue engineering aims to produce fully biological tissues with specific compositions and shapes having the ability to grow their own ECM, and therefore to reduce the immunogenic reactions and other unpredicted complications based on the use of scaffolds [21].

One way of implementing the self-assembly approach is the cell sheet technology, which has been applied clinically for the repair of skin, cornea, blood vessels, and cardiomyocyte patches to repair partial heart infarcts [18, 22, 23]. Another self-assembly-based approach

is founded on the recognition that ‘nature knows best’. This approach relies on the principle that cell aggregates can be used as building blocks, since they have the intrinsic capacity to fuse together, known as tissue fluidity and self-assemble through morphogenetic processes if they are deposited in close spatial organization [24–26]. The engineering of 3D living structures supported by the self-assembly and self-organizing capabilities of cells is commonly termed ‘bioprinting’. Bioprinting is an extension of tissue engineering, where the cells are delivered through the application of additive manufacturing techniques [27, 28]

This chapter focuses on scaffold-free tissue engineering and its adaptation to the technology of three dimensional bioprinting. Further, the importance as well as the challenges for 3D bioprinting using stem cells will be discussed in this chapter.

2 Bioprinting with Live Cells

2.1 2D Patterning and Cell-Sheet Technology

Placing cells into special patterns using the laser light has been one of the first methods used for 2D cell patterning [14]. These laser-based techniques utilize transparent ribbons on which one side is coated with cells that are either adhered to a biological polymer through initial cellular attachment or uniformly suspended in a thin layer of liquid or a hydrogel. A pulsed laser beam is transmitted through the ribbon and is used to push cells from the ribbon to the receiving substrate which is coated with hydrogels.

While laser based approaches enable to pattern living cells on a substrate [30] and to layer multiple cell types [31], laser-based techniques have been also explored for positioning of cells in microarrays [32]. The resolution of laser-assisted bioprinting is affected by different factors such as the laser fluence, the wettability of the substrate, and the thickness and the viscosity of the biological layer [33]. Guillotion and his group studied the effect of the viscosity of the bioink, laser energy, and laser printing speed on the resolution of cell printing [34] as shown in Fig. 1.

By using this method, various cell types including human osteosarcama, rat carcinoma cells and human umbilical vein endothelial cells (HUVEC) could be printed with micrometer accuracy on Matrigel as the absorptive layer [31, 35, 36]. More recently, the biological laser printing was used to print sodium alginate, nano-sized hydroxyapatite (HA) and human endothelial cells [37]. However, most of these methods are limited to two-dimensional patterning and it is difficult to fabricate three-dimensional tissue constructs because of process-induced cell injury. The thermal stress and ultraviolet radiation caused by laser printing could also affect the cell viability.

Similar to 2D patterning, cell sheet technology is another scaffold-free method for construction of 2D and 3D engineered tissues. In this method, cells can be removed from a culture dish as a relatively stable confluent monolayer sheet without destroying cell-cell contacts. In order to build a substantial 3D tissue volume, many sheets need to be culminated in high amount of cells which requires vascularization for cell viability [15].

L'Heureux and his group produced a tissue engineered blood vessel using a cell sheet approach based on cultured human cells. The developed vessel contained all three histological layers such as the endothelium, the media and the adventitia. In

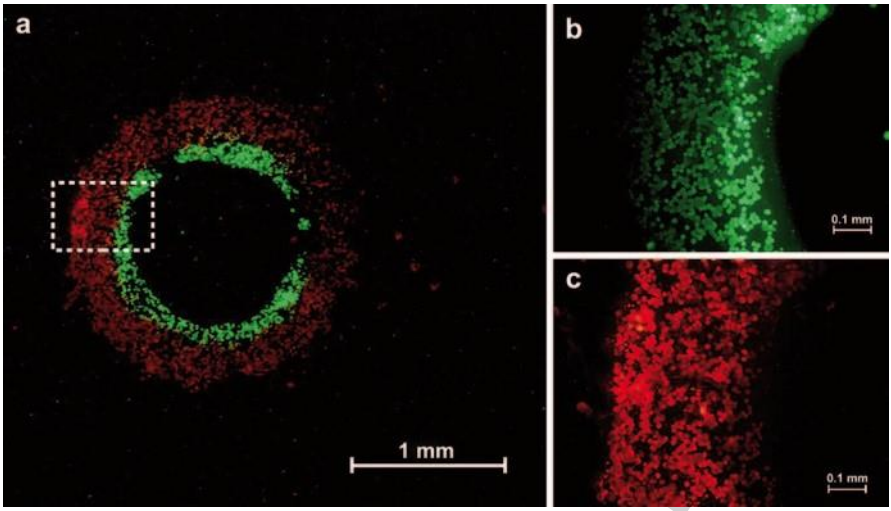

Fig. 1 Laser-assisted sequential two color cell printing in 2D. **a** The two cell suspensions (6·107 cells/ml in DMEM, supplemented with 1% (w/v) alginate were then printed according to a pattern of concentric circles). **b** *Green* Calcein stained cells within the region of interest as defined in 1**a** (*dashed rectangle*). **c** *Red* fluorescent Dil-LDL stained cells within the same region of interest. (Adapted from [34])

this self-assembly approach, smooth-muscle cells and fibroblasts were cultured in medium containing serum and ascorbic acid and produced their own extracellular matrix (ECM). The smooth-muscle cell sheet was placed around a tubular mandrel to produce the media of the vessel. A similar fibroblast cell sheet was wrapped around the media to provide the adventitia after 8 weeks of maturation. Finally, the tubular support was removed and endothelial cells were seeded in the lumen to form the endothelium. The tissue engineered blood vessel has burst strength of over 2500 mm Hg which is significantly higher than that of human saphenous veins (1680 ± 307 mm Hg) [23]. Sheet-based tissue engineering has been used by the same group to produce tissue engineered blood vessel (TEBV) suitable for autologous small diameter arterial revascularization in adult patients. Fibroblasts were cultured in conditions promoting

extracellular matrix (ECM) deposition to produce a cohe[126] sive sheet that can be detached from the culture flask. This approach also eliminates [127] the use of smooth-muscle cells, whose early senescence is related with decreased [128] burst pressures in human models. The decellularized internal membrane (IM) and
[129] living adventitia were assembled by wrapping fibroblast sheets around a temporary [130] Teflon coated stainless steel support tube. After weeks-long maturation and dehy[131] dration to form an acellular substrate, the steel tube was removed and endothelial
[132] cells were seeded in the lumen of living TEBV. The transplantation of these blood [133] vessels into dogs demonstrated good handling, suturability by the use of conven[134] tional surgical techniques. Ultimately, this is an effective approach to produce a [135] completely biological and clinically applicable TEBV in spite of its relatively long [136] production time (≈ 28 weeks) which clearly prevents its urgent clinical use [22].

