Comparison of Biomaterial-Dependent and -Independent Bioprinting Methods for Cardiovascular Medicine

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Abstract

There is an increasing need of human organs for transplantation, of alternatives to animal experimentation, and of better *in vitro* tissue models for drug testing. All these needs create unique opportunities for the development of novel and powerful tissue engineering methods, among which the 3D bioprinting is one of the most promising. However, after decades of incubation, ingenuous efforts, early success and much anticipation, biomaterial-dependent 3D bioprinting, although shows steady progress, is slow to deliver the expected clinical results. For this reason, alternative 'scaffold-free' 3D bioprinting methods are developing in parallel at an accelerated pace. In this opinion paper we discuss comparatively the two approaches, with specific examples drawn from the cardiovascular field. Moving the emphasis away from competition, we show that the two platforms have similar goals but evolve in complementary technological niches. We conclude that the biomaterial-dependent bioprinting is better suited for tasks requiring faster, larger, anatomically-true, cell-homogenous and matrix-rich constructs, while the scaffold-free biofabrication is more adequate for cell-heterogeneous, matrix-poor, complex and smaller constructs, but requiring longer preparation time.

Highlights

- Research in bioprinting for cardiovascular applications is very dynamic and diverse.
- This activity is classified as biomaterial-dependent and -independent ('scaffold-free') bioprinting.
- Both are well represented in bioprinting of cardiac patches, but scaffold-free methods are more advanced in producing pre-clinical vascular grafts.
- Biomaterial-based bioprinting is likely to become successful for larger, faster and less complicated tasks.
- Scaffold-free variant might be preferred for smaller, more compact, cell-heterogeneous constructs.

Abbreviations: 3DBP: 3D bioprinting; EC: endothelial cells; ECM: extracellular matrix; FB: fibroblasts; GelMA: metacrylate gelatin; HUVEC: human umbilical vein endothelial cells; IC: interstitial cells; MSC: mesenchymal stem cells; SMC: smooth muscle cells.

Introduction

Medicine is facing new challenges in a world with an increasingly aged world population. Among them is the massive request of more tissues and organs for transplantation, although fewer than one-third of these patients eventually will receive one [1]. Also, due to their limited efficacy, more robust, possibly radical alternatives to current cell therapy-based methods to treat chronic diseases are needed. Another opportunity for tissue engineering is to replace, or possibly eliminate animal experimentation. This is desirable not only from a bio-ethical standpoint, but also in response to the practical issues derived from species-specific differences in cell function and tissue organization. In addition, more realistic 3D tissue models are increasingly required for toxicological testing and for drug discovery. In all circumstances, tissue engineering is taking a more central position in the emerging bio-medical toolkit [2].

Among the tissue engineering methods, 3D bioprinting (3DBP) holds the promise to become a major revolution in biofabrication of tissues and organs [3•]. This technology might also have an excellent opportunity in the context of deep space exploration: in long-term missions, with very limited resources, the only solution for urgent medical problems could be the on-demand 3D printing of both medical instruments [4] and the required tissues from a patient's own cells [5].

As a form of additive biomanufacturing, 3DBP has been riding so far on the wave of 3D printing. In other words, bioprinting became mainly the biological version of 3D printing [6•]. However, the biomaterials deployed in a layer-wise manner to create the 3D construct, also named 'bioinks' (or 'scaffolds' because of their supporting role), had to coincidently fulfill these often contradictory conditions: i) be printable; ii) protect incorporated cells during bioprinting; iii) sustain their growth and differentiation afterwards; iv) be biocompatible with the recipient organism [7••].

At the interface between scaffold-dependent and scaffold-free bioprinting lies the use of a new generation of 'bioinks' prepared exclusively from natural materials, such as collagen, fibrin or organ-specific extracellular matrices[8•]. Although still experiencing some of the same limitations of their deployment methods as their synthetic correspondents, the latter option is by far more promising in terms

of cell support and biocompatibility. But all these difficulties would be absent if the cellular assembling could be performed with cells capable to produce their own extracellular matrix (ECM), i.e., using biomaterial ('scaffold')-free methods.

