REVIEW

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3D bioprinting of tissues and organs

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Additive manufacturing, otherwise known as three-dimensional (3D) printing, is driving major innovations in many areas, such as engineering, manufacturing, art, education and medicine. Recent advances have enabled 3D printing of biocompatible materials, cells and supporting components into complex 3D functional living tissues. 3D bioprinting is being applied to regenerative medicine to address the need for tissues and organs suitable for transplantation. Compared with non-biological printing, 3D bioprinting involves additional complexities, such as the choice of materials, cell types, growth and differentiation factors, and technical challenges related to the sensitivities of living cells and the construction of tissues. Addressing these complexities requires the integration of technologies from the fields of engineering, biomaterials science, cell biology, physics and medicine. 3D bioprinting has already been used for the generation and transplantation of several tissues, including multilayered skin, bone, vascular grafts, tracheal splints, heart tissue and cartilaginous structures. Other applications include developing high-throughput 3D-bioprinted tissue models for research, drug discovery and toxicology.

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The invention of woodblock printing, and the subsequent development of the industrial-scale printing press in the 15th century, facilitated rapid reproduction of text and images and the dissemination of information. Printing had a revolutionary effect on society, affecting education, politics, religion and language across the globe. Over the past few decades, printing technology has advanced from two-dimensional (2D) printing to an additive process in which successive layers of material are distributed to form 3D shapes[1,2]. The production of 3D structures with complex geometries by printing is being applied both to enable rapid prototyping and manufacturing in industry and to the production of personalized consumer products in the home, such as bicycle parts, jewelry and electrical components[3]. In addition to applications in the manufacturing and consumer sectors, 3D printing is transforming science and education. For example, archeologists and anthropologists produce replicas of rare artifacts or fossils that can be held, shared and distributed[4]. Just as Watson and Crick modeled the structure of DNA using a ball-and-stick model, 3D printing is now being used to model complex molecules and protein interactions, and to fashion customized laboratory tools[5–7]. 3D printing empowers students to design, visualize, hold and test their ideas in real space[8].

3D printing was first described in 1986 by Charles W. Hull. In his method, which he named 'sterolithography', thin layers of a material that can be cured with ultraviolet light were sequentially printed in layers to form a solid 3D structure[9]. This process was later applied to create sacrificial resin molds for the formation of 3D scaffolds from biological materials. The development of solvent-free, aqueous-based systems enabled the direct printing of biological materials into 3D scaffolds that could be used for transplantation with or without seeded cells[10]. The next step was 3D bioprinting as a form of tissue engineering, made possible by recent advances in 3D printing technology, cell biology and materials science. A related development was the application of 3D printing to produce medical devices such as stents and splints for use in the clinic[11].

In 3D bioprinting, layer-by-layer precise positioning of biological materials, biochemicals and living cells, with spatial control of the placement of functional components, is used to fabricate 3D structures. There are several approaches to 3D bioprinting, including biomimicry, autonomous self-assembly and mini-tissue building blocks. Researchers are developing these approaches to fabricate 3D functional living human constructs with biological and mechanical properties suitable for clinical restoration of tissue and organ function. One important challenge is to adapt technologies designed to print molten plastics and metals to the printing of sensitive, living biological materials. However, the central challenge is to reproduce the complex micro-architecture of extracellular matrix (ECM) components and multiple cell types in sufficient resolution to recapitulate biological function.

Here we review the application of 3D bioprinting to tissue and organ engineering. We first consider the main strategies for printing tissue constructs. Next, we describe the different types of bioprinters and their influence on the printed tissue construct. Finally, we discuss the stepwise process of printing a tissue, the limitations of current technologies and the challenges for future research.

3D bioprinting approaches

3D bioprinting is based on three central approaches: biomimicry, autonomous self-assembly and mini-tissue building blocks. We discuss these in more detail below.

Biomimicry. Biologically inspired engineering has been applied to many technological problems, including flight[12], materials research[13], cell-culture methods[14] and nanotechnology[14]. Its application to 3D bioprinting involves the manufacture of identical reproductions of the cellular and extracellular components of a tissue or organ[15]. This can be achieved by reproducing specific cellular functional components

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of tissues, for example, mimicking the branching patterns of the vascular tree or manufacturing physiologically accurate biomaterial types and gradients. For this approach to succeed, the replication of biological tissues on the microscale is necessary. Thus, an understanding of the microenvironment, including the specific arrangement of functional and supporting cell types, gradients of soluble or insoluble factors, composition of the ECM as well as the nature of the biological forces in the microenvironment is needed. The development of this knowledge base will be important to the success of this approach and can be drawn from basic research in fields of engineering, imaging, biomaterials, cell biology, biophysics and medicine.

Autonomous self-assembly. Another approach to replicating biological tissues is to use embryonic organ development as a guide. The early cellular components of a developing tissue produce their own ECM components, appropriate cell signaling and autonomous organization and patterning to yield the desired biological micro-architecture and function[16,17]. A 'scaffold-free' version of this approach uses self-assembling cellular spheroids that undergo fusion and cellular organization to mimic developing tissues. Autonomous self-assembly relies on the cell as the primary driver of histogenesis, directing the composition, localization, functional and structural properties of the tissue[18,19]. It requires an intimate knowledge of the developmental mechanisms of embryonic tissue genesis and organogenesis as well as the ability to manipulate the environment to drive embryonic mechanisms in bioprinted tissues.

Mini-tissues. The concept of mini-tissues is relevant to both of the above strategies for 3D bioprinting. Organs and tissues comprise smaller, functional building blocks[20,21] or mini-tissues. These can be defined as the smallest structural and functional component of a tissue, such as a kidney nephron. Mini-tissues can be fabricated and assembled into the larger construct by rational design, self-assembly or a combination of both. There are two major strategies: first, self-assembling cell spheres (similar to mini-tissues) are assembled into a macro-tissue using biologically inspired design and organization[20,21]; second, accurate, high-resolution reproductions of a tissue unit are designed and then allowed to self-assemble into a functional macro-tissue. Examples of these approaches include the self-assembly of vascular building blocks to form branched vascular networks[22,23] and the use of 3D bioprinting to accurately reproduce functional tissue units to create 'organs-on-a-chip', which are maintained and connected by a microfluidic network for use in the screening of drugs and vaccines or as in *in vitro* models of disease[24–26].

Combinations of the above strategies are likely to be required to print a complex 3D biological structure with multiple functional, structural and mechanical components and properties. The main steps in the bioprinting process are imaging and design, choice of materials and cells, and printing of the tissue construct (**Fig. 1**). The printed construct is then transplanted, in some cases after a period of *in vitro* maturation, or is reserved for *in vitro* analysis.

