

Review

Current methods for fabricating 3D cardiac engineered constructs

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SUMMARY

3D cardiac engineered constructs have yielded not only the next generation of cardiac regenerative medicine but also have allowed for more accurate modeling of both healthy and diseased cardiac tissues. This is critical as current cardiac treatments are rudimentary and often default to eventual heart transplants. This review serves to highlight the various cell types found in cardiac tissues and how they correspond with current advanced fabrication methods for creating cardiac engineered constructs capable of shedding light on various pathologies and providing the therapeutic potential for damaged myocardium. In addition, insight is given toward the future direction of the field with an emphasis on the creation of specialized and personalized constructs that model the region-specific microtopography and function of native cardiac tissues.

INTRODUCTION

According to a report from the American Heart Association in 2020, more than 6 million Americans had suffered from the effects of heart failure. The rate of incidence has seen continual growth over the years despite many advances in the field of cardiac regenerative medicine and disease modeling (Virani et al., 2020). Current conventional treatments are limited as the regenerative properties of cardiomyocytes (CM) are functionally nonexistent, with most of their potential for proliferation long-lost by the time of adulthood (Nakamura and Sadoshima, 2018). Currently, the total heart transplant remains the gold standard of treatment for those suffering the effects of heart failure. However, in recent years, the field of cardiac tissue engineering has directed its time and resources toward the fabrication of cardiac engineered constructs and the development of advanced cardiac patch therapies. Nonetheless, these constructs are often non-distinct and limited in restoring cardiac function. This only emphasizes the need to develop more specific therapies with the hope of replicating certain aspects such as heart chamber-specific microarchitecture and promoting the respective cardiac functions. Patient-derived cardiovascular cells also allow for the personalized modeling of cardiac tissues with genetic variants of interest. These general oversights point cardiac engineered constructs in the direction of region-specific tissue models to not only better understand certain pathological qualities but also provide clinically effective treatments for cardiomyopathies.

The development of next-generation cardiac engineered constructs can be attributed to two key contributions: the advent of induced pluripotent stem cell (iPSC) technologies and the refinement of scaffold generation methods. As more is discovered about the intricacies of cardiac pathologies, it becomes increasingly important to develop methods capable of replicating specific features of the *in vivo* tissue environment with increasing resolution. Like many other cellular processes, the contractility of CMs is regulated by the flux of intracellular Ca^{2+} . These concentrations differ across a variety of cardiac pathologies because of stressors impacting Ca^{2+} transport machinery found in mitochondria and the endoplasmic reticulum (Nan et al., 2021). Factors such as spatial alignment and genetic regulation of transporters can impact Ca^{2+} homeostasis as seen in cardiac hypertrophy and ischemic damage (Zhang et al., 2021). Earlier methods of tissue engineering emphasized the importance of the alignment and contraction of CMs to better match the band-like structures found in “healthy” myocardial tissues. However, these models only replicate basic functions of heart tissues and do not serve to replicate specific contractile deviations among pathologic conditions. Therefore, it is of the utmost importance for cardiac tissue engineers to gain a greater understanding of the mechanistic impacts of pathologic stressors and apply them for the induction of conditions of interest in engineered cardiac tissue constructs.

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<https://doi.org/10.1016/j.isci.2022.104330>



More recent projects have shifted emphasis toward the creation of 3D cardiovascular cell culture environments and modeling changes that may take place because of pathologically induced cardiac tissue remodeling. Similarly, the emergence of iPSC has allowed for an exponential increase in nearly all (sub)types of cardiovascular cells available for testing and does not require the primary cardiac tissues from donors. These reprogrammed cells contain the specific genetic information derived from the patients and can be further differentiated into a plethora of cell types in the heart, such as CMs, endothelial cells (ECs), cardiac fibroblasts (CFs), vascular smooth muscle cells (VSMCs), and immune cells. iPSCs offer a unique opportunity to create personalized cardiac tissue constructs with proper cell genotyping and heterogeneity when incorporated into advanced fabrication methods. This review serves to evaluate the current methods in use and the specific aspects they contribute to recent tissue engineering fabrication techniques of hPSC-derived cardiovascular cells in engineered cardiac constructs as conceptualized in [Figure 1](#). As well-defined cell types must be considered while generating the engineered cardiac constructs, the *in vivo* environment should contain a colorful assortment of cell types all in interconnections with each other. By understanding the roles and various interactions among cell types in the heart, engineering methods can be further refined to incorporate functions derived from these cells and create personalized cardiac engineered constructs.

CELL TYPES IN HEART

The heart houses a diverse population of cell types, such as CMs, ECs, CFs, VSMCs, immune cells, as well as a variety of subtypes ([Carrillo-Salinas et al., 2019](#); [Zuppinger, 2019](#)). They actively crosstalk and contribute mutually to cardiac structure and function, both of which are essential components of cardiac engineered constructs. In this section, we introduce and discuss the important roles of the major cell (sub)types in the heart and cardiac engineered constructs.

Cardiomyocytes

Cardiomyocytes are the primary cell type that provides the contraction of the myocardium. They contain sarcolemma for anchoring the integrin and receptor transmembrane proteins that bind the CM to the extracellular matrix (ECM), including cardiac basement membrane ([Allyson Walker and Francis Spinale, 1999](#); [Yang et al., 2015](#)). The cardiac basement membrane plays key roles in sarcomerogenesis, CM-CM interaction, and cardiac electrophysiology based on the findings in our previous engineering models ([Yang et al., 2014b](#); [Yang et al., 2014](#); [Yang et al., 2015](#)). The sarcoplasmic reticulum in CMs stores the Ca^{2+} used in the excitation-contraction coupling ([Williams, 1997](#)). Action potential in the CM is governed by the flux of K^+ , Na^+ , and Ca^{2+} , with adenosine triphosphate consumption for contraction. This action potential is then utilized by the fundamental contractile unit of the CMs—sarcomeres—which are composed of a thick filament made from myosin, thin filaments made of mostly actin, as well as tropomyosin and troponin complex. The myosin has a filamentous tail and a globular head region which is the site where it binds to actin and where ATPase is catalyzed ([Allyson Walker and Francis Spinale, 1999](#)); when ATP is present, the myosin will bind with the actin and will result in the shortening of the sarcomere which in turn results in the contraction of the CM.