Terminological issues. One of the consequences of the field's rapid expansion, with contribution of many research groups with expertise blended from different disciplines, is the inhomogeneous (and often confusing) terminology [3•]. For example, *bioprinting* is the name given to: i) layer-by-layer deposition of cells dispersed in a biomaterial; ii) biomaterial-dependent assembling of cellular aggregates; iii) formation of cell aggregates (spheroids or larger constructs) by magnetic pull down, or even by centrifugation; iv) biomaterial-independent 3D assembling of cell cords and spheroids. Correspondingly, as the instrument facilitating the act of 'bioprinting', a *'bioprinter'* may have different meanings. Moreover, for some groups the notion of *'bioink'* represents only the embedding biomaterial used for bioprinting, while for others it includes the living entities used for 3D assembling [9]. Also, those procedures where biomaterials are removed shortly after assembling of pre-formed cellular aggregates as building blocks were also called '*scaffold-free'* [10].

Comparative examples of 3DBP for cardiovascular applications.

Commensurate with the exceptional momentum for 3DBP, high-quality reviews of this rapidly evolving field are published almost daily, including many dedicated to cardiovascular applications (e.g. [7,11•,12]) . In what it follows, we will comparatively discuss some recent publications focusing on the cardiovascular field, to help the readers evaluate the strengths and limitations of scaffold-dependent and scaffold-free approaches (see Table 1 for a summary [13•]).

1. **Microvessels**

One of the major roadblocks on the way towards engineering functional tissue constructs is the difficulty to provide them with the needed blood perfusion. A large effort is being conducted in almost every branch of tissue engineering to achieve this goal [14].

Scaffold-dependent bioprinting. In two successive studies, one of the expert groups frontally addressed the problem of micro-vascularization of cell heterogeneous constructs. Illustrating the scale of the problem, in one study they used four bioinks [15,16•], all being extrusion-applied as cylindrical threads, and further embedded in a metacrylate gelatin (GelMA) hydrogel base. Then a sacrificial Pluronic F127 ink was removed, producing empty channels subsequently seeded with human umbilical vein endothelial cells (HUVEC), which formed a monolayer during 9 days of culturing. However, these vascular tubes were not perfusable, thus limiting the thickness of bio-fabricated constructs to 1–2 mm and their survival to less than two weeks of culturing [15].

The same group later bioprinted thicker constructs, perfused for more than 6 weeks, and showing osteogenic differentiation of mesenchymal stem cells (MSC) coupled to oxygen diffusion from the pseudo-vascular supply [16•]. To this end the authors first bioprinted cell-laden bioinks composed of GelMA and fibrinogen, together with a fugitive bioink (Pluronic F127, thrombin and transglutaminase) applied on silicone perfusion chips on a glass substrate. Then the Pluronic-containing, temperature-sensitive ink was removed, leaving behind a network of empty channels, which this time could be both endothelialized and perfused [16•].

Scaffold-free bioprinting. A simpler and more natural approach was used to incorporate EC during assembling of spheroids, as the basic mechanism of scaffold-free biofabrication [17••]. For example, such spheroids made from human cardiomyocytes (CM), FBs and EC were prepared and assembled in a beating, single-spheroid layer, by flotation [18]. When this was applied as a cardiac patch on the surface of the heart in living immunodeficient rats, the construct had not only survived, but after retrieval it was found that blood perfused abundantly the spontaneously-organized capillaries anastomosed to recipient's microvasculature [18••].

As a materialization of the biomaterials-free tissue engineering approach, by facilitating the larger-scale fusion and maturation of spheroids in meaningful tissue constructs, is the use of a set of micro-needles ('Kenzan' in Japanese) as temporary supports [13•,19]. This method was implemented in the *Regenova* 'Bio-3D Printer'. Several such instruments are operational in Japan, and a few more in US. Various cell constructs have been printed on the Regenova robot, of which a small diameter vascular graft is best known [20••], but also tracheal [21] and uretral [22] tubes, as well as liver, nerve and other tissues (http://www.cyfusebio.com/en/regenova.html). Work in progress in several labs (e.g., Novel Stem Cell Therapy for Heart Failure Using 3D Printed Cardiac Tissue, by Ong *et al.*, Circulation 2016;134:A18056) indicate that microvascularization based on this principle is being used for Kenzan bioprinting of cardiac patches.