Imaging and digital design

An essential requirement for reproducing the complex, heterogeneous architecture of functional tissues and organs is a comprehensive understanding of the composition and organization of their components. Medical imaging technology is an indispensable tool used

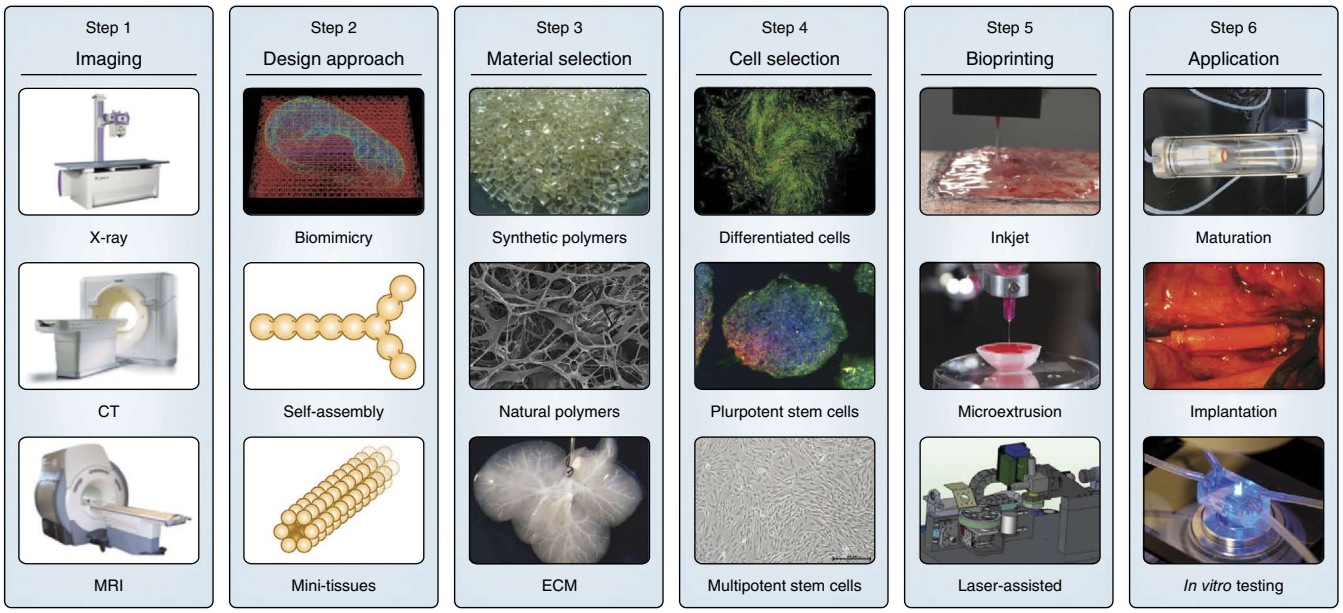

Figure 1 A typical process for bioprinting 3D tissues. Imaging of the damaged tissue and its environment can be used to guide the design of bioprinted tissues. Biomimicry, tissue self-assembly and mini-tissue building blocks are design approaches used singly and in combination. The choice of materials and cell source is essential and specific to the tissue form and function. Common materials include synthetic or natural polymers and decellularized ECM. Cell sources may be allogeneic or autologous. These components have to integrate with bioprinting systems such as inkjet, microextrusion or laser-assisted printers. Some tissues may require a period of maturation in a bioreactor before transplantation. Alternatively the 3D tissue may be used for *in vitro* applications. Self-assembly image is reprinted from Mironov, V. *et al*. Organ printing: tissue spheroids as building blocks. *Biomaterials* **30**, 2164–2174 (2014), with permission from Elsevier; mini-tissue image is reprinted from Norotte, C. *et al*. Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials* **30**, 5910–5917 (2009), with permission from Elsevier; the ECM image is adapted from ref. 132, with permission from Wiley; differentiated cells image is reprinted from Kajstura, J. *et al*. Evidence for human lung stem cells. *N. Engl. J. Med.* **364**, 1795–1806 (2011), Massachusetts Medical Society, with permission from Massachusetts Medical Society; laser-assisted image is reprinted from Guillemot, F. *et al*. High-throughput laser printing of cells and biomaterials for tissue engineering, *Acta Biomater.* **6**, 2494–2500 (2010), with permission from Elsevier.

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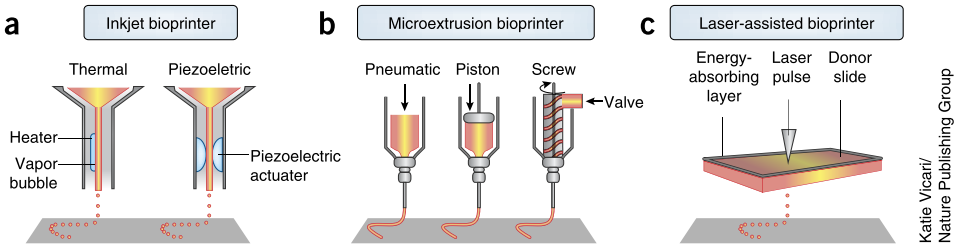

Figure 2 Components of inkjet, microextrusion and laser-assisted bioprinters. (**a**) Thermal inkjet printers electrically heat the printhead to produce air-pressure pulses that force droplets from the nozzle, whereas acoustic printers use pulses formed by piezoelectric or ultrasound pressure. (**b**) Microextrusion printers use pneumatic or mechanical (piston or screw) dispensing systems to extrude continuous beads of material and/or cells. (**c**) Laser-assisted printers use lasers focused on an absorbing substrate to generate pressures that propel cell-containing materials onto a collector substrate. Figure adapted from ref. 146.

by tissue engineers to provide information on 3D structure and function at the cellular, tissue, organ and organism levels. These technologies include most noninvasive imaging modalities, the most common being computed tomography (CT) and magnetic resonance imaging (MRI). Computer-aided design and computer-aided manufacturing (CAD-CAM) tools and mathematical modeling are also used to collect and digitize the complex tomographic and architectural information for tissues.

CT imaging, used for both diagnostics and interventional procedures, is based on the variable absorption of X-rays by different tissues. The X-ray source rotates around the object, and as the X-ray beam penetrates the body, sensors measure the transmitted beam intensity and angle, and record the data as a compilation of pixels that represent a small volume (voxel) of tissue[27]. This imaging modality produces closely spaced axial slices of tissue architecture that, after surface rendering and stereolithographic editing, fully describe the volume of tissue.

A second approach, MRI, also can provide high spatial resolution in soft tissue, with the advantage of increased contrast resolution, which is useful for imaging soft tissues in close proximity to each other, without exposure to ionizing radiation. MRI uses nuclear magnetic resonance: a strong magnetic field causes a small fraction of nuclei in the tissue being imaged to align themselves with the magnetic field[28]. Changes to energy states of nuclei produce radiofrequency signals, which can be measured with receiver coils. The contrast of biological structures can be greatly increased with the use of contrast agents such as barium[29] or iodine[30] for CT scans and iron oxide[31], gadolinium[32] or metalloproteins[33] for MRI scans. These agents attenuate X-rays or enhance magnetic resonance signals that are commonly used to highlight structures, such as blood vessels, which otherwise would be difficult to delineate from their surroundings.

Once raw imaging data have been acquired from these imaging modalities, the data must be processed using tomographic reconstruction to produce 2D cross-sectional images. 3D anatomical representations can be produced for further analysis or modification. This process has been described as the transformation of 'analytical anatomy' into 'synthetic anatomy'[34]. One method to generate computer-based 3D models of organ or tissue architectures is to use CAD-CAM and mathematical modeling techniques[35]. The 3D anatomical representation produces views of organ anatomy while retaining the image-voxel information that can be used for volume rendering, volumetric representation and 3D image representation. Reconstructed images or models can be viewed in multiple ways, including as contour stacks, as wire-frame models, shaded models or solid models with variable lighting, transparency and reflectivity[36].

