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Engineering of human cardiac muscle electromechanically matured to an adult-like phenotype

Kacey Ronaldson-Bouchard[1], Keith Yeager[1], Diogo Teles[1,2,3], Timothy Chen[1], Stephen Ma[1], LouJin Song[4], Kumi Morikawa[4], Holly M. Wobma[1], Alessandro Vasciaveo[5], Edward C. Ruiz[1,5], Masayuki Yazawa[4] and Gordana Vunjak-Novakovic[1,6*]

The application of tissue-engineering approaches to human induced pluripotent stem (hiPS) cells enables the development of physiologically relevant human tissue models for in vitro studies of development, regeneration, and disease. However, the immature phenotype of hiPS-derived cardiomyocytes (hiPS-CMs) limits their utility. We have developed a protocol to generate engineered cardiac tissues from hiPS cells and electromechanically mature them toward an adult-like phenotype. This protocol also provides optimized methods for analyzing these tissues' functionality, ultrastructure, and cellular properties. The approach relies on biological adaptation of cultured tissues subjected to biomimetic cues, applied at an increasing intensity, to drive accelerated maturation. hiPS cells are differentiated into cardiomyocytes and used immediately after the first contractions are observed, when they still have developmental plasticity. This starting cell population is combined with human dermal fibroblasts, encapsulated in a fibrin hydrogel and allowed to compact under passive tension in a custom-designed bioreactor. After 7 d of tissue formation, the engineered tissues are matured for an additional 21 d by increasingly intense electromechanical stimulation. Tissue properties can be evaluated by measuring contractile function, responsiveness to electrical stimuli, ultrastructure properties (sarcomere length, mitochondrial density, networks of transverse tubules), force–frequency and force–length relationships, calcium handling, and responses to β-adrenergic agonists. Cell properties can be evaluated by monitoring gene/protein expression, oxidative metabolism, and electrophysiology. The protocol takes 4 weeks and requires experience in advanced cell culture and machining methods for bioreactor fabrication. We anticipate that this protocol will improve modeling of cardiac diseases and testing of drugs.

Introduction

Advances in stem cell biology and tissue engineering have led to the development of engineered tissue models or 'organs-on-a-chip', intended to serve as physiologically relevant human in vitro models of their in vivo counterparts. Cardiac-tissue engineering aims to emulate the human heart and requires methods for recapitulating the environmental signals inherent in the developing heart. In addition to repair of the damaged or diseased heart, which was the original goal of cardiac-tissue engineering, engineered cardiac tissues are also finding utility for in vitro modeling of heart physiology and disease[1]. The first cardiac tissues were engineered using avian cells in the early 1990s[2], and the field has made major strides since those pioneering efforts[3–11]. Current human cardiac tissue models are starting to enable humanized drug screening, mechanistic biological studies, and regenerative medicine approaches.

The immature phenotype of cardiomyocytes derived from hiPS cells prevents these models from fully realizing their potential[12–14]. The immaturity results in preclinical models that are overly sensitive, causing many drugs to be incorrectly flagged for potentially dangerous side effects, with subsequent removal from further testing. The immaturity is especially limiting when it comes to detecting cardiac arrhythmias at a preclinical stage, at which human cell models could overcome the

[1]Department of Biomedical Engineering, Columbia University, New York, NY, USA. [2]Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal. [3]ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal. [4]Departments of Rehabilitation and Regenerative Medicine, and of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, NY, USA. [5]Department of Systems Biology, Columbia University, New York, NY, USA. [6]Department of Medicine, Columbia University, New York, NY, USA. *e-mail: gv2131@columbia.edu

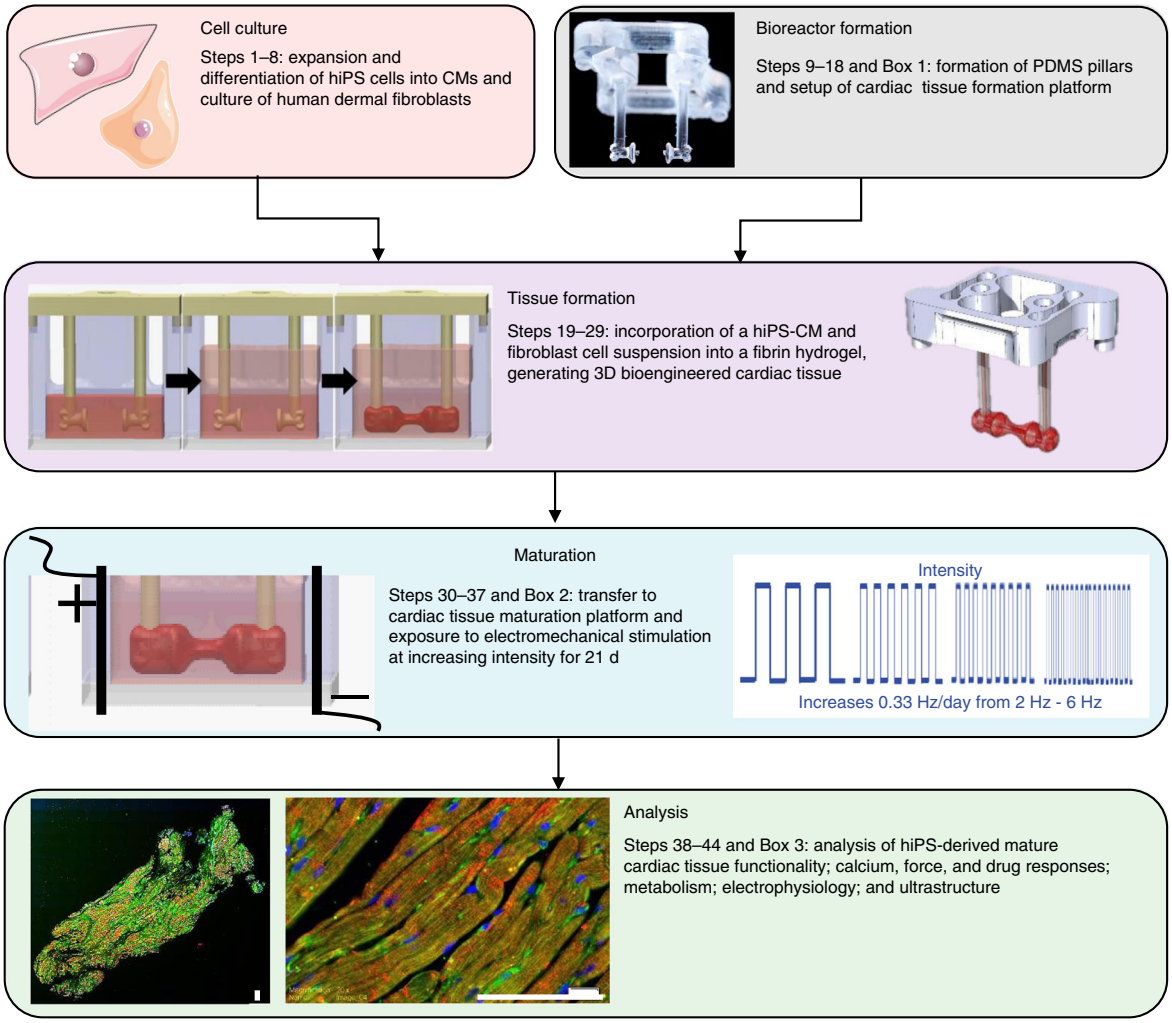

Fig. 1 | Stages involved in the generation and analysis of hiPS-derived cardiac tissues. Analysis assesses their functionality; calcium, force and drug responses; metabolism; electrophysiology; and ultrastructure. Scale bars, 100 μm.

shortcomings that arise when translating results obtained in animal models to the clinic[13]. In addition, the immature hiPS-CMs express the inward 'funny' channel (I_f), which may cause arrhythmias when implanted into an adult heart[14]. We recently developed methods to improve maturation of hiPS-CMs and describe the detailed methodology required to achieve this here[6].

Maturation of engineered human cardiac tissues

In recent studies, we established that adaptive engineering, in which external signals are designed to drive the biological system to its limits, can mature cardiac tissues beyond the levels achieved by any of the previous approaches[3,5,6,8–10,15–19]. The components critical to the formation of adult-like cardiac tissues in vitro are (i) the use of early hiPS-CMs, at a stage of high developmental plasticity; (ii) the combination of hiPS-CMs and supporting human fibroblasts in a native hydrogel; (iii) tissue formation around two flexible pillars, enabling auxotonic contractions; and (iv) electromechanical stimulation at an intensity that is gradually ramped up each day, to constantly force the cardiac tissue to adapt to the increasing workload.

The use of this protocol (Fig. 1) yielded hiPS-CM-derived cardiac tissues of advanced maturity, providing opportunities for cardiac-tissue engineers to overcome the previous limitations of hiPS-CMs immaturity. The utility of the developed mature engineered cardiac tissues in predicting human clinical responses relies on their ability to mimic the physiology, pathology, and pharmacology of the adult human heart (Fig. 2). Matured engineered cardiac tissues were formed from early-stage hiPS-CM cells 10–12 d after the beginning of differentiation (Fig. 2a). These tissues were able to

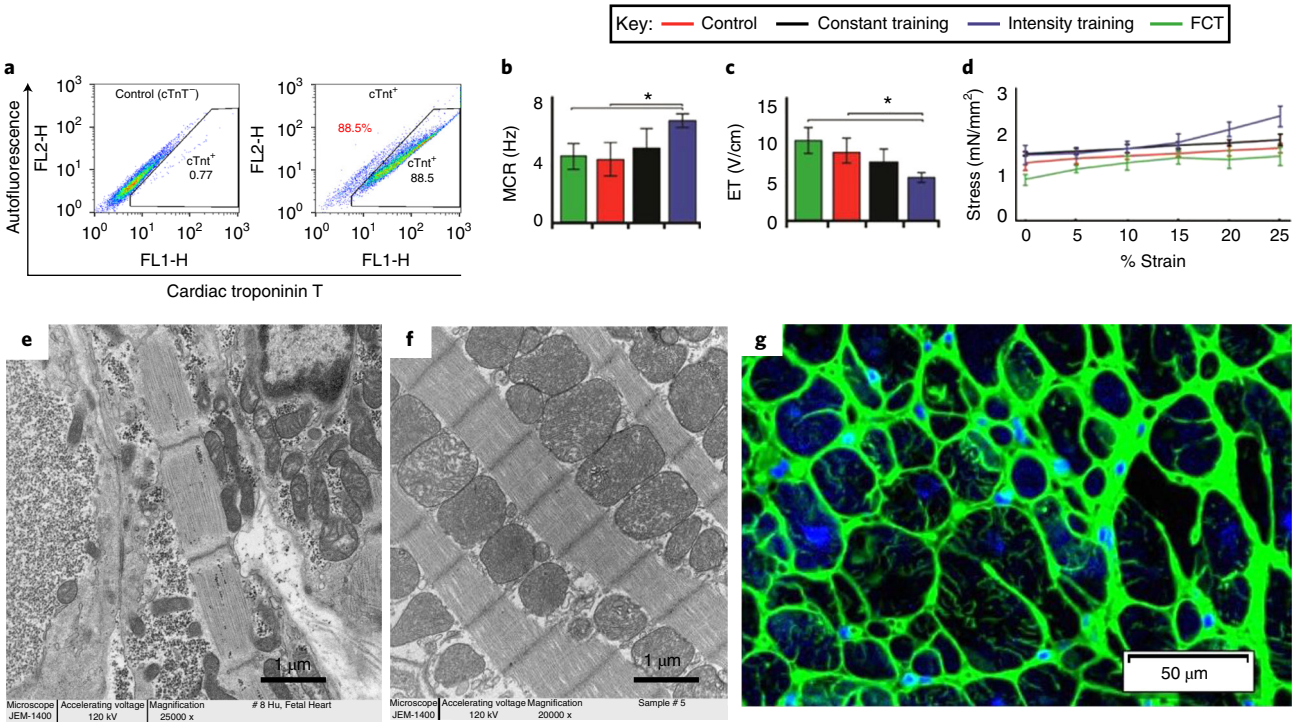

Fig. 2 | Maturation of hiPS-cell-derived cardiac tissues to an adult-like phenotype. a, Flow cytometry of early-stage differentiation of C2A cells labeled with cardiac troponin T (right) and unlabeled cells (left, control) reveals an efficiency of 88.5%. **b**–**d**, Maximum capture rate (MCR; **b**), excitation threshold (ET; **c**), and Frank–Starling curves (**d**) generated in a Muscle Strip Myograph System. Intensity-trained tissues are compared to those trained at a constant frequency (2 Hz, 'constant training'), unstimulated tissues (control), and fetal cardiac tissue (FCT) ($n \geq 12$ per group, mean ± 95% CI; $P < 0.05$; two-way ANOVA followed by Tukey's honestly significant difference (HSD) test). **e**,**f**, Transmission electron microscopy images of a representative 19-week-old fetal cardiac tissue (**e**) and cardiac tissue intensity-trained for 4 weeks (**f**) show ultrastructural alignment and a physiologically high fraction of mitochondria (scale bars, 1 µm) in the electromechanically matured engineered cardiac tissues. **g**, Cross-sectional view of an intensity-trained cardiac tissue after 4 weeks, detailing networks of T-tubules (green, wheat germ agglutinin; blue, DAPI; scale bar, 50 µm). **f** reproduced with permission from ref. [6], Macmillan Publishers Limited. FL1-H, fluorescence 1-height; FL2-H, fluorescence 2-height.

