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Towards chamber specific heart-on-a-chip for drug testing applications

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Abstract

Modelling of human organs has long been a task for scientists in order to lower the costs of therapeutic development and understand the pathological onset of human disease. For decades, despite marked differences in genetics and etiology, animal models remained the norm for drug discovery and disease modelling. Innovative biofabrication techniques have facilitated the development of organ-on-a-chip technology that has great potential to complement conventional animal models. However, human organ as a whole, more specifically the human heart, is difficult to regenerate *in vitro*, in terms of its chamber specific orientation and its electrical functional complexity. Recent progress with the development of induced pluripotent stem cell differentiation protocols, made recapitulating the complexity of the human heart possible through the generation of cells representative of atrial & ventricular tissue, the sinoatrial node, atrioventricular node and Purkinje fibers. Current heart-on-a-chip approaches incorporate biological, electrical, mechanical, and topographical cues to facilitate tissue maturation, therefore improving the predictive power for the chamber-specific therapeutic effects targeting adult human. In this review, we will give a summary of current advances in heart-on-a-chip technology and provide a comprehensive outlook on the challenges involved in the development of human physiologically relevant heart-on-a-chip.

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Y.Z., N.R. and E.Y.W. prepared and edited the manuscript, B.F.L.L., R.X.L., H.S. and M.R. edited the manuscript. M.R. is the corresponding author.

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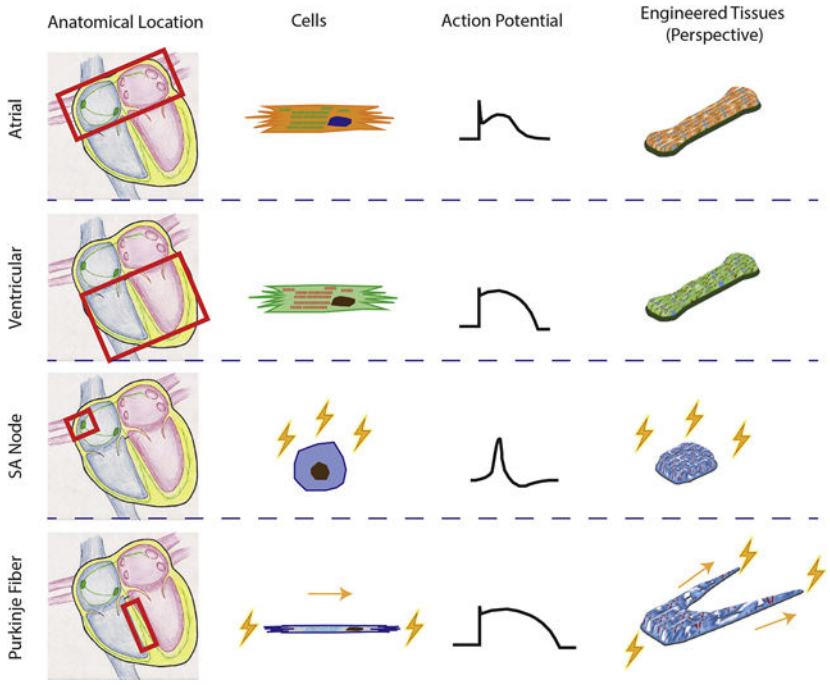
Graphical abstract:



Keywords

drug testing; cardiomyocytes; platform; tissue engineering; chamber-specific; disease modeling; toxicity; atrial; ventricular; purkinje; SA node; cardiotoxicity; screening; heart; maturation; heart-on-a-chip; organ-on-a-chip

On average, the human heart beats 3 billion times during a lifetime, starting as early as 3 weeks of gestation age and stopping right before death.[3]. It is reported that human cardiac muscle has a limited ability to regenerate, with approximately 50% of adult human cardiomyocytes (CM), being throughout a lifetime of 80 years. [4–6] Because of the scarce availability of human adult CMs, cardiac researches have to rely on animal models for studying human cardiac diseases, despite the inter-species differences. However, the emergence of induced pluripotent stem cells (iPSC) and the recent advancement made in differentiation techniques [7] have greatly transformed this conventional research paradigm. Directed differentiation of human pluripotent stem cells (PSCs) can now generate large numbers of cardiomyocytes readily available for research.[8] Thus, cardiac tissue engineering becomes a pivotal conduit to explore the applications of these cells for drug testing and patient-specific disease modeling purposes.

Despite the advances made in tissue engineering to revolutionize the field of therapeutic discovery, the effort to re-create a physiological-relevant heart remains a foreseeable challenge. The human heart is a muscular organ that is characterized by its distinctive chambers orientation and the built-in electrical conducting system that stimulate the muscle contraction. However, conventional bench-top studies lack the capability to mimic this complex 3D orientation. Heart-on-a-chip, one of the more recent tissue engineering

technologies, provides an interesting alternative. It adopts cardiac cell developmental biology and allows precise physical control of microenvironment to create miniaturized 3D cardiac tissue model that offer more reliable clinically relevant biological functions and disease mechanisms. [9]

In reality, current heart-on-a-chip platforms should be viewed as ventricle-on-a-chip platforms since the majority of the current studies use ventricular CMs and only study drug responses and diseases modeling related to ventricle chambers. This is because ventricular dysfunction is the primary cause of heart failure[10] and ventricular CMs is the first chamber-specific CM population that can be differentiated with high purity[11]. Nevertheless, other compartments of the heart, such as the atria, sinoatrial node, and Purkinje fibers, are just as important as ventricles during the investigation of drug-related toxicity and cardiac disease mechanisms (Figure 1). For example, conditions of the atrium, such as atrial fibrillation (AF), can elicit sudden death or severe complications during the medical intervention of ventricular chambers.[12] Failure of sinoatrial node function due to congenital disease or aging results in slowing of the heart rate, inefficient blood circulation and arrhythmia.[13] Therefore, it is imperative to obtain these compartment-specific tissue models and to condition them to reach adult maturity for relevant evaluation of their responses to drug dosage and therapeutic intervention.

This review will highlight the current progress of cardiac tissue engineering of specific heart chambers and discuss the challenges to generate a physiologically-relevant heart-on-a-chip model for drug testing applications and disease modeling.

Brief History of Cardiac Tissue Engineering and Heart-on-a-Chip

The main paradigm tissue engineering is to use cells and other biological materials to build functional tissues. Organ-on-a-chip technology, as the convergence of tissue engineering and microfluidics, aims to build functional miniaturized tissues *in vitro*, simulating the functional characteristics of *in vivo* tissues or organs for the purposes of drug screening and disease modeling.[14, 15]

Long before the invention of the terms “tissue engineering” or “organ-on-a-chip”, Moscona et al were the first to aggregate chicken embryonic myocytes into a spheroid shaped construct by a continous spinning of the fresh cells in flasks, back in 1950s[16]. This culture method is considered the first *in vitro* platform in the cardiac field, that enables 3D tissue culture. It has been adapted and improved for culturing various neonatal animal cardiac cells by many groups. In 1999, Akins et al defined a better and more detailed protocol for culturing sphere-shaped 3D structures, resulting in tissues that were structurally-organized presenting with spontaneous and rhythmic beating patterns[17]. Despite the success of simulating cardiac organogenesis *in vitro*, the morphology and organization of the resulting organoids was not comparable to the native heart muscle.

Heart is a complex organ, which requires sophisticated organization and synchronized behavior of cardiomyocytes. Many efforts were made in the 1990s towards building a functional *in vitro* platforms. Terracio et al performed chronic cyclic stretch on 2D cell

sheets.[18] Carrier et al. used a polyglycolic acid (PGA) scaffold together with laminar flow to form tissue-like structures (50–70 μm thick) in which myocytes were organized in multiple layers in a 3D configuration. [19, 20] Bursac et al seeded cardiomyocytes into patch-shaped PGA scaffolds culturing them in the spinning flask based bioreactor, which enabled improved oxygen supply, thus promoting a better cardiac function. The platform also enabled the electrophysiological assessments on the patch. [19, 20] Similarly, the groups of Li and Leor seeded rat neonatal myocytes into gelatin or alginate scaffolds respectively, which were later engrafted into rat hearts.[21, 22] Tissues seeded on patterned surfaces obtained improved cellular alignment and enhanced tissue function. [23, 24]

Instead of reconstructing the whole heart *in vitro*, an easier solution is to recapitulate the function and morphology of the basic unit of cardiac muscle. With this in mind, in 1997, Eschenhagen et al were the first to invent the biaxial culture platform with two Velcro-covered glass tubes, which created cardiac tissues recapitulating cardiac muscle bundles. The system can provide anchor points for tissue attachment and static stretching forces during cell compaction and tissue formation for cellular alignment.[25] The arrangement allows the measurement of the isometric contraction force generated by the engineered cardiac tissues. [26] They were also the first group to use collagen I gel aiding cardiac tissue formation. [25] In the later work from this group, the rigid tissue anchor was replaced by posts made of flexible and biocompatible materials, PDMS, significantly increasing the throughput. The mechanical properties of PDMS and the bending motion resulting from tissue contraction could be monitored non-invasively for miniaturized tissue culture systems with a higher throughput.[27] The system has been adapted and modified by multiple research groups and became the earliest version of heart-on-a-chip in the field.[28–35]