If the aim is to produce an accurate reproduction of the imaged organ or tissue, 2D cross-sections or 3D representation can be used directly for bioprinting applications. Alternatively, a direct copy of a patients' own organ may not be desirable (due to disease or injury) or might not be economically feasible for large-scale production. In these situations, computer-based models may entirely or partially contribute to anatomical structural design, analysis and simulation[37]. Additionally, computer modeling can assist in predicting mechanical and biochemical properties of fabricated tissue constructs[37–39]. To date, CT and MRI data have been used most often in regenerative medicine to provide specific measurements of tissue dimensions to aid the design of a bioprinted construct.

The completed tissue or organ model is interfaced with numerically controlled bioprinting systems for prototyping and manufacturing. This is achieved by reversing the 2D to 3D reconstruction, such that the 3D-rendered model is divided into thin 2D horizontal slices (with customizable size and orientation) that are imported into the bioprinter system. The anatomical and architectural information contained in the 2D horizontal slices provides the bioprinting device with layer-by-layer deposition instructions. Variations in the available bioprinting technologies also affect tissue and organ design. Some bioprinting systems deposit a continuous bead of material to form a 3D structure. Other systems deposit multiple materials in short interrupted or defined spaces. Tissue design must take into account the capabilities and properties of the bioprinting systems, which we discuss next.

Tissue bioprinting strategies

The main technologies used for deposition and patterning of biological materials are inkjet[40–43], microextrusion[44–46] and laser-assisted printing[47–49] (**Fig. 2**). Different features of these technologies (**Table 1**) should be considered in light of the most important factors in 3D bioprinting, which are surface resolution, cell viability and the biological materials used for printing.

Inkjet bioprinting. Inkjet printers (also known as drop-on-demand printers) are the most commonly used type of printer for both nonbiological and biological applications. Controlled volumes of liquid are delivered to predefined locations. The first inkjet printers used for bioprinting applications were modified versions of commercially available 2D ink-based printers[50,51]. The ink in the cartridge was replaced with a biological material, and the paper was replaced with an electronically controlled elevator stage to provide control of the z axis[40,50] (the third dimension in addition to the x and y axes). Now, inkjet-based bioprinters are custom-designed to handle and print biological materials at increasing resolution, precision and speed. Inkjet printers use thermal[43] or acoustic[50,52,53] forces to eject drops of liquid onto a substrate, which can support or form part of the final construct.

Thermal inkjet printers function by electrically heating the print head to produce pulses of pressure that force droplets from the nozzle. Several studies have demonstrated that this localized heating, which can range from 200 °C to 300 °C, does not have a substantial impact either on the stability of biological molecules, such as DNA[52,53], or on the viability or post-printing function of mammalian

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cells[42,54]. It has been demonstrated that the short duration of the heating (~2 μs) results in an overall temperature rise of only 4–10 °C in the printer head[55]. The advantages of thermal inkjet printers include high print speed, low cost and wide availability. However, the risk of exposing cells and materials to thermal and mechanical stress, low droplet directionality, nonuniform droplet size, frequent clogging of the nozzle and unreliable cell encapsulation pose considerable disadvantages for the use of these printers in 3D bioprinting.

Many inkjet printers contain a piezoelectric crystal that creates an acoustic wave inside the print head to break the liquid into droplets at regular intervals. Applying a voltage to a piezoelectric material induces a rapid change in shape, which in turn generates the pressure needed to eject droplets from the nozzle[56]. Other inkjet printers use an acoustic radiation force associated with an ultrasound field to eject liquid droplets from an air-liquid interface[57,58]. Ultrasound parameters, such as pulse, duration and amplitude, can be adjusted to control the size of droplets and the rate of ejection. Advantages of acoustic inkjet printers include the capability to generate and control a uniform droplet size and ejection directionality as well as to avoid exposure of cells to heat and pressure stressors[59–61]. Additionally, the sheer stress imposed on cells at the nozzle tip wall can be avoided by using an open-pool nozzle-less ejection system[58]. This reduces the potential loss of cell viability and function, and avoids the problem of nozzle clogging. Acoustic ejectors can be combined as multiple ejectors in an adjustable array format, facilitating simultaneous printing of multiple cell and material types[62]. Even so, there remain some concerns regarding the 15–25 kHz frequencies used by piezoelectric inkjet bioprinters and their potential to induce damage of the cell membrane and lysis[43]. Inkjet bioprinters also have limitations on material viscosity (ideally below 10 centipoise) owing to the excessive force required to eject drops using solutions at higher viscosities[63].

One common drawback of inkjet bioprinting is that the biological material has to be in a liquid form to enable droplet formation; as a result, the printed liquid must then form a solid 3D structure with structural organization and functionality. Our group[64] and others[65] have shown that this limitation could be addressed by using materials that can be crosslinked after deposition by printing using chemical, pH or ultraviolet mechanisms. However, the requirement for crosslinking often slows the bioprinting process and involves chemical modification of naturally occurring ECM materials, which changes both their chemical and material properties. Additionally, some crosslinking mechanisms require products or conditions that are toxic to cells, which results in decreased viability and functionality[66]. Another limitation encountered by users of inkjet-based bioprinting technology is the difficulty in achieving biologically relevant cell densities. Often, low cell concentrations (fewer than 10 million cells/ml)[42] are used to facilitate droplet formation, avoid nozzle clogging and reduce shear stress[60]. Higher cell concentrations may also inhibit some of the hydrogel crosslinking mechanisms[67].

Notwithstanding these drawbacks, inkjet-based bioprinters also offer advantages, including low cost, high resolution, high speed and compatibility with many biological materials. Another advantage of inkjet printing is the potential to introduce concentration gradients of cells, materials or growth factors throughout the 3D structure by altering drop densities or sizes[68,69]. Because of the availability of standard 2D inkjet printers, researchers in many labs can readily access, modify and experiment with 3D inkjet–based bioprinting technology. Commercially available inkjet bioprinters are also relatively cost-effective owing to their simple components and readily available design and control software. The wide application of this technology by many groups has accelerated advances in the capacity of inkjet bioprinters to accurately deposit with high resolution and precision controllable droplet sizes with uniform cellular densities. Droplet size and deposition rate can be controlled electronically, and can range from <1 pl to >300 pl in volume[70,71] with rates of 1–10,000 droplets per second[58]. Patterns of single drops, each containing one or two cells, in lines ~50 μm wide, have been printed[61]. Future advances will continue to adapt this technology to handle and deposit other biologically relevant materials, in a manner that both facilitates their printing and provides the essential biological, structural and functional components of the tissue. Additional complexities, such as the requirement for multiple cell types and materials, will also have to be addressed.

Notable examples of the inkjet bioprinting approach include the regeneration of functional skin[72] and cartilage[73] *in situ*. The high printing speed of the approach enables direct deposition of cells and materials directly into skin or cartilage lesions. These applications achieved rapid crosslinking of the cell-containing material via either a biocompatible chemical reaction or a photoinitiator and crosslinking through exposure of the material to ultraviolet light. The inkjet approach facilitated the deposition of either primary cells or stem cell types with uniform density throughout the volume of the lesion, and maintained high cell viability and function after printing. These studies demonstrate the potential of inkjet-based bioprinting to regenerate functional structures.

