Three-Dimensional Bioprinting in Regenerative Medicine

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1 Introduction

In 1993, Langer and Vacanti first defined tissue engineering as an approach of seeding cells to the pre-formed solid and rigid biomaterial scaffolds for tissue fabrication [1]. However, the term of tissue engineering was introduced even earlier by Dr. Fung of the University of California at San Diego in 1985 [2]. In conventional tissue engineering approach, the autologous cells are first cultured in monolayer to expand the cell numbers. The cultured cells are then collected and seeded into the pre-formed porous scaffolds. The scaffolds used for tissue engineering should be biocompatible and degradable. The seeded cells on the scaffold are kept alive and can penetrate or migrate inside the scaffolds instead of staying on the surface. Therefore, the tissue engineering scaffolds should be highly porous with inter-connected pores and safe to the seeded cells. In addition, a customized bioreactor mimicking *in vivo* environment and stimulation is usually desired to maturate the fabricated organ construct before implantation. The goal of tissue engineering is to create the replacements for the lost or diseased organs and eventually solve the crisis of organ donor shortage. Some successes have been achieved in engineering thin and hollow organs [3, 4]. These tissues can survive *in vivo* through nutrients diffusion from the

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host vasculature. However, more than 90% demanding organs are thick and complex, such as kidney, liver, and heart (OPTN & SRTR Annual Data Report 2010). When the size of engineered tissue exceeds 400 μm in any dimension, it will surpass the oxygen diffusion limitation. In this case, functional vasculature must be enabled in the engineered constructs to supply the cells with oxygen and nutrients, and also to remove the waste products generated by the tissue [5]. Unfortunately, the conventional tissue engineering approaches failed to generate these thick, complex and vascularized tissues due to these limitations:

a. The effectiveness of cell seeding and penetration to the biomaterial scaffold is still limited. Although scaffold design has been significantly improved to enhance the cell seeding and migration, the uniform of tissue formation or maturation throughout the scaffold is still far from optimal [6–8].
b. Organs with complex structure are usually composed by multiple cell types and biological factors. However, the precise delivery of cells and biological factors to the desired 3D positions is still far from being resolved.
c. Thick tissues possess complex vascular system [9], which should be enabled within the scaffold. However, the conventional tissue engineering approach has difficulties to construct vascular system within the pre-formed 3D scaffolds.

Additive manufacturing or 3D printing is driving significant innovations in manufacturing, engineering, education and medicine. 3D bioprinting, which was derived by combing biotechnology and 3D printing, is promising to solve these critical issues mentioned above. As one of the most advanced enabling technology in tissue engineering, 3D bioprinting combines solid freeform fabrication and precise placement of cells and other biological factors to the desired 2D and 3D positions. It is described as a precise approach of delivering biomaterials, cells and supporting biological factors to the targeted locations with spatial control to fabricate functional 3D constructs. The key elements of realizing functional bioprinting include capacity of precise positioning, printable biomaterials, and cell sources. In addition, vascularization, innervation, and maturation are also crucial to engineer functional tissues. Bioprinting has promising applications in the field of regenerative medicine, personalized medicine, clinical diagnosis and medicinal development. Although the concept of bioprinting was introduced more than 10 years ago, the current progress of bioprinting is still in its initial stage and far from industrial applications.

The three most common bioprinting mechanisms are inkjet bioprinting [10–21], extrusion bioprinting [22–24], and laser bioprinting [25–27]. Extrusion bioprinting is a contact printing process and typically uses temperature-controlled polymerized materials for scaffold fabrication. This printing process usually causes high cell casualty so it is frequently used in acellular material printing. Sometimes extrusion bioprinting also applies in cell spheroids deposition. This approach does not demand high printing resolution and it is more likely a dispensing process instead of printing. In addition, this approach has difficulties of managing singe cell which is critically important for neuron regeneration or fabricating functional tissues with

higher degree of cell organization of specific anatomic structures [28, 29]. Laser bioprinting offers higher cell viability and printing resolution. Instead of moving cells directly, laser bioprinting uses laser energy to vaporize the solution of biological samples and eject the remaining substances [25]. This approach may cause overdrying leading to the failure for biological systems. Furthermore, the much higher cost of laser printing equipment, as well as the exceedingly low printing efficiency inhibit its application in regenerative medicine [30, 31]. Thus it is mostly applied in the basic research field when single or multiple cell manipulation is needed, instead of tissue construction or other clinical applications demanding higher throughput.