In parallel to developing improved tissue culture systems, significant enhancements in cell sources were also achieved over the past two decades. The first engineered cardiac tissue was generated by murine neonatal myoyctes. However, through generation of human embryonic stem cells (hESC), human stem cell derived cardiomyocytes were gradually introduced into engineered platforms. Caspi et al were among the pioneers who introduced human stem cell derived cardiomyoyctes into 3D structure [36]. Shortly after the discovery of human induced pluripotent stem cells (hiPSC)[37], hiPSC derived cardiomyocytes quickly became available to replace the conventional animal cell sources and hESC derived cardiomyocytes [7, 38] in tissue engineering and organ-on-a-chip applications.[30, 35, 39] Chamber-specific cardiomyocytes became available shortly after to create chamber-specific tissues.[2, 28]

Ventricular Tissue Engineering

Ventricles have thick muscle walls and are the primary driving forces for blood flow through the entire body. The diminished ventricular function negatively impacts the quality of life of the individuals and has become one of the primary research foci on therapeutic strategies. Ventricular differentiation method has been well established since the discovery of iPSCs more than a decade ago [40] and produce consistently reproducible results among species. [41] The emerging challenge is to fully exploit these ventricular CMs for more clinically relevant drug testing. Engineered cardiac tissue targeting drug testing applications has been

envisioned as a miniaturized, functional 3D tissue that not only recapitulates human adult myocardium, but also can be easily reproduced and tested in a high throughput manner.[42]

3D Ventricular Tissue Models

3D cardiac tissue is commonly preferred since it has more physiologically relevant cell-cell cross talk and cell-matrix interactions compared to 2D monolayer cultures. [43] These biological cues are particularly relevant in cardiac tissue engineering, since the coordinated contraction at the tissue level depends on the intercellular ion exchanges, electrical coupling, and mechanical transduction. [44] Cardiac organoids are created by self-organization into a sophisticated architecture that closely resembles the organogenesis of native cardiac tissues. [15, 45–48] The organoids are typically small in size, i.e. 100 μm, with multiple cardiac lineages and are commonly maintained in a relatively high throughput manner. Unfortunately, there is minimal control over the development of organoids in terms of their structural and functional consistency, due to our limited knowledge of cardiac tissue development. More importantly, their functional output, as well as drug responses, suggest a low level of maturity and little resemblance to the adult myocardium.[49–51]

Organ-on-a-chip technology, more specifically heart-on-a-chip, enables precise control of the physical environment that provides topographical guidance, nutrient supply, flow pattern and shear stress and facilitates functional readouts with built-in sensors. [15, 39, 52–55] Muscle thin film[56] (Figure 2A), as an example, uses 3D printing technique to print force sensors within the polymer scaffold that hold orientated CM layers. The sensors can translate forces into electrical signals and therefore potentially facilitate efficient on-line force analysis. However, muscle films are too thin to be considered as 3D tissues and their physiological relevance is not ideal. [57]

An alternative approach to recreate a papillary-like tissue is to use template guided cellular assembly. Soft lithography with polydimethylsiloxane (PDMS) molding is a commonly used technique to fabricate the platform with complex template structures.[28, 39, 58] These templates can be a single wire[39], a single perfusable tube[54], double wires[28, 58], or opposing cantilevers[30, 31, 34, 59], residing in microwells (Figure 2B–F), where they simultaneously serve as tissue anchors and force sensors based on their mechanical properties. When CMs with or without hydrogel are placed into the microwells, these templates allow cell self-organization around the structures and generation of static tension within the tissue. These topographical features promote the elongation and alignment of cells.[60, 61] The resulting tissues resemble the papillary muscle bundles at structural and functional levels.

Ventricular Tissue Maturation towards Adult Phenotype

Although human PSC-CMs in 3D tissues and fetal CMs have similar morphological structures and function, they are drastically different from adult CMs (Table 1) [65] Similar to fetal CMs, PSC-CMs are commonly spherical [66] with small sizes[67] and have a low percentage of binucleated cells [68, 69]. Gap junction proteins, such as connexin-43, (Cx43) in PSC-CMs spread out on the cell edge and do not have anisotropic distribution [70–72], confined to intercalated discs as in the adult CMs, which explains the low conduction

velocity (1–15cm/s[68, 69]) in the tissues. Although large variation exists among studies with different cell lines and experimental conditions, action potential profiles of PSC-CMs are mostly underdeveloped [73–78]. The randomly distributed sarcomeres in PSC-CMs are on average 0.5–0.6 μm shorter than in adult CMs [77, 78] and only have z disc, I- and A-bands[79, 80]. All PSC-CMs exhibit spontaneous beating[81–83] with the low force of contraction (0.15–0.3 mN/mm^2) [84, 85]. Positive force-frequency-relationship (FFR) from 1–3 Hz [86] and pronounced post-rest potentiation (PRP)[87] are two key unique features of adult myocardium, which are not present in either fetal myocardium[88] or PSC-CM derived tissues[89, 90]. Moreover, PSC-CMs and fetal CMs depend on glycolysis for their metabolisms with a minimal level of transition to β-oxidation of fatty acids [91], which is the primary energy source in the adult heart [92]. Mitochondria in PSC-CMs are also less mature, low in quantity and randomly distributed within the cytoplasm. The status of mitochondria confirms the low level of energy production efficiency in PSC-CMs with minimal β-oxidation. [93] Due to these large differences, PSC-CM derived tissues are considered poor approximations of adult myocardium. Further improvements are vital to produce clinically relevant results through the use of these tissues for drug testing applications.

Native cardiomyocytes reach full maturity after 6–10 years of development [70]. Tissue models for drug testing, however, should achieve ideal maturity within a much shorter time frame, e.g. weeks, after differentiation for an appropriate throughput. To facilitate better tissue maturation within a shorter time frame, many maturation strategies have been investigated over the years. To date, advances of cardiomyocyte maturation have been made through the prolonged culture[78, 119], non-myocyte co-cultures[60, 101, 120], mechanical[31, 35, 101], electrical[32, 39, 121], chemical[33, 122] conditioning and the combinations of these treatments, which all attempt to emulate native physiological microenvironment (Table 2).

Bioreactor systems have also been demonstrated to improve functional cardiomyocyte phenotypes and tissue maturation. Godier-Furnemont *et al* demonstrated that electro-mechanical stimulation on the rat engineered heart muscle at a physiological frequency is a necessity to obtain a positive FFR.[123] The combination of mechanical and electrical stimulation was used to mimic the complexities of the native tissue environment.[124] The timing between mechanical and electrical stimulation is important for cardiomyocyte and myocardial tissue maturation in a dual electromechanical bioreactor system.[125] Bioreactors can also provide chemical and mechanical cues, such as hypoxic or dynamic flow conditions, for certain specialized tissue culture models. For example, the rocking/rotating shaker promotes dynamic flow in the bioreactor and enhanced delivery of nutrients, compared to static systems.[126] Hypoxic conditions in the bioreactors have been used to simulate cardiac ischemic conditions *in vitro*.[127]

Studies have shown that long-term (up to 6 months) monolayer culture can gradually promote human PSC-CMs maturation by improving morphology, myofibril density, alignment, Z-disc registration and action potential (AP) profiles[78]. Jackman and his colleagues have demonstrated that increased nutrient availability by dynamic cultures (rocking the plate) enhanced the force of contraction (23.2 mN/mm^2) to the half of the force

in adult heart (51 mN/mm^2). [31] Tiburcy *et al.* have shown that fully defined medium can improve the FFR from negative to positive and increase sarcomere length in comparison to fetal CMs.[33] Adding triiodothyronine also improves the force of contraction and AP parameters.[77, 128] β-adrenergic stimulation was shown to improve cellular hypertrophy and the force of contraction through increased expressions of structural proteins.[129] Co-culture with cardiac fibroblasts significantly improves tissue morphology and conduction velocity.[60] While static stretching has moderate effects on increasing contractility, tissue stiffness, cell alignment and size [101, 129], a combination of stretching and low-frequency electrical pacing synergistically enhance cardiac tissue functional development.[130, 131] Nunes et al have shown that gradual increase of the electrical pacing frequency up to 6 Hz in a week can greatly improve calcium handling, AP profiles and cell morphology. Ronaldson-Bouchard *et al.*[32] slightly decreased the frequency step-up from 0.83 Hz per day to 0.33 Hz per day. The modified electrical stimulation regime was shown to facilitate the formation of cardiac tissues with many hallmarks of adult myocardium, such as positive FFR, T-tubule formation, partially anisotropic gap junction protein distribution and myofibril and mitochondria organization, reporting the M-line presence for the first time and full sarcomere length achievement in engineered cardiac tissues. Another study with a slower frequency ramping up protocol obtained tissues with over 200% PRP, faster conduction velocity, and AP profiles with I*to* notch and better refractory phase.[28]

However, many of these studies only assess a subset of these maturation parameters and can hardly perceive the actual maturity of their tissues in the natural cardiac developmental process. The level of maturation in PSC-CMs requires multifaceted evaluations, which should include intracellular structure development, tissue function assessment such as contractile forces, conduction velocity, calcium handling, and electrophysiology; metabolic activities and cellular morphology. The benchmarks of native CMs would allow us to better evaluate the level of CM maturation and determine whether they are improved towards the adult myocardium and therefore will be more suitable for drug testing applications.