CMs can be isolated from adult mammalian myocardium (e.g., small animal, large animal, and human cardiac biopsy) for further biomedical applications. Adult CMs from the adult myocardium retain the rod shape structure after isolation, but they begin to diminish over time ([Woodcock and Matkovich, 2005](#)). Drawbacks to the use of adult CMs could be their vulnerability and instability in long-term culture, and very limited proliferation rate after isolation ([Robertson et al., 2013](#)). Neonatal CMs are more commonly used in cardiac tissue engineering by easier and faster isolation protocol than adult CMs ([Louch et al., 2011](#)). Currently, CMs are much more conveniently and efficiently derived from iPSCs for the applications of regenerative medicine and disease modeling in our lab and also in other labs ([Fine et al., 2013](#); [Wu et al., 2019](#); [Yang et al., 2019, 2021](#)). The pluripotent stem cells (PSCs), including iPSC and embryonic stem cells (ESC), can be differentiated not just into CMs but also into ECs, CFs, VSMCs, etc., by several well-established protocols ([Kattman et al., 2011](#); [Ardehali et al., 2013](#); [Zwi-Dantsis et al., 2013](#); [Palpant et al., 2017](#)). The freshly isolated hPSC-CMs are considered as fetal CM-like in need of further maturation. We have previously cultured CMs to capture dynamic myofibrillar remodeling under static stretching, an unexplored process in cardiac hypertrophy (H. [Yang et al., 2016a](#)). Stretching of CMs has also been shown to increase the expression of mature CM genes such as troponin-T and caveolin-3 ([Abilez et al., 2018](#)). The incorporation of CMs in the cardiac engineered constructs is necessary to add the fundamental functions of a heart, such as an accurate representation of the contraction mechanism, calcium cycling, intercellular interactions, and ECM deposition as seen in the myocardium. To address the contraction mechanism, greater

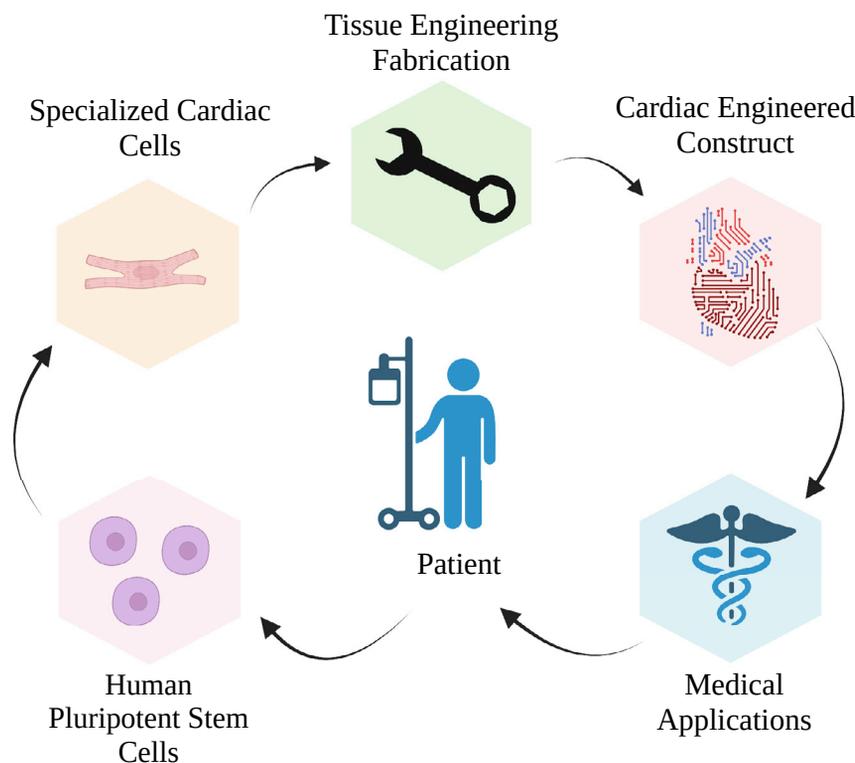


Figure 1. The general concept of hPSCs-derived cardiac engineered constructs for medical applications

hiPSCs sourced from patients are applied for differentiating into various cardiovascular cell lineages, which are further incorporated with specific engineering techniques to fabricate the heart tissue construct that emulates the structure and function of cardiac tissue *in vivo*. These constructs are then specified to the targeted biomedical applications of modeling and treating cardiac diseases. Created with [BioRender.com](https://www.biorender.com).

emphasis on chamber-specific action potentials (AP) could be used to better understand the chamber-specific cardiac diseases. As we know, there is a significant difference in AP between the subtypes of CMs, whereas the ventricular CMs have a longer AP than the atrial CMs (Mummery et al., 2003; Moretti et al., 2010; Chen et al., 2017b). Ideally, by incorporating the subtypes of CMs with their respective Aps, we broaden the application of the cardiac engineered constructs in region-specific regeneration, drug responses, and pathological phenotypes.

Vascular smooth muscle cells

Vascular smooth muscle cells play a role in the vascular wall by adding structural support. Their main function is to regulate vasoconstriction and relaxation to regulate blood pressure and flow (Proudfoot and Shanahan, 2012). They also participate in the process of vascular development and after injury or in the presence of growth factors and mitogens. When any of these occur, VSMCs change phenotype and take up lipids resulting in a foam-like mixture of cells called foam cells in the vascular wall. They secrete ECMs and are associated with matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (TIMPs), all of which are necessary for correct remodeling of vessel walls. The regulation of VSMCs depends on the way the cells are organized; besides, multiple VSMCs function using autonomic sympathetic innervations which release neurotransmitters, whereas individual VSMCs function using autocrine and paracrine hormones. VSMCs use Ca^{2+} binding protein calmodulin (CaM) which when bound with Ca^{2+} begins the contraction procedure of the cells. The incorporation of VSMCs into the cardiac engineered constructs provides the possible vascular structure to be more comparable to the *in vivo* cardiac tissue (Wang et al., 2014).

Human VSMCs are usually isolated by removing the endothelial layer from the medial layer of blood vessels and then removing the underlying sheet of VSMCs. These cells then dedifferentiate and become “synthetic” cells. It is also noted that different phenotypes of VSMCs could be obtained depending on the origin of the blood vessel from which they are being extracted from (Proudfoot and Shanahan, 2012).

VSMCs can also be obtained by differentiating iPSCs. The iPSCs are cultured in an ultralow attachment dish to generate embryoid bodies which are then transferred into a Matrigel-coated dish with a smooth muscle growth medium to differentiate into the smooth muscle cells (Wang et al., 2014). Several other methods for differentiating iPSCs to VSMCs have been developed, including the use of GSK3 inhibition and BMP4 treatment followed by exposure to PDGF-BB (Patsch et al., 2015) or the implementation of CHIR99021 with BMP-4 and RPMI1640 to differentiate either iPSCs or ESCs (Yang et al., 2016b). The incorporation of VSMCs into engineered cardiac tissue constructs can lead to a vascularized tissue that is not limited in size because of limited oxygen diffusion. Previous studies have found that once tissue thickness exceeds 100 μm , the diffusion of oxygen and nutrients into the core of the tissue decreases significantly (Sekiya and Shimizu, 2017). For this reason, other cell types such as ECs and pericytes that make up the blood vessels are also necessary.

Endothelial cells

Endothelial cells exist in the endocardium, coronary arterial, venous, capillary, and lymphatic in the heart. ECs create a monolayer of endothelium and lines the lumen of the vascular beds as well as coronary blood vessels and the cardiac chambers and functions as the barrier that separates the blood from the vascular walls while also being a sensor and modulator for the myocardial structure and function (Lerman and Zeiher, 2005; Kamo et al., 2015). Endothelium generates nitric oxide which is a vasodilator that can affect vasculature in many ways including platelet adhesion and aggregation, proliferation, and vasoconstriction (Wilson, 2011). ECs are in direct contact with CMs, VSMCs, and CFs and actively secrete proteins, small molecules, and peptides that are used in intercellular communications. Proteins secreted by ECs can modulate cardiac contractility and cardiac remodeling. For instance, periostin is a part of normal fibrogenesis but also plays a part in pathological fibrosis (Snider et al., 2009). Endothelin-1 is a vasoconstrictive peptide that is secreted by ECs and is known to induce hypertrophy in CMs (Ito et al., 1991). Having ECs present in cardiac engineered constructs can lead to better modeling with vascular components and understanding of how these proteins, peptides, and small molecules affect the heart in both normal and disease states.