recapitulate both the force–frequency[6] and force–length relationships of the heart (Fig. 2b–d). This is a strong indicator of their increased physiological relevance, as current preclinical small-animal models and previous hiPS-CM models lack this fundamental force–frequency relationship, which is characteristic of human cardiac physiology[20,21]. Similarly, the mature cardiac ultrastructure attained showed increased sarcomere alignment, intercalated discs, M-line structures, and dense mitochondrial populations, as compared to those of fetal cardiac tissues (Fig. 2e,f); we also observed the development of T-tubules in hiPS-CM-derived cardiac structures for the first time (Fig. 2g). The biological fidelity of the developed model was further supported by the demonstrated shift from glycolysis to fatty acid oxidation–based metabolism and a switch in gene expression from fetal α-myosins to adult β-myosins[6]. The development of T-tubules enables rapid exchange of calcium through the cell membrane, where the calcium elicits a calcium-induced calcium response (CICR) by triggering the ryanodine receptor to release stored calcium in the sarcoplasmic reticulum. Immunostaining of matured engineered cardiac tissues revealed a colocalization of these calcium-handling proteins, and functional assays demonstrated increased ryanodine function, increased calcium loading within the sarcoplasmic reticulum, and corresponding changes in gene expression that further validate an adult-like phenotype[6]. The level of maturation attained provided an opportunity to demonstrate its predictive capacity by recapitulating the well-characterized ionotropic (Fig. 3a) and lusitropic (Fig. 3b) responses clinically seen from the β-adrenergic agonist isoproterenol, a response notably missing from current hiPS-CM protocols[21]. This ionotropic response to isoproterenol and enhanced ultrastructure are seen in multiple cell lines when the protocol described herein is used (Fig. 3c,d).

In addition, comparison of the transcriptome of our hiPS-CM-derived cardiac structures with those of matured ventricular heart tissue from the Genotype Tissue Expression Consortium (GTEx) and human fetal cardiac tissue from a recent study by Holden demonstrated closer similarities to the adult tissue (Fig. 4, Supplementary Table 1; ref. [22]).

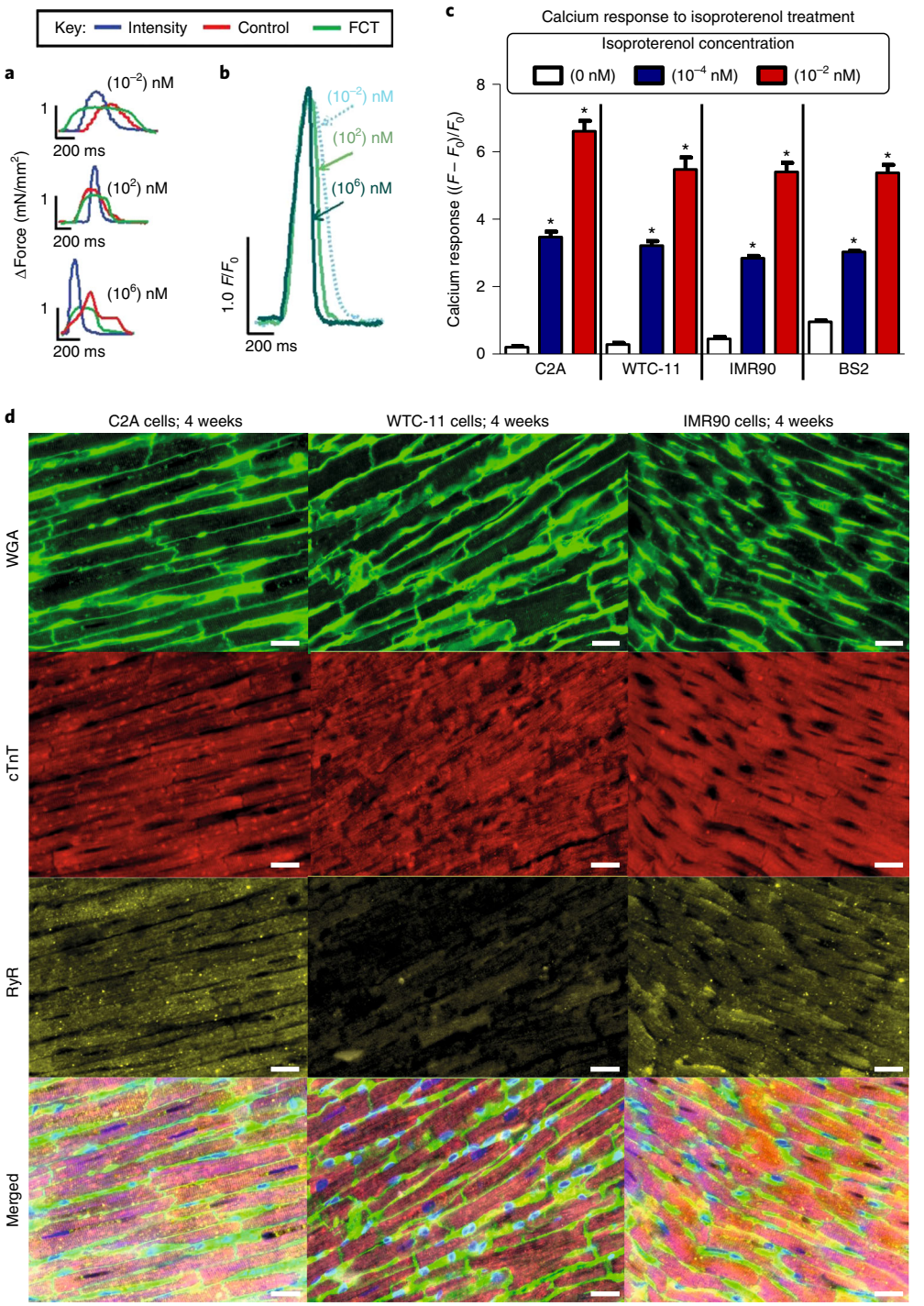

Fig. 3 | Enhanced isoproterenol response and ultrastructure of tissues formed from early-stage hiPS-CMs from different cell lines. a,**b**, Isoproterenol-induced positive ionotropic (**a**) and lusitropic (**b**) dose-dependent responses within cardiac tissues from the C2A cell line. **c**, Calcium transient peak heights after exposure to isoproterenol at the indicated concentration within intensity-trained early-stage cardiac tissues from four different cell lines after 4 weeks of culture ($n \geq 7$; mean + s.e.m., $P < 0.05$ by ANOVA followed by Tukey's multiple comparison test). **d**, Representative immunofluorescent images from early-stage hiPS-CMs from three different cell lines after 4 weeks of maturation (wheat germ agglutinin (WGA): green; cardiac troponin T (cTnT): red; ryanodine receptor (RyR): yellow; scale bars, 20 μm). **a**,**b** reproduced with permission from ref. [6], Macmillan Publishers Limited.

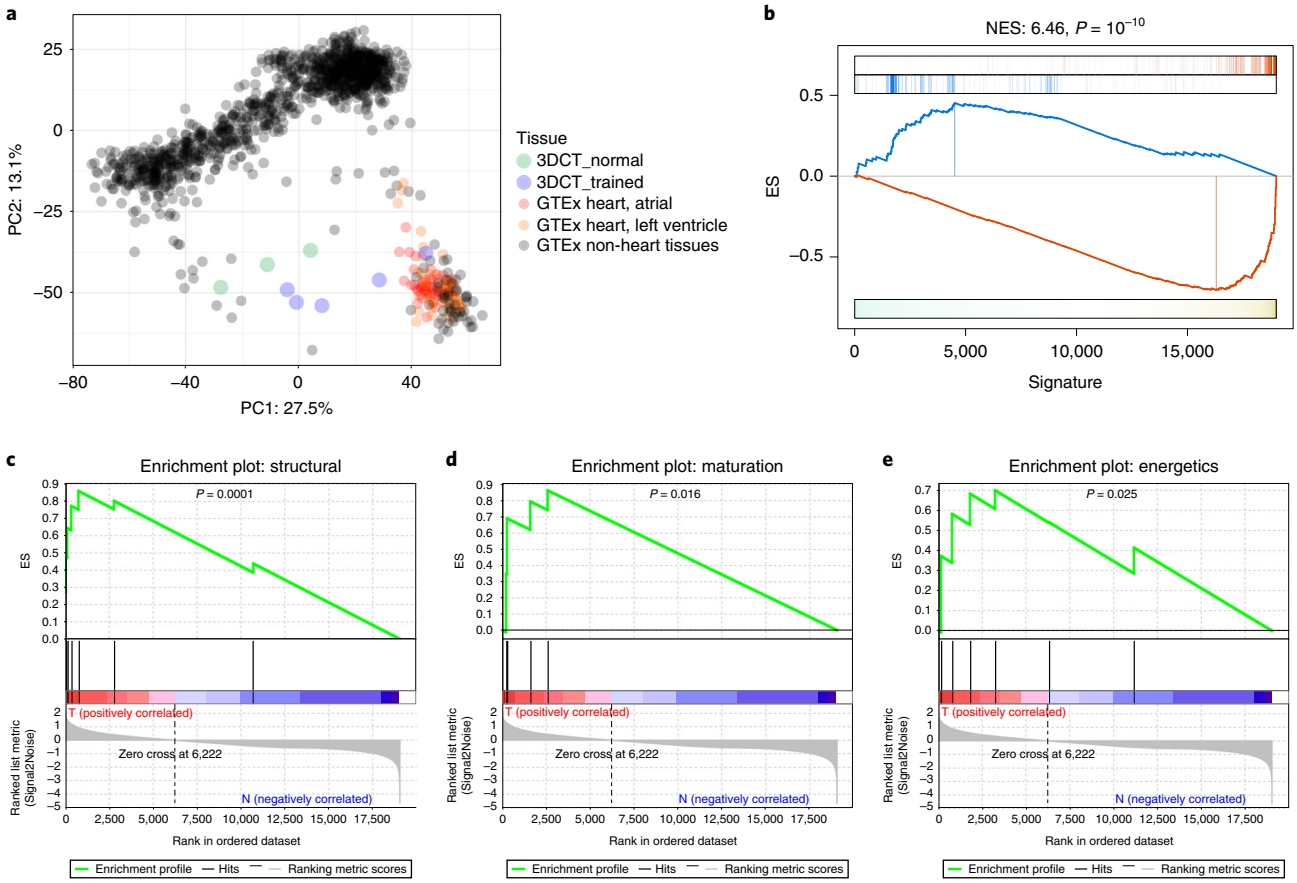

Fig. 4 | Gene expression analysis of bioengineered heart tissues. a, 2D principal component analysis (PCA) of normalized gene expression for $n = 26$ GTEx tissues, electrically stimulated 3D bioengineered cardiac tissues ('3DCT_trained', $n = 5$), and nonstimulated 3D bioengineered cardiac tissues ('3DCT_normal', $n = 3$) showing that PC1and PC2 account for 27.5% and 13.1% of the variance, respectively. Gene expression was normalized using variance-stabilizing transformation from the R package DESeq2. The top 500 genes by variance are plotted across all samples. GTEx tissue samples were randomly subsampled 50 times from each GTEx tissue ($n = 26$) for a total of 1,300 GTEx samples. In PCA, 3DCT_trained samples cluster more closely to adult heart tissue samples from GTEx than to 3DCT_normal samples. **b**, Two-tailed gene set enrichment analysis (GSEA) plot of $n = 50$ randomly subsampled GTEx left ventricle heart tissues versus $n = 50$ randomly selected non-heart GTEx samples (from $n = 23$ randomly selected GTEx tissues) for the top 100 positively (red bars) and negatively (blue bars) differentially expressed genes between electrically stimulated and nonstimulated (normal) bioengineered cardiac tissues. NES and Bonferroni-corrected P values are indicated in the plot. **c**–**e**, One-tailed gene set enrichment analysis plots of electrically stimulated bioengineered cardiac tissues versus nonstimulated bioengineered cardiac tissues, produced using the GSEA software available from the Broad Institute[33]. Gene sets include structural genes (**c**), maturation genes (**d**), and energetics genes (**e**). Gene sets are detailed in Supplementary Table 1. All gene sets show statistical enrichment for electrically stimulated versus nonstimulated bioengineered cardiac tissues (false-discovery rate < 5%). ES, enrichment score; NES, normalized enrichment score.