Inkjet printing is also known as drop-on-demand printing. It is a non-contact printing technology that reproduces digital patterns onto a substrate using tiny ink drops [32]. Inkjet printing is based on thermal, piezoelectric, or electromagnetic mechanisms [33]. In thermal inkjet printers, small air bubbles generated by heating in the printhead collapse to provide pressure pulses to eject tiny drops out of the nozzle [34–36]. The droplet size varies from 10 to 150 pL, which is determined by the applied temperature gradient, frequency of current pulses, and viscosity of the ink [34–36]. As for the piezoelectric inkjet printers, the actuator of polycrystalline piezoelectric ceramic in each nozzle provides the transient pressure to eject the ink drops [37]. These printing technologies have already been widely used in printing electronic materials and complex integrated circuits in industry [38]. Although biological substances are usually considered sensitive, fragile DNA molecules have been directly printed using commercially available inkjet printers for high-density DNA microarray fabrication [39, 40]. Challenges still exist when printing cells using inkjet technology. The working frequency of piezoelectric inkjet printers is 15–25 kHz, which is within the well-documented sonification damage to the cell membrane [41]. Although the heating element in thermal inkjet printers raises the local temperature to 300 °C [36], the ejected mammalian cells are only heated for 2 μs with a temperature raise of 4–10 °C above ambient and an average cell viability over 90% [11]. In addition, the development, operation, and maintenance of thermal inkjet is usually more convenient than piezoelectric printing. Therefore, the majority successes in tissue bioprinting are based on thermal inkjet printing instead of piezoelectric inkjet printing. One limitation of inkjet bioprinting is the strict requirement of bioink viscosity. This issue has recently been minimized by using water based biomaterials or combination of various printing technologies. Water based bioink allows the printer to freely deliver cells from single to multiple cells by simply adjusting the bioink concentration and the digital patterns. Cells are usually well-protected in the aqueous environment during the printing process therefore it is assumed to be the safest strategy to deliver living systems.

Based on the discussion above, bioprinting based on thermal inkjet printing is so far the most appropriate approach for regenerative medicine and tissue engineering applications. Researchers keep developing this technology as an optimal approach for cell delivery and scaffold fabrication. Therefore, we will mainly focus on the advancement and applications using this bioprinting technology in this chapter.

2 Cell Printing

Although the term of bioprinting can be used on printing any biological systems, it usually involves living cell patterning in tissue engineering and regenerative medicine applications. Therefore, the capacity of printing living cells is critical to evaluate a bioprinting platform or system.

Although bioprinting based on thermal inkjet printing technology has many successful applications, there were concerns that the printing process may cause damages or cell death. The small printhead nozzle size is necessary for high printing resolution. Due to the thermal heat and mechanical stresses applied to the cells during printing, it is possible that the cells may be damaged or their phenotype may be altered [42]. Therefore, a comprehensive evaluation of cell viability, apoptosis, heat shock proteins production, cell membrane damages of the printed cells is desired to confirm the bioprinting safety. Using a modified Hewlett-Packard (HP) thermal inkjet printer, cell viability at various cell concentrations was between 85 and 95%. No significant difference in apoptosis and heat shock protein expression was observed between printed and non-printed cells [11]. Quantitative cell seeding can be achieved by adjusting the cell concentration in bioink. The inkjet printing process does alter the cell membrane of printed cells. Fluorescent labeled dextran dye with molecular weight (MW) up to 40,000 can penetrate into the printed cells. No dye was found in the non-printed cells even with the lowest MW (3000) (Fig. 1). The cell membrane pore size was estimated as 105 Å according to the Stokes diameter of these dye molecules [11].