Okano and colleagues have engineered a long-lasting cardiac tissue based on 137 a similar self-assembled sheet based approach. In their method, culture dishes are 138 first coated with a temperature-responsive polymer, poly (N-isopropylacrylamide) 139

(PIPAAm). The surface is relatively hydrophobic at 37 °C allowing cells to attach 140 and proliferate, while cooling below 32 °C (typically 20 °C for 30 min) makes the 141 surface hydrophilic and causes the cells to detach without the use of enzyme di142 gestion reagent. When grafted PIPAAm layer thickness is between 15 and 20 nm, 143 temperature-dependent cell adhesion and detachment can be observed. Once the 144 cells spread and confluent on the surface, they can be spontaneously detached as a 145 contiguous cell sheet by reducing the temperature. This process does not disrupt the 146 cell-cell junctions because no enzymes like trypsin are required. Additionally, basal 147 surface extracellular matrix (ECM) proteins such as fibronectin are preserved after 148 detachment which enables easy attachment of cell sheets to host tissues and even 149 wound sites with minimal cell loss. In order to obtain tissue constructs with char150 acteristic physiological cellular functions in vitro, heterotypic cell-cell interactions 151 are inevitable. As shown in Fig. 2, it is possible to modify the above-mentioned 152 technique in order to develop patterned cell sheets using two or more kinds of cell 153 source. Domains on petri dishes were grafted by using area-selective electron beam 154 polymerization of PIPAAm. After cells were cultured on the patterned grafted sur155 faces at 37 °C, the temperature was decreased to 20 °C. Cells on the PIPAAm sur156 face are detached where other cell types were seeded subsequently by increasing the 157 temperature to 37 °C. Therefore, two cell types can be co-cultured in desired places 158 which improve cellular functions [18, 38, 39]. Three-dimensional myocardial tubes

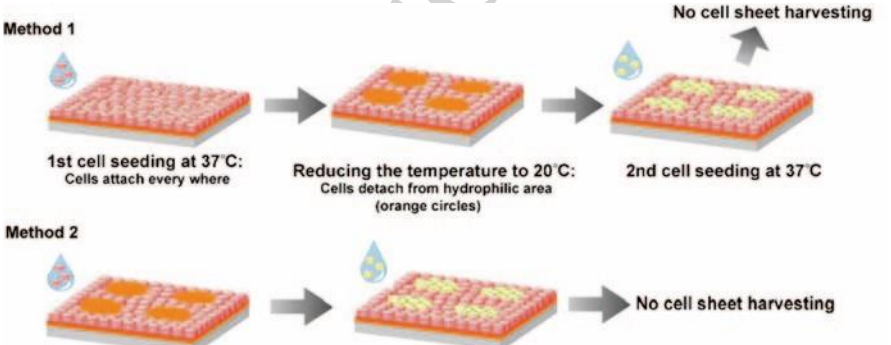

Fig. 2 Schematic diagram of methods of cell seeding for patterned co-culture on PIPAAm-grafted surfaces. (Adapted from[40])

159 were fabricated using neonatal rat cardiomyocyte sheets cultured on temperature160 responsive culture dishes [40, 41]. Due to the functional gap junction formation, 161 electrical coupling of cardiomyocyte sheets was obtained quickly and the construct 162 was implanted [42]. Four weeks after the implantation, the myocardial tubes were 163 integrated with the host tissues showing spontaneous and synchronized pulsation 164 [38, 43]. Using this versatile method, functional and transplantable tissue sheets are 165 produced from different cell types including epidermal keratinocytes [44], kidney 166 epithelial cells [45] and periodontal ligaments [46, 47].

167 Two-dimensional cell patterning or cell-sheet based approaches have been suc-
168 cessful tissue engineering approaches. However, the engineered constructs fabri169 cated with these methods are limited to 2D cell patterns or simple shapes because 170 of the flat and uncontrolled shape of the cell sheets. In addition, many sheets also 171 need to be culminated in high amount of cells which requires pre-vascularization 172 of the sheets for 3D tissue constructs. Therefore, several bioprinting approaches 173 have been developed for fabricating 3D tissue constructs with live cells. Two major 174 approaches, ink-jet based and extrusion based 3D bioprinting methods will be ex175 plained in details below.

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178 *2.2 Inkjet-Based Bioprinting*

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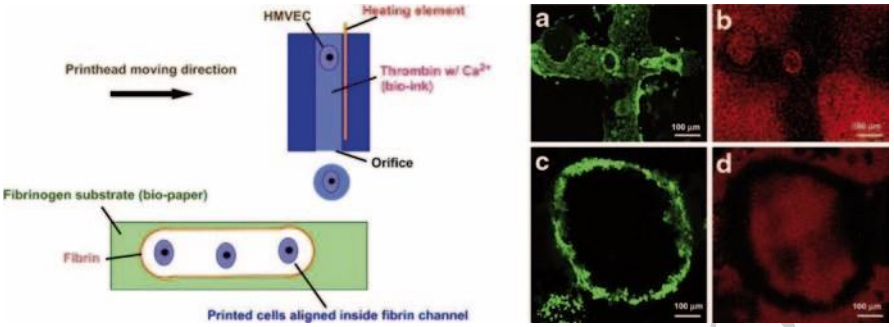
180 In inkjet-based bioprinting, a bioink made of cells and biomaterials are printed in 181 the form of droplets through cartridges onto a substrate. There are two types of 182 inkjet printing including continuous inkjet printing (CIJ) and drop-on-demand ink183 jet printing (DOD). In CIJ mode, a jet is obtained by forcing the liquid through an 184 orifice under an external pressure and it breaks up into a stream of droplets. In DOD 185 mode, a pressure pulse is applied into the fluid which generates drops only when 186 needed. For ink-jet printing of cells, there are two most commonly used approaches: 187 thermal and piezo-electric inkjet printing. For thermal inkjet printing, small vol188 umes of the printing fluid are vaporized by a micro-heater to create the pulse that 189 expels droplets from the print head. In piezoelectric inkjet printing, a direct me190 chanical pulse is applied to the fluid in the nozzle by a piezoelectric actuator, which 191 causes a shock wave that forces the bioink through the nozzle [48]. However, there 192 have been only a few examples of cell deposition by piezoelectric ink-jet printing 193 due to the electrically conducting ink formulations and contamination concerns on 194 ink recycling [24].