Layered cartilage constructs have also been fabricated *in vitro* using a combination of electrospinning and inkjet bioprinting[74]. The hybrid electrospinning–inkjet bioprinting technique enabled the fabrication of a layered construct that supported cell function and maintained suitable mechanical and structural properties. Inkjet bioprinters have also been used to fabricate bone constructs[75], matured *in vitro* before implantation into mice. These constructs continued to mature *in vivo* and formed highly mineralized tissues with similar density as endogenous bone tissue.

Microextrusion bioprinting. The most common and affordable nonbiological 3D printers use microextrusion. Microextrusion bioprinters usually consist of a temperature-controlled material-handling and

Table 1 Comparison of bioprinter types

	Bioprinter type			
	Inkjet	Microextrusion	Laser assisted	Refs.
Material viscosities	3.5–12 mPa/s	30 mPa/s to >6 × 10^7 mPa/s	1–300 mPa/s	48,63,78,107
Gelation methods	Chemical, photo-crosslinking	Chemical, photo-crosslinking, sheer thinning, temperature	Chemical, photo-crosslinking	64,85,106,110
Preparation time	Low	Low to medium	Medium to high	38,64,94,107
Print speed	Fast (1–10,000 droplets per second)	Slow (10–50 μm/s)	Medium-fast (200–1,600 mm/s)	49,58,76,90
Resolution or droplet size	<1 pl to >300 pl droplets, 50 μm wide	5 μm to millimeters wide	Microscale resolution	49,68,69,76
Cell viability	>85%	40–80%	>95%	42,54,80,104
Cell densities	Low, <10^6 cells/ml	High, cell spheroids	Medium, 10^8 cells/ml	42,49,88,89
Printer cost	Low	Medium	High	77

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dispensing system and stage, with one or both capable of movement along the *x*, *y* and *z* axes, a fiberoptic light source to illuminate the deposition area and/or for photoinitiator activation, a video camera for *x*-*y*-*z* command and control, and a piezoelectric humidifier. A few systems use multiple print heads to facilitate the serial dispensing of several materials without retooling[20,76]. Nearly 30,000 3D printers are sold worldwide every year, and academic institutions are increasingly purchasing and applying microextrusion technology in tissue and organ engineering research[77]. Industrial printers are considerably more expensive but have better resolution, speed, spatial controllability and more flexibility in the material they can print.

Microextrusion printers function by robotically controlled extrusion of a material, which is deposited onto a substrate by a microextrusion head. Microextrusion yields continuous beads of material rather than liquid droplets. Small beads of material are deposited in two dimensions, as directed by the CAD-CAM software, the stage or microextrusion head is moved along the *z* axis, and the deposited layer serves as a foundation for the next layer. A myriad of materials are compatible with microextrusion printers, including materials such as hydrogels, biocompatible copolymers and cell spheroids[38]. The most common methods to extrude biological materials for 3D bioprinting applications are pneumatic[65,78–80] or mechanical (piston or screw)[44,81,82] dispensing systems. Mechanical dispensing systems might provide more direct control over the material flow because of the delay of the compressed gas volume in pneumatic systems. Screw-based systems might give more spatial control and are thought to be beneficial for the dispensing of hydrogels with higher viscosities, although pneumatic systems could also be suited to dispense high-viscosity materials[78]. Pneumatically driven printers have the advantage of having simpler drive-mechanism components, with the force limited only by the air-pressure capabilities of the system. Mechanically driven mechanisms have smaller and more complex components, which provide greater spatial control but often at reduced maximum force capabilities.

Microextrusion methods have a very wide range of fluid properties that are compatible with the process, with a broad array of biocompatible materials described in the literature. Materials with viscosities ranging from 30 mPa/s to $>6 \times 10^7$ mPa/s (ref. 77) have been shown to be compatible with microextrusion bioprinters, with higher-viscosity materials often providing structural support for the printed construct and lower-viscosity materials providing a suitable environment for maintaining cell viability and function. For microextrusion bioprinting, researchers often exploit materials that can be thermally crosslinked and/or possess sheer-thinning properties. Several biocompatible materials can flow at room temperature, which allows their extrusion together with other biological components, but crosslink into a stable material at body temperature[83,84]. Alternatively, materials that flow at physiologically suitable temperatures (35–40 °C), but crosslink at room temperature may also be useful for bioprinting applications[76,85]. Materials with shear-thinning properties are commonly used for microextrusion applications. This non-newtonian material behavior causes a decrease in viscosity in response to increases in shear rate[86]. The high shear rates that are present at the nozzle during biofabrication allow these materials to flow through the nozzle, and upon deposition, the shear rate decreases, causing a sharp increase in viscosity. The high resolution of microextrusion systems permits the bioprinter to accurately fabricate complex structures designed using CAD software and facilitate the patterning of multiple cell types.

The main advantage of microextrusion bioprinting technology is the ability to deposit very high cell densities. Achieving physiological cell densities in tissue-engineered organs is a major goal for the bioprinting field. Some groups have used solutions comprised only of cells to create 3D tissue constructs with microextrusion printing[87]. Multicellular cell spheroids are deposited and allowed to self-assemble into the desired 3D structure[20,88,89]. Tissue spheroids are thought to possess material properties that can replicate the mechanical and functional properties of the tissue ECM. Depending on the viscoelastic properties of the building blocks, the apposed cell aggregates fuse with each other, forming a cohesive macroscopic construct. One advantage of the self-assembling spheroid strategy is potentially accelerated tissue organization and the ability to direct the formation of complex structures. This approach shows promise in enabling the generation of an intraorgan branched vascular tree in 3D thick tissue or organ constructs by patterning self-assembling vascular tissue spheroids, in 3D bioprinted organs. The most common technology used for scaffoldless tissue spheroid bioprinting is mechanical microextrusion.

Cell viability after microextrusion bioprinting is lower than that with inkjet-based bioprinting; cell survival rates are in the range of 40–86%, with the rate decreasing with increasing extrusion pressure and increasing nozzle gauge[76,80]. The decreased viability of cells deposited by microextrusion is likely to result from the shear stresses inflicted on cells in viscous fluids. Dispensing pressure may have a more substantial effect on cell viability than the nozzle diameter[90]. Although cell viability can be maintained using low pressures and large nozzle sizes, the drawback may be a major loss of resolution and print speed. Maintaining high viability is essential for achieving tissue functionality. Although many studies report maintenance of cell viability after printing, it is important for researchers to demonstrate that these cells not only survive, but also perform their essential functions in the tissue construct.

Increasing print resolution and speed is a challenge for many users of microextrusion bioprinting technology. Nonbiological microextrusion printers are capable of 5 μm and 200 μm resolution at linear speeds of 10–50 μm/s (ref. 75). Whether these parameters can be matched using biologically relevant materials while maintaining high cell viability and function is yet to be seen. Use of improved biocompatible materials, such as dynamically crosslinked hydrogels[91,92], that are mechanically robust during printing and that develop secondary mechanical properties after printing might help to maintain cell viablity and function after printing. Single-phase, dual-phase and continuous-gradation scaffolds are also being designed using similar principles. Additionally, improvements in nozzle, syringe or motor-control systems might reduce print times as well as allow deposition of multiple diverse materials simultaneously[82].