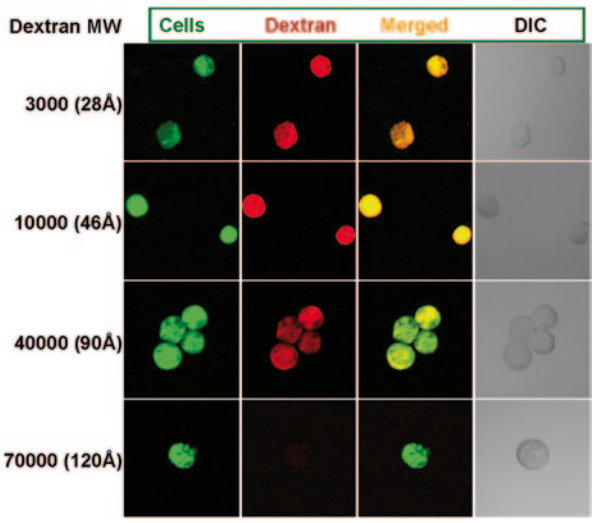

Fig. 1 Printed cell membrane pore evaluation using dextran dye penetration study

The pores developed during printing were transient and can be repaired by the cells in just a couple hours. The transient nature of the cell membrane pores as well as the self-repair mechanism can be utilized for targeted gene delivery during the printing process [11, 43].

3 Microvasculature Printing

Although the concept of tissue engineering was introduced more than two decades ago, the current tissue engineering strategies still cannot create fully vascularized tissue constructs. The current tissue engineering paradigm is that successfully engineered thick tissues must include vasculature. As biological approaches alone, such as VEGF or co-culture of vessel cells, have fallen short of their promises, one may look for an engineering approach to build microvasculature. Layer-by-layer approaches for customized fabrication of cell and scaffold constructs have shown great potential in building complex 3D structures [44]. With the advent of cell printing, one may be able to build precise human microvasculature with suitable bioink. Human microvascular endothelial cells (HMVECs) and fibrin scaffold were utilized as bioink for microvasculature construction [12].

A standard inkjet printer was modified to simultaneously deposit HMVECs and fibrin scaffold to form the microvasculature. The bioink and biopaper components for fibrin bioprinting were carefully evaluated for optimal condition of simultaneous deposition of cells and scaffold [12]. The printed microvasculature was incubated for 10–15 min after the printing to finalize the crosslinking and enhance the cell attachment.

After 3 weeks in culture, the printed HMVECs aligned themselves in the fibrin channel and proliferated to form a confluent lining. Confocal laser scanning images at the z-axis demonstrated tubular structure of the printed human microvasculature. The endothelial cells were forming a vessel-like structure in the printed fibrin channel [12]. This demonstrates the printed and proliferated endothelial cells possessed the crucial angiogenesis function. The simultaneous deposition of endothelial cells and fibrin using thermal inkjet printing technology can be used for human microvasculature fabrication (Fig. 2).

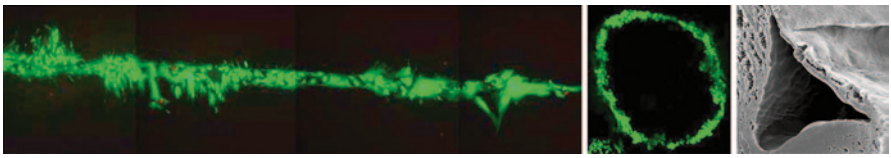

Fig. 2 Inkjet bioprinted human microvasculature using HMVEC and fibrin

4 Muscle Printing

Biological microelectromechanical system (Bio-MEMS) devices conjugated with biological components are promising for the development of novel bioengineering microdevices, such as motors and actuators [45], heart pumps [46], and biosensors [47]. Muscle cells have been widely used in these applications by generating force activated by actin-myosin motors regulated by excitation-contraction coupling [48]. These muscle powered microdevices utilizing energy generated by biochemical reaction are promising to save energy, resources, and spaces [49]. C2C12 skeletal muscle cells possess the advantages of infinite proliferation and differentiation into multinucleated myotubes [50]. As a well established cell line, the overall properties of C2C12 cells cultured and differentiated *in vitro* have been tested to closely mimic the properties of skeletal muscle *in vivo* [51]. Although C2C12 cells have been widely used to incorporate with bio-microdevices for many applications, it is important that the muscle cells and microdevices are consistently conjugated to produce reliable and reproducible results. The traditional methodology for Bio-MEMS fabrication is to manually seed cells on or into the microdevices [52]. However, the randomly deposited cells through this approach were uneven and further affected the cell proliferation and differentiation. Therefore, it is critical to incorporate a precise cell seeding technology to develop the Bio-MEMS constructs with consistent cell arrangement.