195 Inkjet-based bioprinting (Fig. 3) enables to deposit different cell types in precise 196 orientations relative to the print surface and to each other at micrometer resolution 197 by

controlling the ejection nozzles and timing of spray [49]. Wilson and Boland first [198] adapted the ink-jet printers for the manufacture of cell and protein arrays, which [199] have the advantage of being fully automated and computer controlled [50]. In their [200] next study, cell aggregates were printed onto thermosensitive gels layer-by-layer [201] in order to demonstrate the fusion between the closely-placed cell aggregates [51].

[202] The same group deposited CHO cells and rat embryonic motoneurons as an 'ink'



AQ1 **Fig. 3** Schematic diagram of inkjet bioprinting methods of cells with fibrin channel scaffold printed microvasculature. **a** Printed perpendicular microvasculature cultured for 14 days. **b** Integrity of the printed structure stained using Texas *Red* conjugated dextran molecules of 3000 MW. **c** Printed *ring* shaped microvasculature cultured for 21 days. **d** Integrity of printed structure cultured for 21 days

[203] onto several 'bio-papers' made from soy agar and collagen gel. They demonstrated [204] also that the mammalian cells can be effectively delivered by a modified thermal [205] inkjet printer onto biological substrates and that they retain their ability to function [206]

[52]. Cui and Boland used also thermal inkjet printing to produce cell containing [207] fibrin channels by printing human microvascular endothelial cells onto thin layers [208] of fibrinogen as shown in Fig. 3. During the incubation period, the cells proliferated [209] and formed branched tubular structures mimicking simple vasculature [53]. [210]

Inkjet bioprinting has been progressed to fabricate 3D biological structures by [211] the use of readily crosslinked hydrogels such as alginate. In published studies, cells [212] have been mixed with alginate solutions and crosslinked with calcium chloride to [213] create cell encapsulating hydrogels having defined 3D structures [54–56]. In a more [214] recent study, alginate has been used as a constituent of bioink and it was mixed with [215]

NIH 3T3 mouse fibroblasts cell suspension in order to fabricate zigzag cellular [216] tubes with an overhang structure using a platform-assisted 3D inkjet bioprinting

system [57].

Although inkjet bioprinting has been one of the most commonly used method in printing living cells and biomaterials, cell aggregation, sedimentation and cell-damage because of the high shear stresses are common drawbacks of this method.

Cell aggregation and sedimentation may be prevented by frequent stirring of the cell mixture, which can result in reduced cell viability if the cells are sensitive to the shear forces [58]. Another problem limiting the inkjet bioprinting is the clogging of the nozzle orifice. Low viscosity surfactants can be added to the ink which can cause additional challenges such as cell damage [59].

Recently, two research groups successfully address the sedimentation and cell aggregation problem during the inkjet bioprinting. Chahal and coworkers used a surfactant (Ficoll PM400) create neutrally buoyant MCF-7 breast cancer cell suspensions, which were ejected using a piezoelectric drop-on-demand inkjet printing system. They demonstrated that Ficoll PM400 did not have adverse effects on cell viability. Moreover, neutrally buoyant suspension greatly increased the reproducibility of consistent cell counts, and eliminated nozzle clogging. [60].

Ferris et al. used two different commercially available drop-on-demand printing systems in order to reproducibly print several different cell types over long printing periods. The bio-ink based on a novel microgel suspension in a surfactant-containing tissue culture medium can prevent the settling and aggregation of cells, while meeting the stringent fluid property requirements needed for many-nozzle commercial inkjet print heads. They could print two cells types simultaneously from two different inkjet print heads, which is a innovative way to biofabricate more complex multi-cellular structures [61].

2.3 Self-Assembly Based Bioprinting

The autonomous organization of components from an initial state into a final pattern or structure without external intervention is called self-assembly. The aim of the self-assembly-based bioprinting is the use of the inherent organizational capacity of cells into tissues and eventually organs by mimicking natural morphogenesis. The best examples of tissue self-organization and self-assembly are in the field of developmental biology and scaffold-free biomimetic approach has deep roots in developmental biology [20]. Malcolm Steinberg published papers, in which he formulated fundamental thermodynamic rules determining tissue self-assembly and developed

differential adhesion hypothesis (DAH) explaining the fluidic nature of cell sorting and tissue self-assembly [62–64]. Therefore, the novel scaffold-free biomimetic tissue engineering paradigm relies on the principle that in vitro tissue assembly from single cells or tissue aggregates is feasible.