Microextrusion bioprinters have been used to fabricate multiple tissue types, including aortic valves[93], branched vascular trees[94] and *in vitro* pharmokinetic[95] as well as tumor models[96]. Although the fabrication time can be slow for high-resolution complex structures, constructs have been fabricated that range from clinically relevant tissue sizes down to micro-tissues in microfluidic chambers.

Laser-assisted bioprinting. Laser-assisted bioprinting (LAB) is based on the principles of laser-induced forward transfer[97,98]. Initially developed to transfer metals, laser-induced forward transfer technology has been successfully applied to biological material, such as peptides, DNA and cells[99–102]. Although less common than inkjet or microextrusion bioprinting, LAB is increasingly being used for tissue- and organ-engineering applications. A typical LAB device consists of a pulsed laser beam, a focusing system, a 'ribbon' that has a donor transport support usually made from glass that is covered with a laser-energy-absorbing layer (e.g., gold or titanium) and a layer of biological material (e.g., cells and/or hydrogel) prepared in a liquid

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Box 1 Ideal material properties for bioprinting

The selection of appropriate materials for use in bioprinting and their performance in a particular application depend on several features. These are listed below.

- Printability
  Properties that facilitate handling and deposition by the bioprinter may include viscosity, gelation methods and rheological properties.
- Biocompatibility
  Materials should not induce undesirable local or systemic responses from the host and should contribute actively and controllably to the biological and functional components of the construct.
- Degradation kinetics and byproducts
  Degradation rates should be matched to the ability of the cells to produce their own ECM; degradation byproducts should be nontoxic; materials should demonstrate suitable swelling or contractile characteristics.
- Structural and mechanical properties
  Materials should be chosen based on the required mechanical properties of the construct, ranging from rigid thermoplastic polymer fibers for strength to soft hydrogels for cell compatibility.
- Material biomimicry
  Engineering of desired structural, functional and dynamic material properties should be based on knowledge of tissue-specific endogenous material compositions.

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solution, and a receiving substrate facing the ribbon. LAB functions using focused laser pulses on the absorbing layer of the ribbon to generate a high-pressure bubble that propels cell-containing materials toward the collector substrate.

The resolution of LAB is influenced by many factors, including the laser fluence (energy delivered per unit area), the surface tension, the wettability of the substrate, the air gap between the ribbon and the substrate, and the thickness and viscosity of the biological layer[103]. Because LAB is nozzle-free, the problem of clogging with cells or materials that plague other bioprinting technologies is avoided. LAB is compatible with a range of viscosities (1–300 mPa/s) and can print mammalian cells with negligible effect on cell viability and function[104–106]. LAB can deposit cells at a density of up to 10^8 cells/ml with microscale resolution of a single cell per drop using a laser pulse repetition rate of 5 kHz, with speeds up to 1,600 mm/s (ref 49).

Despite these advantages, the high resolution of LAB requires rapid gelation kinetics to achieve high shape fidelity, which results in a relatively low overall flow rate[107]. Preparation of each individual ribbon, which is often required for each printed cell or hydrogel type, is time-consuming and may become onerous if multiple cell types and/or materials have to be co-deposited. Because of the nature of the ribbon cell coating, it can be difficult to accurately target and position cells. Some of these challenges might be overcome by using cell-recognition scanning technology to enable the laser beam to select a single cell per pulse. This so-called 'aim-and-shoot' procedure could ensure that each printed droplet contains a predefined number of cells. However, statistical cell printing can be achieved using a ribbon with very high cell concentrations, avoiding the need for such specific cell targeting[49]. Finally, metallic residues are present in the final bioprinted construct, owing to the vaporization of the metallic laser-absorbing layer during printing. Approaches to avoid this contamination include the use of nonmetallic absorbing layers and modifying the printing process to not require an absorbable layer[108,109]. The high cost of these systems is also a concern for basic tissue-engineering research, although as is the case with most 3D printing technologies, these costs are rapidly decreasing.

The application of LAB to fabricate a cellularized skin construct demonstrated the potential to print clinically relevant cell densities in a layered tissue construct, but it is unclear whether this system can be scaled up for larger tissue sizes[110]. *In vivo* LAB has been used to deposit nano-hydroxyapatite in a mouse calvaria 3D defect model[111]. In these studies, a 3 mm diameter, 600 μm–deep calvarial hole was filled as a proof of concept. Laser 3D printing has been used to fabricate medical devices, such as a customized, noncellular, bioresorbable tracheal splint that was implanted into a young patient with localized tracheobronchomalacia[11]. Future studies might use materials that can directly integrate into a patient's tissue. Additionally, incorporating the patients' own cells may facilitate the applicability of these types of constructs to contribute to both the structural and functional components of the tissue.

Materials and scaffolds

Initially, 3D printing technologies were designed for nonbiological applications, such as the deposition of metals, ceramics and thermoplastic polymers, and generally involved the use of organic solvents, high temperatures or crosslinking agents that are not compatible with living cells and biological materials. Therefore, one of the main challenges in the 3D bioprinting field has been to find materials that are not only compatible with biological materials and the printing process but can also provide the desired mechanical and functional properties for tissue constructs.

Materials currently used in the field of regenerative medicine for repair and regeneration are predominantly based on either naturally derived polymers (including alginate, gelatin, collagen, chitosan, fibrin and hyaluronic acid, often isolated from animal or human tissues) or synthetic molecules (polyethylene glycol; PEG[112–115]). The advantages of natural polymers for 3D bioprinting and other tissue-engineering applications is their similarity to human ECM, and their inherent bioactivity. The advantage of synthetic polymers is that they can be tailored with specific physical properties to suit particular applications. Challenges in the use of synthetic polymers include poor biocompatibility, toxic degradation products and loss of mechanical properties during degradation. Even so, synthetic hydrogels, which are both hydrophilic and absorbent, are attractive for 3D bioprinting regenerative-medicine applications owing to the ease of controlling their physical properties during synthesis.

As the variety of biological materials for medical applications increases, the list of desirable traits for printable materials has become more specific and complex (**Box 1**). Materials must have suitable crosslinking mechanisms to facilitate bioprinter deposition,

must be biocompatible for transplantation over the long-term, and must have suitable swelling characteristics and short-term stability. Short-term stability is required to maintain initial mechanical properties, ensuring that tissue structures such as pores, channels and networks do not collapse. As bioprinted tissues develop *in vivo*, they should be amenable to remodeling, facilitating the formation of structures driven by cellular and physiological requirements. Most importantly, materials must support cellular attachment, proliferation and function[64]. We now discuss in more detail the key attributes of printability, biocompatibility, degradation kinetics and byproducts, structural and mechanical properties, and material biomimicry.

Printability. An important property of a suitable material is that it can be accurately and precisely deposited with the desired spatial and temporal control. Some types of bioprinting technology, such as inkjet, have limitations on material viscosity, whereas others, such as microextrusion, may require the material to have specific crosslinking mechanisms or shear-thinning properties. Processing parameters, such as nozzle gauge, determine the shear stress to which cells are exposed[90] as well as the time required for the material to be deposited to form a 3D structure[64]. For example, inkjet printing requires materials with a rapid crosslinking time to facilitate the layering of a complex 3D structure. Microextrusion, however, can incorporate highly viscous materials to maintain a 3D shape after deposition, with final crosslinking occurring after fabrication.