By comparing with the benchmarks of native CMs, several maturation strategies can successfully improve a subset of parameters to the level of neonatal CMs or adult CMs (Table 1 and 2). Thus, ventricular engineered tissues after maturation regimes are ideally comparable to post-natal stages with several aspects approaching adult myocardium. [28, 31, 32, 119, 135]Although the tissue model would be more suitable as a surrogate for drugs targeting infants[88], it would be interesting to compare the drug testing results from engineered tissue model and adult myocardium to understand the differences.

We have compiled a list of commonly used drugs and the comparison of drug responses in PSC-CMs, matured PSC-CMs, and adult myocardium (Table 3). Isoproterenol is a potent β-adrenergic agonist that induce positive chronotropic and inotropic responses. The inotropic EC50 in matured engineered cardiac tissues and increased maximum force are comparable to these in adult myocardium. However, inotropic EC50 is much higher [34] with minimal force increase[30, 119] in non-matured cardiac tissue. Dofetilide, an hERG channel blocker, has a high risk of causing QT interval prolongation and arrhythmias. In adult CMs, prolongation of the action potential duration (APD) by 20% is observed at 220 nM [136]. Matured PSC-CMs have approximately 20–40% longer APD at 100–1000nM[28] which is

comparable with the adult CMs responses. On the other hand, non-matured PSC-CMs are highly sensitive to dofetilide and have approximately 1000% APD increase at 1000 nM [137]. Oversensitivity of non-matures PSC-CMs is a critical issue in drug development, as it may potentially lead to inappropriate elimination of potentially useful compounds. Lidocaine, a sodium channel blocker, has a negative chronotropic effect on matured PSC-CMs with EC50 at 10–20μM range[28], which is at the same magnitude as EC50 of sodium channel activity in adult CMs [138]. Diltiazem and verapamil, calcium channel blockers, also have similar EC50s in matured CMs[28] and adult CMs [139, 140]. This evidence suggests that the engineered cardiac tissue with maturity approaching adult level can be used to capture some drug responses in adult cardiac muscle strips. However, there are also several exceptions. Both non-matured and matured CMs have different sensitivity of Ca^{2+} compared to adult CMs. It is also unexpected to see matured CMs becomes much less sensitive to nifedipine compared to adult CMs. To sum up, PSC-CMs with higher maturity have indeed improved the reliability of the drug response evaluation, nevertheless, more sophisticated strategies should be developed to further improve the cell maturity, which eventually closes the gap between matured engineered tissues and adult myocardium.

Atrial Tissue Engineering

CMs from atrial and ventricular myocardium have profound physiological differences, such as genetic profiles[154], calcium handling [155], electrophysiology [156–160], and structural and functional protein expressions [154] (Figure 1 B, C). Compared to ventricular cells, atrial cells are smaller in size and surface area. They have thinner and fewer traverse tubules with calcium handling machinery different from ventricular cells. In atria, calcium propagation is delayed and shorter than in ventricles, shows lower expression of Ryanodine receptor (RYR), higher expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and sarcolipin (SERCA inhibitor). [155]

Atria and ventricles show a distinct pattern of gene expression. For example, atria express MLC2a, sarcolipin or connexin 40 whereas ventricles express MLC2v and phospholamban [154]. Atrial cells have differential channel activities and expressions which result in a triangle-like AP shape[156]. (Figure 1B) More specifically, potassium channel KCNA5 and its current I_{kur} are responsible for fast repolarization in atrial AP. Low activity of I_{K1}, I_{Ks}, I_{Kr}, and I_{Na} in atrial cells, also contributes to atrial specific AP shape. I_{KACh}, T type Ca^{2+} channel and Ca^{2+} activated K^+ channels are also atrial specific channels [157–160]. Thus, several compounds are atrial-selective due to these atrial-specific ion channels. Carbachol (Figure 3A–B), an M2 muscarinic receptors agonist which acts on I_{KACh}, reduces action potential duration[161]; serotonin, acting through the atrial-specific 5HT4 receptor, increases calcium current and induces an inotropic response in atrial cells[162]. These divergent drug responses in different heart chambers, increases the complexity of the drug screening process. The ideal therapeutic strategy for the ventricular disease should have minimal impact in atria, and vice versa. Off-target effects should be actively screened and identified for drugs like adenosine, which targets supraventricular tachycardias with off-target effect on I_{KACh} potentially inducing atrial arrhythmia (off-target effect of known compounds on atria is reviewed elsewhere [163]).

Therefore, chamber-specific engineered tissues are imperative in drug screening to minimize the cardiac off-target effect in human. While significant progress has been made in ventricular tissue engineering, generation of engineered atrial tissues is still at its infancy stage. This lag is partly due to the limited availability of atrial specific differentiation protocols [164]. Recently, the use of retinoic acid (RA) has been studied to direct atrial CMs differentiation from hPSC cells [160, 165–167]. Zhang et al added noggin and retinoic acid in cardiac differentiation protocol, and obtained impure atrial population. A purer population of atrial cells was generated by applying retinoic acid after induction of mesodermal stage[6, 166]. Lee et al reported an efficient atrial differentiation protocol for the generation of atrial linages through the use of developmental signaling gradients that specify atrial mesoderm precursors.[6] These advances provide a strong foundation for the creation of functional engineered atrial tissues.

Several groups have reported engineered atrial tissues. Zhao et al have successfully cultured and improved the maturity of atrial tissues[28] on Biowire II platform with two opposing elastic wires as a template. Cyganek and Lemme have created ring-shaped and rod-shaped atrial cardiac tissues using the two post platform [2, 116] (Figure 3C). These tissues showed distinct AP profiles with much shorter APD30 relative to ventricular tissue, resembling adult atrial APD_{30}, measured both by potentiometric dyes and sharp microelectrode recording. (Table 4) When compared with atrial adult myocardium atrial phenotype of these tissues was confirmed by atrial specific carbachol response which reduces APD and hyperpolarizes membrane potential (Figure 3D–I). Tissues from Lemme et al[201] and Zhao et al[28] showed unique responses to low doses of 4AP, I_{Kur} blocker by APD extension. These atrial tissues had less force compared to their ventricular counterparts and did not show positive FFR. Instead, they showed flat FFR, a characteristic of atrial tissue [2, 28, 116]. All reported atrial tissues had around 100 times less force than human adult atria. [168] Atrial tissue from Zhao's study had approximately the same level of post rest potentiation compared to that of atrial adult myocardium. Atrial myocytes are known to have faster calcium handling. They show smaller post rest twitch and in a shorter time frame[139,140]. The AP parameters, including APD30 and APD30/90, are also slightly improved towards adult level when atrial PSC-CMs are incorporated in engineered tissues. Moreover, atrial PSC-CMs and atrial engineered tissues both have higher than 1Hz spontaneous beat which shows both immaturity and contamination with a small population of pacemaker cells. The engineered tissues are also showing AP profile of immature myocytes, with lower Upstroke velocity, a sign of lower adult I_{Na} activity, hyperpolarized membrane potential which indicates lower I_{K1} activity. The APD is also shorter than adult atrial CMs. To date, the ability to generate pacemaker free atrial population and development of a maturation protocol that would generate quiescent cells remain as challenges in atrial tissue generation.

Tissue engineering of the conduction system

Development of pacemaker has great therapeutic importance for patient suffering from brachychardia, in this regard, several groups applied different approaches to purify and characterize cells from the cardiac conduction system; sinoatrial node, atrioventricular node or Purkinje fibers (Figure 1A). The primary attempts in the generation of nodal cells were the introduction of genes with an active role in conduction systems in stem cells

differentiation, expression of mink-Lacz under chicken GATA6 enhancer in mouse ES, expression of HCN2 /4 in NKX2.5 myocytes and differentiation HCN4 expressing cells.[175, 176] Addition of certain chemicals, like B_{12} or SKCa activator 1-ethyl-2-benzimidazolinone to cardiac differentiation, increased the population of nodal cells.[177, 178]. A mixed population of pacemaker cells was generated by induction of C-MYC regulated $NK_{X2–5}$ and TGF-beta inhibition[165] or by controlling the expression of BMP and Wnt at specific time points from the mesoderm stage. Portze et al developed the first SA nodal specific differentiation protocol by treating mesoderm differentiated PSCs by bFGF and low amount of activin A, BMP4 then inhibition bFGF, TGF-β, and Wnt signaling in the presence of BMP4 and RA.[13]

ESC reporter line derived from Contactin2:EGFP BAC gene was first used to separate small population of cells in cardiac differentiation that show Purkinje fiber like gene expression and AP profile, using small molecule screening strategies in ventricular differentiation. It was reported that the addition of sodium nitroprusside between day 6 to 11 increases Purkinje cells population from less than 1% to more than 33%.[179, 180]

The first engineered tissue of the conduction system was made using neonatal rat ventricular myocytes co-cultured with HCN1 transfected human mesenchymal cells. The tissue showed hyperpolarized membrane potential and higher spontaneous beating rate [181]. Another 3D structure was generated from cells with sinoatrial or atrioventricular AP and gene expression profile. These tissues were generated by selection of cells based on SHOX2 promoter and Cx30.2 enhancer and constructing spheroid shaped aggregates [182]. Co-culture of endothelin treated embryonic rat cardiac cells with endothelial progenitor cells in Matrigel generated high spontaneous beating vascularized tissue like pacemaker [183]. Further efforts need to be done on the direct differentiation protocol for Purkinje fibers and AV nodal cells to enable a complete setup of cardiac conductive tissue model.