Comparison. In the case of the material-based 3D constructs we see the direct, potentially anatomically correct (although this still has to be demonstrated) channel formation, followed by endothelial colonization, and possibly by perfusion. However, such soft biomaterial-dependent constructs could not be tested *in vivo* yet, while the prospect to eliminate all the supportive materials in the constructs is still remote (Fig. 1A1, Design flexibility vs. Architecture). In the scaffold-free method the endothelial cells (EC) are directly incorporated in pre-formed cell spheroids, with subsequent self-organization in microvascular networks which spontaneously connect (anastomose) with the recipient's capillaries. However, their direct connection to larger vessels is still to be demonstrated (Fig. 1A2, Anastomosis vs. Connectivity). Thus, at face value, the latter method seems to be closer to pre-clinical testing. With some improvements, this approach could become a viable solution to the micro-vascularization needs of scaffold-free tissue engineered constructs in general. In addition, lymphatic cells and even neural cells could be introduced in a similar way.

2. Large vessels

Scaffold-dependent bioprinting. Tissue engineering of vascular grafts was traditionally an active area of research, with notable recent progress in use of natural (such as fibrin [23]) or artificial (e.g., fibrillar polycaprolactone [24]) biomaterials, or of decellularized vessels [25]. However, we could not

find convincing demonstrations of free-standing scaffold-dependent bioprinted vascular grafts ready to be tested *in vivo*. The reason is simple: the mechanical properties of hydrogels which are needed for bioprinting may not be compatible with this application, even with a post-printing hardening step, but the search for this 'holy grail' continues nevertheless. A recent example of cell incorporation in layered biomaterial tubes is the work by Wilkers *et al*. [26••]. Multilayered cylindrical constructs were obtained by deposition of biomaterials on rotating mandrels of different diameters, made of fugitive (removable) alginate. The authors could print tubes with lumens in the 0.5-6 mm diameter range, with layers of 1-400 μm from GelMA, alginate and chitosan. Only HUVEC were added to these layers (e.g., to a 20–30 μm intima), which demonstrated good viability and proliferation. No additional assessments besides microcopy were reported, since the authors noted the extreme fragility of the construct (Fig. 1B1, Precision vs. Resistance). In addition, they acknowledged the limited range of polymerization parameters explored, constrained by requirements to maintain cell viability.

Scaffold-free bioprinting. In the scaffold-free camp there is more convincing progress: a generic vascular-like tube obtained by the Kenzan method from human smooth muscle cells (SMC), fibroblasts and EC, with about 1.5 mm in thickness and 5 cm in length [20••]. This tube, completely made of living cells, could be grafted in abdominal aorta in rats, while remaining patent for five days. Although having a burst pressure ten times more than a human vessel of same caliber, this vascular graft still eventually failed, likely because of slow expansion and remodeling, due to the lack of organized elastic elements [20••](Fig. 1B2, Cell composition vs. Biomechanics). Previously reported small-diameter vascular tubes [27••] or torroids [28] were obtained by 'hybrid' bioprinting (i.e. using fugitive alginate molds).

Another biomaterial-free approach exploits the versatility of magnetic force, deployed via magnetic nanoparticles. Since the internalization of commonly used magnetite (iron oxide) has some toxicity on cells, different alternative strategies have been proposed. By separating the cell-rich and magnetite-rich domains within cellular spheroids (creating the so-called 'Janus' spheroids [29•]), spheroids with lesser magnetite incorporation were used to assemble rings reminiscent of vascular tubes [30]. Alternatively, the use of the more biological-compatible reagent magnetoferritin, which has fewer

adverse effects on cells for up to 1 week, has been suggested by the same group [31]. Cellular spheroids labeled with this reagent were magnetically brought and maintained in contact and fused into tissue rings [31].