Development of the protocol

Cell populations

Dissociation of hiPS-CM monolayers into a single-cell suspension using cell-protective methods is key for proper tissue formation. Large cell clusters lead to heterogeneous contracting patches, whereas long or harsh dissociation harms the cells. The early-stage hiPS-CMs (until days 10–12 of cardiac differentiation) are easier to dissociate to a single-cell suspension because they have not yet deposited much extracellular matrix. We found that the resulting cardiac tissues were more responsive to electromechanical stimuli, presumably because these cells are still developmentally plastic. Interestingly, studies have found that hiPS-CMs exhibit a bifurcation event between days 14 and 20, at which miRNAs responsible for pluripotency are turned 'off' and miRNAs associated with cardiomyocyte development and function are turned 'on'[23]. Similarly, long-term culture reveals that the related miRNAs plateau by day 60 post-differentiation. The use of early-stage hiPS-CMs may also coincide with their innate developmental timing, toward cardiac development. The culture of hiPS-CMs in a native-like hydrogel (fibrin) containing the supporting fibroblasts is critical to the development of functional cardiac tissues. The inclusion of a fibroblast cell population facilitated tissue formation and stabilization. Without fibroblasts, cardiac tissues comprising only cardiomyocytes would beat

too strongly and break apart. A ratio of 75% hiPS-CMs and 25% fibroblasts was found to yield cardiac tissues of increased robustness. A ratio of fibroblasts >25% limited the contractile ability of the cardiac tissues, whereas hiPS-CM ratios >75% resulted in tissue breakage during the maturation phase.

Cardiac tissue formation

Collagen, which has been used in the generation of cardiac tissues, can facilitate the development of a necrotic core in these tissues, possibly due to long crosslinking times and changes of pH. Instead, a combination of fibrinogen and thrombin, which is used to crosslink fibrinogen quickly, creates a fibrin hydrogel without affecting pH. In addition, the spontaneous contractions seen in fibrin hydrogels may facilitate the transport of fluid to the tissue interior. Fibrin hydrogels do not compact as much as collagen hydrogels and thus require a smaller amount of fibrin than collagen for cardiac tissues of a similar size. We form tissues in a specialized bioreactor containing wells machined from polycarbonate and coated with a hydrophobic solution to prevent the hydrogel from attaching. The pillars were designed to control the formation of the tissue around the head of the pillar. Other methods to generate 3D cardiac tissues around flexible polydimethylsiloxane (PDMS) pillars are similar to the protocol described herein with respect to cell numbers and the steps of tissue formation[2,10,15]. However, they do not utilize electromechanical stimulation of an increasing intensity to achieve maturation. To our knowledge, the method described here results in the most mature cardiac tissue phenotype to date.

Bioreactors

For tissue formation and maturation, we developed an individual support structure for PDMS pillars and two different platforms, one for tissue formation and another for tissue maturation (Fig. 5 and Box 1). The PDMS pillars are 1 mm in diameter, 9 mm in length, and spaced 6 mm apart, center to center (Fig. 5a).

We have adapted the pillar design to increase the robustness of tissue formation and precise positioning on the pillars. The head of the pillar is shaped so that the tissue aligns with the pillar head and the greatest force is exerted in the middle of the tissue. This is critical when using the deflection of the pillar as a measure of the force generated on the basis of beam-bending theory[24,25], as the bending equation depends on the height of the pillar and therefore the location of the tissue deflecting the pillar (Fig. 5b).

To increase the robustness of tissue formation, we designed a single pillar support so that the tissues can be removed or separated without affecting other tissues attached to the same multi-supportive structure (Fig. 5c,d,g–j). This feature is different from that used in other platforms, including those we previously published, and reduces the time and reagents needed in comparison to those previously used on tissues that do not form properly[6]. We also developed a standard mold for the cardiac tissue (Fig. 5e,k). With six wells, it allows concurrent generation of six individual tissues. After the tissues are formed, they can be transferred to the maturation platform (Fig. 5f,m).

We found that the actual mechanical properties of the PDMS pillars can vary greatly depending on the time allowed for curing, the accuracy and thoroughness of mixing the base and the curing agent, the humidity in the environment, and the flux in the temperature of the oven. To standardize these variables, we make PDMS pillars in large batches under the same conditions and ensure that the parameters are precisely maintained. To obtain accurate force readouts, we used a commercially available Muscle Strip Myograph System with a force transducer to record the force generation of the cardiac tissue. This enabled us to check for any differences that may arise from the mechanical properties of the pillars and provided the means to precisely measure the force–length relationships.

Medium composition

During maturation, the cardiac tissues were cultured in a large volume of medium, which enabled crosstalk between the tissues, exchange of nutrients and secreted factors, and dilution of any reactive oxygen species resulting from electrical stimulation. To further advance maturation, we included fatty acids (supporting oxidative metabolism) and thyroid hormone in the cardiac tissue culture (TC) medium by adding the cell culture B-27 Supplement[26,27]. The combination of these environmental cues enabled the development of a biomimetic cardiac tissue that can be driven to adapt to the imposed contractile demands and yield cardiac tissue with adult-like maturity. Aprotinin was supplemented within the culture medium for the first week following cardiac tissue formation to prevent rapid enzymatic digestion of the fibrin hydrogel and allow the fibroblast population time to secrete

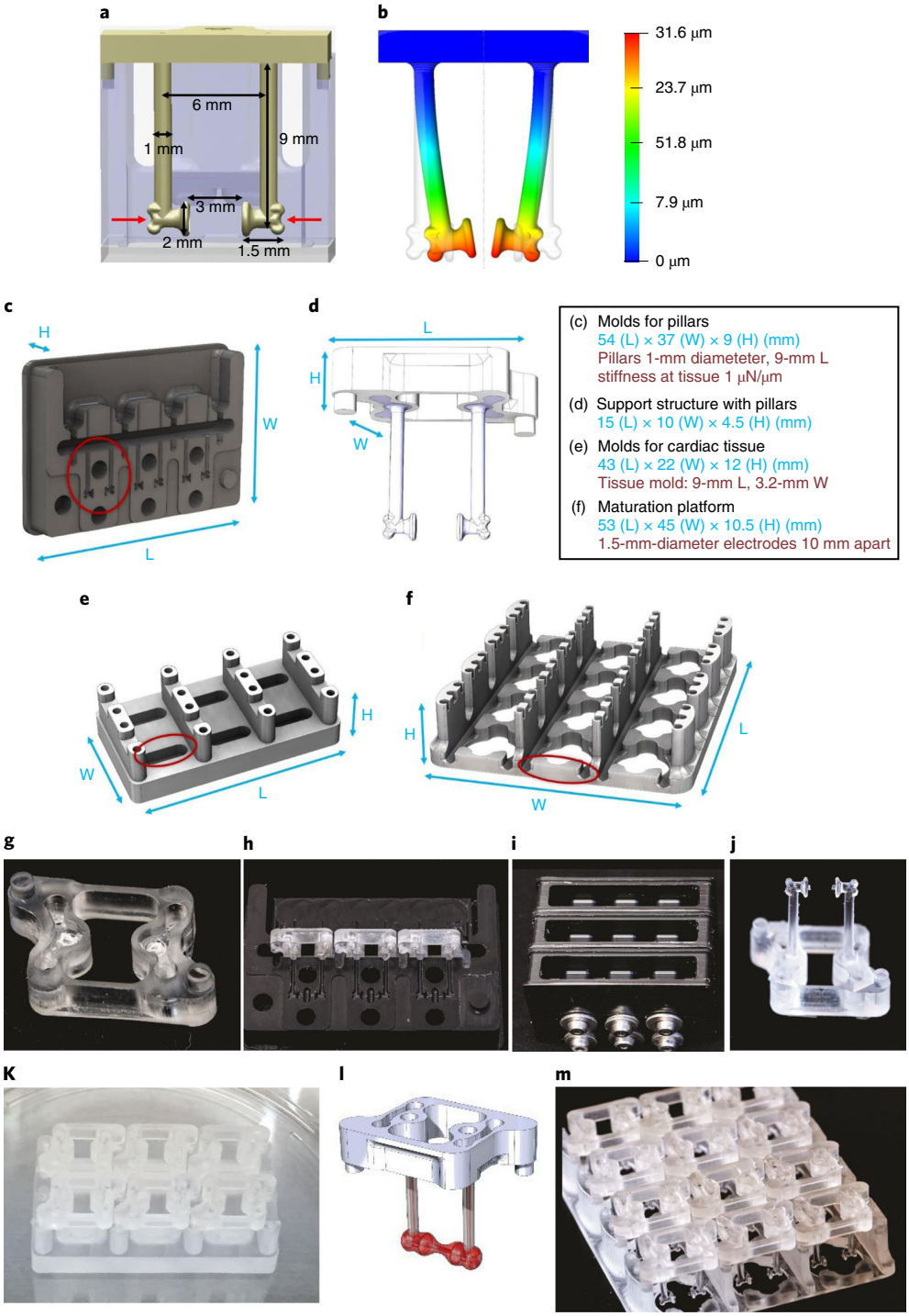

Fig. 5 | Platforms for tissue formation and maturation. a, Dimensions of pillars and details of a notch on each pillar's head (indicated by red arrows). **b**, The design of the pillars herein enables a consistent modeling of the PDMS deflection at a set height on the pillar (i.e., where the notch is located) to facilitate online force readouts based on pillar deflection. **c**, Delrin molds for PDMS casting of pillars. The red circle indicates the pillars mold. **d**, Dimensions of support structure for the pillars. **e**, Tissue-formation platform. The red circle highlights the tissue molds. **f**, Maturation platform. The red circle indicates the grooves for the electrodes and the distance between them. **g**, Polycarbonate support structure for pillars with mating features. **h**, Three polycarbonate support structures can fit within one mold. **i**, Three Delrin molds, containing three polycarbonate support structures each, are filled with PDMS in the top chamber and screwed tightly shut to prevent flashing. **j**, The polycarbonate support structure containing the cured PDMS pillars after removal from the Delrin mold. **k**, Six polycarbonate sets of pillars can fit on one cardiac tissue–formation platform. **l**, The polycarbonate support structure and PDMS pillars contain mating features for proper alignment with the cardiac tissue–formation and maturation platforms. **m**, Twelve polycarbonate sets of support structures with pillars can fit on one cardiac maturation platform. H, height; L, length; W, width.