The choice of material may also be influenced by the ability of the material to protect cell viability during the printing process. Thermal inkjet printing and LAB both involve the localized heating of the material to deposit cells. Materials with either low thermal conductivity[116] or the ability to cushion the cells during delivery may increase cell viability and function after printing[117]. Although post-printing cell viability can range markedly based on printer specifications, material properties, resolution and cell types, inkjet bioprinting studies usually quote cell viabilities in excess of 85%, microextrusion printing studies report viability ranges of 40–80% and LAB studies report viability in excess of 90%[42,54,80,104].

Biocompatibility. With the advent of tissue engineering, the goal for biocompatibility has changed from needing an implanted material to coexist with the endogenous tissue without eliciting any undesirable local or systemic effects in the host, to implanted materials being expected to passively allow or actively produce desirable effects in the host[118]. Biocompatibility in bioprinting includes the expectation of an active and controllable contribution to the biological and functional components of the construct. This could include interaction with endogenous tissues and/or the immune system, supporting appropriate cellular activity and facilitation of molecular or mechanical signaling systems, all of which are essential for successful transplantation and function.

Degradation kinetics and byproducts. As a material scaffold degrades, the embedded cells secrete proteases and subsequently produce ECM proteins that define the new tissue[119]. The degradation kinetics of the materials must be understood and controlled. There are several aspects of degradation that must be considered. The first is the ability to control degradation rates, ideally matching the rate of degradation with the ability of cells to replace the materials with their own ECM proteins. This is challenging because materials with suitable functional and mechanical characteristics for a given tissue may not match the ability of the cellular components to replace the material upon degradation. Degradation byproducts are also important because they often define the biocompatibility of any degradable material. The degradation products should be nontoxic, readily metabolized and rapidly cleared from the body. Toxic products can include small proteins and molecules but also nonphysiological pH, temperature or other factors that can be detrimental to cell viability and function. For example, some large-molecular-weight polymers that are initially inert can be broken down into oligomers or monomers that can be recognized by cells and cause inflammation and other detrimental effects. Swelling and contractile characteristics of materials are especially of concern in the fabrication of tissue-engineering products. Overly swelling materials can potentially result in absorption of fluid from the surrounding tissues, and contraction may result in the closing of pores or vessels that are essential for cell migration and nutrient delivery. Moreover, it is important to understand these responses when applying multiple materials with dissimilar swelling or contractile behavior because this could potentially result in loss of layer integrity or deformation of the final construct.

Structural and mechanical properties. If a material is essential for the maintenance of a 3D structure, in resisting or producing specific forces or as an anchoring point for mechanical leverage, then maintenance of these properties is essential for continued function of the construct. Materials must be carefully selected based on the required mechanical properties of the construct, and different structural requirements will be needed for diverse tissue types ranging from skin[64,102] and liver[120] to bone[121]. One approach to overcome this limitation is the use of sacrificial materials that can provide the required structural and mechanical properties over a given period of time. This sacrificial material either may be used at the time of printing to allow sufficient crosslinking to occur in the construct[122,123] or alternatively could be incorporated into the construct, functioning until the endogenously produced materials can sufficiently carry out this function. With this approach, care must be taken to design a material with specific structural and degradation properties while avoiding potential foreign body responses or toxic degradation byproducts in the construct.

Material biomimicry. The importance of biomimicry for biocompatibility has only recently been studied. The ability to incorporate biomimetic components into a bioprinted construct can have an active effect on the attachment, migration, proliferation and function of both endogenous and exogenous cells. It is well established that materials have a large influence on cell attachment[104,124,125] as well as cell size and shape[126], and these principles may be useful in controlling the proliferation and differentiation of cells in a scaffold. The addition of surface ligands to a material has the potential to increase cell attachment and proliferation on the material substrate[125]. The presence of nanoscale features such as ridges, steps and grooves also affects cell attachment, proliferation and cytoskeletal assembly[127,128]. The 3D environment in a tissue-engineered construct can influence cell shape and affect the differentiation process[129,130]. Nanoscale characteristics of materials can affect cell adhesion, cell orientation, cell motility, surface antigen display, cytoskeletal condensation and modulation of intracellular signaling pathways that regulate transcriptional activity and gene expression[131].

A biomimicry approach to engineer materials with specific physiological functions requires an understanding of the naturally occurring tissue-specific composition and localization of ECM components in the tissue of interest. Recent advances in tissue decellularization methods[132] could provide intact ECM scaffolds for detailed analysis of ECM compositions, localization and biological functions. This process involves the lysis and removal of the cellular components

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of a tissue, usually via perfusion with deionized water or mild detergents, while leaving behind the tissue-specific ECM. The ability to reproduce identical ECM scaffolds using a bioprinting approach would be useful in tissue engineering and regenerative medicine.

Challenges in tissue decellularization include striking a balance between complete removal of cellular components and maintenance of the fine vascular and other tissue structures. Additionally, some toxicity has been observed when cells are grown on decellularized tissue scaffolds, potentially due to the retention of the decellularization detergent within the ECM[133]. In mammals, there are more than 300 ECM proteins as well as multiple ECM-modifying enzymes, ECM-binding growth factors and other ECM-associated proteins[134]. The most abundant and understood proteins are the collagens, proteoglycans and glycoproteins. These proteins provide strength and space-filling functions, bind growth factors, regulate protein complexes, promote cell adhesion, participate in cellular signaling and may have additional functions. A 'scaffold-free' approach to bioprinting may be another interesting take on the concept of material biomimicry. As cells produce and deposit the tissue ECM, bioprinted self-assembling cellular spheroids may produce an ECM environment best suited for their own function. Engineering these dynamic ECM mechanisms into materials offers further control over cell behavior. One challenge is to develop methods to incorporate these materials into constructs using bioprinting technology, ensuring that the materials have suitable degradation times and byproducts, and that these materials have well-understood and controllable structural and functional biological effects in the construct.

Cell sources

The choice of cells for tissue or organ printing is crucial for correct functioning of the fabricated construct. Tissues and organs comprise multiple cell types with specific and essential biological functions that must be recapitulated in the transplanted tissue. In addition to the primary functional cell types, most tissues also contain cell types that provide supportive, structural or barrier functions, are involved in vascularization or provide a niche for stem cell maintenance and differentiation. Current options for printing cells involve either the deposition of multiple primary cell types into patterns that faithfully represent the native tissue or printing stem cells that can proliferate and differentiate into required cell types. Cells chosen for printing should closely mimic the physiological state of cells *in vivo* and are expected to maintain their *in vivo* functions under optimized conditions[135].