Based on the self-assembly principle, it is possible to fabricate reliable and reproducible 3D tissue constructs having defined topology and functionality in vitro when combined with bioprinting techniques. The disadvantage of this method is that the development of the natural ECM is time consuming and in vitro self-assembly may vary with fully physiological conditions. The bioprinting of 3D tissue constructs is achieved via a three-phase process: (1) preprocessing or bio-ink preparation; (2) processing, i.e. the actual automated deliver/printing of the bio-ink particles into the bio-paper by the bioprinter; and (3) postprocessing, i.e. the maturation/incubation of the printed construct in the bioreactor [19]. Self-assembly occurs in an *in vivo* like, fully controllable cell environment (bioreactor) by the differentiation of cells at the right time, in the right place and into the right phenotype and eventually the assembly of them to form functional tissues. Based on this approach, a perfusion reactor is used for the maturation of a bioprinted macrovascular network in order to obtain the required mechanical properties. Microvascular units consisting of cylindrical or spherical multicellular aggregates were fabricated by the parenchymal and endothelial cells. Afterwards, microvascular units were located in the macrovascular network for the perfusion supporting self-assembly and the connection to the existing network. Multicellular spherical and cylindrical aggregates have been

constructed by using 3D printing methods, which enable to achieve flexibility in tube diameter and wall thickness and to form branched tubular structures. However, the printed cell aggregates should be perfectly supported by hydrogels for 3D printing [21]. Forgacs and his group employed this novel technology to print cellular topologically defined structures of various shapes. Cardiac constructs were built using embryonic cardiac and endothelial cells and their postprinting self-assembly resulted in synchronously beating solid tissue blocks, where the endothelial cells were organized into vessel-like conduits [65]. In their more recent study, the same group utilized the self-assembly approach in order to bioprint small-diameter, multilayered, tubular vascular and nerve grafts using bio-ink composed of aortic smooth muscle cells (HASMC), human aortic endothelial cells (HAEC), human dermal fibroblasts (HDFb) and bone marrow stem cells (BMSC), respectively as shown in Fig. 4 [25]. Similarly, in another study, self-assembled cell-based microtissue blocks were used to generate small diameter tissue-engineered living blood vessels (TEBV). Microtissues composed of human-artery-derived fibroblasts (HAFs) and endothelial celss (HUVECs) were cultured for 7 and 14 day under pulsatile flow/mechanical stimulation in a designed bioreactor or static culture conditions with a diameter of 3 mm and a wall thickness of 1 mm. Self-assembled microtissues

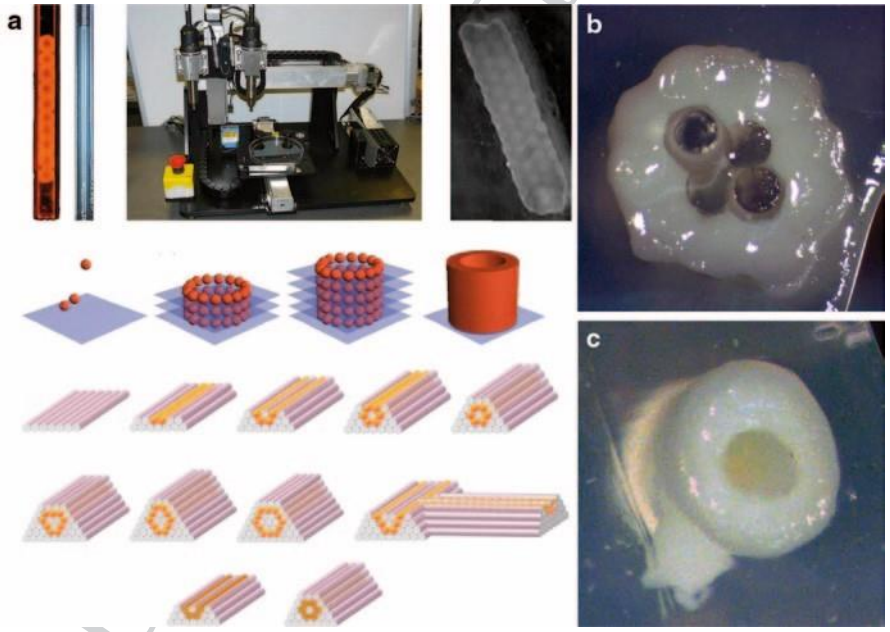


Fig. 4 a Organovo bioprinter with cell and hydrogel printing heads and schematics to print tubular structures with cellular spheroids: layer-by-layer deposition of spheroids into the hydrogel. **b** Cross-section of a vascular graft printed with four central rods 12 h post-printing. All cellular cylinders have fused to form a continuous conduit. **c** A vascular construct (ID =600 μm) at 14 days post-perfusion. (Adapted from [25])

Fig. 5 Bioprinting of aortic valve conduit. **a** Aortic valve model reconstructed from micro-CT images. The root and leaflet regions were identified with intensity thresholds and rendered separately into 3D geometries into STL format (*green* color indicates valve root and *red* color indicates valve leaflets); **b**, **c** schematic illustration of the bioprinting process with dual cell types and dual syringes; **b** root region of first layer generated by hydrogel with SMC; **c** leaflet region of first layer generated by hydrogel with VIC; **d** fluorescent image of first printed two layers of aortic valve conduit; SMC for valve root were labeled by cell tracker *green* and VIC for valve leaflet were labeled by cell tracker *red*. **e** as-printed aortic valve conduit. (Reproduced with permission [75]).

293 composed of fibroblasts showed accelerated ECM formation and a layered tissue AQ2 formation was obtained only in flow/mechanical stimulation conditions [66] Fig. 5.

295 An alternative approach for multicellular spheroid assembly technique for bio296 fabrication was developed by Nakayama and his group. In their technique, they 297 used a so called needle-array system instead of using a bioprinter. A robotic system 298 was developed in order to skewer the multicellular spheroids into medical-grade 299 stainless needles, which served as temporal fixators until multicellular spheroids 300 fused each other. They could fabricate complex 3D scaffold-free cell constructs 301 with different types of cells including chondrocyte, hepatocyte, cardiomyocyte and 302 vascular smooth muscle cell. One of the advantages of this technique is the easy 303 removal of the temporary supports without contamination with exogenous materi-

304 als [3].