Bioprinting was able to print and align C2C12 cells onto the tiny cantilevers at a resolution at 300 dpi (85 μm). In order to control the cell proliferation and differentiation with minimal variations, same amount of cells were printed to evenly cover each cantilever of the microdevices. The viability of printed C2C12 cells was 91.2 ± 2.6 % and the printed cells aligned closely with each other forming confluent myotubes on almost all the cantilevers. Conjugated myotube and cantilever constructs responded synchronously to the electric pulses of 2 V with 40 ms duration up to 5 Hz (Fig. 3). This showed the bioprinted microdevices possessed equal

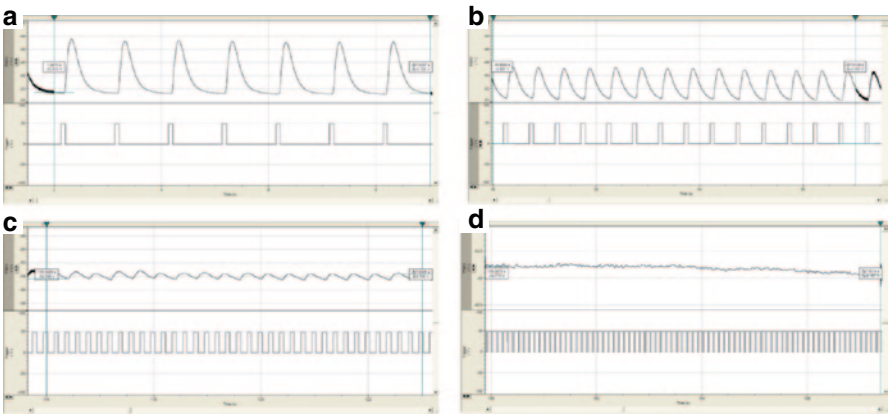

Fig. 3 Printed myotube construct responds synchronously to the applied electronic field with 2 V and 40 ms duration for each pulse. **a** 1 Hz. **b** 2 Hz. **c** 5 Hz. **d** 10 Hz.

or even better physiological properties comparing to the conventionally fabricated constructs in term of the spontaneous responses to the stimulation with significantly less culture time. Moreover, the bioprinted myotubes can also be used for muscle exercise studies with electric stimulations at various frequencies, which demonstrates the versatility of this work.

5 Cartilage Printing

Cartilage defects resulting from osteoarthritis, aging, and joint injury are a major cause of joint pain and chronic disability [53]. Mature cartilage cannot heal spontaneously because of its avascular, aneural, and alymphatic nature. The most common clinical treatments for cartilage repair include microfracture, osteochondral transfer, and autologous chondrocyte implantation. All these invasive and complicated treatments are still not able to restore the long lasting healthy cartilage [54]. Although articular cartilage was predicted to be one of the first tissues to be successfully engineered [55], the current cartilage tissue engineering strategies still cannot fabricate new tissue that is indistinguishable from native cartilage with respect to the zonal organization, extracellular matrix (ECM) composition, and mechanical properties [56]. In addition, most current cartilage repair strategies involve removing healthy cartilage tissue around the lesion site to create artificial defects for further treatment [57]. This procedure in fact causes additional necrosis to the existing healthy cartilage and leads to ultimate cartilage degeneration and failure of implanted tissue [58].

Inkjet bioprinting is able to directly repair cartilage tissue with closely mimicked native cartilage anatomy to the lesion site without additional damage. The ideal implanted tissue is expected to integrate with existing native cartilage and to repair lesions of different sizes and thicknesses. The multifaceted nature of this challenge requires a technique adaptable to variable physical dimensions and properties for tissue repair; bioprinting technology, based on inkjet printing, provides the necessary capabilities.

A standard thermal inkjet printer was modified to precisely deposit human articular chondrocytes and poly(ethylene) glycol dimethacrylate (PEGDMA; MW, 3400) layer-by-layer into a cartilage defect within an osteochondral plug for cartilage repair (Fig. 4). For a representative defect of 4 mm diameter and cartilage thickness of 2 mm, a nominal 0.23 μL of bioink estimated to contain 1140 human chondrocytes (5×10^6 cells/mL) was printed and photopolymerized for each layer to repair the cartilage defect in a layer-by-layer assembly. The thickness of each printed layer was about 18 μm. Total firing time of printhead was 1.1 s and the whole printing process completed less than 2 min. Compared to manual zonal cartilage fabrication which requires at least 11 min for UV exposure [59], bioprinting reduced UV exposure to the cells by 80%. The viability of human chondrocytes printed with simultaneous photopolymerization increased 40% than that when exposed to the same UV light source continuously for 10 min in manual fabrication [60].