Box 1 | Bioreactor design and fabrication

The cardiac platform comprises three main components: elastomeric pillars for tissue attachment, a tissue-formation well platform, and a tissue-maturation platform. Design and fabrication aspects of each component are outlined below. Depending on what facilities are accessible, the platform can be made in-house or outsourced to any machine shop with plastics experience. A general recommendation for plastics machining is to avoid the use of oil coolants (air is best; water is also acceptable). We have found that immersing components in a beaker of water and running the autoclave liquid cycle (121 °C for at least 30 min using saturated steam under at least 15 p.s.i.) helps to remove any unintentional surface contamination. A subsequent dry autoclaving (121 °C for at least 30 min using saturated steam under at least 15 p.s.i., 30 min drying time) in standard packaging should be done before use.

Elastomeric pillars

PDMS pillars are overmolded onto a polycarbonate support structure. CNC machining is utilized to fabricate molds for overmolding the pillars onto the support structure, which are themselves also machined. The supportive structure was designed in 3D computer-aided design software; we used SolidWorks. There is an open window from the top to provide a clear path for imaging. There are embossments on the base of the structure that are utilized as alignment features. There are recessed pockets on the top and bottom sides of the structure with through-holes that allow the PDMS to anchor to the support structure. Molds are machined from Delrin (acetal) resin and are recessed to allow a two-part mold over the support structure. Molds must be burr free and incorporate through-holes for the shoulder screws that align the halves and provide clamping force. These precautions minimize the chances of PDMS flashing (thin film curing where there should be open space). Tolerances on components should be such that full clamping is not prevented (undersized supports and oversized mold cavities prevent this). To form pillars, support structures are inserted into the mold (a three-cavity mold is pictured) and clamped together. They are mounted onto a centrifuge plate normally used for multiwell plates. PDMS is added to the mold reservoirs. Pairs of molds/centrifuge plates are centrifuged together for balance and should weigh within a 0.5-g difference to minimize imbalance. Before centrifugation (5 min at 300*g* at room temperature), molds are placed inside a vacuum chamber for 30 min (29 mmHg). After vacuum degassing and centrifugation, the molds are transferred to an oven at 60 °C for 12 h, cured, and opened to remove the pillars formed over their support structures; the pillars are autoclaved before use.

Cardiac tissue–formation platform

To provide consistent tissue, the pillars for initial attachment were sized to allow space for cells to attach around the pillar heads. The wells should be machined in polycarbonate and contain alignment features that interface with the support structure of the pillar component (see schematic). This ensures consistent positioning of the pillars within the mold volume. Wells are machined using standard techniques and autoclaved before use. It is helpful to assemble pillars within formation wells before autoclaving, to reduce manual manipulation.

Cardiac tissue–maturation platform

The maturation platform interfaces with the PDMS pillars with cardiac tissue attached. The platform contains features for aligning pillars and tissues with the carbon electrodes. This ensures a spatially consistent stimulation environment. Electrodes are placed within grooves with a slight undertolerance to provide a transition fit. Additional recesses were designed into the platform so as to maximize medium volume proximal to the tissue (see schematic). Platforms should be machined in polycarbonate using standard techniques; electrodes should be assembled with platinum wires attached and the assembly should be autoclaved before use.

extracellular matrix. Serum supplementation was avoided because it is not well characterized and contributes to rapid fibrin degradation.

Cardiac tissue maturation

To develop an in vitro human-based cardiac system capable of drug screening and disease modeling, we combined some of the best practices in the field for both generating 3D cardiac tissues[3] and using electromechanical stimulation for maturation[8,9,28]. For mechanical stimulation, we adapted the use of auxotonic pillars to mechanically constrain the forming tissue and provide mechanical preload[2,10,18] (Fig. 5l). To induce mechanical contractions, we applied electrical signals via an electrical field established between two carbon rods placed in parallel to the cardiac tissue and connected to an external stimulation source. This setup enabled the user to control electromechanical stimulation. Pacing the tissues at a regular rate (2 Hz) helped synchronize contractility, but it did not lead to substantial cardiac maturation. To accelerate maturation, we developed an electromechanical stimulation regimen termed 'intensity training', in which the cardiac tissues were forced to continuously adapt to electromechanical signals. The protocol was designed to slowly increase the stimulation frequency (0.33 Hz per d, over 2 weeks) so that the cardiac tissue had sufficient time to develop capacity to keep up with the increasing workload. To further accelerate the maturation process, we constantly forced the tissue to reestablish homeostasis with each increasing stimulation frequency. This regimen advanced cardiac maturation beyond the levels achieved previously[5,8–11]. To facilitate the

implementation of electromechanical stimulation, we also developed an Arduino-based electrical stimulator, which is a much more affordable option for broad use than commercial units (Box 2; Fig. 6).

Analysis of mature cardiac tissues

To measure the cardiac contractility online, we developed a MATLAB code for analyzing pixel movement within a predefined area in time-lapse videos of the contracting cardiac tissues (Box 3). For analyses of gene expression, protein content, histomorphology, and ultrastructure, we used tissue samples and standard methodologies[16,19,29,30]. We developed additional methods to detail the presence of T-tubules, including staining before permeabilization, evaluating both longitudinal and axial cross-sections, and using a virtual slide scanner microscope for imaging. We found that immunostaining and confocal imaging of ultrastructural striations in intact tissues, rather than histological sections, is a more robust method for analyzing structural maturity. Regarding force, we used a Muscle Strip Myograph System to analyze the force generation of the cardiac tissues. For several other assays (cytometry, metabolic function using Seahorse Analyzers, and electrophysiological measurements), tissues needed to be dissociated into single cells, a process that is more difficult for mature and compact cardiac tissues. We therefore developed a papain-based dissociation method to minimize cell damage and obtain a viable single-cell suspension (Steps 39–43). This enabled electrophysiological characterization, which is critical for validating the function of hiPS-CMs. To examine the maturity of hiPS-CMs in cardiac tissues, whole-cell patch-clamp recordings for action potentials and inward rectifier current (I_{K1}) were taken. The time window for recording electrical activities in such mature hiPS-CMs was narrower than in fetal cardiomyocytes, with the strongest activity occurring within 72 h of dissociation.

Applications of the protocol

The described methods result in a mature human cardiac tissue grown in vitro that is suitable for many applications, including drug screening, disease modeling, and developmental biology studies. The need for a mature cardiac phenotype is most critical when studying contractile dysfunction and diseases related to contractility. A mature cardiac phenotype also provides a more accurate representation of the adult cardiac function for drug screening. In particular, there is a need for human-tissue models capable of predicting drug-induced cardiac arrhythmias for drugs in the development pipeline. Mature human cardiac tissues can also be used to alleviate the growing burden of heart failure through testing of potential therapeutics in a patient-specific manner. The use of hiPS-CMs enables inclusion of material from patients with genetic mutations with known causal relationships to heart failure, such as myosin heavy chain 7 (*MYH7*) mutations that lead to dilated cardiomyopathy and eventual heart failure[31].

The mechanisms underlying cardiac development can also be investigated to better understand congenital heart malformations. The methods described herein provide a 3D human model for studying cardiac development, particularly during the transition from an immature to an adult-like phenotype. The mechanistic approaches may help elucidate the signals governing cardiac development and cardiac disease. Thus, a mature cardiac tissue model is necessary to capture transitions from an adult-like phenotype to a fetal-like phenotype and negative force–frequency responses (FFRs) in cardiac disease states. The cardiac maturation protocol is applicable to a number of other experimental setups. Overall, the key consideration when developing and utilizing a cardiac bioreactor is to mimic the native environment (i.e., hydrogel matrix, culture media supplements, passive tension, electromechanical stimulation).

Comparison with other methods

Tissue engineering is becoming increasingly successful at more authentically representing the native tissue milieu[2,17]. Instead of attempting to recapitulate the entire complexity of an organ, a reachable goal would be to replicate tissue-specific architecture and a subset of the most relevant functions, in the form of the simplest functional tissue unit, as a predictive screening platform[14]. Human cardiac muscle, engineered with the biological fidelity necessary for predictive use in high-throughput settings, would be transformative to drug testing and modeling of disease. Despite major advances[2–5], engineered tissues formed using other methods do not physiologically emulate the adult heart, largely due to the immature phenotype of human cardiomyocytes derived from hiPS cells. To our knowledge, the method detailed here represents the only current protocol capable of recapitulating the hallmarks of adult cardiac muscle within hiPS-CM-based engineered tissues: excitation–contraction (E–C)

Box 2 | Electromechanical stimulation of cardiac tissues ● Timing 21 days

To build the electrical stimulation bioreactors described herein, tools to control and deliver the electrical stimulation regimen need to be obtained or created. In this protocol, we use commercial electrical stimulators, which are expensive if they are not already available in your lab. To overcome a potential hurdle to implementation of the electrical stimulation regimen described in the protocol, we have developed an alternative electrical stimulator that is based on an Arduino microcontroller and off-the-shelf electrical components.

Arduino microcontrollers serve as mini-computers that can be programmed to control and deliver various stimuli to cells and tissues. The Arduino platform is advantageous because of its low cost and capability of performing a multitude of functions and complex data acquisition protocols, all on the small mobile device. The Arduino programming language is based on running programs, called 'sketches', which are loaded into the Arduino microcontroller's memory and can be operated without being connected to a computer. To facilitate implementation of electrical stimulation regimens, we include here an Arduino-based microcontroller and corresponding code to create an electrical stimulator that automatically increases the stimulation intensity according to the protocol herein. It is coupled to a liquid crystal display (LCD) screen, which also enables the user to set custom stimulation regimens without having to change the parameters in the code manually. Although the system is advantageous in cost and ease of use, it is limited by a maximum voltage output of 5 V. This limitation may affect ET and MCR studies, if tissues require higher voltages to be controlled, which is not normally the case.

This program controls the electrical stimulation of cells by generating monophasic waves. The adjustable parameters are voltage, frequency, and pulse time (the duration for which the voltage is maintained at a high level). These parameters are controlled via the LCD screen. The setup of the circuit involves the use of the Arduino Uno microcontroller board, an Analog Devices AD5206 digital potentiometer, and a Texas Instruments TLV4110 operational amplifier. The serial peripheral interface (SPI) library on the Arduino microcontroller is used to control the digital port via the connection between Arduino pins 13 (SCK), 11 (MOSI), and 10 (SS) to pins 8, 7, and 5, respectively, on the AD5206 digital potentiometer. Stimulation is achieved by setting the digital potentiometer to a specific resistance to achieve the desired voltage for cell stimulation. The frequency is achieved by switching the resistance on the digital potentiometer between the set level and the maximum resistance to obtain a monophasic wave. The pulse time is achieved by controlling when the switch occurs. The voltage can be set to a number between 0 and 5 V, the frequency can be set to between 0 and 100 Hz, and the pulse duration time must be less than the period duration and less than 2 ms. Overall, the main parameters can be controlled through the LCD screen, with the option of either running the intensity-training electrical stimulation protocol described herein (Program 1) or a custom input from the user (Program 2).