ECs can be isolated from the human umbilical vein vascular wall using collagenase treatment, the seeding cells on fibronectin-coated plates, and cultured to make human umbilical vein ECs (HUVECs) (Baudin et al., 2007). Besides the primary ECs isolated from human vessel biopsies, ECs can be also differentiated from iPSCs (Homma et al., 2010; Ikuno et al., 2017; Palpant et al., 2017; Chen et al., 2021; Luo et al., 2021). ECs were actively cocultured with CMs to create advanced cardiac engineered constructs (Weinberger et al., 2016; Vollert et al., 2014).

Current limitations to tissue engineering arise from the inadequate vascularization of tissue, resulting in a lack of oxygen diffusion and nutrient perfusion into the cells deeper in the tissue (Lovett et al., 2009). The cardiac engineered constructs containing EC had a larger diameter and generated vessel-like structure, which was not achievable with CMs alone (Maiullari et al., 2018). ECs serve along with the VSMCs in the blood vessels for supporting oxygen and nutrient exchange in the heart. By incorporating the ECs, better vascularization can be achieved which in turn results in larger cardiac engineered constructs that become more clinically relevant to the regenerative therapies.

Cardiac fibroblasts

Cardiac fibroblasts (CFs) provide structural interstitial structures for myocardial formation by secreting ECM, which is regulated from crosstalk between CFs and CMs. CFs secrete regulatory molecules that aid in the biochemical and electrical properties of the myocardium (Radisic et al., 2008). CFs can be differentiated into myofibroblasts upon injury. In this stage, they are responsible for the production of growth factors, cytokines, ECM proteins, and proteases for cardiac remodeling.

CFs can be isolated from primary myocardial tissue. For example, the CFs can be isolated from 3-month-old to 5-month-old murine hearts. To isolate these CFs, a similar protocol with mincing the tissue and digesting it with collagenase was used (Li et al., 2017). These murine cells were then used to explore the structural and functional effects the different maturation of CFs has on CMs. CFs are also able to be derived from iPSCs by first differentiating into epicardial cells then further driving the commitment of iPSC-CFs using commercial fibroblast growth medium in the presence of FGF and TGF-beta inhibitor, SB431542 (Zhang et al., 2019).

The incorporation of CFs in the cardiac engineered constructs becomes highly significant for further assembling, condensing, and maintaining the 3D cardiac architecture in cardiac development and function. One study explored the effects of CF age on CM function and maturation and found that adult CFs induce changes resembling fibrotic disease (Li et al., 2017). The incorporation of CFs could be used to model cardiac fibrosis through excessive ECM secretion, myofibroblast transition, and crosstalk between CFs and CMs.

Immune cells

The immune cells in the heart include macrophages, T cells, monocytes, and B cells (Pinto et al., 2016). Cardiac macrophages that arise from yolk-sac and fetal monocyte progenitors play an important role in tissue repair after cardiac injury, coordinate cardiac inflammation, and regulate compartment size (Epelman et al., 2014). There are several subtypes of cardiac macrophages that have specialized functions such as stimulating T cells, immunosurveillance, the ability to uptake antigens and cells, and the release of interleukin-1 β (IL-1 β) and inflammatory responses. T cells are necessary for adaptive immunity but can affect the heart in other ways such as having a positive correlation with left ventricular dysfunction (Fukunaga et al., 2007). Subsets of T cells such as the regulation T cells have been shown to play a role in the modulation of inflammation and immune response and when not regulated or functioning properly can lead to a variety of cardiac diseases (Meng et al., 2016). One of the most abundant immune cell types in the heart are neutrophils, which function as the innate immune system. Neutrophils have also recently been shown to have a positive correlation with the severity of coronary damage in patients with coronary artery disease as well as a positive correlation with infarction size and a negative correlation with left ventricular function when the number of neutrophils increased in the blood (Chia et al., 2009; van Hout et al., 2015; Sharma et al., 2017). Although there are a very limited number of studies involving immune cells, more accurate and complex cardiac engineered constructs with immune cells are expected for gathering more insights into how heart tissue responds to the injury in cardiac repair and regeneration.

Chamber-specific cardiovascular cells

Recent advances in developing more complex cardiac engineered constructs with subtypes of chamber-specific cardiovascular cells further allow for modeling and recapitulating the cardiac diseases more precisely and accurately. The atrial and ventricular CMs can be specifically differentiated from PSCs by using bone morphogenetic protein 4 (BMP4) and retinoic acid inhibitors (RAi). For example, hESCs could be differentiated into the ventricular-like CMs with Noggin (BMP4 inhibitor) and RAi, whereas more atrial-like CMs with RA treatment during differentiation (Zhang et al., 2011). Recently, hESCs were more precisely differentiated into chamber-specific CMs by changing the concentrations of BMP, activin-nodal, Wnt, and RA signaling pathways (Goldfracht et al., 2020a). Moreover, atrial CFs have significant differences from ventricular CFs. Morphologically atrial fibroblasts are more elongated with more alignment across that axis and can grow at a faster rate than the ventricular fibroblasts (Burstein et al., 2008). The incorporation of chamber-specific cardiovascular cells produces the cardiac engineered constructs more complex and relevant for modeling and regenerating the chamber-specific cardiomyopathies.

ENGINEERING METHODS FOR FABRICATING CARDIAC ENGINEERED CONSTRUCTS

In this section, we review and summarize the eight most commonly utilized engineering methods as shown in Figure 2 for successfully fabricating cardiac engineered constructs.

Micropatterning

Micropatterning is the method of applying a specialized topography to a surface or substrate. This is done by either manipulation of the existing surface or by the addition of another layer with a preconstructed pattern. Cells are receptive to their immediate environment and the surrounding ECM as they utilize these structures to cue essential functions such as proliferation and differentiation. CMs derived from iPSC have been cultured by utilizing stenciled patterns to produce functional cardiac cell groupings (Batalov et al., 2021). In addition, sarcomere development can be improved by using micropatterning, a key component of contractility and a marker of cardiac output (Salick et al., 2014). Through controlled alignment and increased cardiac output, tissue engineers can look toward generating more complex and realistic cardiac models to supplement the development of new technologies related to disease modeling and regenerative medicine.