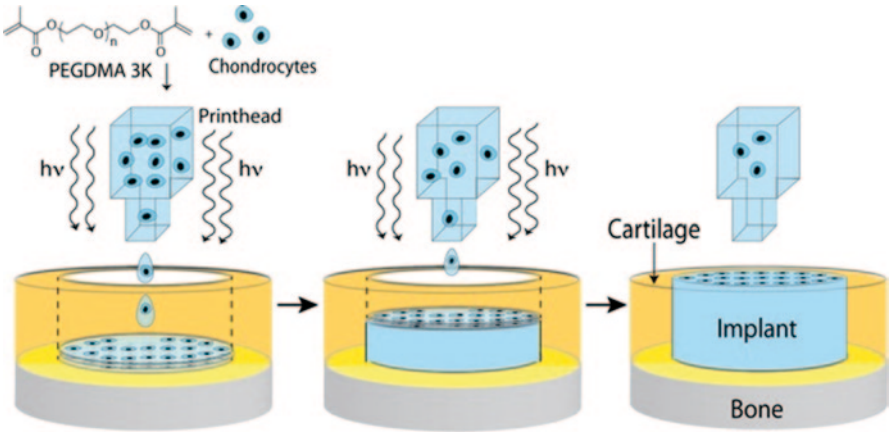

Fig. 4 Schematic of bioprinting cartilage with simultaneous photopolymerization

Printed cartilage implant attached firmly with existing tissue and greater proteoglycan deposition was also observed at the interface of implant and native cartilage. Printed cartilage in 3D biopaper had elevated glycosaminoglycan (GAG) content comparing to that without biopaper. This study indicates the importance and feasibility of direct cartilage repair and bioprinting successfully controlled placement of individual cells, preserved cell viability, maintained chondrogenic phenotype, and demonstrated integration with host tissue.

6 Bone Printing

Although bone is well known for its self-healing capacities [61], the body cannot completely heal the bone defect without intervention when it is beyond the critical size [62, 63]. Large-scale bone loss resulting from tumor resections and high impact trauma is the major cause for bone repair and implantation in clinic. The availability and functionality of bone autografts and allografts are limited to restore the normal operations. The inert implants fail over time due to repetitive loading. Therefore, tissue engineered bone which can ideally be remodeled into new bone to restore, maintain or improve its functions is becoming increasingly attractive [64].

Thermal inkjet bioprinting has been developed as an enabling technology to simultaneously deposit cells, growth factors, and biomaterial scaffolds to the desired 2D and 3D locations [10–14, 17–21]. The ejected ink drops through the nozzles are smaller than 0.03 mm in diameter, which guarantees excellent printing resolution [34, 35]. Many inkjet printed scaffolds were natural hydrogels for the enhanced

biocompatibility to the cells [12, 13, 65–67]. These scaffolds usually lacked mechanical strength due to the properties of material and crosslinking methods, limiting their applications to soft tissues. Previous work also showed bone grafts created using natural hydrogels such as fibrin or alginate [68–71]. Although the cells proliferated and differentiated well in these natural hydrogels, the compressive modulus of these scaffolds is less than 5 kPa even after 4 weeks in culture, which is not ideal for bone tissue engineering [69–71].

A 3D bioprinting platform with simultaneous photopolymerization using a synthetic polymeric hydrogel was recently developed. The compressive modulus of the printed PEGDMA using layer-by-layer assembly exceeds 500 kPa, which is 100 times more than the compressive modulus of the natural hydrogels [14, 21] and in the same order of magnitude as human musculoskeletal tissue [72]. In addition, PEG hydrogel has been demonstrated to maintain cell viability and promote ECM production [14, 21, 73, 74].

Bone marrow derived stem cells are capable to migrate to the skeletal sites, proliferate and differentiate at the local injured area. Isolated human mesenchymal stem cells (hMSCs) can maintain their osteogenic potential during monolayer cell expansion *in vitro* [75]. These cells are therefore commonly used to reconstruct skeletal tissues in orthopedic tissue engineering [76–78]. hMSCs isolated from bone marrow or adipose tissue can be induced for osteogenic differentiation and form bone tissue when stimulated by ceramic scaffold [79–81]. Bioactive glass (BG) and hydroxyapatite (HA) were also reported to promote bone tissue formation [70, 82].