305 Apart from the above mentioned applications, a new method is presented to rap306 idly self-assemble cells into 3D tissue rings without the use of additional equip307 ment. This method enables fabrication of engineered tissue constructs entirely from 308 cells by seeding cells into custom made annular agarose wells with 2, 4 or 6 mm 309 inside diameters. Different cell types including rat aortic smooth muscle cells and

310 human smooth muscle cells are used with varying seeding conditions and culture length to form tissue rings. The strength and modulus of tissue rings increased with

311 ring size and decreased with culture duration. Rat smooth muscle cell rings with an 312 inner diameter of 2 mm are cohesive enough for handling after 8 days incubation 313 and they yield at 169 kPa ultimate tensile strength. Furthermore, it is also pos314 sible to fabricate tissue tubes by transferring the rings onto silicone tubes, sliding 315 them into contact with one another and incubating them for an additional 7 days. 316 Although these rings are not as strong as ring segments of native blood vessels or 317 TEBV fabricated from cell sheets for 2–3 months, the presented method allows de318 veloping 3D tissue constructs from aggregated cells within an experimentally useful 319 time frame (1–2 weeks) [67]. Likewise created smooth muscle cell tissue rings and 320 rings fabricated from cells seeded in fibrin or collagen gels are compared based on 321 their relative strength and utility for tissue engineering. All tissue rings were cul322 tured for 7 days in supplemented growth medium which includes ε-amino caproic

323 acid, ascorbic acid, and insulin-transferrin-selenium. Ultimate tensile strength and 324 stiffness values of tissue rings were two-fold higher than fibrin gel and collagen gel 325

rings. Tissue rings cultured in supplemented growth medium exhibit a three-fold increase in tensile strength and stiffness in comparison to the tissue rings cultured in standard growth medium [68].

The approach of using microtissues as building blocks to form larger structures is further used by other research groups in order to investigate the reassemble capacity of cell aggregates. After a preculture period for 7 days of HUVEC spheroids, they were mixed with NHF cells and were able to reassemble and form microtissues with the NHF cells on the inside and coated with HUVEC on the outside [69]. Additionally, the kinetics of the cellular self-assembly also differs from one cell type to the other. While H35 cells formed relatively stable rod structures inside the recesses of micromolded agarose gels, NHF cells reassembled quickly the initial rod structures to a final spheroid structure [70].

2.4 Extrusion-Based Bioprinting

Bioprinting methods based on extrusion of cell or cell-laden biomaterials use self-assembly cells to construct 3D biological constructs. The main principle of extrusion-based bioprinting techniques is to force continuous filaments of materials including hydrogels, biocompatible copolymers and living cells through a nozzle with a help of a computer to construct a 3D structure [27]. Extrusion-based printers usually have a temperature-controlled material handling and dispensing system and stage with the movement capability along the x, y and z axes. The printers are directed by the CAD-CAM software and continuous filaments are deposited in two dimensions layer-by-layer to from 3D tissue constructs. The stage or the extrusion head is moved along the z axis, and the printed layers serve as a base and support for the next layer. Pneumatic or mechanical (piston or screw) are the most common techniques to print biological materials for 3D bioprinting applications [33]. Additionally, novel multi-nozzle biopolymer deposition systems were developed for freeform fabrication of biopolymer-based tissue scaffolds and cell-embedded tissue constructs [71, 72].

An extrusion-based printer was used to deposit living cells by Williams and co-workers. Instead of using a thermally crosslinked biomaterial, which can flow at room temperature, but crosslink into a stable material at body temperature, they used Pluronic F-127 and type I collagen to encapsulate human fibroblasts and bovine aortic endothelial cells (BAECs) separately. These materials flow at physiologically suitable temperatures (35–40°), but crosslink at room temperature. They demonstrated the availability of CAD/CAM technology to fabricate anatomically correct shaped constructs and also examined several environmental factors with respect to the viability of the extruded cells [73, 74]. Recently, different research groups used the similar extrusion systems in order to fabricate anatomically accurate and mechanically heterogeneous aortic valves as shown in Fig. 1 [75, 76].

Several groups used high resolution extrusion systems to print different type of cells encapsulated in various hydrogels. For instance, Chang et al. printed HepG2

cell encapsulated sodium alginate through a pneumatically powered nozzle and examined the process parameters, the dispensing pressure and the nozzle diameter, regarding to the cell viability and recovery [77]. In another study, alginate hydrogel was used with calcium sulfate as a crosslinking agent to fabricate pre-seeded implants of arbitrary geometries and the printed constructs showed high viabilities [78]. Although the cell viability after printing is important, it is also important that the cells perform their essential functions in the tissue constructs.

Extrusion-based printing allows the construction of organized structures within a realistic time frame, and hence it is the most promising bioprinting technology.

The main advantage of extrusion-based bioprinting is the ability to print very high cell densities. Some groups developed 3D bioprinters in order to use multicellular spheroids or cylinders as bioink to create 3D tissue constructs [19, 21, 25, 79–81]. However, preparing bioink requires time-consuming manual operation and makes totally automated and computer-controlled 3D bioprinting impossible in earlier studies. Therefore, our group focused on the development of a continuous bioprinting approach in order to extrude cylindrical multicellular aggregates using an extrusion-based bioprinter, which is an automated, flexible platform designed to fabricate 3D tissue engineered cell constructs. In order to bioprint anatomically correct tissue constructs directly from medical images, the targeted tissue or organ must be biomodeled. In the following section, the details of modeling and developing path planning for automated direct cell bioprinting will be explained.

2.4.1 Biomodeling

In order to obtain an anatomically accurate tissue constructs, several imaging methods for data acquisition of tissue organ such computed tomography (CT) and magnetic resonance imaging (MRI) could be used. The obtained medical images are then transferred to a special segmentation software, where the images are represented with stack of numerous planar scan captures (Fig. 6a). The segmented 3D