Any cell type chosen for printing should be able to expand into sufficient numbers for printing. Precise control of cell proliferation *in vitro* and *in vivo* is important for bioprinting. Too little proliferation may result in the loss of viability of the transplanted construct, whereas too much proliferation may result in hyperplasia or apoptosis. Efforts to control cellular proliferation in the transplanted construct are essential to achieve physiological ratios of functional and supporting cells. In addition, the timing of cellular proliferation is important. Initially, a high cellular proliferation rate may be desirable to populate the construct, but over the long term, proliferation must be maintained at a rate suitable to achieve tissue homeostasis, albeit without hyperplasia. Some approaches to solve this problem involve viral transfection[136] or use of small molecules[137,138] to induce cell proliferation and prevent senescence. *In vivo*, endogenous stem cells function to replace terminally differentiated cells following normal cell turnover or injury. For a bioprinted construct to maintain function long-term, after transplantation, bioprinted tissues must be able to maintain cellular homeostasis, self-renew and respond to tissue damage or injury. Improved understanding of the nature and

Box 2 Next steps for 3D bioprinting

For 3D bioprinting to realize its potential, advances are needed in several aspects of the technology and in our understanding of the biology and biophysics underlying regenerative processes *in vivo*. **Table 2** details some of the specific areas where further research is needed.

Table 2 Issues to be addressed

Area	Focus for future research
Bioprinter technology	Compatible with physiologically relevant materials and cells Increased resolution and speed Scale up for commercial applications Combining bioprinter technologies to overcome technical challenges
Biomaterials	Complex combinations or gradients to achieve desired functional, mechanical and supportive properties Modified or designed to facilitate bioprinter deposition, while also exhibiting desired postprinting properties Use of decellularized tissue-specific ECM scaffolds to study ECM compositions, and/or as printable material
Cell sources	Well-characterized and reproducible source of cells required Combinations of cell phenotypes with specific functions Greater understanding required of the heterogeneous cell types present in the tissues Direct control over cell proliferation and differentiation with small molecules or other factors
Vascularization	Well-developed vascular tree required for large tissues May have to be engineered in the bioprinted construct Capillaries and microvessels required for tissue perfusion Suitable mechanical properties for physiological pressures and for surgical connection
Innervation	Innervation is required for normal tissue function May be inducible after transplantation using pharmacologic or growth factor signaling Simulation before transplantation could be achieved using bioreactors
Maturation	Time required for assembly and maturation Bioreactors may be used to maintain tissues *in vitro* Provide maturation factors as well as physiological stressors Potential for preimplantation testing of constructs

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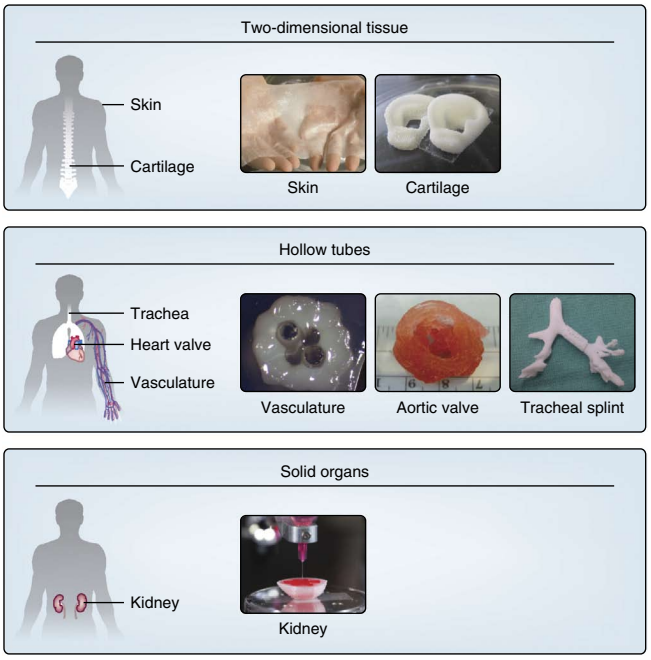

Figure 3 Examples of human-scale bioprinted tissues. Skin (unpublished; Wake Forest Institute for Regenerative Medicine) and cartilage[73] substitutes developed using inkjet bioprinting systems, capable of fabricating tissues either *in vitro* or *in situ*. A vascular graft construct manufactured using microextruded and fused cellular vascular rods[89] and a microextrusion-bioprinted aortic valve fabricated with dual cell types, aortic root sinus smooth muscle cells and aortic valve leaflet interstitial cells[93]. A laser bioprinted bioresorbable airway splint[11] and an early stage kidney prototype, manufactured using microextrusion bioprinting (unpublished; Wake Forest Institute for Regenerative Medicine). All of these bioprinted tissues required integration of multiple components for the fabrication of functional, appropriately sized tissue constructs. Vasculature image is reprinted from ref. 89 with permission; aortic valve image is reprinted from ref. 93 with permission from Wiley; tracheal splint image is reprinted from Zopf, D.A., Hollister, S.J., Nelson, M.E., Ohye, R.G. & Green, G.E. Bioresorbable airway splint created with a three-dimensional printer, *N. Engl. J. Med.* **368**, 2043–2045 (2013), Massachusetts Medical Society, with permission from Massachusetts Medical Society.

composition of endogenous stem cells and their niches will be beneficial in engineering tissues that can maintain their long-term function after transplantation.

As with any transplanted tissues or organs, rejection of bioprinted constructs by the host immune system is a potential problem that can be overcome by using an autologous source of cells or by using tolerance-induction strategies. Autologous sources of cells may be obtained from biopsies, from the generation and differentiation of autologous stem cells or through reprogramming approaches. However, if a patient is already ill or has either genetic or metabolic disorders, it may not be possible for the patient to undergo an invasive surgical procedure, and the isolated cell types may not produce the desired function in the bioprinted construct.

Many primary cell types are difficult to isolate and culture, and their finite lifespan is a limitation for the long-term functionality of any bioprinted constructs[139]. Stem cells are a promising cell type for tissue-engineering applications owing to their ability to proliferate in an undifferentiated but multipotent state (self-renewal) and their capability to generate multiple functional tissue-specific cell phenotypes. Embryonic stem cells and induced pluripotent stem cells are capable of indefinite self-renewal and have demonstrated their longevity by maintaining their undifferentiated state for over 80 passages[140]. The capacity of pluripotent stem cells to generate large numbers of cells highlights the potential of these cells for bioprinting (and other) therapeutic applications; further work to ensure the safety of these cells would be a major benefit for the field. Other types of stem cells—such as adult stem cells from bone marrow[141–143] and fat[144] or perinatal stem cells from amniotic fluid[75] or placenta[145]—are thought to have a more limited multipotent differentiation potential but are considered safer for clinical transplantation and have the potential for autologous applications. With established protocols for the isolation, expansion and differentiation, mesenchymal stromal cells (MSCs) may also be a promising cell source for bioprinted constructs. Clinically relevant numbers of MSCs have been successfully generated *in vitro* for clinical trials, and future advances in cell-culture techniques are likely to make use of other stem cell populations for bioprinting clinical applications a realistic possibility.

The cells used for bioprinting applications must be robust enough to survive the bioprinting process and withstand physiological stresses once transplanted, including physical forces such as shear stress and pressure as well as biological stressors including presence of toxins, enzymes and nonphysiological pH. Many published bioprinting studies use cell lines that are known to be capable of substantial proliferation and are very robust, such as fibroblasts or transformed cell lines. Although these cells are suitable for proof-of-concept studies, it is important to realize that bioprinting technology may have to be adapted to incorporate cell types that are more sensitive to forces such as shear stress or culture conditions as well as the time it takes to prepare the construct. Advances in cell culture techniques as well as in reprogramming and directed differentiation methods will be important for providing highly proliferative, functional, nonimmunogenic and robust cell populations that are suitable for bioprinting applications.