Additional equipment

- Arduino Starter Kit Multi-Language (Arduino; https://store.arduino.cc/usa/arduino-starter-kit) or Arduino Uno Rev3 (Arduino, cat. no. A000066)
- LCD keypad shield for Arduino Duemilanove Uno Mega 2560 Mega 1280 (SainSmart, cat. no. SKU 101-50-104)
- Digital potentiometer (Analog Devices, model no. AD5206)
- Operational amplifier (Texas Instruments, model no. TLV4110)
- Electrical wiring (e.g., male jumper wires (Arduino, part no. C000036; https://store.arduino.cc/usa/10-jumper-wires-150mm-male)
- Breadboard (SparkFun Electronics, cat. no. PRT-12002)
- Alligator clips
- Rubber feet (Adafruit, product no. 550)
- (Optional) Oscilloscope

Procedure

1. *Setup of Arduino electrical stimulator*. Connect the LCD screen to the Arduino microcontroller by physically inserting the LCD screen on top of the microcontroller so that the corresponding pins line up (i.e., pin 'A5' on the bottom right of the LCD screen will insert into receptacle 'A5' on the Arduino board.
2. Plug the digital potentiometer and operational amplifier into a breadboard according to the circuit diagram (Fig. 6a).
3. Use wiring to connect the Arduino microcontroller to the breadboard (or solder the components for a more durable connection) according to the PnP diagram (Fig. 6a).
4. *Uploading of code to the Arduino stimulator*. Put the rubber bumpers on the bottom of the board or put the stimulator in a plastic case. This will protect it from spills and is essential if the table is made out of metal.
5. Download and install the IDE software (available on the Arduino website: https://www.arduino.cc/en/Main/Software).
6. Plug the Arduino microcontroller into the computer (via the provided USB cable) to power up.
7. Double-click the Arduino software icon to open up the workspace.
8. Configure the Arduino software for the correct chip. Look at the chip and determine the board (i.e., Arduino/Genuino Uno). Go to 'Tools' > 'Microcontroller' and select your chip.
9. Configure the serial port by going to 'Tools' > 'Serial Port' and selecting the appropriate port.
10. Open the 'electrical_stimulation_cardiac_maturation sketch' (sketches are little scripts that can be sent to the Arduino microcontroller to direct it to act) provided with this protocol in Supplementary Code 1.
11. The first step to getting a sketch ready for transfer to the Arduino microcontroller is to verify/compile it. This means checking for mistakes (editing) and translating it into an application that is compatible with the Arduino hardware. To verify and compile the code, press the check mark icon in the top left of the window. If the verification and compilation steps were successful, you will see the text 'Done compiling' in the bottom left of the screen.
12. Make sure the Arduino Uno Rev3 microcontroller is plugged in, the green light is on, and the correct serial port is selected. Press the 'Reset' button now, just before selecting the upload menu item. Select 'Upload to I/O Board' from the 'File' menu.
13. To run the intensity electrical stimulation regimen detailed in this protocol, select '1' for Program (Fig. 6b). On the following screens, input the voltage and pulse duration. We recommend a voltage of 4.5 V and a pulse duration of 2 ms. Press 'select', using the bottom left button to start the intensity-training electrical stimulation. To run a custom electrical stimulation regimen, select '2' for the program and input the proper stimulation parameters as directed by the LCD screen prompts (Fig. 6c). Press 'select' to start the stimulation.
14. Connect the Arduino microcontroller to an oscilloscope to verify that it is outputting signals properly.
15. *Connecting the stimulator to the cardiac tissue reactor and applying the intensity-training regimen*. Using an alligator clip, connect one end to the GND (ground) wire of the Arduino electrical stimulator and connect the other end to one of the platinum wires in the cardiac bioreactor.
16. Using a separate alligator clip, connect one end to the other wire connected to pin 6 of the TLV4110 operational amplifier (the remaining free wire) of the Arduino electrical stimulator and connect the other end to the other platinum wire in your cardiac bioreactor.
17. Plug the Arduino microcontroller in and reset it to start the stimulation by following the instructions on the LCD screen.
    ▲ **CRITICAL STEP** If bubbles form in the cardiac medium, disconnect the reactor and ensure none of the connections have come loose.

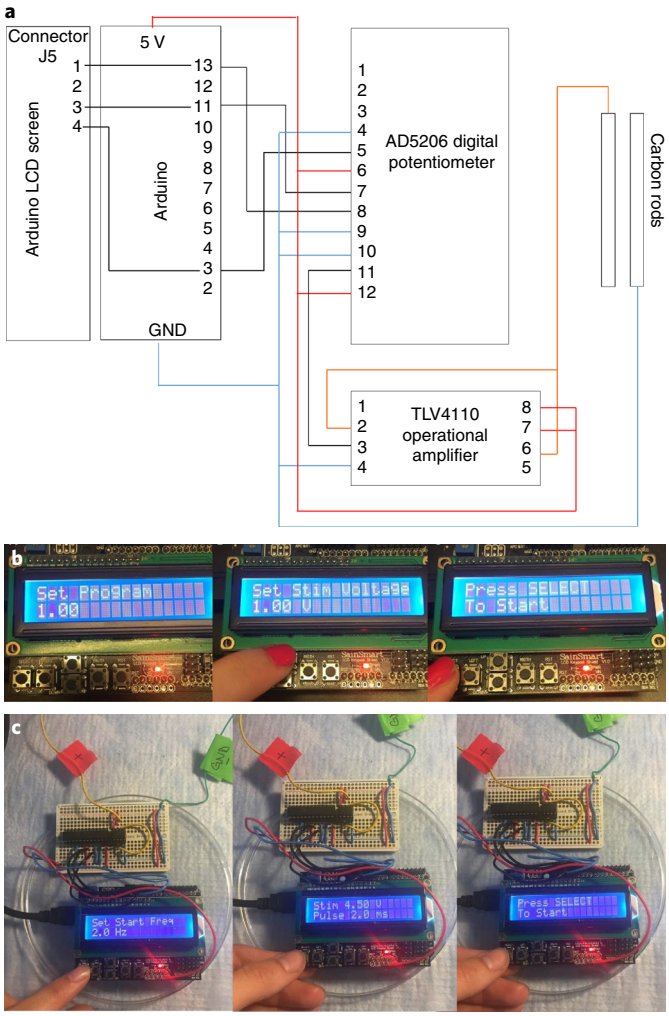

Fig. 6 | Setup of the intensity stimulation program in an Arduino electrical stimulator. a, Circuit diagram of the Arduino stimulator. The numbers refer to pin locations on each component for connections/wiring between components. **b**, Select Program '1' for the intensity stimulation regimen. Use the 'UP' and 'DOWN' buttons at the bottom left to select the stimulation parameters and then press 'SELECT' at the bottom left. Upon selecting the parameters, press 'SELECT' to begin the program. **c**, For setting up a custom program, input the desired frequency by using the 'UP' and 'DOWN' buttons at the bottom left and then press 'SELECT' at the bottom left. Then press the 'SELECT' button to start the program.

coupling (requiring networks of T-tubules), calcium homeostasis (requiring a functional sarcoplasmic reticulum), and positive force–frequency relationships[6].

The protocol described here is biomimetic in nature but also forces the cardiomyocytes to adapt to increasing electromechanical demands, inducing biological adaption toward a more mature phenotype to sustain function. Cardiomyocytes in the heart are exposed to electrical signals starting at 3 weeks into gestation and continuing throughout life, with cascades of Ca^{2+}-mediated events triggering the ensuing mechanical contractions. Replicating these combinatory signals in vitro transitioned hiPS-CMs toward a physiologically relevant adult-like phenotype. The maturation demonstrated here via intensity training results in calcium homeostasis and E–C coupling through the combined development of a functional sarcoplasmic reticulum for intracellular Ca^{2+} storage and an enhanced ultrastructural organization for utilizing this Ca^{2+} storage for increased contractile efficiency. As in the native heart, the translation of synchronous cell membrane depolarization into contractile forces is recapitulated, enabling control of the rate of contraction, as each electrical signal forces the cardiac muscle to exert energy when contracting against mechanical forces imposed by the elastic pillars. Cell alignment and force generation lead to physiological hypertrophy, with postnatal increases in cardiomyocyte mass and establishment of a sarcomere length optimized for force production[6]. By mimicking this process in vitro at an increased level of electromechanical conditioning,

Box 3 | Assessment of contractile motion within engineered cardiac tissues ● Timing 1 min/video

Methods for analysis of the contractile motion of beating cardiac cells or tissues require the generation of custom data scripts or the use of open-source scripts. Open-source scripts that rely on optical flow-based vector mapping[34,35] can be computationally expensive and therefore time consuming for large data files. Other approaches include analyzing the change in pixels over time in reference to a baseline frame or using edge detection methods to make a mask of the tissue and subsequently tracking the overall change in area during contracted versus relaxed states[34–36]. The pixel-based approach was recently demonstrated to be comparable to contractile measurements of pillar deflection, sarcomere shortening, optical flow, and edge detection algorithms[36]. The edge detection approach defines a decrease in the area of the cardiac tissue as a contraction, whereas the subsequent increase in area corresponds to the cardiac tissue relaxation, to create a trace of the cardiac tissue area over time. These approaches are reliable but can have high noise due to background movement when analyzing overall pixel motion or low detection of contractile motion when tracking the change in area overall as deciphered by the imposed mask. The limitations of these approaches are apparent when analyzing cardiac tissues with decreased contractile motion, fluctuating or uneven illumination sources during video acquisition, or low video resolution. To overcome these, our analysis utilizes both approaches to subsequently decrease the noise by focusing on only the tissue area (using edge detection techniques) and then enhancing detection of the contractile motion signal by analyzing the pixel motion within this defined tissue area.

To enable analysis of the contractile motion within the cardiac tissues described herein, we provide custom MATLAB code to analyze cardiac contractility on the basis of both (i) cell edge detection to narrow the cardiac tissue analysis region and (ii) subsequent analysis of changes in pixel motion over time, in reference to a baseline frame. Overall, the approach detailed is simple and efficient and increases the detection of small changes in contractile motion to facilitate comparisons between tissue samples. This increase in resolution is particularly useful when analyzing the effects of drugs that reduce cardiac contractility.

To adequately assess the contractile motion at electrical stimulation frequencies of up to 6 Hz, video acquisition speeds should be set to a minimum of 100 f.p.s. Similarly, a minimum of 20 s is recommended to analyze the parameters of multiple contraction cycles within each video and capture beating abnormalities or missed beats that may arise. Because ImageJ plugins have limited available memory to run large data files, the contractility analysis script was written in MATLAB to facilitate the large video files generated by high-resolution imaging at fast frame rates. The MATLAB contractility code is provided in Supplementary Code 2.

Our script, CardiacContractileMotion (Supplementary Code 2), analyzes changes in pixel motion from a reference baseline frame within a defined region (dictated by a mask of the cardiac tissue area during a relaxed baseline state) to create a trace of pixel motion over time. The traces generated by each approach are used to calculate the resulting contractile parameters and output them into an Excel spreadsheet.

We provide our code in Supplementary Code 2, with detailed instructions for use below (Supplementary Fig. 1).

Additional equipment

- Phase-contrast/bright-field videos (20-s duration, 100 frames/s recommended) containing the entire tissue within the field of view (.tif or .nd2 format)

Procedure

1 Load the 'CardiacContractileMotion.m' file and set the MATLAB path to the folder containing the files in Supplementary Code 2.

2 Within the MATLAB Editor window, input the data directory where your file can be found on line 5 of the code within the single quotation marks, followed by a '.tif' or '.nd2':

```
% input file path information
dataDirectory = 'D:/folder containing your video/';
```

3 Within the MATLAB Editor window, input the filename of your file on line 6 of the code within the single quotation marks, followed by a '`.tif`' as follows:

```
% input file path information
scanName = 'Filename of your video.tif';
```

4 Specify the input data format by setting the '`andorFlag`' variable (line 9 of code) to 0 if loading TIFF stack data (.tif) or set the '`andorFlag`' variable to 1 if loading Andor Zyla data (.nd2).

5 Save the file and press 'Run' to run the code.

6 Specify the frame rate (f.p.s.) used when acquiring the video in the dialog box. Press 'ok'.

7 Choose the region of interest (ROI): select '1' if there is one tissue in the field of view and, using the cursor, select the top left and bottom right region of interest containing the tissue.

8 Choose the baseline frame: select the time at which the baseline occurs (relaxed state) and enter it into the pop-up window. Note: this is to correct for a video being started in the middle of a contraction.

9 Choose the peak amplitude threshold by selecting a value above which there are only peaks. Note that this is to facilitate samples with high noise.

? TROUBLESHOOTING

Anticipated results

The code will determine the beat frequency, contraction parameters (10%, 50%, and 90% contraction times indicated in green), relaxation parameters (10%, 50%, and 90% relaxation times indicated in red), peak width, time between beats, and number of beats and output these variables into an Excel spreadsheet, along with images of the trace with and without contraction parameter lines and a histogram plot of the peak-to-peak times.

and combining the best cardiac-tissue engineering approaches of others in the field[2–5,8], this protocol is able to mature the engineered cardiac tissues beyond currently achievable levels and reverse the characteristically negative force–frequency relationships seen in hiPS-CMs.