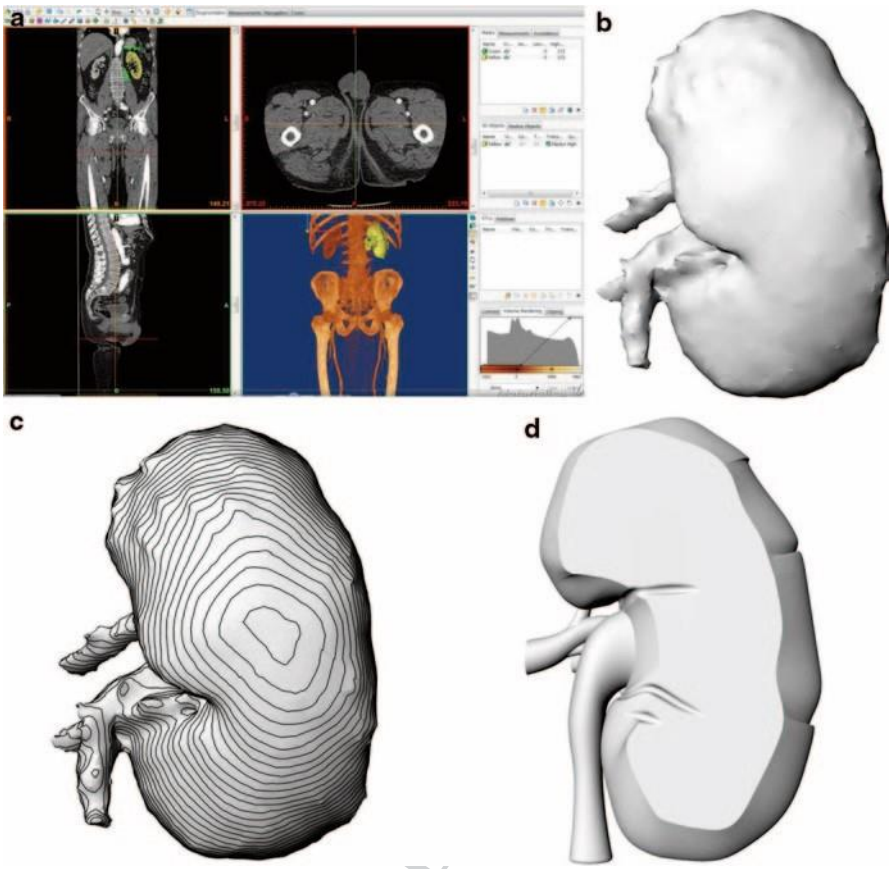


Fig. 6 Biomodeling of the bioprinted tissue/organ **a** segmentation of the targeted tissue/organ **b** mesh model of segmented model **c** slicing of CAD model

surface geometry is transformed to a CAD model which is a mesh model of the object (Fig. 6b). In order to generate bioprinting path planning as well as the topology optimization for bioprinting processes, the resultant mesh models need to be represented by smooth parametric surfaces. The mesh model is then sliced with consecutive planar cross-sections, resulting in closed contour curves for each thin layer slice [28] (Fig. 6c). Those contour curves are basically the surface boundaries of tissue constructs. Obtained contour curves need to smoothed by B-spline curve fitting from their control points, in order to generate smooth parametric surfaces and finer surface geometry (Fig. 6d). The resultant CAD model is then ready to be used for path planning and topology optimization purposes for biomimetic 3D bioprinting.

Recently, novel computer-aided algorithms and strategies are developed to model and 3D bioprint a scaffold-free human aortic tissue construct biomimetically by our group. Medical images obtained from magnetic resonance imaging (MRI) are used

Fig. 7 Biomimetic modeling of aorta directly from medical images **a** segmentation from medical images **b** Conversion to a CAD model

to obtain the geometric and topological information of the targeted aorta. In order to obtain 3D computer models of the aortic tissue, MRI images are segmented using a segmentation software and converted into CAD model (Fig. 7). For tool path planning as well as for optimization of 3D bioprinting, the resultant mesh model of aorta converted to a CAD model with smooth parametric surfaces. Three-dimensional bioprinting path planning and parameter optimization are then developed. The developed self-supporting methodology is used to calculate corresponding tool paths for both cell aggregates and the support structures, which control the bioprinter for 3D printing of a biomimetic aortic construct [74].

2.4.2 Path Planning and Optimization for Bioprinting

In order to bioprint an anatomically correct tissue constructs with live cells layer-by-layer, the generated computer model of this construct needs to be sliced by planar cross-sections, resulting in closed contour curves for each layer. Then those layers need to be filled by appropriate types of cellular aggregates with supportive hydrogel walls surrounding them for keeping the biomimetic form. In our recent work, multicellular cell aggregates are 3D bioprinted based on computer-aided continuous and, interconnected tool-path planning methodologies. Continuous bioprinting enables to design and 3D bioprint extruded multicellular aggregates according to the computer model of the targeted tissue. The Zig-zag and Contour Offsetting pattern tool-path methodologies are developed to 3D bioprint different shaped structures with multiple layers. A CAD software package was used for developing algorithms for continuous and connected bioprinting path plans. In order to keep the 3D forms of printed structures during the maturation period, a biocompatible and bio-inert agarose-based hydrogel was used as a support material [75]. The developed bioprinting process starts from the bottom layer and follows the generated path plan for each particular layer consecutively through the top layer. At a layer, support material enclosing the cellular aggregates are printed first, and then cellular aggregates are deposited to fill the respective contour areas.

In Fig. 8, schematic view of pat planning strategies are showed for a layer. Figure 8a-b shows Zig-zag whereas Fig. 8c-d shows Contour Offsetting path planning.