Optical lithography has emerged as a cornerstone for developing more advanced microtopography to better facilitate the modeling and development of cardiac engineered constructs. The method utilizes

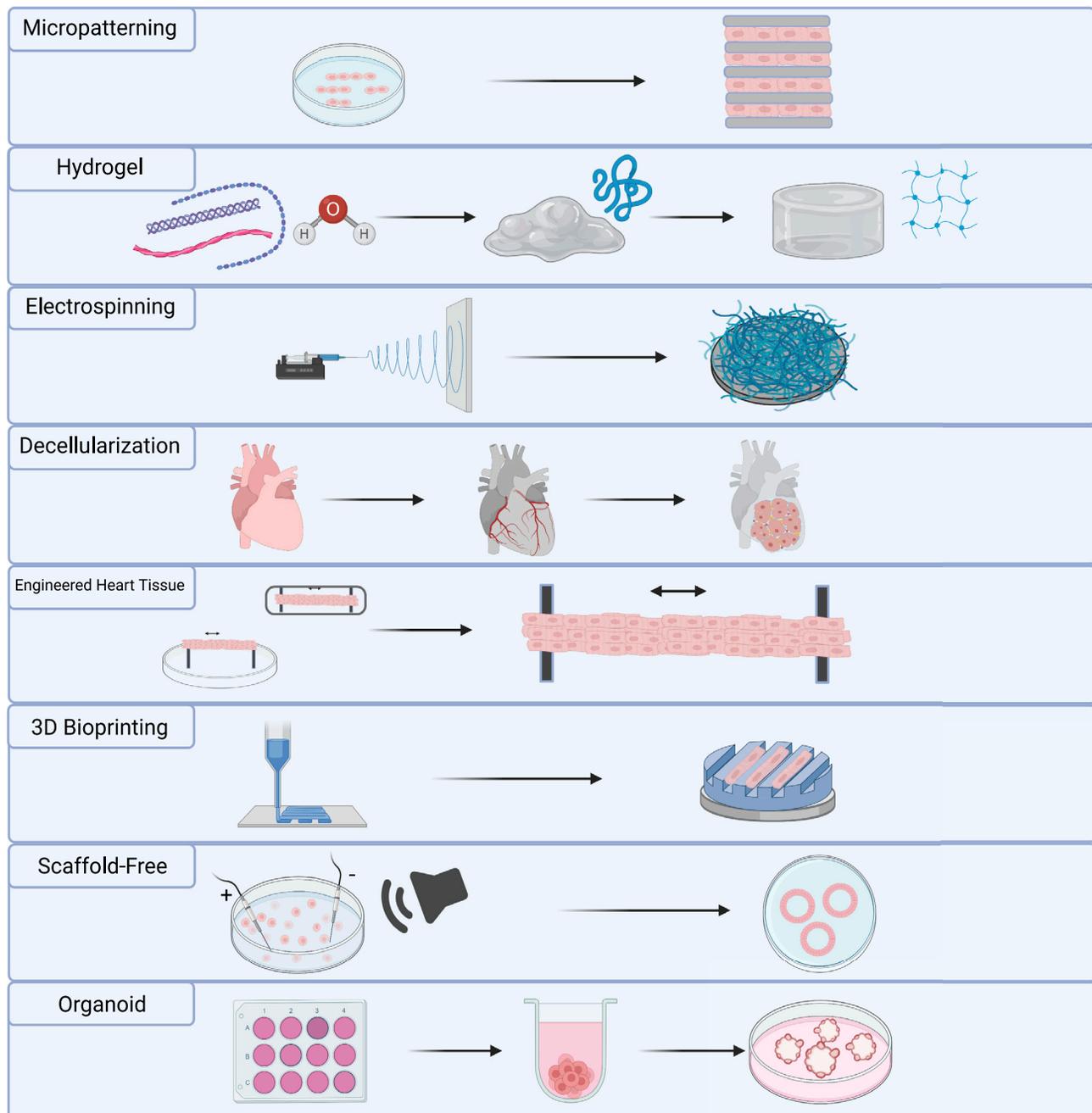


Figure 2. Engineering methods for fabricating 3D cardiac engineered constructs

Micropatterning: cells are aligned on a substrate by the guidance of grooves or other pattern techniques. Hydrogel: a polymer is chosen as base material and mixed with cells to form a gel, which is then hardened to desired stiffness through crosslinking. Electrospinning: a polymer solution is ejected onto a platform to form a fibrous scaffold. Cells are seeded on the scaffold. Decellularization: cells are stripped from natural tissues by surfactants and replaced with cells from the patient. Engineered heart tissue: CMs are suspended and attached in an aligned fashion. 3D bioprinting: layered printing of cells in hydrogels (“bio-ink”) to produce a detailed scaffold with mechanical assistance. Scaffold-free: the depicted patterning is organized using acoustic waves to move cells. Organoid: aggregates of pluripotent stem cells in ultralow attachment well plates are differentiated into cardiac tissues. Created with [BioRender.com](https://www.biorender.com).

various spectra of light to mechanically stencil a micropattern into the desired material or to help create a mold for later transfer of that micropattern (Kitsara et al., 2006). Building off the success of optical lithography, soft lithography utilizes the fabrication of a flexible and reusable stamp—usually consisting of an elastomeric material—for the micropatterning of culture materials. One of the most widely used

elastomeric materials for cardiac cell culture is polydimethylsiloxane (PDMS), which is primarily attributed to the material's low cytotoxicity, high optical transparency (Kitsara and Ducreé, 2013), affordable cost, and ease of manipulation. However, these polymers typically have limited resolution using these methods and cannot replicate important microstructure required to influence certain cell behaviors of cardiovascular cells *in vitro* (Wang et al., 2017). Both techniques have opened the door to many engineering possibilities as this method allows for high degrees of control when stenciling to better recreate advanced topographic features of the heart and has inspired the pivotal features of microfluidic devices (Zhao et al., 2001).

Other approaches relevant to micropatterning are less accessible as they require specialized equipment. However, their unique properties allow them to create topography with a higher degree of specificity for cardiac engineered constructs. One such is the use of laser tweezers for single-cell micropatterning. A study utilized this technology to prepare the pattern and identify interactions between CMs, specifically at their gap junctions (Yang et al., 2014a). These laser etching techniques offer the unique ability to fabricate cardiac tissue structure with high degrees of resolution both consistently and efficiently through the vaporization of the material (Gittard and Narayan, 2014). The use of low-pressure plasma has been increased in recent years in combination with current methods, offering a new tool for controlled pattern generation (Trantidou et al., 2014). The emergence of these unique microfabrication methods is promising for the field going forward and helps to offer insight on the horizon of next-generation micropatterning methods to isolate key features of cardiac tissues *in vitro*.

Hydrogel

Hydrogels have emerged as one of the most widely utilized methods for fabricating tissue models in recent years. Primarily, hydrogels consist of a porous network of either natural or synthetic polymers with the remaining space occupied mostly by water. These gels are solidified by interactions between the polymer molecules known as the process of "crosslinking," allowing for the formation of a sponge-like network (Malda et al., 2013). Cells can then be cultured either on top of the gel or interlaced within the matrix. Hydrogels offer multiple promising ways to recapture and transfer the intricacies of native tissue environments, such as the unique advantage of replicating tissue stiffness (Plotkin et al., 2014). The transportation of nutrients can also be facilitated by hydrogels, a useful feature for increasing the size of engineered tissue constructs. Modulation of both matrix geometry and substrate stiffness allows for flexibility of application and offers optimized ways to mimic both healthy and diseased tissue environments (Caliari et al., 2016).