Conductive biomaterials aiding functional maturation

Other than the biological niches used in differentiation protocols, specialized biomaterials, especially conductive biocompatible materials, can potentially be incorporated as scaffolding materials to promote the electrical function of the engineered conduction systems.

Cardiac muscle fibers throughout the entire heart rely on the adequate electrical signal propagation to generate chamber-specific synchronized contraction. In case of large injuries caused by ischemic myocardial infarction, conductive materials can be incorporated into the scaffolds of large implantable cardiac tissue patches to quickly re-establish the signal propagation through the infarct sites. The use of conductive materials can potentially accelerate the electromechanical integration of the grafts with the host tissues, compensating for the functional loss of cardiomyocytes in the infarct site and minimizing the possibility of arrhythmia.[184, 185] For *in vitro* applications, miniaturized engineered cardiac tissues can benefit from the incorporation of conductive materials at the early phase of the tissue formation and functional maturation. Shortly after cell seeding, cell-cell junctions and gap junction proteins are not well-developed to provide sufficient conduction for adequate intercellular communication. Some cardiomyocytes begin to spontaneously contract, but

they are unable to propagate the electrical impulses to the adjacent cells for synchronized contraction. Thus, introducing conductive materials at the early stage can be beneficial to ameliorate the conductive properties of the cardiac tissues and help with the initiation of tissue level synchronized contraction. [186]

The biocompatible materials and conductive materials are commonly chemically or covalently incorporated together as composite materials for cardiac tissue engineering. Carbon nanotubes blended with collagen hydrogel[187], GelMA[188] or polyesters[189], and reduced graphene oxide dispersed in GelMA[190] or silk materials[191, 192], have been previously reported and shown to promote functional improvements in the engineered cardiac tissues. Conductive and electroactive polymers work in the similar fashion. Polypyrrole has been incorporated with polycaprolactone[193], chitosan[194] or silk[195] as scaffold materials to improve electrophysiology of cardiomyocytes, synchronization of tissue contraction and myocardial electrical impulse propagation across the tissue constructs. PEDOT:PSS[196] as well as polyaniline[197] were both tested in a similar fashion for *in vitro* cardiac tissue engineering.

Disease models

Disease models are essential to allow a better understanding of disease mechanisms and accelerate drug development. Conventional *in vitro* cardiac disease studies rely mostly on the use of heterologous cell culture models[9]. However, these cell sources are often accompanied by marked interspecies differences and confounding genetic information, thus cannot fully elucidate human pathology[198]. Human-derived disease models have great potential in precisely dissecting disease mechanisms and developing compounds for treating patient-specific disorders, and identifying individualized therapies[199].

Genetic diseases present as major causes of cardiac conditions, and have been the focus of personalized disease modeling[200]. The advent of iPSC technology presents exciting opportunities for generating personalized diseased cardiac tissues[9]. As human iPSC-derived cardiac cells inherit physiological and pathological traits of the differentiation origin[201], they often possess disease-specific genotypes that can significantly affect disease onset and progression[201–203]. Besides using patient-specific cells, cardiac myocytes carrying disorders can also be generated from healthy individuals through genetic editing on specific loci. The emergence of genome editing techniques such as the clustered regularly interspaced short palindromic repeat (CRISPR) system has provided another useful approach to create disease alleles into healthy cells[204].

Various genetic cardiac models have been generated using iPSC lines from patients with defined genetic disorders[28, 201]. Among these models, monogenic diseases have been intensively studied[205]. Monogenetic diseases normally present high penetrance and clear phenotypes[205]. Hereditary arrhythmia-related cardiomyopathies are a family of well-characterized monogenic conditions [199]. Gene mutations in ion channels associated with the repolarization process can cause long QT syndrome (LQT), which is characterized by prolonged QT intervals on the electrocardiograms and severe ventricular arrhythmias[206]. LQT syndrome type 1 (LQT1) is caused by a mutation in the KCNQ1 gene, leading to

abnormalities in encoding the channels that regulate potassium current[206]. Moretti et al generated iPSCs-cardiomyocytes derived from patients with LQT1 with mutations in KCNQ1, which demonstrated prolonged APD, increased the occurrence of catecholamine-induced tachyarrhythmia, and negative trafficking defect associated with IKs reduction[207]. Many other patient-specific PSCs based disease models have been used to study different types of model LQT syndrome and demonstrated the applicability of patient-derived stems in modeling cardiac disease[206]. As in the case of other cardiac diseases, Lan at al reported the generation of patient-specific PSCs from HCM patients carrying a mutation in the MYH7 gene. Abnormal Ca^{2+} transients, arrhythmias and morphological remodeling were observed[208]. More diseases, such as Timothy syndrome[209] with mutation in the L-type calcium channel CaV1.2; LEOPARD syndromes[210] with a mutation in the PTPN11 gene; and arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) [211] with desmosome gene mutations, have been investigated to demonstrate the development of pathological phenotypes in terms of cellular morphology, intercellular structural protein organization, calcium transients, electrophysiology and contractile behaviours.

More importantly, iPSCs offer the possibility to recapitulate and investigate the pathogenesis of cardiac disease with 3D cardiac microtissues to model cardiomyopathy *in vitro*. Two recent studies investigated the diseased phenotypes human iPSC derived CMs in the engineered tissue environment. In recent work, human engineered cardiac tissues from a post-based platform were used for evaluating the pathogenicity of sarcomeric protein titin gene variants in four patients[212] (Figure 4A). The effect of titin truncations can be successfully demonstrated with affected sarcomerogenesis-related remodeling, impaired intrinsic contractility, reduced responses to stress and altered gene and protein expressions, which all contributes to the progression of dilated cardiomyopathies[212]. In another study, two patient-specific PSCs were differentiated into cardiomyocytes and were constructed using thin muscle film tissue model to recapitulate the pathophysiology underlying Barth syndrome (BTHS) cardiomyopathy [213] (Figure 4B). The abnormalities associated with mitochondrial protein taffazin mutation were captured at metabolic, structural and functional levels. The platform enabled the study to capture the changes of twitch forces (Figure 4B) due to the impairment of sarcomeres organization and metabolic activity resulted from mitochondrial dysfunction. [213]

Polygenetic diseases, one the other hand, originate from a set of genes and involve various unknown loci[205]. Although polygenic diseases are the most common form of genetic disorders, they present complex disease phenotypes and are challenging to model using PSC cells[205]. Polygenic disorders often involve mutations in genes that have not been fully identified thus cannot be easily corrected using genomic editing methods. Some of these challenges can be overcome in order to model certain complex disorders. For example, cardiac hypertrophy generally occurs as a polygenic disease. It is poorly understood how different variants in sarcomeric proteins can lead to changes underlying this condition. To model this polygenic disease, it is critically required to provide a chronically increased workload to the cardiac tissue over a prolonged time period. [28] Tissue maturation methods are proven to facilitate the modeling of polygenic cardiac hypertrophy[214]. Zhao et al found electrical conditioning for up to 8 months enabled modeling of polygenic left ventricular hypertrophy starting from patient cells[214].

The cardiac disease can be potentiated through environmental factors. Polygenic conditions are particularly prone to be affected by external cues. The onset of polygenetic disease often remains unclear, owing to environmental modifications on phenotype development. The interplay between genetic and environmental factors is crucial to disease progression. For example, hypertrophy-promoting factors, such as energy depletion, the increased workload associated with prolonged hypertension can both accelerate cardiac hypertrophy progression and eventually cause left ventricular dysfunction and heart failure[99]. It is important to generate 3D tissue models that enable prolonged biophysical stimulation through tissue cultivation. The environmental factors can be mimicked through modulating electromechanical stimulation, metabolism, matrix stiffness, and oxidative conditions[198]. The applications of PSCs in differentiating cardiac cells combined with external pathological cues have important implications in constructing functional and diseased cardiac tissues[201].

Currently, most of the disease models are generated using ventricular cardiomyocytes derived from patient cells[6]. Given their major role in supplying blood to the human body, ventricular functional inefficiency is a significant cause of heart failure. Great strides have been made in modeling of ventricular arrhythmia[199], left ventricular hypertrophy[9], dilated cardiomyopathy[203] and channelopathy[215]. However, atrial disease modeling has not yet been extensively studied. Atrial originating cardiac diseases such as AF present as rising healthcare challenges. AF, which is a cardiac electrical conduction system disorder which leads to irregular and fast heartbeats, is emerging as an epidemic heart disease of aging population[216]. AF generally increases mortality and hospitalization rate as it is associated with a higher risk of stroke, heart failure, sudden cardiac death, and premature dementia[217, 218]. It is important to develop platforms that enable the creation of chamber-specific tissues for disease modeling. Several atrial disease models have been studied, enabled by the development of atrial specific protocols. Laksman et al generated a circular sheet of confluent Hes3 derived cells[71]. Arrhythmia was induced in the cell sheet by fast rate pacing, and the behaviour of the formed rotor was studied with two known antiarrhythmic drugs flecainide and dofetilide[219]. In another study, Olsen et al created a KCNA5 mutation model of atrial iPSC derived myocytes on Matrigel coated coverslip[220]. Although these cells had extended APD at baseline, they did not have arrhythmic effects. The addition of cholinergic agonist charbachol was found to shrink APD and eventually induce arrythmia in this model[221]

Cardiac Tissue Engineering for Drug Discovery

A major focus in drug discovery has to be set on avoiding a drug-induced fatal ventricular arrhythmia - torsades de pointes (TdP)[222–224]. Binding to ether-a-go-go-related (hERG) potassium channel has been used to predict the risk of drug inducing TdP[224] through the use of patch-clamp systems or binding assays[225]. However, drug cardiotoxicity can be multifaceted and the screening methods cannot detect cardiotoxicity related to multiple ion channels and other mechanisms. Chamber-specific toxicity should also be a factor to consider in screening potential drug candidates.[28] More comprehensive approaches are being developed to detect drug-induced, chamber-specific, structural and contractile cardiotoxicity[28] to avoid unexpected failure at later stages of drug discovery.