Table 1 | Comparison of different cardiac models generated with cells at different time points and submitted to different environments

Culture settings	2D culture	3D tissues				Adult cardiomyocytes
		Late cells	Early cells			
		Intensity stimulation	Control	Constant stimulation	Stimulation intensity	
Cell morphology						
Cell size	+	++	+	++	+++	+++
T-tubules	−	−	−	−	++	+++
Intercalated discs	−	−	−	+	++	+++
Sarcomere length	++	−	−	+	++	+++
Membrane potential	+	+	+	++	+++	+++
Metabolism						
Primary metabolic function	Glycolysis	Glycolysis	Glycolysis	Glycolysis	Fatty acid oxidation	Fatty acid oxidation
Mitochondrial density	+	+	+	+	++	+++
Physiology						
Force–frequency relationship	−	−	−	−	+	+
Calcium-induced response	+	+	+	++	+++	+++
Isoproterenol response					Ionotropic, lusitropic, chronotropic	Ionotropic, lusitropic, chronotropic
Gene expression						
Gene expression profile	Fetal-like	Fetal-like	Fetal-like	Fetal-like	Adult-like	Adult

Advantages and limitations

The advantages of the described method are that it enables the generation of human models of advanced maturity, allowing study of healthy and diseased cardiac tissues (Table 1). The time line for achieving maturity is only 4 weeks, which is efficient considering the maturity attained was benchmarked to be beyond that of the fetal phenotype seen at 14–19 weeks of in vivo development. However, the maturity level achieved is still not fully at the level of an adult in all parameters. Specifically, the force values generated are lower than would be expected for adult cardiomyocytes. In addition, the method requires a high number of cells during the setup, which is a disadvantage for its utilization in high-throughput screening.

Experimental design

This protocol, outlined in Fig. 1, describes a step-by-step process for cell preparation, engineered cardiac tissue formation, fabrication of bioreactors used to generate and mature the cardiac tissues, the electromechanical stimulation parameters for accelerated tissue maturation, and the analysis techniques that have been optimized specifically for mature engineered cardiac tissues.

The first steps involve expanding, differentiating, and culturing the cells needed to make the cardiac tissues (cardiomyocytes and fibroblasts). There are multiple cardiac differentiation techniques that relatively easily produce cardiac troponin-T-positive ($cTnT^{+}$) cells from hiPS cells by mimicking the embryonic development signals that induce mesoderm and cardiac specification. In our previous work, we differentiated hiPS cells into cardiomyocytes with multiple methods before adopting the GiAB protocol developed in the Palecek lab at the University of Wisconsin–Madison, where directed cell differentiation is achieved using a glycogen synthase kinase (Gsk) 3 inhibitor, activin A, and bone morphogenic protein 4 (BMP4)[7]. During differentiation, cells are cultured in RPMI 1640 medium supplemented with B27 without insulin, ascorbic acid, or antibiotics. Over the first 24 h, this base medium is supplemented with activin A and BMP4. From 24 to 72 h, it is supplemented with vascular endothelial growth factor (VEGF165). Beyond 72 h, supplements are not added. In recent studies, we routinely differentiated hiPS cells using the chemically defined protocol developed in the Wu lab at Stanford University[4]. This protocol requires fewer medium components to successfully differentiate hiPS cells and does not require the strict 24-h time point characteristic of the GiAB protocol. The

chemically defined medium (three components; CDM3) described by the Wu lab consists of RPMI 1640 medium supplemented with human recombinant albumin and ascorbic acid. In the first 48 h, this base medium is supplemented with CHIR 99021, a potent and highly selective inhibitor of Gsk 3, to activate the Wnt/beta-catenin signaling pathway. From 48 to 96 h, the medium is changed to CDM3 supplemented with Wnt-C59, a Wnt/beta-catenin pathway inhibitor. Beyond this time point, cells are cultured in the base differentiation medium. This protocol provided us with a reproducible and scalable method for generating cardiomyocytes and is the protocol detailed herein. Overall, any cardiac differentiation that results in ≥85% hiPS-CM efficiency can be used for the tissue-engineering protocol proposed here. We found that the cardiac differentiation protocols that use cell monolayers are more reproducible than those using embryoid bodies (EBs) and that dissociation of cell monolayers is more robust and less damaging to the cells. Cardiomyocytes are harvested at an early stage (days 10–12) of cardiac differentiation. At this stage, the collagenase digestion times needed to obtain a single-cell suspension are relatively short compared to those needed at later cell differentiation stages, and the cells are highly responsive to external stimuli. This timing is critical for applying electromechanical stimulation protocols of an increasing intensity, as required for cardiac maturation. Cardiac tissues formed from hiPS-CMs beyond day 28 were not as responsive to the electromechanical stimuli and lacked the adaptive ability to mature in response to the stimuli shown by early-stage hiPS-CMs.

To form cardiac tissues, three bioreactor platforms were developed: elastomeric pillars for tissue attachment, a cardiac tissue–formation platform, and a cardiac maturation platform (Fig. 5). All bioreactor components were machined in house, but they can also be outsourced to a machine shop for fabrication if machining expertise or equipment is limited. Elastomeric pillars for tissue attachment are formed by centrifugal casting of PDMS around a polycarbonate supportive structure that the resulting pillars are later attached to. The polycarbonate supportive structure was designed with mating features to facilitate proper alignment of the pillars within the bioreactor chambers. A two-part Delrin mold contains the negative imprint of the pillar structure on either side so that both sides of the Delrin mold can be closed around the polycarbonate supportive structure to leave a negative area in the shape of the elastomeric pillars where PDMS is inserted (Fig. 5c). This mold enables easy removal of the cured PDMS and allows repeated exposure to high temperatures without deforming. The molds are tightened with screws to prevent flashing of PDMS around the pillars (Fig. 5i). The mold contains a reservoir at the top to facilitate loading of PDMS into the mold. Injection molding of PDMS requires high pressure to push the polymer into the narrow channels of the mold. Centrifugal force was chosen to push the PDMS into the Delrin mold, because it is an easy and accessible method that utilizes a common piece of lab equipment in cell culture–based labs.

To produce fibrin gels consistently around the elastomeric pillars, we developed a cardiac tissue–formation platform, machined out of biocompatible polycarbonate and autoclave-sterilized to facilitate ease of use (Fig. 5e). The platform contains two rows of three cylindrical wells that each hold a desired amount of hydrogel for tissue formation. Above the wells are shelves with mating features that match those of the pillar polycarbonate supportive structure to facilitate proper positioning of the pillars in each well. The body of the bioreactor is coated with a hydrophobic solution to prevent the hydrogel from attaching to the sides of the wells (Fig. 7a). The PDMS pillars are inserted after this step so that they are not coated (Fig. 7b).

The dissociated hiPS-CMs and fibroblasts are combined at a 75:25 ratio in a fibrinogen solution. Half of the overall amount of thrombin needed per tissue is added to the bottom of each tissue formation bioreactor well. The cell suspension in fibrinogen is immediately added to each well and subsequently crosslinked by adding the remaining half of the thrombin. Once the cardiac constructs have fully crosslinked, cardiac medium is added and supplemented with aprotinin to slow the degradation of the fibrin hydrogel (Fig. 7c).

The hydrogel compacts over 7 d to form a cardiac construct stretched between the pillars. It is then transferred to the cardiac maturation bioreactor, where it is maintained in a larger amount of culture medium and electromechanically stimulated at an increasing intensity (Fig. 7d,e). The bioreactor is placed into a 100-mm-diameter Petri dish and enclosed (for sterility during transfer to and from the incubator) in a covered 150-mm-diameter Petri dish.

The cardiac tissues are matured by exposure to electromechanical stimulation at an intensity that increases from 2 Hz by 0.33 Hz per d until a 6-Hz frequency is reached. Following this regimen, the stimulation frequency is set back to 2 Hz and the tissues are stimulated for an additional 7 d. The use of this regimen was largely influenced by the pioneering work of the Radisic lab at the University of Toronto, where they used electrical stimulation at rates that increased up to either 3 or 6 Hz to

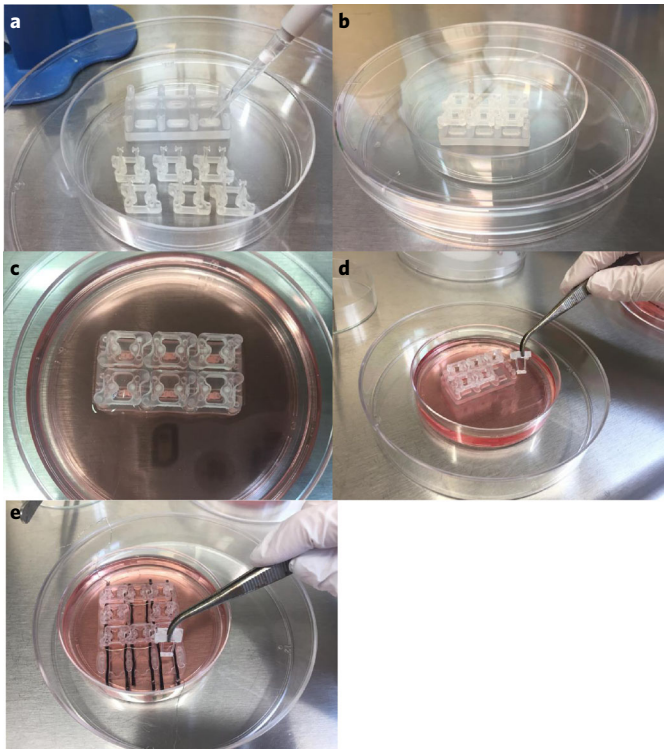

Fig. 7 | Overview of cardiac tissue formation. a, Hydrophobic coating of tissue formation chamber platform. **b**, PDMS pillars are placed back into the tissue formation reactor after coating. **c**, The cardiac tissue is formed around the pillars and allowed to compact over 7 d. **d**,**e**, After 7 d, the cardiac tissues in the tissue formation reactor (**d**) are transferred to the cardiac maturation platform (**e**).

mature cardiac biowires[8]. Because the best results were achieved for the 6-Hz group, we adopted this frequency as the high end for electromechanical stimulation.

The resulting cardiac tissue maturity can then be assessed for functionality (video-based analysis of contractile motion, response to electrical pacing and voltage threshold, force–frequency and force–length measurements), calcium handling (calcium transients, drug response), and ultra-structure (paraffin-embedded immunohistochemistry and slide-scanner imaging of tissue sections, immunostaining and confocal imaging of whole-tissue constructs). To enable analysis of metabolic functionality, electrophysiology, and cell populations, the cardiac tissues should be dissociated into single cells using a papain dissociation protocol. This dissociation protocol was developed specifically for mature cardiac tissues and optimized to reduce the time required for dissociation and minimize damage to the cells. Here, we describe detailed protocols for deriving, maturing, and analyzing the engineered cardiac tissues at the cellular and tissue levels, online and in end-point assays.

Materials

Biological materials

! CAUTION When handling cells and tissues, take the necessary precautions by wearing personal protective equipment and performing the work within a class II biosafety cabinet.

- hiPS cells. We have used C2A, WTC-11, IMR90, and BS2 cell lines obtained through material transfer agreements from S. Duncan, University of Wisconsin (C2A line); B. Conklin, Gladstone Institute (WTC-11 line); and M.Y., Columbia University (IMR90 line). The BS2 line was derived at Columbia University. With informed consent, one vial of peripheral blood was taken from a healthy volunteer. The Columbia University's Stem Cell Core Facility reprogrammed the cells and characterized the final hiPS cells. The protocol for the BS2 cell line was approved by the Columbia University Institutional Review Board **! CAUTION** The cell lines used should be regularly tested for mycoplasma, karyotyped, and assessed for expression of pluripotency markers.
- Normal human dermal fibroblasts (NHDFs; Lonza, cat. no. CC-2509) **! CAUTION** The cells should be regularly tested for mycoplasma, karyotyped, and assessed for expression of fibroblast markers.