Success has been reported across a variety of different hydrogel materials in fabricating cardiac engineered constructs. One such study was conducted by using iPSC-derived CMs, SMCs, and ECs in a fibrin scaffold for implantation into nonhuman hearts, resulting in a clinically relevant graft of cardiac muscle patch (Gao et al., 2018). Another instance utilized hydrogels containing type I collagen, a critical component of the ECM. During this study, the restoration of cardiac function in infarcted regions of nonhuman hearts with a reduction of fibrotic conditions was reported (Serpooshan et al., 2013). Collagen is a unique example as the protein is typically degraded by the body when used for implantation, making it favorable for temporary scaffolds to assist in cardiac repair (Yang et al., 2020). The use of methacrylated hyaluronic acid seems to incorporate both high biocompatibility and robustness in its use as a crosslinking agent (Hahn et al., 2006). Although hydrogels serve as a solid base for the next generation of cardiovascular therapies to arise, it is important to realize their limitations for use. Hydrogels are typically very fragile and can pose some challenges while handling. The relative stability of these scaffolds is significantly lower as compared to other fabrication methods. Nonetheless, the highly reproducible nature of this method and industrial availability have made them especially attractive for use.

Although replication of the ECM is critical to facilitate cellular processes, the iPSC-CMs produced do not typically present adult-like phenotypes. In search of increased clinical viability, some creative methods have resulted in improved CM maturity through additional stimulation via mechanical and electrical stimulations (Kroll et al., 2017). Biochemical means would be also suggested for the stimulation of developmental cues in the cardiac microenvironment such as the supplementation of fatty acids to promote the metabolic maturation of iPSC-CMs (Horikoshi et al., 2019). The downregulation of *HIF-1 α* , typically associated with increased fatty acid oxidation, has been shown to increase iPSC-derived CM maturity when cells were supplemented with inhibitory postnatal factors (Gentillon et al., 2019). Hydrogels also show promise as a method to generate chamber-specific tissue constructs. Through the embedding of atrial and ventricular specific CMs in a ring-like pattern on a collagen hydrogel, a model to observe arrhythmia in the presence of

pharmaceuticals was established (Goldfracht et al., 2020b). The next generation of cardiac engineered constructs and cardiac therapies will look to expand upon additive manufacturing with hydrogels.

Electrospinning

Electrospinning is a method that utilizes high voltage to electrostatically eject a polymer solution out of a nozzle (Taylor Cone) toward a platform to generate fibers via elongation of the solution. Although the platform can be rotating or static, these fibers stick to the desired surface and serve to mimic the microscaled network structure of ECMs. Although these fibers do not contain the exact components of ECMs, they often retain biocompatible and conductive properties required for the proper function of cardiac tissues (Bertuoli et al., 2019). Scaffolds derived from electrospinning offer two key advantages: the fibrous characteristics of the scaffold and the simplistic nature of the method, making it highly modular.

Myocardial tissue consists of interweaving collagen fibers interlaced with highly fibrous cells (Kitsara et al., 2017). The importance of a fibrous scaffold is highlighted with both CMs and CFs relying on this specific pattern of organization for direction-dependent factors such as contractility and electrical signal propagation (Engelmayr et al., 2008). These aligned fibers have also been proven to provide structural cues to promote cellular processes such as dictating the cell fate and function of CMs in the cardiac engineered constructs (Ding et al., 2020) as well as displaying increased calcium transience and cell-cell interactions (Zhu et al., 2018). In addition, the modification of polymers used allows for the alteration of fiber characteristics such as fiber morphology, diameter, and biocompatibility (Fang et al., 2010). The focus of newly developed polymers has shifted toward increasing biocompatibility of the scaffold, preserving CM contractility (Gouveia et al., 2017), and increasing polymer conductance (Marsudi et al., 2021) to optimize cardiac output and viability of cardiac engineered tissue models.

The streamlined nature of this fabrication method allows for combination with other existing methods, holding great potential for realizing cardiac regeneration and drug screening applications. For example, the use of hydrogels as a spun material allows for selective stiffness of substrate and facilitates diffusion of nutrients (Majidi et al., 2018). However, because of the complexity of myocardial tissues and their difficulty of accurate replication, overcoming this substantial hurdle will require the fabrication of more complex electrospun materials for biomimetic cardiac engineered constructs. Incorporating features on the nanoscale that induce cell proliferation or deliver signaling molecules will be influential in overcoming current limitations (Abdollahiyan et al., 2021). Therefore, tissue engineers must find new ways to compact the physical and biochemical features of cardiac tissues using emerging nanotechnologies and other advanced means.

Decellularized scaffolds

Tissue-derived decellularized scaffolds have emerged as an effective tool for generating cardiac engineered constructs. This method utilizes an original tissue sample and strips the native cells to leave a preserved cardiac ECM scaffold. The derived scaffold can then be reintroduced with cardiovascular cells obtained from a patient or other alternate sources, such as iPSC derivatives. Decellularization of tissues is typically conducted chemically through perfusion-decellularization, a series of surfactant washes that include SDS and Triton X-100 (Guyette et al., 2016). Additional means include enzymatic and physically induced decellularization (Tang-Quan and Karis et al., 2018). Bioactivity of the scaffolds is generally very high and further treatment of the material is rarely necessary for seeding. However, it is also important to keep in mind that each method is not completely efficient and results in some degree of ECM degradation, offering a baseline for improvement in recent years (Haupt et al., 2018).

One critical advantage of decellularized scaffolds is that they contain region-specific ECM microstructure, as the sample is taken directly from cardiac tissue. This method of engineered tissue fabrication is particularly of interest for use as cardiac grafts. The use of these biomaterials for cardiac grafts would allow for increased restoration of function because the structural uniqueness of the scaffold can match that of the damaged region. Much like in embryonic development, the exact coordination among cells in 3D space allows for the development of differential tissue functionality because each region of the heart is responsible for a different task (MacGrogan, Münch and de la Pompa, 2018). Although the usage of any scaffold for repair would result in possible regeneration and function restoration of damaged myocardial tissues, region-specific scaffolding would promote matching functionalization of the area and give the best chance for restoring the lost cardiac muscle in higher efficiency. The unique perspective of recapturing distinct regions of the heart is the primary focus of many ongoing studies on cardiac asymmetry and the abnormal

patterning that can arise from various pathologies (Desgrange et al., 2018). Replicating these features artificially has been difficult, acting as a barrier in the field in the past (Iop et al., 2017), and offers an area for improvement going forward.

There are two classifications of grafts used by cardiac tissue engineers: allogeneic and xenogeneic (Zia et al., 2016). Allografts are samples derived from human tissue, meaning, ECM composition and specialization are ideal for therapeutic use. One study highlighted the capability of decellularized allografts to aid in right ventricular reconstruction (da Costa et al., 2017). The results showed that region-specific scaffolding must be considered as well as the origin of the sample. Although the use of allografts demonstrates promising results, they are typically scarce, considering the limited number of willing donors and the invasiveness of collection. Therefore, the use of xenografts—samples derived from animals of other species—may offer an attractive alternative. Decellularized scaffolds derived from porcine and bovine sources have been proven to display minimal immune rejection and excellent biocompatibility even after implantation (Li et al., 2018; Al-Hejailan et al., 2021). Similar studies report prevention of ventricular wall thinning and an increase in M2 macrophage concentrations at the site of implantation, a marker of implant acceptance (Shah et al., 2019). Although these results are promising, the use of xenogenic grafts must be explored in extensive detail and on a much larger scale before widespread clinical use can occur.