The evaluation of electrophysiological influence of new drug candidates is critical in understanding drug safety and mechanisms for clinical translation. For instance, electrophysiological responses of iPSC-CMs to 28 blinded compounds were used to predict the risk of lethal ventricular tachyarrhythmia by using microelectrode array, computational model[226] and intracellular recording[227]. On the other hand, contractility is another pivotal functional index in drug discovery, since changes in cardiac contractility are representative of the changes in ejection fraction in clinical setting. In patients, changes in contractility can indicate altered ventricular wall movements and post treatment cardiac hemodynamics.[228] Unwanted contractility related toxicity may also result in systemic hemodynamic effects such as drug-induced orthostatic hypotension[229]. Heart-on-a-chip models can serve as an ideal *in vitro* platform and provide these functional parameters for drug assessment.

To employ heart-on-a-chip in the development of drug discovery, a key focus should be set on developing simplistic, high-throughput, automated cell culture and testing systems to screen large libraries of drug leads. Agarwal *et al* developed PDMS-based micron-size cantilevers through semi-automated laser-based fabrication technique to engineer 2D anisotropic cardiac tissues, allowing measurement of diastolic and systolic stresses of the tissues[230]. The positive inotropic effect of isoproterenol on cardiac contractility was demonstrated with the platform. The platforms can quantify contractility and tissue structure, and have a throughput of 35 tissues on one chip device. A muscle-on-a-chip (MTF) model developed as a thin muscle film to monitor muscle contractility was fitted in a 24-well plate and can be easily scalable to 96-well plate format[231].

In terms of 3D tissue, the throughput of culture and testing are slightly lower[33] due to the complexity of the platform. However, compared to monolayer cultures and MTF, the 3D engineered tissues have demonstrated advanced maturation in genetic, functional and structural aspects, which can improve the physiological relevance of the results. With non-invasive long-term monitoring, a chronic cardiotoxicity from catecholamine was demonstrated with this platform recapitulating hallmarks of heart failure (e.g., contractile dysfunction, cardiomyocyte death, and release of N-terminal pro B-type natriuretic peptide) [33]. Biowire II platform has been scaled up from 8 microtissues per 10 cm culture dish, to the format of 96-well plate, which facilitates tissue culture and drug testing in a much higher throughput[58]. Schneider *et al* [232] demonstrated a centrifugally-assisted cell loading approach to robustly construct and culture 8 individual cardiac microtissues at single seeding. Tissues cultured in the centrifugal chip can be maintained for 1 month, with calculation of temporal and spatial beating kinetics observed by an optical method. This study innovated an automated cell loading system, which standardizes the tissue culture procedures, eliminates the inconsistency of the conventional manual cell seeding method, and enables the generation of multiple reproducible cardiac tissues. Nevertheless, more non-invasive functional assessments need to be incorporated into the system. [232]

There are still many remaining challenges to the use of heart-on-a-chip technology for drug discovery. First is to standardize the tissue culture and testing protocol for reliable tissue manufacture with reproducible, high throughput, and high content functional readouts. Culture and testing platform should be amenable to chamber-specific cardiac tissues for

targeted drug responses. At the same time, the connections between functional readouts from *in vitro* platform and clinical assessments should be established. With the proper translation, drug safety and toxicity can be obtained and understood prior to the clinical trials and therefore significantly improve the success rate.

Microscale fabrication technologies and their applications in heart-on-a-chip

Significant advances have been made in current microscale technologies, such as additive manufacturing and micropatterning methods. These powerful fabrication methods can build complex microstructures with various designed chemical and mechanical properties to create customized microphysiological environment for heart-on-a-chip, resulting in the significant advances for cardiac tissue models.

Lind et al developed a cardiac microphysiological device by multimaterial 3D printing with six customized inks.[233] By an innovative combination of these ink materials, a high-conductance carbon black/thermoplastic polyurethane composite was used to fabricate the device. The device has a biocompatible culture surface for cardiomyocytes, and a highly conductive inner material with piezo-resistive shield to sense the contractile function of the seeded cardiac tissues. 3D printing technology can also incorporate cells directly. Lee *et al* demonstrated 3D bioprinting of cardiac ventricles using cardiomyocytes and collagen hydrogel. The resulting constructs had synchronized contractions and directional action potential propagation.[234] To further increase the cell seeding density to a physiologically relevant level, organoids in hydrogels were printed instead of cellular suspensions using sacrificial writing. [235] The resulting tissue constructs had high cell density of 180 million/mL and customized perfusable vascular channels.

Another commonly used technology is soft-lithography. The method is commonly used to produce micropatterned molds with ultra-high resolutions. For example, Ribeiro *et al* demonstrated Matrigel micropatterns generated by microcontact printing from PDMS molds. Customized shape generated from Matrigel micropatterns and appropriate stiffness of polyacrylamide substrate induced myofibril alignment and affected the contractility of single iPSC-derived cardiomyocytes by increasing translation of sarcomere shortening.[236] The platform also allows the *in situ* force measurement through analyzing videos of live-cell imaging.[237] Huebsch *et al* presented a cardiac microphyciological system with 2500 cardiomyocytes per construct on micropatterned PDMS template. This miniaturized cardiac system promotes cardiomyocyte alignment and uniaxial tissue contraction.[238] “I-wire” platform provided two conductive rigid wires as anchor points. The force probe was placed at the center of the cardiac tissue to measure the force of contraction.[239–241]

Challenges and Future Steps

Technological challenges

To date, ventricular tissue models are the most established and most intensively investigated in vitro models. Because of their similarities in tissue organization and functions, atrial tissue models can be improved by adaptation and modification of the existing ventricular tissue models, including the tissue formation on platforms and maturation strategies.

However, proper adjustments are needed based on the atriums physiological characteristics. For example, due to the shorter refractiveness of atrial cells, maturation of atrial tissues should be electrically conditioned at a much faster ramp up protocol.[28] Similarly, cells from SA node have automaticity and will require different maturation strategies and evaluation criteria. Thus, developing standard strategies to mature cardiac cells is therefore utmost important, especially when understanding disease models. This is because most of the cardiac diseases associated with aging and environmental factors are often manifest to adult patients. Nevertheless, native adult CMs from different cardiac compartments should always be used as the benchmarks for tissue maturity.

Taking a step further, once these matured engineered tissues from different cardiac compartments are readily available, building integrated tissue with cells from different chamber origins can improve drug screening efficiency and set foundations for an integrated heart-on-a-chip platform. The recently published paper in Cell is the first to report a heteropolar tissue model made with functional atrial and ventricular zones using Biowire II platform.[28] This model is suitable for duo assessments of drug responses on AP and calcium transients (potentiometric or calcium dye) in single preparation. Serotonin and ranolazine have been used to demonstrate their atrial-selective drug responses on the heteropolar tissue. By monitoring the calcium transients, this heteropolar tissue model is the first to demonstrate the inotropic responses of these drugs. However, the atrial and ventricular chambers in the heart are connected with atrioventricular conduction system (Purkinje fiber). Therefore, a better heteropolar platform that incorporates the conductive system to electrically connect two chamber-specific tissues is needed and will provide a more physiological-relevant drug response.

Furthermore, conventional drug testing protocol introduces pharmaceutical compounds by directly bathing the tissues in different doses of the drugs in culture media. In reality, these drugs are delivered to the tissue through the vascular system. Interactions between drugs and endothelial cells can provide new insights on cardiovascular adverse events. Therefore, a sophisticated functional vasculature network would be beneficial in emulating a more physiological-relevant drug delivery. However, cardiac tissue with vasculature is a more complicated system to recapitulate. Simple co-culture of CMs with endothelial cells will only result in premature lumen structures embedded within the tissue.[101] These micro-vasculatures are not sufficient to support perfusion.[101] On the other hand, designing a defined vasculature within the parenchymal space requires biomaterials with adequate mechanical stability.[53, 55] The rigidity of the materials reduces the flexibility of the cellular organization and the tissue shortening during contraction would be obstructed. Moreover, the setup is difficult to fabricate and standardize. The functional readouts for drug testing with these vascularized cardiac tissues is more difficult to interpret and less likely to enable high throughput assessment. Therefore, more effort should be focused on incorporating stable, native-like vasculatures within cardiac tissue while facilitating its highly compacted, aligned, fully contractible cellular organization.