Comparison. Scaffold-free methods seem to be again more advanced in their ability to produce meaningful, testable vascular grafts of surgical interest. Although these are also still not enough resistant to blood pressure, they are readily amendable to improvements, for example by additional extracellular matrix-targeted engineering [30], or by hybrid methods, such as incorporating additional - albeit temporary - biodegradable supporting scaffolds.

3. Cardiac valves

Heart valves are anatomically complex and cell-heterogeneous layered tissues prone to substantial damage [32]. Cardiac valves are comprised of three cellular layers: two layers of EC on the surface of valvular leaflet, sandwiching a layer of interstitial cells (IC) within a complex ECM. Valvular EC convey signals from bloodstream, mediate their lipid uptake and the anti-inflammatory and anti-thrombotic responses, and maintain the IC quiescent. IC have a phenotype intermediate between fibroblast and SMC, and are mainly responsible with the secretion of a structural ECM. Interaction between these cell types is also instrumental for valve function. Valvular EC injury induces inflammation, thrombosis, and lipid and/or calcium accumulation, coincident with IC activation, increased smooth muscle-type actin expression, and ECM remodeling. These factors trigger in IC an osteoblastic phenotype, leading to valvular calcification and stenosis [32].

In spite of the remarkable progress in surgical replacement with either inorganic or animal-derived prostheses, there is still a large need for improvement in heart valve tissue engineering. 3DBP could in principle address the limitations of current valve replacement options [12]. Also desirable would be valvular image-driven constructs with a personalized geometry, or in pediatric patients, valves capable to grow and remodel. However, despite significant progress in other areas of valvular tissue engineering [33,34] to our knowledge no functional testing has been reported of any of the bioprinted valves.

Scaffold-dependent bioprinting. An anatomically relevant tri-leaflet valve model was developed by extrusion bioprinting, using rigid (root) and soft (leaflets) hydrogels [35••,36]. Aortic root SMC embedded into the root and aortic valve IC in the leaflet portions of the printed valve remained viable for 7 days in culture, and expressed α-smooth muscle actin and vimentin, respectively. IC deposited their own collagen- and glycosaminoglycan-rich ECM. Although EC were not included in this work, it nevertheless demonstrated that complex and cell-heterogeneous cardiac valves could be bioprinted. The main problem remains the biomaterial, because cell survival was suboptimal and needed improvement [37], while the anticipated replacement with native matrix apparently did not progress too far, sine no biomechanical testing of these constructs has been reported yet (Fig. 1C1, Geometry vs. Material).

Scaffold-free bioprinting. Layered co-cultures of aortic valve cells (EC and IC) were prepared and cultured using magnetic levitation [38]. This method has been employed previously to create a variety of other 3D culture models (vascular smooth muscle [39•], pulmonary [40], tumoral [41], and adipose [42] stem cells). To this end, the cells were first incubated with a proprietary (commercial) reagent consisting of poly-L-lysine, magnetite and gold nanoparticles, and formed a gel that attached reversibly by electrostatic interactions with the cell surface, making it less toxic than other magnetic particle reagents. After three days in culture, the cells maintained their phenotype, as shown by staining for the EC marker CD31, endothelial nitric oxide synthase, von Willebrand factor and prolyl-4-hydroxylase, and for smooth muscle actin in the IC. The increase in endothelial nitric oxide synthase and von Willebrand factor expression by EC in the construct, as compared to normal cultures, suggested that they might be less thrombogenic in the presence of IC cells. Quiescence of the IC as compared to 2D cultures was demonstrated by reduced expression of the collagen I, lysyl oxidase and smooth muscle actin genes. The ECM proteins collagen type I, laminin and fibronectin were detected within the construct by immunostaining. A major limitation of this study was the simple, two-layered geometry of the construct, which again did not permit integration in an anatomically meaningful construct and biomechanical testing (Fig. 1C2, Cell organization vs. Integration).

Interestingly, although vascular rather than valvular cells have been used, the tri-laminar structure of a cardiac valve leaflet was produced using the 'Janus' magnetic cell spheroids [30]. Given the overall potential of spheroids-based tissue engineering, these early attempts are definitely worth continuing with valvular cells as well.