Engineered heart tissue

In native cardiac tissues, CMs join through end-to-end connections and side-by-side bundles, forming elongated structures. The adjoined cells serve to facilitate mechano-electro activation and propagate signals for the regulation of contractility. Several techniques have attempted to connect chains of CMs to form myocardial tissues in 3D space. One such study utilized a system based on a well containing CMs and matrix proteins to generate the engineered heart tissues (EHT). This reservoir contained two posts meant to facilitate adjoining CMs to form a suspended bridge of tissue, creating a 3D model as it is not limited by substrate attachment and promotes cell alignment similar to that seen *in vivo*. Transcriptional analysis of iPSC-CM formed EHTs revealed that hypoxic conditions were replicated more accurately in the 3D EHT versus the 2D monolayer culture shown in our recent study (Yang et al., 2021). Similarly, the bio-wire method seeks to replicate the unique features of the myocardium by running a long singular wire across the length of a culture reservoir (Xiao et al., 2014). In this reservoir, cells are suspended and allowed to attach to the wire, allowing for unidirectional alignment across the surface of the wire to form elongated cultures resembling cardiac tissue structure. In addition, the degree of control for stressors applied on the model is greatly increased, as mechanical and electrical stimuli can be applied along the direction of the wire (Nunes et al., 2013). CMs cultured using this method have well-developed sarcomere structures and action potentials expected of high functioning cardiac phenotype and activities (Wang et al., 2019). Heart chamber specificity has also been achieved using Biowire II, exercising the ability to reliably discriminate between the two subtypes of CMs (Zhao et al., 2019). Here, the modeling of atrial tissue paves the way for the induction of pathologies, such as atrial fibrillation, to better understand the conditions and what drugs may offer effective treatments (Lemoine et al., 2020).

3D bioprinting

In recent years, the attention of many has turned to 3D bioprinting for the next generation of advanced tissue models and cardiac patches. 3D bioprinting uses an additive layering of biocompatible materials and interlaid cells to achieve a tissue construct that more closely resembles the microstructure of specific organs. The fabrication method grants previously unavailable degrees of control and allows for the generation of additional layers of complexity to engineered tissues by allowing for the modulation of certain factors, many of which include qualities such as cell density, heterogeneity, matrix stiffness, and construct size. The general principle of 3D bioprinting follows the conventional standards of current 3D printing techniques. Typically, this includes a 3D printer with a heated nozzle on a mechanical rail system which allows for precise control when depositing a substrate. A common method for cardiac tissue bioprinting is known as micro-extrusion printing, where the bio-ink residing within the nozzle is pneumatically extruded using a plunger, displacing the material for printing on a surface (Ong et al., 2017).

Factors contributing to the widespread use of 3D bioprinting include the ability to replicate intricate tissue structures and the placement of controlled cell densities. One study that was conducted focused on the bioprinting of CM dense cardiac spheroids within a self-healing hydrogel (Daly et al., 2021). This strategic placement of spheroids allows for the replication of cell density and heterogeneity of cardiac tissues,

potentially attributing to the formation of structure-dependent organoids. The high resolution of 3D bioprinting has also prompted methods for inducing angiogenesis in engineered tissue constructs, increasing the scalability of models and efficacy of regeneration (Saberianpour et al., 2018; Lee et al., 2019). To better improve this degree of spatial control, additional manufacturing techniques have been explored such as the use of magnetic nanoparticles embedded in bio-inks to influence the orientation of cells through induced scaffold remodeling (Buyukhatipoglu et al., 2010), showing promise in the separation of cell lines within an engineered tissue construct. Additional control can be achieved using acoustic waves to orient cells into the desired pattern after they have been printed onto a scaffold (Zhu et al., 2017). These methods seek to overcome some of the major challenges facing current bioprinted tissue constructs, such as limited regulation of cell clumping and inadequate oxygen and nutrient transport to facilitate larger organ models. Improvement of the mechanical stability of printed constructs must also be investigated further; besides, similar to hydrogels, they are often delicate and can receive damage during handling or physical manipulation.

Scaffold-free fabrication

To circumvent the limitations of conventional scaffolding techniques, some methods have been investigated that do not require direct physical contact with cells to produce an intended pattern or ideal cell density. Typically, scaffold-reliant methods induce cellular processes and functions through morphological guidance as opposed to scaffold-free methods which depend more heavily on cell-cell interactions. These constructs can be fabricated on low-attachment substrates and rely on the formation of cardiac spheroids. However, one promising technique using faraday waves to induce patterning of CMs offers a much more directed approach. These clusters contained ideal cell density and displayed contractility of that found in mature myocardium, providing evidence that these methods are just as effective (P. Chen et al., 2017a). Spheroids of different cell types can be fabricated and then pieced together to create heterogeneous tissue constructs with more organ-like functions (Arai et al., 2018). Scaffold-free methods of cardiac tissue fabrication hold promise as a highly efficient and reproducible technique with extended applications, especially because the biocompatibility of materials used is a nonfactor.

Organoids

Similar to the scaffold-free methods detailed before, the use of organoids for cardiac tissue development has promise because of the natural and dynamic cell-cell interactions. Organoids are hPSC-derived tissue models with self-organized structures that resemble *in vivo* organs/tissues. There are already a variety of organoids that mimic other organs in the human body such as liver organoids (Nahmias and Odde, 2006) or kidney organoids (Takasato et al., 2015). Over the years, there have been several studies using cardiac organoids and further perfecting their protocols to model an *in vivo* heart more accurately. Several studies demonstrated the differentiation of cardiac organoids with spontaneous beating, which feature chamber-like structures (Lee et al., 2008). One of the more recent studies demonstrated the development of cardiac organoids that could be used to model myocardial infarction as well as drug cardiotoxicity (Richards et al., 2020). There have been cardiac organoids that have expressed early hallmarks of both regeneration and heart disease when injured and could be used to model congenital heart defects (Hofbauer et al., 2021).

One of the methods of fabricating cardiac organoids is by casting a mixture of hiPSC-CMs and hiPSC-ECs into nonadhesive agarose hydrogel molds containing microwells that allow for the formation of the spherical microtissues (Richards et al., 2017). Cardiac organoids can also be directly derived by micropatterned iPSC differentiation using thin PDMS stencils where cells are seeded (Hoang et al., 2021). These methods of fabrication can optimize contraction duration and diastolic functions as well as developmental toxicity for pharmaceutical drugs. One of the most common methods for cardiac organoid generation is direct differentiation of hiPSC spheroids through WNT pathway modulation in U-shaped, ultralow-attachment 96-well plates (Drakhlis et al., 2021).

In addition, the side-by-side comparison of the techniques for the fabrication of cardiac engineered constructs is provided in Table 1.