To sum up, an ideal heart on a chip system should be envisioned as a multi-compartment tissue with pacemaker cells, Purkinje conduction system, and several chamber-specific myocardium integrated with a built-in vasculature for proper drug delivery. By utilizing

standardized maturation protocol, tissues from each compartment would achieve adult-like phenotypes and functions to provide drug testing results relevant to human native physiology. During drug testing, tested compound perfused through the vasculature network will emulate the actual drug entry route and interactions between the drug and endothelial cells. At the same time, chamber-specific tissues will be assessed and analyzed for chamber-specific drug responses. In addition, uniquely designed conditioning protocol, such as tuning the cardiac workload, hormone level, blood sugar level, would be customized after the maturation process to trigger disease phenotypes in these tissues by emulating environmental factors of polygenic diseases.

To realize this ultimate goal, a multi-disciplinary collaboration between cell engineers and platform engineers is therefore needed. Cell engineers are tasked with developing more sophisticated strategies to obtain matured, purified cardiac cell population, whereas, platform engineers are assigned to create a versatile platform that can provide physiological relevant microenvironment while maintaining user-friendly interface with an aim for high throughput culturing and testing efficiency.

Commercialization challenges

Heart-on-a-chip technology poses superior advantages for the applications in drug screening and personalized medicine. Many pioneers in the field have recognized the growing opportunity and have initiated the commercialization efforts of the heart-on-a-chip products in the past 5–10 years, through companies such as TARA Biosystems, myriamed, EHT Technologies, NovoHeart etc. Novoheart, EHT technologies and myriamed are using post-based platforms, whereas TARA biosystems exploits the platform using elastic wires for cultivation of strips of human myocardium. These companies can provide both products as well as testing services for academic collaborators and industrial partners. During the early technological development, the start-up companies commonly seek interest and collaborations from pharmaceutical industry and regulatory departments to tailor the technology development for better usability and clinical translation. At this stage, tissue-based drug screening platforms can effectively be used to answer specific questions on the mechanism of action or drug toxicity. However, they cannot be used alone in the process of bringing a drug to the market due to the current regulatory framework and the need to further validate these systems.

One persisting question from the pharmaceutical industry is focused on proving that the organ-on-a-chip platforms are better than the current approaches that rely on cell lines in 2D culture and animal models. Moreover, as commercial products, reproducibility among batches of hearts-on-a-chip is essential. In some cases, this may be difficult to achieve due to the large biological variability of various iPSC lines and iPSC derived cell products. Although cell suppliers, such as Cellular dynamics and Ncardia, have done an impressive quality control, biological variability still contributes significantly to the variability of the tissue-based platforms despite the incorporation of GMP. Patient-specific tissue models have become the primary focus of several companies, however, many disease phenotypes only manifest after the adult-like maturation or aging, which may be difficult to achieve over a

short time in culture (e.g. several weeks) in these tissue-based platform. These are the challenges for the field going forward.

Conclusions

In summary, this review highlighted and discussed the current progress in cardiac tissue engineering of different compartments of the heart, ventricular, atrial and SA nodal and their uses in drug testing applications. As the most established cardiac tissue model, ventricular tissues have shown similar maturity and drug responses that are comparable to bench mark of native adult CMs. Recent progress in stem cell differentiation has demonstrated atrial CMs can be generated in high purity and atrial tissues generated from these cells displayed atrial-like features and response to atrial specific drugs. However, better maturation strategies and removal of pacemaker cells side populations are still critical to produce high fidelity atrial tissue. More work remains before the generation of physiological-relevant Purkinje fiber-like tissues and SA nodal tissue. Finally, proof-of-concept study demonstrated the possibility to model polygenic diseases in vitro, which further substantiate the necessity of matured CMs for disease modeling and personalized medicine.

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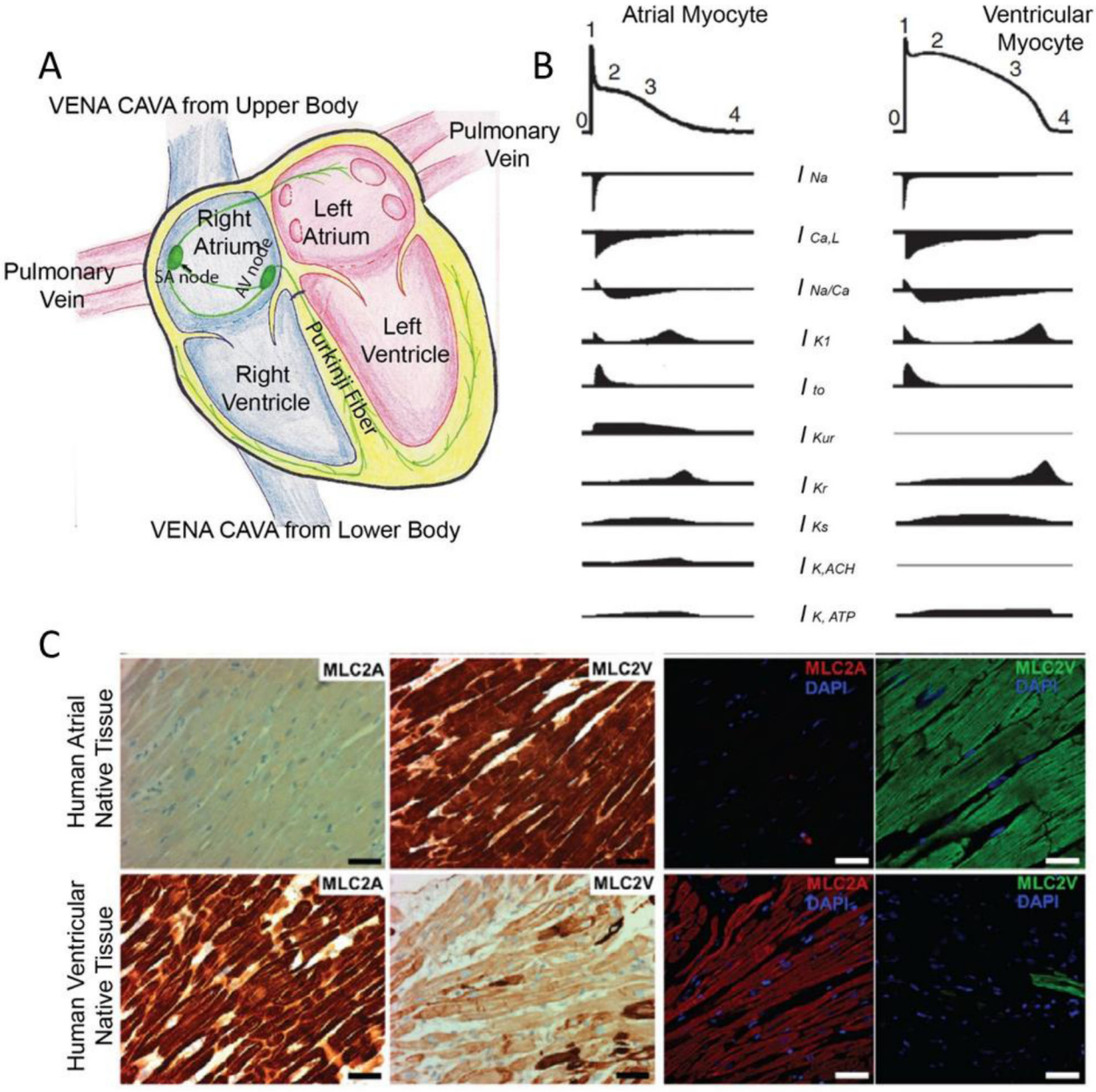

Figure 1. Heart anatomy and chamber-specific physiology.

A) Anatomy of the heart. B) Chamber-specific action potential profiles and ion channels. [1] C) Atrial-specific protein, MLC2a, and ventricular-specific protein, MLC2v, in native human tissues. [2] Figures are adapted with permission from their original references.