Reagents

! CAUTION When handling reagents, use proper personal protective equipment and refer to specific reagent material safety data sheets for additional information. **! CAUTION** Room temperature is defined as 20–25 °C. **▲ CRITICAL** We have not tested medium, supplements, or small molecules from other vendors.

- mTeSR1 medium (StemCell Technologies, cat. no. 85850)
- Penicillin–streptomycin (Gibco by Life Technologies, cat. no. 15070063)
- Y-27632 dihydrochloride (Tocris, cat. no. 1254)
- Matrigel growth factor reduced basement membrane matrix (Corning, cat. no. 354230)
- Ethanol (100% (vol/vol); Decon Laboratories, cat. no. 2701)
- PBS (Corning, cat. no. 21-040-CV)
- EDTA (0.5 M; Thermo Fisher Scientific, cat. no. 15575020)
- RPMI 1640 medium (Gibco by Life Technologies, cat. no. 11875093)
- L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich, cat. no. A8960)
- Recombinant human albumin (Sigma-Aldrich, cat. no. A9731)
- CHIR 99021 (Tocris, cat. no. 4423)
- Wnt-C59 (Tocris, cat. no. 5148)
- DMEM, high glucose (Thermo Fisher Scientific, cat. no. 12430-054)
- FBS (Atlanta Biologicals, cat. no. S11150)
- Trypsin–EDTA (Gibco by Life Technologies, cat. no. 25200072)
- Sylgard 184 Silicone Elastomer Kit (Krayden, cat. no. DC2065622)
- Collagenase, Type 2 (collagenase II; Worthington, cat. no. LS004176)
- Hanks' Balanced Salt Solution (HBSS; no calcium, no magnesium; Gibco by Life Technologies, cat. no. 14170112)
- Fibrinogen from human plasma (Sigma-Aldrich, cat. no. F3879)
- Thrombin from human plasma (Sigma-Aldrich, cat. no. T6884)
- Poly-L-lysine grafted with PEG (PLL-g-PEG; Surface Solutions, cat. no. PLL(20)-g[3.5]-PEG(2))
- Aprotinin from bovine lung (Sigma-Aldrich, cat. no. A3428)
- Tyrode's solution (Sigma, cat.no. T2145)
- B-27 Supplement (50×; serum free; Gibco by Life Technologies, cat. no. 17504044)
- Fluo-4 Direct Calcium Assay Kit (Thermo Fisher Scientific, cat. no. F10471)
- (−)-Blebbistatin (Sigma-Aldrich, cat. no. B0560)
- Papain dissociation system (Worthington-Biochem, cat. no. LK003150)
- Papain (20 U/mL; Sigma-Aldrich, cat. no. 76220)
- Barium chloride ($BaCl_2$; Sigma-Aldrich, cat. no. 342920)
- Nisoldipine (Sigma-Aldrich, cat. no. N0165)
- E-4031 dihydrochloride (Tocris, cat. no. 1808)
- Potassium D-gluconate (Sigma-Aldrich, cat. no. G4500)
- Potassium hydroxide (KOH; Sigma-Aldrich, cat. no. P1767)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045)
- Adenosine 5′-triphosphate magnesium salt (MgATP; Sigma-Aldrich, cat. no. A9187)
- Guanosine 5′-triphosphate sodium salt (NaGTP; Sigma-Aldrich, cat. no. G8877)
- Phosphocreatine disodium salt hydrate (Na_2-phosphocreatine; Sigma-Aldrich, cat. no. P7936)
- EGTA (Sigma-Aldrich, cat. no. E3889)
- Calcium chloride ($CaCl_2$; Sigma-Aldrich, cat. no. 21115)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P9333)
- Magnesium chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M2670)
- Ammonium chloride (NH_4Cl; Sigma-Aldrich, cat. no.A9434)
- D-(+)-glucose (Sigma-Aldrich, cat. no. G7021)
- MEM non-essential amino acids (Thermo Fisher Scientific, cat. no. 11140050)
- *N*-methyl-D-glucamine (NMDG; Sigma-Aldrich, cat. no. 66930)
- 2-Mercaptoethanol (Gibco by Life Technologies, cat no. 21985-023)
- L-Cysteine hydrochloride monohydrate (L-cysteine HCl; Sigma-Aldrich, cat. no. C7880)
- Hydrochloric acid (HCl; Sigma-Aldrich, cat. no. H1758)
- Earle's Balanced Salt Solution (EBSS; Thermo Fisher Scientific, cat. no. 24010043)
- Paraformaldehyde solution (PFA; 4% in PBS; Santa Cruz Biotechnology, cat. no. sc-281692)
- HEPES buffer (Corning, cat. no. 25-060-CI)
- UltraPure DNase/RNase-free distilled water (Thermo Fisher Scientific, cat. no. 10977-015)

- BSA (Millipore Sigma, cat. no. 820451)
- Formalin (Fisher Scientific, cat. no. SF100-4)
- CitriSolv (Decon Laboratories, cat. no. 1601)
- Dako target retrieval solution (Agilent, cat. no. S2368)
- Triton X-100 (Sigma-Aldrich, cat. no. X100)
- Vectashield antifade mounting medium with DAPI (Vectorlabs, cat. no. H-1200-10)
- Tween 20 (Sigma-Aldrich, cat. no. P1379)
- Dimethyl sulfoxide (DMSO; Corning, cat. no. MT-25950CQC)
- BD Cytofix/Cytoperm Kit (BD Biosciences, 554714)
- Geltrex (Thermo Fisher Scientific, cat. no. A1413302)
- DMEM/F-12–GlutaMAX supplement (Thermo Fisher Scientific, cat. no. 10565018)
- DAPI stain (Thermo Fisher Scientific, cat. no. D1306)
- Goat serum (Thermo Fisher Scientific, cat. no. 16210072)
- Seahorse XFe96 FluxPak (Agilent Technologies, Seahorse Bioscience, cat. no. 102416-100)
- DNase solution (25 U/mL; Worthington, cat. no. LS002145)
- Stain buffer (BD, cat. no. 554656)
- Tris-HCl (Thermo Fisher Scientific, cat. no. 15567027)

Antibodies

- Anti-sarcomeric α-actinin (1:200; Abcam, cat. no. ab9465)
- Anti-cardiac troponin T (cTnT, 1:100; Thermo Fisher Scientific, cat. no. MS-295-P1)
- Anti-ryanodine receptor 2 (1:100; Abcam, cat. no. ab2827)
- Anti-CACNA1C (1:200; Abcam, cat. no. ab58552)
- Anti-BIN1 (1:100; Abcam, cat. no. ab137459)
- Anti-mitochondria (1:50; Abcam, cat. no. ab3298)
- Anti-OXPHOS (1:100; Acris, cat. no. MS601-720)
- Alexa Fluor 350 phalloidin (1:40, when dissolved in 1.5 mL of methanol according to the manufacturer's protocol; Thermo Fisher Scientific, cat. no. A22281)
- NucBlue Fixed Cell ReadyProbes Reagent (Molecular Probes, cat. no. R37606)
- Wheat germ agglutinin, Alexa Fluor 488 conjugate (WGA; 1 μg/mL; Molecular Probes, cat. no. W11261)
- Di-8-ANEPPS (1:100; Life Technologies, cat. no. D-3167)
- Donkey anti-mouse Alexa Fluor 488 (1:400; Invitrogen, cat. no. A21202)
- Goat anti-rabbit Alexa Fluor 568 (1:400; Invitrogen, cat. no. 81-6114)
- Goat anti-mouse Alexa Fluor 635 (1:400; Invitrogen, cat. no. A31574)
- Anti-vimentin Alexa Fluor 647 (1:5,000; Abcam, cat. no. ab195878, clone V9)
- Anti-cardiac troponin T antibody (1:50; Abcam, cat. no. ab105439, clone 1C11, FITC)
- Anti-human CD42b (1:200; BD Pharmingen, cat. no. 551061, clone HIP1, APC)
- Anti-MLC2a (1:100; BD Pharmingen, cat. no. 565496, clone S58-205, RUO)
- Anti-MLC2v (1:100; Miltenyi Biotec, cat. no. 130-106-184, clone REA401)
- Goat anti-mouse Alexa Fluor 488 (1:2,000; Abcam, cat. no. ab150113, IgG (H&L))

Equipment

- Water bath (Thermo Fisher Scientific, cat. no. 2302)
- Liquid nitrogen tank (Thermo Fisher Scientific, cat. no. CY509106)
- Biological safety cabinet (Labconco, cat. no. 3621304)
- Centrifuge tubes (15 and 50 mL; Corning, cat. nos. 352097 and 352098)
- Clear flat-bottom TC-treated multiwell cell culture plate (6 well; Corning, cat. no. 353046)
- T75 and T150 TC-treated flasks (Fisher Scientific, cat. nos. 353136 and 355001)
- Glass-bottom dish (35 mm; MatTek, cat. no. P35G-1.5-7-C)
- Automated cell counter (Thermo Fisher Scientific, cat. no. C10281)
- Metallized hemocytometer (Hausser Scientific, cat. no. 3200)
- Centrifuge (Eppendorf, cat. no. B1-022628092)
- Incubator (Thermo Fisher Scientific, model no. Heracell 150i)
- Inverted microscope (Olympus, model no. IX81))
- Single-channel pipettors (Thermo Fisher Scientific, cat. nos. 07-764-701, 07-764-702, 07-764-703, 07-764-705)
- Pipettor barrier tips (Denville Scientific, cat. nos. P1126, P1122, P1096-FR, P1121)
- Pipette controller (Thermo Fisher Scientific, cat. no. 07-202-350)

- Serologic pipettes (Thermo Fisher Scientific, cat. nos. 357558, 357551, 357543, 357525)
- Steriflip filter (50 mL, 0.22 μm; Millipore Sigma, cat. no. SCGP00525)
- Vacuum filter/storage bottle system (500 mL; Corning, cat. no. 431097)
- CNC (computer numerical control) milling machine using uncoated carbide endmills (3-axis; e.g., Haas Automation, model no. CM-1)
- Polycarbonate (McMaster-Carr, cat. no. 8574K321)
- Delrin acetal resin (McMaster-Carr, cat. no. 8575K142)
- Vacuum chamber (Corning, cat. no. 3121-150)
- Lab oven (Thermo Fisher Scientific, cat. no. 51028112)
- Autoclave/steam sterilizer (Tuttnauer, cat. no. 2540E)
- Carbon rods (1.5 mm; Graphitestore, cat. no. BL001601)
- Cell culture dishes (Corning, cat. nos. 430599, 430591)
- Stimulators (Grass, cat. no. S88X; and ADInstruments, cat. no. DMT100273)
- Platinum wire (Ladd Research, cat. no. PW3N5)
- Forceps (Fine Science Tools, cat. nos. 11274-20, 1100-12)
- Alligator test leads (Uxcell, cat. no. 0700955248310)
- Camera (Andor, model no. Zyla 5.5 sCMOS)
- Muscle Strip Myograph System (DMT, cat. no. 840MD)
- Hydrophobic barrier pen (Vector Laboratories, cat. no. H-4000)
- Slide-scanner microscope (virtual slide microscope; Olympus, model no. VS120)
- Confocal microscope (we used a Leica, model no. TCS SP5 confocal and multiphoton microscope and an Olympus, model no. Fluoview FV1000 confocal microscope)
- Myograph chamber stimulation lids (ADInstruments, cat. no. DMT100238)
- Data acquisition device (PowerLab 4/35; ADInstruments, cat. no. PL3504)
- Patch-clamp amplifiers (Molecular Devices, model nos. MultiClamp 700B and Digidata 1440)
- Inverted microscope (Nikon, model no. Ti-U)
- EMCCD camera (512 × 512 EMCCD digital monochrome; Photometrics Evolve Intelligent, cat. no. EVO-512-M-FW-16-AC)
- Micropipette puller (Sutter Instrument, model no. P-97)
- Borosilicate glass (Sutter Instrument, cat. no. BF150-110-10)
- Heating system (Warner Instrument, cat. no. TC324C)
- Quick-exchange platform (for 35- to 40-mm dishes; Warner Instrument, cat. no. QE-1)
- Flow cytometry tubes (Corning, cat. no. 352235)
- Flow cytometer (BD Bioscience, model no. FACSCanto II)
- Microcontroller board (Arduino for orders from the United States, Genuino for orders elsewhere, model no. Mega 2560)
- Parafilm (Sigma-Aldrich, cat. no. P7793)

Software

- FlowJo (FlowJo: https://www.flowjo.com/)
- ImageJ (National Institutes of Health: https://imagej.nih.gov/ij/)
- MATLAB (MathWorks https://www.mathworks.com/products/matlab.html)
- pClamp (Molecular Devices: https://www.moleculardevices.com/products/axon-patch-clamp-system/acquisition-and-analysis-software/pclamp-software-suite)
- MetaMorph microscopy automation and image analysis software (Molecular Devices: https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy)
- LabChart v.8 (ADInstruments: https://www.adinstruments.com/products/labchart)
- IDE (Arduino for orders from the United States, Genuino for orders elsewhere: https://www.arduino.cc/en/main/software)
- SolidWorks (Dassault Systèmes: https://www.solidworks.com/)

Reagent setup

hiPS cell medium

Add together mTeSR1 basal medium, 5× mTeSR1 supplement, and 1% penicillin–streptomycin. Filter-sterilize the medium by passing it through a 0.22-μm filter. Store at 4 °C for up to 1 week. Prewarm to 37 °C before use.