Comparison. Apparently, both biomaterial-dependent and scaffold-free bioprinting of artificial valves lag behind other scaffold-based versions of valvular tissue engineering. This is not surprising, given the need for a printable material similar to valve's heterogeneous ECM, which is critical for its structure and biomechanics.

4. Myocardial tissue

At cellular level, heart's basic units (myofibers) are organized in rather parallel fashion, an alignment that combined with the contractile synchronization of myocytes, promotes their electric activity. Conceptually, this structure could be relatively easily implemented by bioprinting.

Scaffold-dependent bioprinting. Several forms of cardiac patches were prepared by bioprinting so far. For example, Gaebel *et al*. used laser-assisted bioprinting to seed HUVEC and human MSC in a pattern used as cardiac patch [43]. Similarly, human fetal CM progenitors were bioprinted in an alginate base to fabricate a cardiogenic patch with defined pore size and with satisfactory viability [44]. However, neither of these attempts achieved the cell density required for a functional myocardial analog [45••].

A substantial advance in this regard was recently reported, which relies on a high resolution photochemistry-based 3D printing method (two-photon photolithography) to generate a pattern in the scaffold, extracted directly from microscopic images of the architecture of native myocardial ECM, then seeded with human induced pluripotent stem-derived cardiac cells. This cardiac patch promoted high levels of cell engraftment, and improved cardiac function, vascularity, and cell proliferation in the adjacent recipient tissue, thus reducing infarct size in a murine model of myocardial infarction [46••].

However, scaling-up this construct to patch a human infarced heart is the next big challenge (Fig. 1D1, Matrix structure vs. Scaling).

Scaffold-free bioprinting. Several research groups are engaged in the use of Kenzan method to produce a scaffold-free cardiac patch (e.g. Ong *et al.*, Circulation 2016;134:A18056). In fact, its building blocks, i.e., spheroids prepared from cardiomyocytes, fibroblasts (FB) and EC, have been already assembled in a beating, viable cardiac patch, and implanted in rats. This patch showed excellent cell survival and perfusion, consecutive to anastomosis with the recipient capillaries of the built-in microvascular primordia self-assembled during the maturation phase of the construct. However, as described before, this construct was obtained by simple flotation of one layer of spheroids on the surface of culture dish, rather than using the bioprinter. For this reason it had a thickness (given by the diameter of the spheroids) of only about 0.5 mm, a limitation that several teams are currently aiming to surpass by actual Kenzan bioprinting in multiple spheroid layers.

In another significant development, cell spheroids were assembled within a microfluidic device by direct inter-cell 'click' ligation, a liposome-based technology which displays bio-orthogonal functional groups on cell membranes [47]. This method could be one day used to create larger cardiac patches, because the same group already applied it to the engineering of cell-to-cell contacts between liver cells [48] and amongst all cardiac cell types [45••]. When compared with 2D co-culture monolayers, these 3D cardiac tissue 'chips' showed increased cardiac markers, electromechanical coupling, beating rates and reduced toxicity of tested drugs [45••](Fig. 1D2, Cell density vs Size).

Comparison. Apparently, incorporation of cardiomyocytes into biomaterials for creation of cardiac patches with pre-clinical relevance is the most advanced cardiovascular application of both scaffold-dependent and independent bioprinting to date. However, scaling up of the method, biocompatibility of the materials, as well as micro-vascularization of the construct still makes uncertain the ultimate clinical fate of this otherwise promising scaffold-dependent approach. At the same time, scaffold-free methods also take speed, with use of the microneedle technology and other versions of direct engineering of the cell-to-cell interactions.

Conclusion

In a domain as complex as cardiovascular tissue engineering, it is hard to predict which of these emerging technologies will succeed, and/or will become dominant. Most likely, both will soon occupy their best fitting application niches. By corroborating developments from other branches of bioprinting discussed elsewhere [13•], we anticipate that (pending transcending a number of remaining roadblocks), scaffold-dependent bioprinting may be taking the lead for constructs which are larger, more cell-homogenous, high-matrix tissues, such as the musculoskeletal system and myocardial tissue (and possibly cardiac valves).