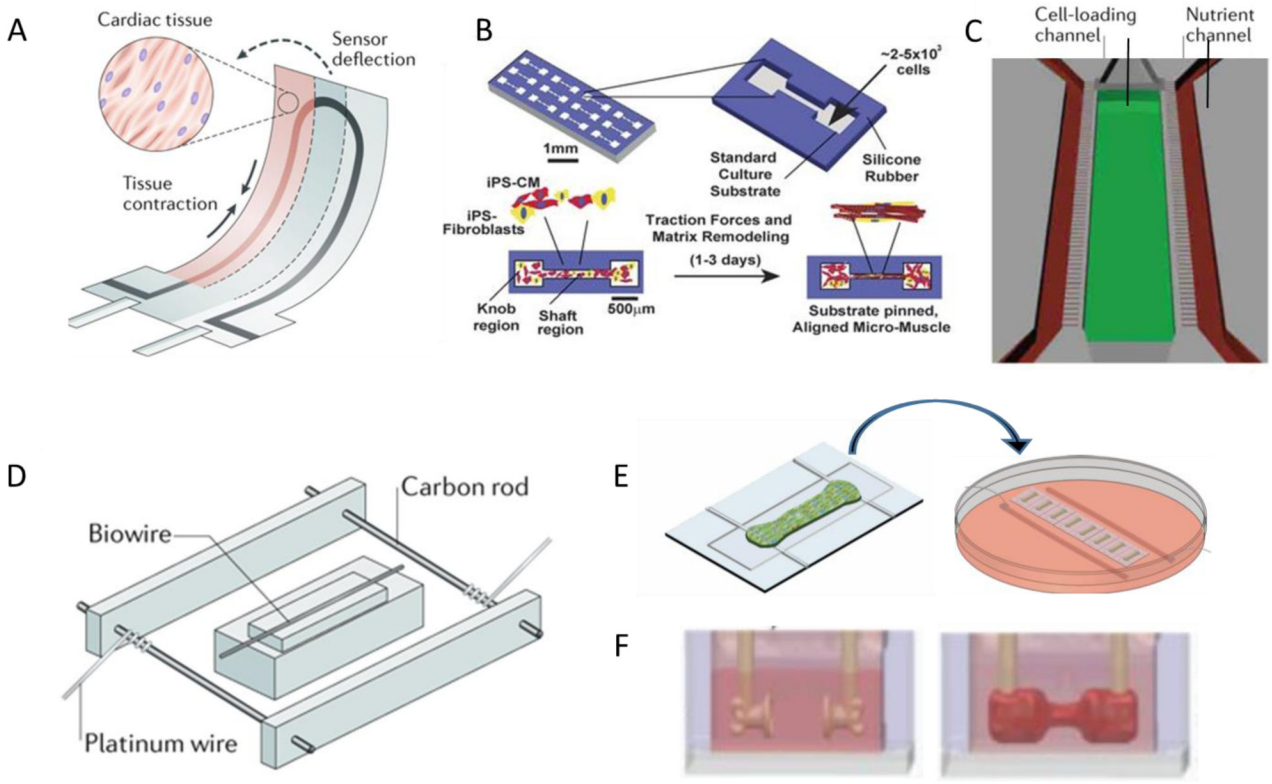

Figure 2. Representative template-guided heart-on-a-chip devices.

Cardiomyocytes can compact A) adhering as muscle thin films on top of 3D printed sensors[57, 62], B) within microgrooves[59], C) along patterned perfusable channels [62, 63], D) around single suture template [62, 64], E) around double elastic force sensors[28], and F) upside down force-sensing posts[32]. Figures are adapted with permission from their original references.

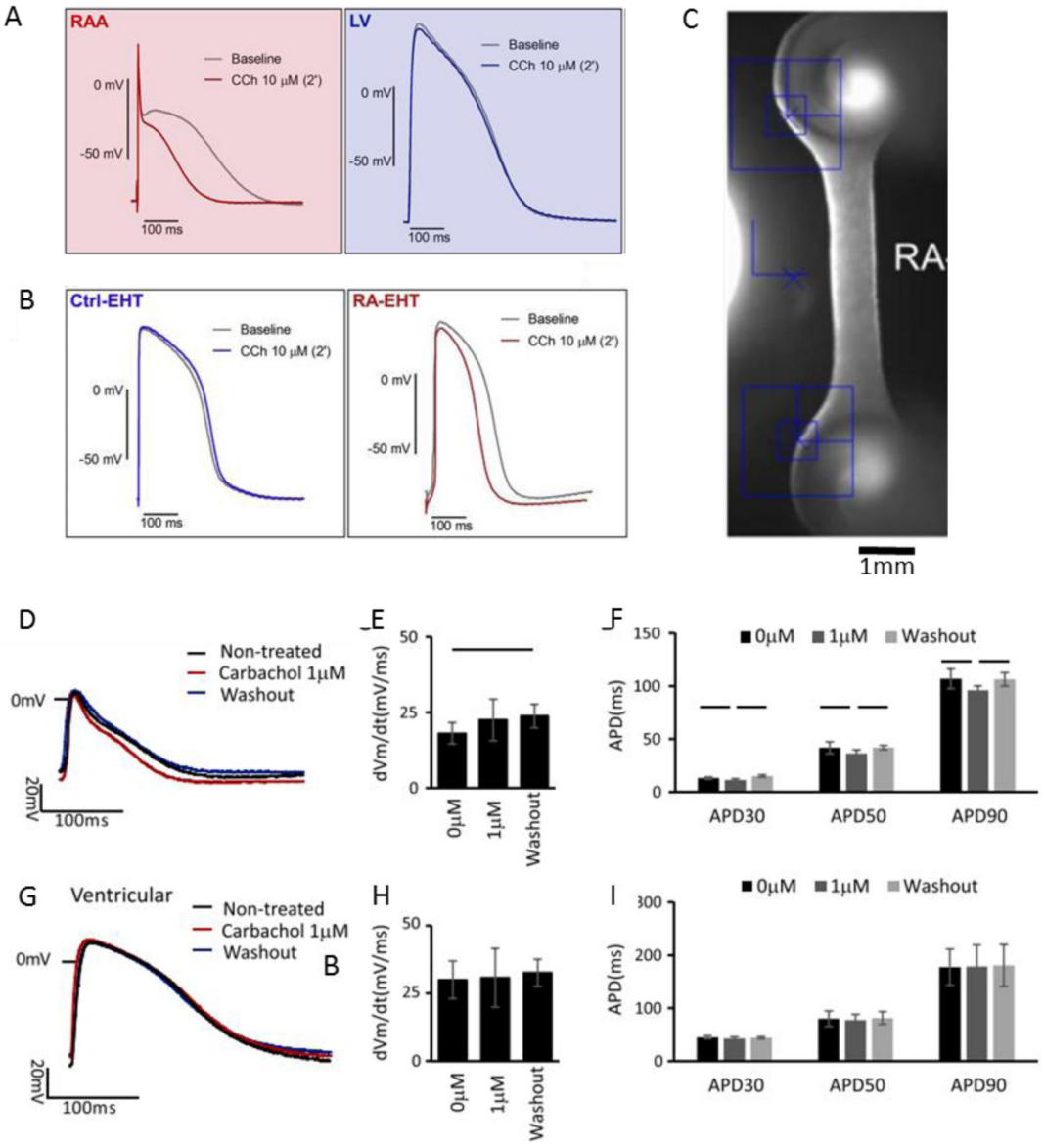

Figure 3. Atrial tissue engineering and drug testing.

Carbachol-induced atrial-specific responses on action potential parameters in A) native human heart tissues; engineered human heart tissues on the B, C) post-based platform[2], and D-I) Biowire II tissues[28]. Figures are adapted with permission from their original references.

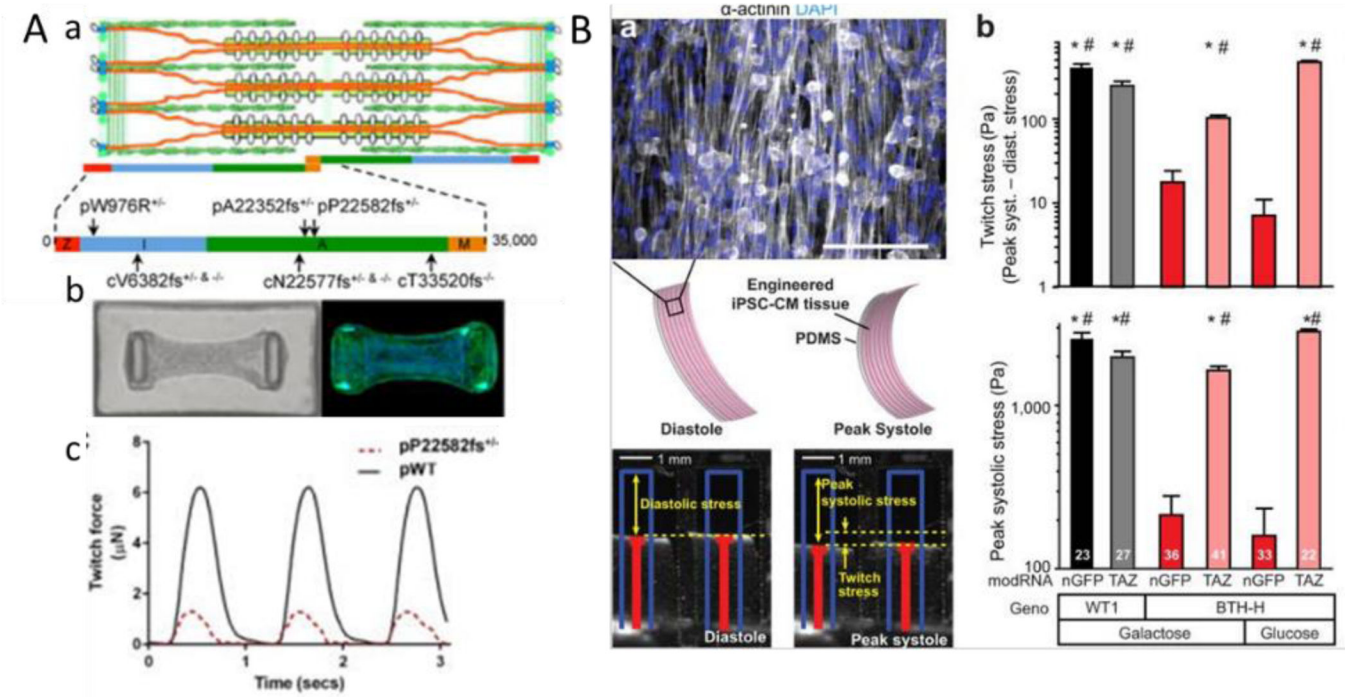

Figure 4. Disease modeling with heart-on-a-chip.

A) Engineered PSC-CM microtissues with titin mutations have impaired intrinsic contractility and responses to stress. [212] a) Schematic of the cardiac sarcomere with titin (orange), b) Images of bright field and Phalloidin labelled tissues suspended between two PDMS pillars, c) twitch forces comparison between wild type and mutant tissues. B) Depressed contractile stress generation [213] by a) diseased myocardial tissue constructs (mitochondrial protein taffazin mutation) demonstrated with muscle thin film (MTF) platform. Cardiomyocyte stress generation reduces the radius of curvature of the construct as it contracts from diastole to peak systole. b) Twitch stress and peak systolic stress generated by MTFs from patient-derived PSC-CMs and control PSC-CMs. Figures are reproduced with permission from their original references.