BIOMEDICAL APPLICATIONS

Although it is common to assume that all these methods serve the sole purpose of creating fully functional artificial tissue constructs, that would be only partially correct. The development of advanced cardiac

Table 1. Evaluation of fabrication methods of engineered cardiac constructs

Technique	Advantage	Limitation	References
Micropatterning	<ul style="list-style-type: none"> Promotes cell alignment Mediates cell density High reproducibility Combine with other techniques 	<ul style="list-style-type: none"> Tissue constructs limited to 2D or 2.5D Constructs not suited for implantation Limited complexity in tissue organization 	<ul style="list-style-type: none"> (Gittard and Narayan, 2014) (Trantidou et al., 2014) (Batalov et al., 2021) (Wang et al., 2017)
Hydrogel	<ul style="list-style-type: none"> Replicates tissue stiffness High biocompatibility Custom design Chamber-specific^a 	<ul style="list-style-type: none"> Fragile Limited cell directionality 	<ul style="list-style-type: none"> (Plotkin et al., 2014) (Goldfracht et al., 2020b) (Yang et al., 2020)
Electrospinning	<ul style="list-style-type: none"> Low cost High reproducibility Facilitates proper cell alignment 	<ul style="list-style-type: none"> Limited flexibility Low biocompatibility Limited complexity in tissue organization 	<ul style="list-style-type: none"> (Zhu et al., 2018) (Gouveia et al., 2017) (Majidi et al., 2018)
Decellularized scaffolds	<ul style="list-style-type: none"> Recaptures 3D organ-specific architecture Low cytotoxicity Chamber-specific^a 	<ul style="list-style-type: none"> Difficult/limited production efficiency Limited sample availability 	<ul style="list-style-type: none"> (Li et al., 2018; Al-Hejailan et al., 2021) (Iop et al., 2017)
Engineered Heart Tissues	<ul style="list-style-type: none"> Accurately recaptures myocardial structure Promotes proper contractility of CMs 	<ul style="list-style-type: none"> Limited applications Low modularity of apparatus 	<ul style="list-style-type: none"> (Lemoine et al., 2020) (Yang et al., 2021) (Wang et al., 2019)
3D Bioprinting	<ul style="list-style-type: none"> Creates desired microarchitecture with high accuracy and reproducibility Modularity of bio-inks 	<ul style="list-style-type: none"> Limited print resolution Size of prints limited to diffusion 	<ul style="list-style-type: none"> (Ong et al., 2017) (Saberianpour et al., 2018) (Lee et al., 2019)
Scaffold-Free Approaches	<ul style="list-style-type: none"> No extrinsic biomaterials used Naturally derived cell-cell interactions High efficiency and reproducibility 	<ul style="list-style-type: none"> Dependence on self-organizing spheroids Limited regenerative medical applications Limited accessibility 	<ul style="list-style-type: none"> (Chen et al., 2017a) (Arai et al., 2018)
Cardiac Organoids	<ul style="list-style-type: none"> Naturally derived cell-cell interactions Recaptures cell (sub)types, compositions, and networks in cardiac development Existing organoids used for disease modeling 	<ul style="list-style-type: none"> Immature at the early heart developmental stage Low reproducibility with large variations among labs and protocols 	<ul style="list-style-type: none"> (Lee et al., 2008) (Hofbauer et al., 2021; Richards et al., 2017)

^aIndicates the chamber-specific applications reported in those fabrication methods. It does not refer to those other methods which cannot be chamber-specific. There is just no corresponding report yet.

engineered constructs opens the door to a multitude of new possibilities not only in cardiac regeneration but also in disease modeling and drug screening methodologies as summarized in [Figure 3](#).

These modeling techniques have been most recently addressed by efforts to create organ-on-chip devices. By delivering an oversimplified model of heart structure and function, these devices take advantage of induced native tissue characteristics such as multidimensional cell interactions and mimicked vasculature perfusion to model complex systems in a concise manner. These devices can be created using iPSC-derived cardiovascular cells from a healthy donor or a patient to give insight into the specific response of an individual to a given drug (Chan and Huang, 2021). Other methods best suited for the creation of cardiac disease models include hydrogels, electrospinning-derived scaffolds, and 3D bioprinting. The

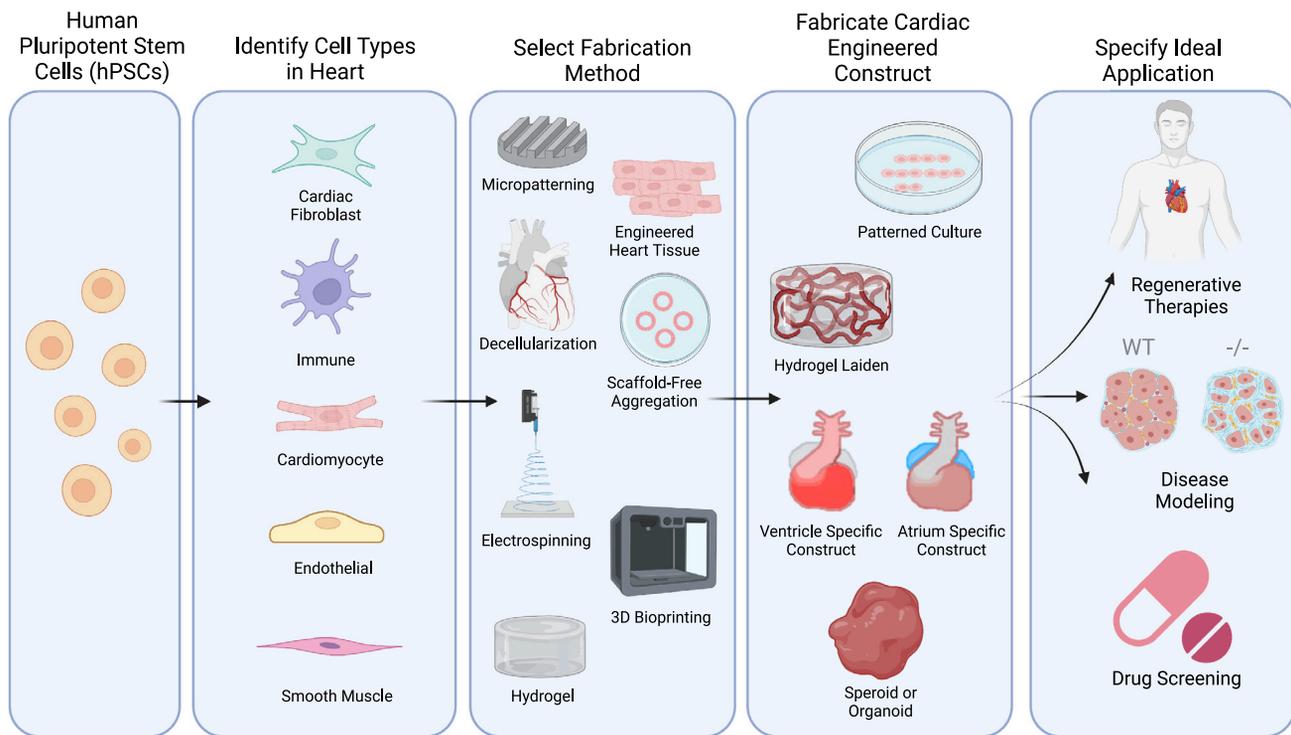


Figure 3. Overview of fabrication methods and applications for cardiac engineered constructs

First, human pluripotent stem cells are sourced from donors through reprogramming or from direct extraction as embryonic stem cells. The pluripotent stem cells are then differentiated into various cardiac-specific cell types, such as the myocardium (fibroblasts, cardiomyocytes), vasculature (endothelial, smooth muscle), and immune cells. The derived cells are then combined with various fabrication methods, ranging from the use of additive biopolymers to utilizing natural components to generate ECM-like scaffolds using biocompatible materials. The use of these engineering methods enables growth and maturation of the cell culture to generate a cardiac engineered construct, some of which are capable of replicating chamber-specific conditions. The cardiac engineered constructs maintain important properties of *in vivo* tissues, allowing for various applications. These include (but are not limited to): the creation of *in vitro* disease models, evaluation of drug efficacy through screening, and regenerative therapies such as through cardiac patches. Created with [BioRender.com](https://www.biorender.com).