Outlook

Many of the challenges facing the 3D bioprinting field relate to specific technical, material and cellular aspects of the bioprinting process (**Box 2** and **Table 2**). Although the field is at an early stage, it has already succeeded in creating several tissues at human scale that are approaching the functionality required for transplantation (**Fig. 3**). Technological challenges include the need for increased resolution, speed and compatibility with biologically relevant materials. As we move away from the modification of preexisting technology and begin to design 3D bioprinters to handle specific biological components, the range of compatible materials can be extended, and methods to deposit materials and cells with increasing precision and specificity can be developed. The speed of fabrication must be increased to manufacture constructs of clinically relevant sizes. One way to achieve this would be to generate miniature functional tissue blocks that could be scaled to a clinically relevant size by using a macro-scaffold to join blocks. Commercialization may require scalable automated robotic technologies that incorporate each of the components of the biofabrication production line[88]. This may include not just the bioprinting device but also the manufacture of materials, cells and other supporting components.

Currently, the materials being used for printing are selected either because of their compatibility with cell growth and function or because of their crosslinking or extrusion characteristics. For this reason, many published studies use a limited range of materials, including collagen, hyaluronic acid, alginate, modified copolymers and photocured acrylates. The main physiochemical parameters that

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determine the printability of a hydrogel are its rheological properties and crosslinking mechanisms[146]. A useful material for bioprinting should be biocompatible, easily manipulated by the bioprinter technology to be dispensed in complex 3D structures, and maintain cellular viability and function, thereby providing structural and mechanical support to the structure. Almost all human tissues have complex combinations and gradients of ECM components, each with specific biological and mechanical influences. It is likely that by reproducing the biomaterial environment of the tissue or organ of interest, many of the desired mechanical and functional properties will also be reproduced. It seems unlikely that any single material will have all the properties required to recapitulate tissue function. One interesting approach is the development of functionally adaptive materials that reprogram their shape, properties or functionality on demand, based on external stimuli. Such materials modify their function in response to stimuli from the body as the organ matures, in response to physiological cues, or following externally administered stimuli designed to change the tissue[91,147].

One approach to improve understanding of the material environment that is required would be to analyze the composition and distribution of ECM proteins in decellularized tissue scaffolds[132,148,149]. The ability to image, map and reproduce complex 3D structures composed of biologically relevant ECM proteins would be a major advancement for the field. In addition to using decellularized tissues to gain a greater understanding of physiological ECM compositions, ECM derived from decellularized tissues may serve as a useful biomaterial for bioprinting applications. Other approaches may include the combination of rigid thermoplastic polymer fibers with soft hydrogel constructs[150]. In addition, structural materials could be modified with natural or synthetic factors to affect the surrounding biological behavior. This approach may satisfy the functional requirements of the cells as well as the structural requirements of the 3D tissues.

Bioprinting requires sources of cells that are readily available, easy to expand in culture, nonimmunogenic and that can reproduce all the functions of the tissue or organ system. Potentially, combinations of various mature and/or multipotent cell sources can be applied to efficiently reproduce the cell phenotypes needed for specific tissues. For example, a stem cell population derived from the functional component of the tissue of interest could be used to generate the functional building blocks of the construct, whereas MSCs derived from bone marrow or gestational tissue could efficiently generate the connective tissue that forms the structural components of the organ. Additionally, recent advances in the application of small molecules[151] to cell culture suggest that we are heading toward a future in which we have more direct control over cell proliferation and differentiation, with several studies now describing the directed differentiation of cells using small molecules[152–154].

The field of bioprinting also faces other challenges shared by all researchers in the fields of tissue engineering and regenerative medicine. Ensuring sufficient vascularization of the engineered construct is essential for the long-term viability of any bioprinted tissue construct. Several studies have demonstrated generation of a branched vascular tree for bioprinted organ constructs[94,155,156]. A challenge with this approach is the compatibility of the process with the materials and cells and other components of the printing system. In addition, the time required for assembly and maturation of a perfused vascular network throughout the entire tissue construct may be longer than the cell survival time. Bioreactors can help to maintain viability of tissue constructs and 'buy' time necessary for postprocessing tissue fusion, remodeling and maturation. Bioreactor processing can be used in combination with factors that promote angiogenesis and innervation[157]

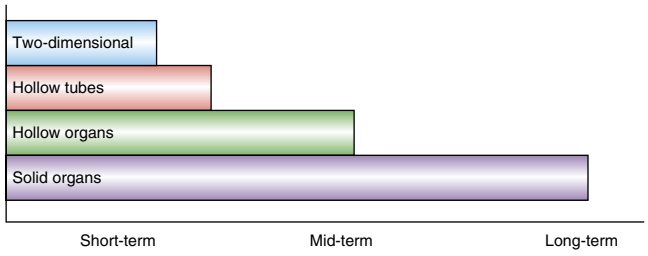

Figure 4 Timeframe for the development of various types of 3D bioprinted tissues. There are four main types of tissues that can be ranked from simple to complex; 2D tissues, such as skin; hollow tubes, such as blood vessels; hollow nontubular organs, such as the bladder; and solid organs, such as the kidney. As the complexity of tissues increases, new approaches will be needed to overcome the challenges of creating them by bioprinting. 2D organs have already been fabricated and tested, and these will likely be one of the first types of bioprinted tissues to be transplanted in patients. Hollow tubes, including blood vessels, tracheas and urethras are currently in development and are likely to closely follow 2D tissues in clinical application. Hollow organs are more complex and may take longer to develop. Solid organs are the most complex, and there are still many challenges to overcome, especially in achieving vascularization and innervation.

as well as factors that can maintain or preserve cell viability[158]. Additionally, bioreactors have an essential role in maintaining microenvironmental parameters such as temperature, pH, nutrient and gas concentrations as well as regulation of specific mechanical stimulations[159]. These parameters will require design and engineering for each specific tissue type and developmental goal.

An alternative approach to the bioprinting-transplantation paradigm is *in vivo* bioprinting, in which cells and materials are directly deposited on or in a patient. Currently, this approach has been used in our laboratory to bioprint skin directly into wound or burn defects and by others to bioprint bone into calvaria defects in mice[111]. With the increasing speed and resolution of 3D bioprinters, this approach may become viable for the *in vivo* regeneration of tissues immediately after injury or during surgery. One interesting direction is the potential integration of 3D bioprinters into minimally invasive, robotic surgical tools. A combined robotic surgical tool and 3D bioprinter might be able to remove and replace tissues during the same surgery or perhaps be applied to accelerate the healing of the tissues removed by the surgical intervention.

3D-bioprinted tissue constructs are being developed not only for transplantation but also for use in drug discovery, analysis of chemical, biological and toxicological agents, and basic research. As we progress toward printing tissues with increasing complexity, beginning with 2D tissues such as skin, through to hollow tubes such as blood vessels, to hollow nontubular organs such as the bladder, and finally to solid organs such as the kidney (**Fig. 4**), we will have to address increasingly difficult challenges, including cell and material requirements, tissue maturation and functionality, and appropriate vascularization and innervation. Multidisciplinary research will be needed to meet these challenges and realize the potential of 3D bioprinting to transform the field of regenerative medicine.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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