At the same time, the creation of smaller, cell-heterogeneous, low-matrix tissues, e.g. micro-vascularization of a variety of tissues such as glands and sensory organs, will probably better be served by scaffold-free biofabrication approaches. Not unlikely, these versions of biofabrication will share the same application landscape depending on the required speed to completion and complexity. For example, if a large skin surface needs to be made fast to cover a burned dorsal area, the scaffold-dependent bioprinting may better help. However, if the time and conditions permit, skin patches containing not only the protective dermal layers in an appropriate ECM embedding, but also glands, hair, capillaries and nerves could someday be better made using the scaffold-free approach.

In summary, neither one of the two modes of performing bioprinting (biomaterial-dependent or 'scaffold-free') has convincingly shown pre-clinical examples of success yet. However, as discussed here, the less known scaffold-free methods (specifically the Kenzan and magnetic nanobead-assisted) methods show promising advances to complement, and in some areas to surpass, the fast-pacing scaffold-dependent bioprinting.

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Figure legend:

Figure 1. Graphical comparison of biomaterial-dependent and -independent cardiovascular constructs. Selected examples illustrate the major benefits (green highlight) and limitations (red highlight), in our opinion, of the respective methods. **A. Microvasculature**: from https://en.wikipedia.org/wiki/Capillary#/media/File:Capillary_system_CERT.jpg; **A1**: Kolesky et al. [15]; **A2:** Noguchi et al. [18] **B. Large vessels:** from Blausen.com. "Blausen gallery 2014". *Wikiversity Journal of Medicine*. DOI:10.15347/wjm/2014.010; **B1**: Wilkers et al. [26]; **B2**. Itoh et al. [20], **C. Cardiac valves:** from http://cnx.org/content/col11496/1.6/; **C1:** Cai/Duan et al. [36], **C2:** Mattix et al. [29], **D. Myocardial muscle:** from Patrick J. Lynch, medical illustrator; C. Carl Jaffe, MD, cardiologist. http://creativecommons.org/licenses/by/2.5/; **D1:** Gao et al. [46]; **D2**: Rogozhnikov et al. [45] (Reproduced with permission). See explanations in text.

	BIOMATERIAL-DEPENDENT		BIOMATERIAL-FREE	
	Attributes	**Comments**	**Attributes**	**Comments**
OBJECT CONFIGURATION	Direct image input via CAD	Similar to 3D printing	Approximate	Larger 'voxel' size, limited resolution
STRUCTURAL COHESION ('glue')	Obtained by non-universal, sometimes proprietary and/or expensive bio-inks	New biological bio-inks emerging (e.g. collagen or fibrin based)	Cells produce their own matrix; constructs are dependent on cell type and quality	Matrix deposition can be unpredictable or insufficient
BIOMECHANICS	Hydrogels are essentially soft; hardening can be cell-damaging	'Hybrid' bioprinting as alternative: incorporation of a second (fibrillar) biomaterial	Construct biomechanics less predictable and controllable	Hybrid versions are also likely to be developed
EFFICIENCY	Substantial cell death, for a variety of method-specific reasons	Milder methods are being tested (e.g. laser-assisted bioprinting)	Less or no cell damage Cell-type dependent	By using large spheroids, speed can become comparable or even higher than laser-assisted bioprinting
CELLULAR CROSS-TALK	Material-limited inter-cellular communication ('encapsulation')	Not a problem for matrix-rich tissues such as bone, cartilage	Direct cellular interactions	Optional addition of hydrogels into or between spheroids still possible
TISSUE STRUCTURE	Simplistic cellular architecture	Biomaterial dissolution allows more spontaneous cell rearrangements	Follows developmental principles	Incorporation of endothelial cells in spheroids may promote micro-vascularization
BIO-COMPATIBILITY	Cytotoxicity possible, foreign-body reactions likely	Less serious if biological bio-inks are used	Patient-specific cells: MSC, iPSC	Possibly fully autologous constructs
COMMON TECHNICAL PROBLEMS	Nozzle clogging	Limited to ink-jet and micro-extrusion methods	Time of pre-printing preparations	Post-printing maturation time comparable between the two approaches
SCALABILITY	Excellent	***Good for large, cell-homogenous, matrix-rich tissues***	More limited	***Recommended for small, cell-heterogeneous, matrix-poor tissues***

Table 1. Comparative features of biomaterial-dependent and independent bioprinting methods.