method of 3D bioprinting lends specific talents as it allows for the precise depositing of specific cell types and formation of proper cell densities in the cardiac engineered constructs, key features to aid in the creation of personalized cardiac organoid models (Ma et al., 2018). Human cardiac organoids in a closer heart complexity offer unrivaled screening capabilities as more is understood about cardiovascular development, and advanced methods for directing the differentiation of progenitor cells are explored (Takebe and Wells, 2019). Much like 3D bioprinting, the EHT and Bio-Wire platform offers unique ways to obtain 3D myocardial assemblies, leading to another potential method for specific evaluation of drug effects on the myocardium and calcium signal propagation among others. The creation of these 3D myocardial assemblies offers an effective model for drug screening by offering more closely replications of *in vivo* morphology, cell composition, and tissue function, paving the way for personalized medicine using cardiac engineered tissue models.

Because myocardial tissue is constantly remodeled in response to both development and disease, utilizing personalized models would provide an opportunity to better capture these changes for purposes of replicating cardiac disease states *in vitro*. The stiffness of myocardial tissue plays a significant role in the expression of pathologies as a direct biomarker and can be used as a tool to assess cardiac output (Villalobos Lizardi et al., 2022). Highly customizable hydrogels offer the most promising for use in studying cardiac development and pathology as their stiffnesses and active compositions can be modulated to replicate the ECM of a specified condition (Crocini et al., 2020). Disease states of interest, such as cardiac fibrosis, stand to be better understood by combining technologies such as decellularization and bioprinting by taking advantage of their strengths and rescuing the shortcomings of the others. One such study was able to derive a bio-ink from cardiac decellularized materials to provide further resolution of the printer through the assistance of mechanical cues to improve the tissue-level organization of the cardiac construct (Shin et al., 2021).

Cardiac engineered constructs have had a tremendous impact in the field of regenerative medicine through the development of cardiac patches. Patches typically operate by providing temporary scaffolding in an area of damaged cardiac tissue. This engineered scaffolding can either be cell-containing or cell-free, is specifically designed to facilitate the proliferation of multiple cell types, and induce restoration of function. They are especially of interest for use in aiding patients to recover from myocardial infarction and treating the tissue damage that ensues (Izadifar et al., 2018). Methods such as using decellularized scaffolds and using ECM like-hydrogels have been proven useful in supporting partial regeneration of cardiac tissues, although the degree is still limited because of the proliferative abilities of CMs. Ideal qualities still need to be realized such as conductivity, the robustness of the material, and increased cardiac functionality. However, these materials display superior biocompatibility and provide a temporary ECM that allows for some restoration of tissue where it normally would not occur, a promising result that many cardiac tissue engineers are eager to build off. Many of which are exploring ways to optimize regenerative capabilities and induce vascularization of cardiac tissue, such as through alteration of scaffold stiffness (Plotkin et al., 2014), subsection to mechanical stretching (Yang et al., 2016a), and direct engraftment of vasculature from decellularized hearts (Dahl et al., 2011). Improving the design of these materials allows for the generation of more mature cardiac tissues with native cell types, regardless of application.

PERSPECTIVES

Although the field of cardiac tissue engineering is accelerating forward with the contributions of many, there is still debate as to where the focus should be prioritized. To date, the ability to generate tissue constructs with limited functionality and cell heterogeneity is an established feat of the field. However, these models still lack the complexity and specificity needed to identify the trajectory of heart development and causes of region-specific cardiomyopathies. Because the heart is asymmetric, the morphology of each half is unique to execute the required function of that region. Atrial and ventricular CMs have been shown to display distinct phenotypes and pharmacological reactions, indeed confirming significant regional differences among CMs. Thus, identification of these regional differences may be instrumental in developing more tailored therapeutics. One such example is pulmonary arterial hypertension, which is known to be dictated by right ventricular function and impact the prognosis of the patient (Groeneveldt et al., 2019). It can also be assumed that CM alignment and activity changes as a function of structural differences, for example between the thinner-walled right ventricle and the left ventricle. Intracellular peak calcium transience has been shown to differ with right ventricular retaining a slightly lower value, although no differences in calcium handling protein expression were detected (Walker et al., 2013). Although an older study suggests the possibility that myosin isozymes are responsible for alterations in calcium handling (Brooks et al., 1987), further studies must be conducted to better understand cardiac output on a cellular level and translate the findings to be replicated on cardiac tissue engineering platforms. There is even concern that among these region-specific cells, hiPSC-derived tissues can vary from adult *in vivo* tissues to a significant enough degree to impact drug response observations. Similar to engineered atrial tissue constructs, the repolarization capabilities of hiPSC-derived cells are questioned as potassium ion efflux has been observed to occur in a minor divergence from those of natural sources (Horváth et al., 2020). Besides, worth citing is a lack of studies incorporating immune cells into the cardiac engineered constructs, which must also be addressed to develop more complex heart tissue with extended understanding and applications. Utilization of multiple cardiovascular cell types is imperative for these cardiac engineered constructs to be considered as an alternative for cardiac disease modeling and regenerative medicine.

Once these differences have greater definition and are better understood, the methods to recapture these conditions *in vitro* need to deliver in both complexity and efficiency required for next-generation cardiac engineered constructs. Much like the lucrative combination of decellularization and bioprinting, methods outside of microenvironment design must be explored. Currently, cardiovascular cell types utilize genetic modifications to replicate certain facets of pathological conditions. This means that the methodology can provide high efficiency in obtaining the desired phenotype, a task that is typically achieved through rigorous testing of physical or chemical remodeling methods. Perhaps in the future, genetic modulators in conjunction with microenvironment regulation can be combined to further refine tissue construct quality and improve overall functionality in the heart through providing reliable and accurate models for cardiac development and diseases.

CONCLUSIONS

Implementation of multiple cardiovascular cell types and the development of more advanced bioengineering techniques are equally critical for the capture of *in vivo* conditions and functionality among cardiac

engineered constructs. The role of cell heterogeneity in heart tissue cultures allows for more complex and accurate communications between different cell types that are only present in the cardiac tissue. In conjunction, the advancement of engineering techniques allows for these cell types to obtain proper organization and concentration in space to allow for optimal heart-like functions to be achieved. Further studies can then utilize this high degree of control to replicate the microtopographic differences between heart chambers, paving the way for personalized tissue models of an ultra-specific region of the heart to evaluate drug efficacy or seamlessly integrate with surrounding tissue in the form of a curing patch. No matter the application, the capacity to control fully functional tissues would impact those relying on heart transplants and decrease the toll of heart-related diseases on society. Last but not the least, owing to the limited space and rapid growth of the cardiac engineered constructs field, we likely missed several important works in this review.

ACKNOWLEDGMENTS

We like to thank our funding sources: University of North Texas (UNT) Biomedical Engineering, Start-up funding (H.Y.), and UNT Research Seed Grant (H.Y.)

DECLARATION OF INTERESTS

The authors declare no competing interests.

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