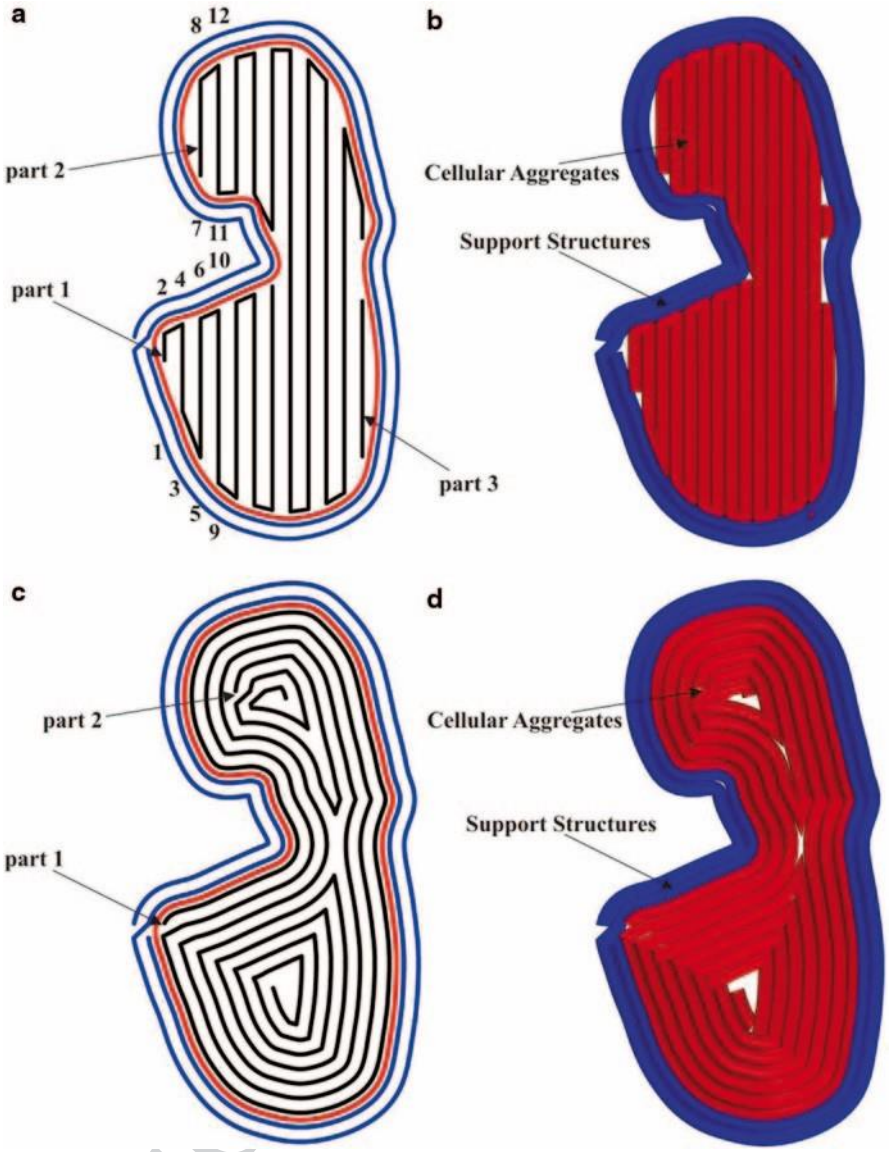


Fig. 8 Path planning for bioprinting **a**–**b** Zig-zag pattern **c**–**d** Contour offsetting path planning

446 In both Zig-zag and Contour Offsetting patterns, outer supportive hydrogel walls' 447 tool path are generated with offsetting the contour curve with a deposited cellular 448 or hydrogel extrusion diameter. Placement of support structures before the cellular 449 aggregates provides necessary conditions for cell fusion and structure conservation. 450 In Zig-zag pattern path planning strategy, contour curves are

crossed with par451 allel consecutive lines, each separated by extrusion diameter, and an intersection 452 point is generated for each cross as shown in Fig. 8a. Zig-zag pattern path planning 453 strategy aims to fill the contour area by following a zig-zag patterned path way be454 tween the generated intersection points as uninterrupted as possible.

455 In Contour Offsetting path plan strategy, cellular aggregates' path plan is formed 456 by offsetting the contour curve to fill the entire area. After the necessary amount 457 of offset curves is generated, they are joined by small line segments to enable con458 tinuous bioprinting. That strategy also aims to fill the contour area with minimum 459 number of cellular aggregate as shown in Fig. 8c).

460 After the path plan is calculated, the coordinates of these movements are trans461 ferred to bioprinter to guide the deposition path plan in order to obtain anatomically

462 correct tissue constructs. In Fig. 8b and Fig. 8d, the finalized generated path plans 463 of cellular aggregates (red) and support structures (blue) are shown for both path 464 planning strategies.

465

466

467 2.4.3 Continuous Cell Printing

468

469 In our recent work, a novel bioprinting method is used for precise deposition of mul470 ticellular aggregates composed of different combinations of mouse aortic smooth

471 muscle cells (MOVAS), NIH 3T3 mouse fibroblast cells, human umbilical vein 472 endothelial cells (HUVEC), and human dermal fibroblast (HDF) cells according 473 to computer-generated paths as shown in Fig. 9 [83]. The proposed methodology 474 increases the contact of cylindrical multicellular aggregates in adjacent bioprinted 475 layers, facilitates the fusion of cells and accelerates the maturation process. More 476 significantly, this procedure reduces the human intervention at forming of cylindri477 cal multicellular aggregates and therefore, increases the reproducibility.

478 The printed 3D multicellular structures are examined for their mechanical 479 strength, shape deformation with time, cell viability and cell fusion. The printed 480 constructs having different shapes deformed during the incubation period (up to

481 10-days) and generally a shrinking between 20 – 38 % was observed. After 4 or 7 482 days incubation, the support structures of well-defined and random-shaped printed 483 structures composed of MOVAS, HUVEC and NIH 3T3 multicellular aggregates 484 were manually removed and the fused cell structures could be transferred with for485 ceps into a falcon filled with PBS (Fig. 10a). It is remarkable that the stripe shaped 486 constructs composed of HUVEC/HDF cell aggregates had a small deformation per487 centage 85 % after 3-days incubation) and were sufficiently sturdy to be handled 488 and transferred as shown in Fig. 10b.[83]. MOVAS/HUVEC/NIH 3T3 multicellular 489 aggregates fused within 3 days, which corresponds to earlier studies [25, 81]. The 490 cell viability upon implementation was high (97 %) showing that the cellular bio491 ink preparation method is successful in comparison to other studies in literature. It

492 seems that multicellular aggregates composed of human cells have better mechanical properties.