Table 1.

Maturation Benchmarks Set by Native CMs and Current Progress of PSC-CM

Maturation Parameters	Human Adult CMs	Human Neonatal CMs	Human Fetal CMs	PSC-CM	PSC-CM after Maturation
Cell Volume (μm^3) (Area μm^2)	⩾80000 [94] (Area 1000–2500 [95])	5,854 ± 818 (0–1yr) [94]	Similar to PSC-CM[77]	(Area 500–600 [39, 67, 96])	(Area 917–1700 [39, 67, 96])
Cell Shape	Rod-like[66]	Rod-like[66]	Rod-like[97]	Spherical[66]	Rod-like [39]
Cell Surface Area (μm^2)	10,212–14,418 [98]	4395 ±436 [98]	1171–1261 [77]	1445 ± 74.39 [77]	2169 ± 110.2 [77]
Binucleation	25%–30%[4, 99]	25% [4]	None[65, 100]	~0–15% [68, 69]	~0–20% [32, 68, 101]
Sarcomere Structure	Z-, I-, A-, H-, and M-bands[102]	Z-, I-, A-, H-, and M-bands[81, 103]	Missing M-band [81, 103]	Z-, I- and A-bands[80, 104]	Z-, I-, A-, H-, and M-bands o
Sarcomere Length (μm)	2.15 [105]	2.2 [88]	1.8 [77]	1.6 – 1.7 [78]	2.2 [32]
T-tubule	Fully Developed	Fully developed by 2–3 weeks [106]	Minimal [107]	None [106, 108]	Present [28, 32]
Gap Junction	Anisotropic [70–72]	Gradually more anisotropic [71, 106, 109]	Cell edges, punctate [106]	Cell edges [70–72]	Cell edges [28]; Anisotropic ends [32]
Conduction Velocity (cm/s)	30–100 [29]	30 [110]	40–70 [111]	1–15 [73, 74]	31.8 ± 7.9 [28]
Action potential Duration (ms)	228–259 [112]		324–416 [76]	200–500 [73–77]	100–150 [39]
Action potential Amplitude (mV)	102–110 [112]		20–27 [76]	77–116 [75–77]	~70 [39] or ~100 [28]
Vmax (V/s)	254–303 [112]	150 [113]	5–13 [76]	6–40 [75–77]	176–201 [78] or 125 [39] Or 108.8 ± 19.6 [28]
Resting Membrane Potentials (mV)	−90 [114]		−37–40 [76]	37–71 [76–78]	−66–70 [78] or −100 [39]
Automaticity	No[81]	No[82]	Yes[82]	Yes[81–83]	No[32] or minimal [39]
Contractile Forces (mN/mm^2)	51±8[105]	0.8–1.7 (<2week neonatal)[88, 108]	0.4 (second trimester) [77]	0.15–0.30 [84, 85]	0.05–23.2 [28, 31–33]
FFR (1–3 Hz)	yes[86]	Improving from flat to positive [88]	Flat[88]	Negative[89, 90, 115]	Positive [28, 32, 33, 90, 116]
PRP	yes[87]	Improving from minimal	Minimal [32]		yes [28, 32, 90]
Metabolic activity	β-oxidation of fatty acids [92]	β-oxidation of fatty acids after 7 days [117, 118]	Glycolysis [93]	Glycolysis[91]	Glycolysis and β-oxidation of fatty acids [28, 32]
Presence of Mitochondria	Highly organized and occupy 20–40% intracellular space[67]	More developed, ovular shape[117, 118]	Immature, round [93]	Immature and randomly distributed	More developed, packed along the sarcomeres. [32]

~estimated from graphs in the original reference

Table 2.

Maturation Strategies and Tissue Improvement

External Stimulus	Parameter approaching the adult level	Other improved Parameters
Static or Dynamic Stretch	N/A	Better cellular alignment, cell area and higher expression of MYH7, TNNT2, NPPA, NPPB, CACNA1C, RYR2 and ATP2A2[129] More Cx43 and TnT proteins [132]
Mechanical and Electrical Stimulation (Fixed pacing frequency at 1–2 Hz)	N/A	Improved cell areas and Frank-Starling mechanism and FFR, improved expression of SR-related proteins [131] Higher contractile forces and better structural proteins[130]
β-adrenergic	N/A	Improved contractile forces, structural proteins, gene expression of ANF and MYH7/MYH6 [129] Cell Hypertrophy[104]
Triiodothyronine	APD90	Contractile stress and cell surface area are higher than human fetal control[77]; Improved AP parameters and sarcomere length[77]
Dynamic Culture (nutrient availability and shear stress)	Near adult level contractile force 23.2 ± 1.6 mN/mm^2 and conduction velocity 25.8±1.2 cm/s [31]	Cellular hypertrophy [31]
Electrical Stimulation (Step-up frequency from 2 to 6 Hz)	Positive FFR, t-tubule formation, M-line, Oxidative metabolism, Mitochondria density 30% [32] Conduction velocity 31.8±7.9 cm/s [28]	Pronounced Post-rest potentiation, better action potential profiles with I_{to} notch [28]
Prolonged culture	Rod-like shape (90 day EB)[133] M-lines (360 days EB)[119]	Improved AP profiles, cell morphology, % multinucleation, and sarcomere structure[29, 119, 133]
Co-culture with cardiac fibroblasts	Higher conduction velocity 25.1 ± 7.7 cm/s[60]	Better tissue formation and improved gene expression of MYL7, MYL2, ANP, BNP, and MYH7/MYH6 [60] Improved APD[134]

~estimated from graphs in the original reference

Table 3.

Drug response comparison among PSC-CMs, matured PSC-CMs and Adult CMs.

Drug	Matured PSC-CMs in 3D Tissues	Human Adult CMs	PSC-CMs in 2D or 3D Tissues
Isoproterenol	Both chronotropic and inotropic EC50~100nM [32] Inotropic EC50 =10±1 nM while paced [33] (>100% force increase)	EC50 = 11–80nM [141–144] EC50 = 20–120nM [139] (>100% force increase)	Minimal inotropic effect Chronotropic effect at 1–10μM [30, 119] Chronotropic EC50=12.9nM[145] Inotropic EC50=750nM [34]
Dofetilide	EC50 between 10nM and 100nM (APD) 20–40% elongation of APD50 and APD90 at 100 and 1000 nM[28]	20% increase in repolarization at 220nM[136]	Increased APD60 by 99 ± 77% at 30 nM [146] Increased APD90 by 1000% at 1000 nM [137]
Lidocaine	Reduced frequency by half at 20μM[28]	Na+ EC50=38 μM [138]	Cessation of beating at 50 μM - 1mM [147]
Verapamil	APD30 and APD50 EC50 ~ 0.1-lnM[28]	Ca^{2+} EC50=4.2–24.2μM[148, 149] Inotropic EC50= 0.14–0.79μM[139]	Inotropic EC50=0.61μM [34]
Diltiazem HCL	Negative inotropic effect EC50 between 10–20 μM[28]	EC50= 0.18–0.69 μM [139] Inotropic EC50 is around 10μM [140]	
Ca^{2+}	EC50~0.4–1.2 nM [32] EC50~0.5mN [33]	EC50=2.47±0.1μM (pCa=5.61±0.02) [105] pCa=5.67±0.02[150]	EC50 = 0.8–1.0mM [35, 151] or 1.8mM[34] or 0.4mM [152]
Nifedipine	EC50=4.5±1.4μM for force and 3.1±1.9μM for Ca^{2+} transients[28]	Ca^{2+} EC50=16nM [148] EC50 = 50–200nM (inotropic; ventricular muscle)[139, 153]	Ca2+ EC50=39 nM [146] EC50 <100nM (inotropic)[151]

~estimated from graphs in the original reference

Table 4.

Parameter comparison among atrial PSC CMs, atrial PSC-CM in 3D tissues and CMs in atrial adult tissue

Parameters	Adult Atrial CMs or Myocardium	Atrial PSC-CMs in Engineered Tissues	Atrial PSC-CMs
Force (mN/mm^2)	8–10[168]	0.12 [2] or 0.25 [116] or 1μN/ tissue at 1Hz[28]	NA
FFR (1–3 Hz)	Positive from 0.5–1.5 Hz and negative between 1.5Hz to 3Hz[169]	Flat [2, 28]	NA
PRP	Max at 10s after rest Increased over 50%[168]	100% [2, 28]	NA
APD90 (ms)	150–500 [157]	90–120 [28]	150 [166] or ~200 [6]
APD30 (ms)	~10–20 [170]	~13–19[28]	~15[6]
APD50 (ms)	~100 [170]	~40–66[28]	~50 [6]
APD30/90	0.1[171–173]	~0.14[28]	<0.3[6]
Action potential Vmax	150–300[157]	30[28] or 97[2] (measured by dye)	45[166]
MDP	−65 to −80[157]	−65 and −70 [2, 28]	−65[166]
Amplitude	Around 100 [174]	85–90 [2, 28]	80[166]

~estimated from graphs in the original reference