In bone printing, the approaches mentioned above were integrated into a novel bioprinting setup, in which hMSCs and PEGDMA combined with BG or HA or both BG and HA nanoparticles were simultaneously printed to form the homogeneous bone constructs in a layer-by-layer approach. Biochemical analysis showed significantly higher total collagen production and alkaline phosphatase (ALP) activity in hMSCs printed within PEG-HA scaffold. The higher collagen production in PEG-HA scaffold was also observed in histology studies (Fig. 5), which was consistent with the previous work by Patel et al. that HA presence increased cell ALP activity and promoted osteogenesis [83]. Collectively, HA in PEG hydrogel maintained hMSCs viability, promoted hMSCs osteogenic differentiation and biosynthetic function.

This work demonstrates the feasibility of fabricating a neobone tissue by delivering hMSCs and osteogenic factors such as HA and BG nanoparticles in strong PEG scaffold for bone tissue engineering. Using layer-by-layer assembly, the deposited hMSCs were fixed at their initially deposited positions using simultaneous photopolymerization with reduced phototoxicity. HA in scaffold significantly stimulated hMSCs osteogenic differentiation as well as osteogenic ECM production with minimal cell toxicity. Combining with previous success in cartilage bioprinting [14], it is promising to construct osteochondral interface, which is one of the most important and difficult subjects in bone tissue engineering [84].

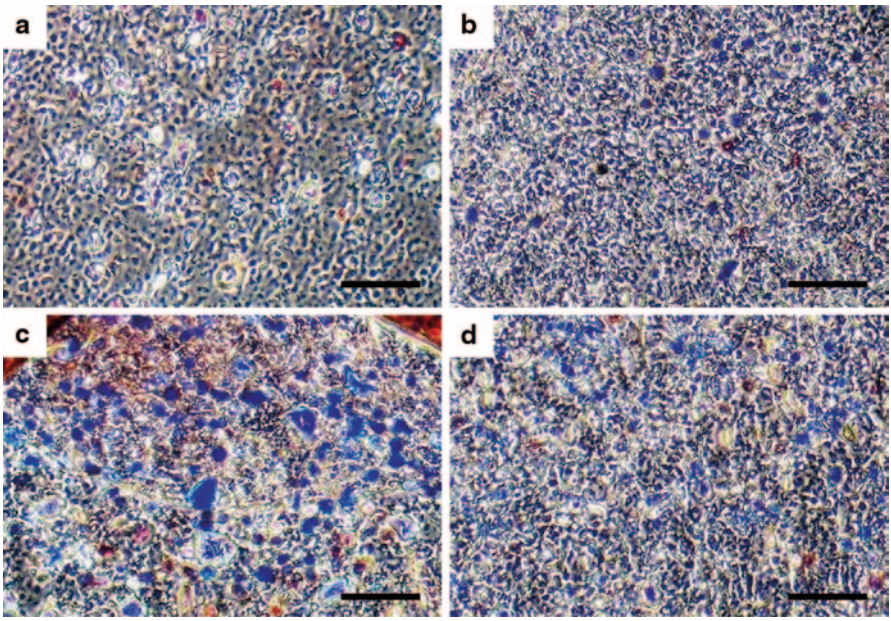

Fig. 5 Masson's trichrome staining for collagen production of hMSCs under osteogenic differentiation in various scaffolds after 21 days in culture. **a** PEG. **b** PEG-BG. **c** PEG-HA. **d** PEG-BG-HA. Scale bars: 50 μm

7 The Future

Taken together, bioprinting based on thermal inkjet printing demonstrates great feasibility of printing living systems and the flexibility of printing various subjects from soft to hard tissues with minimal side effects. In fact, the benign effects to the printed cells can be used for many other attractive applications, such as gene transfection and targeted drug delivery. The bioprinting system is versatile for 2D and 3D tissue application as well as avascular and vascular tissue construction. One promising clinical application is to develop a hand-held printer or printhead with digital control for direct tissue repair. By using 3D reconstructions of scanned lesions, bioprinting is able to precisely deliver cells, growth factors, and biomaterial scaffolds to repair the lesion with various shape and thickness. One promising direction is to combine the bioprinting approaches based on various mechanisms to meet the different challenges. Ultimately, the successful application in microvasculature fabrication also revealed the bioprinting may be the only solution to engineer thick and complex tissues with fully functional vasculature and innervation.

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