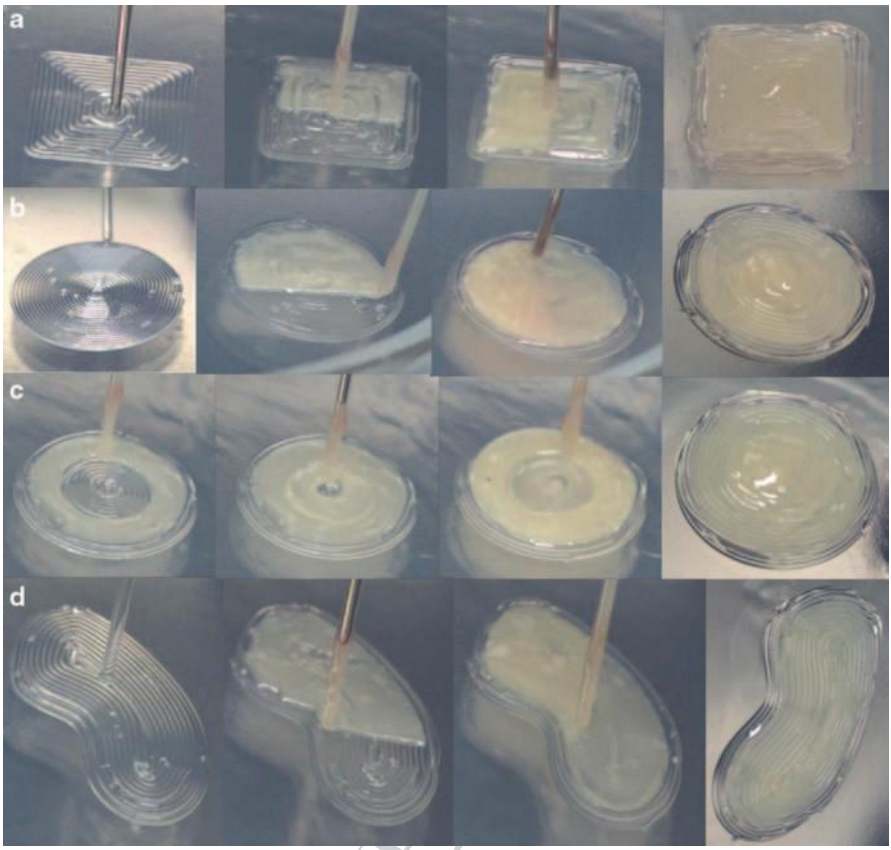


Fig. 9 Continuous bioprinting of live cells directly from computer models **a** *Rectangular* shaped **b** Random-shaped **c** Zig-zag patterned *circular* **d** Spiral patterned circular printed structures [83]

3 Conclusion and Discussion on Stem Cell Printing

Bioprinting is one of the most promising techniques in tissue engineering, where living cells are deposited layer-by-layer with or without biomaterials in user-defined patterns to build 3D tissue constructs. However, there are some challenges related with technical, material and cellular aspects. 3D bioprinting technology requires increased resolution, speed and compatibility with biologically relevant materials. Especially, the fabrication speed must be increased to create structures of clinically relevant sizes. Even the cells used for bioprinting applications are robust enough to survive the bioprinting process and withstand the physiological stresses; a large cell construct in an open environment may not survive a slow and therefore long printing process. For bioprinting, well-characterized and reproducible source of cells is required and any cell type selected for printing should be able to be proliferated into sufficient numbers for printing. Additionally, the proliferation

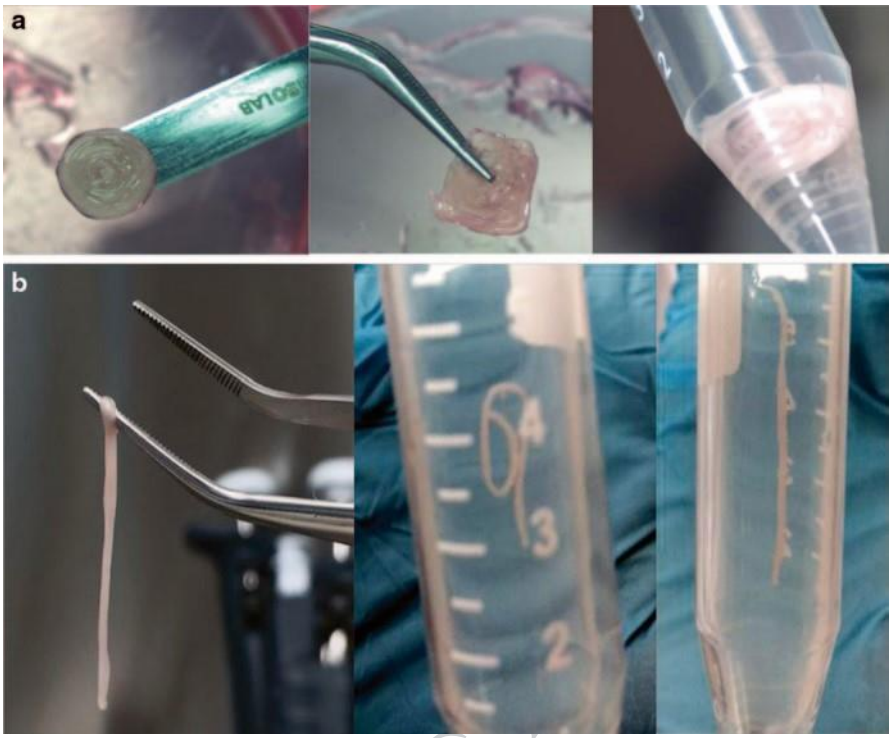


Fig. 10 Bioprinted 3D totally biological tissue constructs without any biomaterial. **a** *Circular* and *square* shaped bioprinted with MOVAS, HUVEC and NIH 3T3 multicellular aggregates. **b** Stripe shaped bioprinted with HUVEC/HDF cell aggregates [83]

rate and the differentiation with small molecules or other factors should be controllable. Furthermore, sufficient vascularization and capillaries/microvessels are required for long-term viability of the fabricated construct and for tissue perfusion, respectively. The engineered structure should have suitable mechanical properties for physiological pressures and for surgical connection in case of transplantation. Bioreactors are used to maintain tissues in vitro and to provide maturation factors as well as physiological stressors for assembly, differentiation and ECM production prior to in vivo implantation.

Stem cells such as mesenchymal, induced pluripotent and embryonic stem cells could be a great source for bioprinting. Especially, printing differentiated or progenitor cells precisely and spatially could lead to multi-functional tissue constructs or even organs. However several challenges need to be overcome. First, bioprinting with stem cells requires large number of cells. Culturing this amount of stem cells could be really difficult. Especially, growing large number of stem cells on a feeder layer or special growth medium is really challenging. Even after culturing enough number of stem cells, bioprinting stem cells precisely and spatially accurate manner would require highly precise and special bioprinters. During bioprinting, the effect of compression on the viability and differentiation of stem cells should be considered. Since the stem cells are more susceptible to bioprinting conditions, more gentle and short printing procedures should be developed. Although the current 3D

bioprinting technology shows a great deal of promise to generate 3D layered constructs using live mixed cell populations, there is still a long way to go to create a fully-functional organ.

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