(reproduced with permission from Moldovan et al., 2016[13]).

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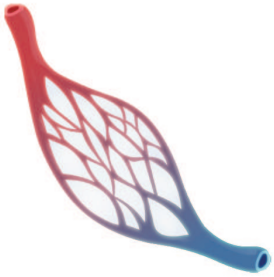
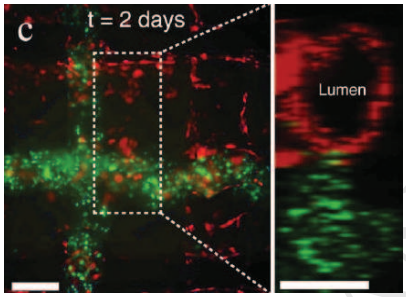
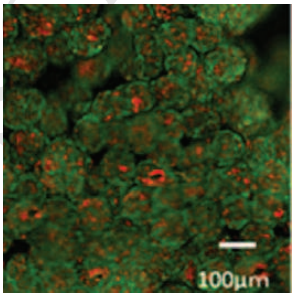
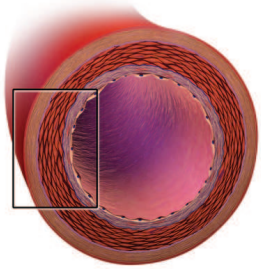
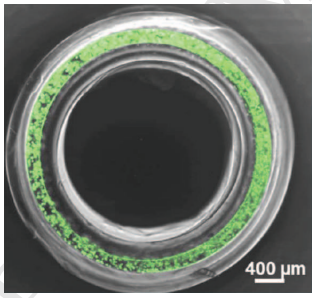
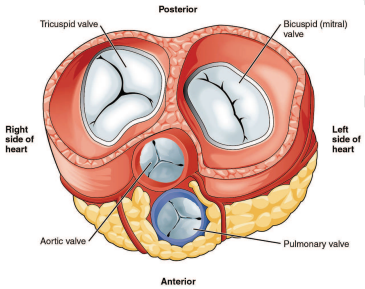
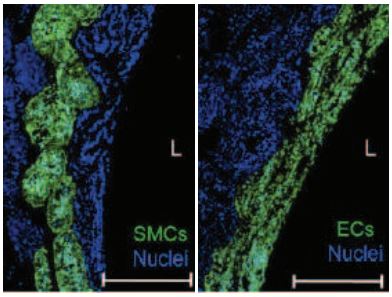
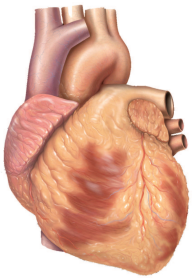
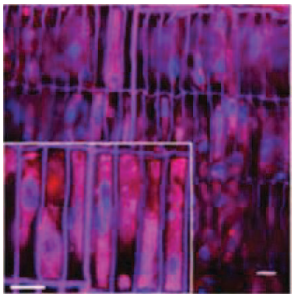
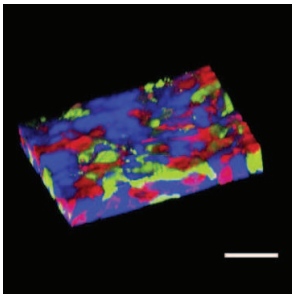
GOALS	EXAMPLES	
	BIOMATERIAL-DEPENDENT	BIOMATERIAL-FREE
	Major benefits ... Major limitations	
A Microvessels 	**A1**  Design flexibility ... Architecture	**A2**  Anastomosis ... Connectivity
B Large vessels 	**B1**  Precision ... Resistance	**B2**  Cell composition ... Biomechanics
C Cardiac valves 	**C1**  Geometry ... Material	**C2**  Cell organizarion ... Integration
D Myocardium 	**D1**  Matrix structure ... Scaling	**D2**  Cell density ... Size