Y-27632 dihydrochloride stock
Dissolve at 5 mM in UltraPure DNase/RNase-free distilled water. Make 150-µL aliquots of the Y-27632 dihydrochloride stock and store them at −20 °C for up to 1 month. Avoid freeze–thaw cycles.

Matrigel growth-factor-reduced basement membrane matrix stock
Thaw Matrigel at 4 °C. Make 600-µL aliquots and store them at −20 °C. Avoid freeze–thaw cycles and work with it on ice. ▲ **CRITICAL** Matrigel will rapidly begin to solidify near room temperature, so it is vital to work quickly, on ice, and with cold tips.

0.5 mM EDTA solution
Add 0.5 M EDTA to PBS to make a 0.5 mM solution. Filter-sterilize by passing it through a 0.22-µm filter and store at 4 °C for up to 6 months. Prewarm to 37 °C before use.

CDM
Add together RPMI 1640 medium, 213 µg/mL L-ascorbic acid 2-phosphate, 500 µg/mL recombinant human albumin, and 1% penicillin–streptomycin. Filter-sterilize the medium by passing it through a 0.22-µm filter. Store at 4 °C for up to 1 week. Prewarm to 37 °C before each medium change.

L-Ascorbic acid stock
Dissolve L-ascorbic acid at 21.3 mg/mL in UltraPure DNase/RNase-free distilled water. Filter-sterilize by passing it through a 0.22-µm filter. Make 5-mL aliquots and store at −20 °C for up to 1 month. Avoid freeze–thaw cycles.

Recombinant human albumin stock
Dissolve at 50 mg/mL in UltraPure DNase/RNase-free distilled water. Filter-sterilize it by passing it through a 0.22-µm filter. Make 5-mL aliquots and store at −20 °C for up to 6 months. Avoid freeze–thaw cycles.

CHIR 99021 stock
Dissolve CHIR 99021 at 3 mM in DMSO. Make 150-µL aliquots and store at −20 °C for up to 1 month. Avoid freeze–thaw cycles.

Wnt-C59 stock
Dissolve Wnt-C59 at 2 mM in DMSO. Make 150-µL aliquots and store at −20 °C for up to 1 month. Avoid freeze–thaw cycles.

Fibroblast medium
Add together high-glucose DMEM, 5% FBS, and 1% penicillin–streptomycin. Filter-sterilize the medium by passing it through a 0.22-µm filter. Store at 4 °C for up to 1 week. Prewarm before each medium change.

Collagenase II solution
Dissolve collagenase II at 2 mg/mL in HBSS. Mix thoroughly. Filter-sterilize by passing through a 0.22-µm filter. Make fresh each time and prewarm to 37 °C before use.

Fibrinogen stock
Dissolve fibrinogen at 33 mg/mL in 20 mM HEPES buffer in 0.9% saline over several hours at 37 °C. Filter-sterilize by passing it through a 0.22-µm filter and store at 4 °C. Make 1-mL aliquots and store at −20 °C for up to 2 months. Avoid freeze–thaw cycles.

Thrombin stock
Dissolve thrombin at 25 U/mL in 0.1% BSA in PBS. Mix thoroughly. Make 100-µL aliquots and store at −80 °C for up to 1 year. Avoid freeze–thaw cycles. ▲ **CRITICAL** Thrombin solution adsorbs to glass, so it is recommended to store aliquots of the solution in plastic tubes.

PLL-g-PEG
Dissolve PLL-g-PEG at 1 mg/mL in HEPES buffer. Mix thoroughly. Filter-sterilize by passing it through a 0.22-µm filter and store at 4 °C for up to 1 month.

Aprotinin stock
Dissolve aprotinin at 33 mg/mL in deionized water. Mix thoroughly. Filter-sterilize it by passing it through a 0.22-μm filter. Make 50-μL aliquots and store at −20 °C for up to 1 year. Avoid freeze–thaw cycles

Cardiac medium
Add together RPMI 1640 medium, 213 μg/mL L-ascorbic acid 2-phosphate, 2% B-27 Supplement, and 1% penicillin–streptomycin. Filter-sterilize the medium by passing it through a 0.22-μm filter. Store at 4 °C for up to 1 week. Prewarm to 37 °C before each medium change.

Blebbistatin solution
Mix 10 μL of blebbistatin into 7 mL of prewarmed (44 °C), vigorously agitated, oxygenated Tyrode's solution. Add extra Tyrode's solution until a final volume of 17 mL and a final concentration of 10 μM are reached. Make fresh each time.

250 mM Stock probenecid solution
Add 1 mL of Fluo-4 Direct calcium assay buffer to a 77-mg vial of water-soluble probenecid. Vortex until dissolved. Store at 20 °C for up to 6 months.

2× Fluo-4 Direct calcium reagent loading solution
Add 10 mL of Fluo-4 Direct calcium assay buffer and 200 μL of 250 mM stock probenecid solution (prepared above) to one bottle of Fluo-4 Direct calcium assay reagent. Vortex briefly and allow the prepared solution to sit for 5 min to ensure it is completely dissolved; then vortex again before proceeding. Make fresh each time.

Glass-dish-coating solution
Dissolve Geltrex in DMEM/F-12–GlutaMAX-I at a 1:100 dilution. Store at 4 °C for up to 2 weeks.

Modified Tyrode's solution
Add together 129 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM D-(+)-glucose, and 25 mM HEPES buffer. Check that the pH is 7.4 and adjust if needed. Add 2% B-27 Supplement. Prepare fresh each time.

Quenching solution
Add together 0.5 M NH_4Cl, PBS, and 0.1% BSA. Prepare fresh each time.

Blocking buffer
Add together PBS, 0.1% BSA, and 10% goat serum and store aliquots of the solution at −20 °C for up to 1 year.

DNase stock solution
Dissolve DNase in 0.15 M $MgCl_2$, 0.001 M NaCl and 0.1 M Tris-HCl (pH 7.5) in distilled water for a concentration of 10 U/μl. Filter the solution using a 0.2-μm filter. Gently mix the solution with sterile 50% glycerol in equal volumes (1:1 ratio) and make small aliquots of the solution for long-term storage at −20 °C. The concentration of DNase stock solution is 5 U/μL DNase in 25% glycerol.

Cardiac tissue dissociation solution
Mix together 20 U/mL papain, 1.1 mM EDTA, 67 μM 2-mercaptoethanol, 5.5 mM L-cysteine HCl, and 1× EBSS. Filter-sterilize the medium by passing through a 0.22-μm filter. Prepare fresh each time and use immediately. The working solution for cardiac tissue dissociation is made by adding 5 μl of DNase stock to 1 ml of papain solution to yield a dissolution solution of 25 U DNase per ml.

Post-papain digestion cardiomyocyte culture medium
Mix together 500 mL of DMEM/F-12–GlutaMAX-I, 56 mL of FBS, 5.6 mL of penicillin–streptomycin, 5.6 mL of MEM non-essential amino acids, and 3.5 μl of 2-mercaptoethanol. Filter-sterilize the medium by passing it through a 0.22-μm filter. Store at 4 °C for up to 1 week. Prewarm before each medium change.

External action potential recording solution
Mix together 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1.8 mM $CaCl_2$, and 10 mM HEPES. Use NaOH to adjust the pH to 7.4 at 25 °C. The solution can be stored at 4 °C for up to 6 months.

Internal (pipette) action potential recording solution
Mix together 120 mM potassium D-gluconate, 25 mM KCl, 4 mM MgATP, 2 mM NaGTP, 4 mM Na_2-phosphocreatine, 10 mM EGTA, 1 mM $CaCl_2$, and 10 mM HEPES. Use KOH to adjust the pH to 7.4 at 25 °C. The solution can be stored at −80 °C for up to 2 years or at 4 °C for up to 7 d.

External I_{K1} recording solution
Mix together 160 mM NMDG, 5.4 mM KCl, 2 mM $MgCl_2$, 10 mM D-glucose, 10 μM nisoldipine, 1 μM E-4031, and 10 mM HEPES. Use HCl to adjust the pH to 7.2 at 25 °C. The solution can be stored at 4 °C for up to 6 months.

Internal I_{K1} recording solution
Mix together 150 mM potassium gluconate, 5 mM EGTA, 1 mM MgATP, and 10 mM HEPES. Use KOH to adjust the pH to 7.2 at 25 °C. The solution can be stored at −80 °C for up to 2 years or at 4 °C for up to 7 d.

XF assay medium
For 100 mL of XF assay medium, add to 100 mL of XF Base Medium 400 μL of 2.5 M glucose, 1 mL of 100 mM sodium pyruvate, and 1 mL of 200 mM L-glutamine (all from the Seahorse XFe96 FluxPak) to yield final concentrations of 1 mM pyruvate and 2 mM glutamine. Warm the medium and subsequently adjust the pH to 7.4 with a pH meter by adding 1 N NaOH in 2-μL increments. Filter-sterilize the medium by passing it through a 0.22-μm filter before use. Store at 4 °C for up to 18 months.

Oligomycin stock
Dissolve oligomycin (from the Seahorse XFe96 FluxPak) in DMSO to yield a final concentration of 100 μM and store aliquots at −20 °C for up to 3 months.

FCCP stock
Dissolve FCCP (from the Seahorse XFe96 FluxPak) in DMSO to yield a final concentration of 100 μM and store aliquots at −20 °C for up to 1 month.

Rotenone–antimycin A stock
Dissolve rotenone and antimycin A (from the Seahorse XFe96 FluxPak) in DMSO to yield a final concentration of 50 μM (each) and store aliquots at −20 °C for up to 1 month.

Oligomycin
Add 450 μL of oligomycin stock to 2,350 μL of XF assay medium to yield 3 mL of oligomycin at a final concentration of 1.5 μM. Prepare fresh each time.

FCCP
Add 700 μL of FCCP stock in 2,800 μL of assay medium to yield 3 mL of FCCP at a final concentration of 1.5 μM. Prepare fresh each time.

Rotenone–antimycin A
Add 300 μL of rotenone–antimycin A stock to 2,800 μL of XF assay medium to yield 3 mL of rotenone–antimycin A at a final concentration of 1.5 μM. Prepare fresh each time.

Perm Wash buffer
Dilute the Cytoperm reagent 1:10 with deionized water to make a working concentration of Perm Wash buffer. Prepare fresh each time.

70% Ethanol
Dilute 100% ethanol 7:10 with water to make a working 70% ethanol solution. The solution can be stored at room temperature for up to 1 year.

Coating protocols

Matrigel coating of cell culture plates

Thaw Matrigel growth-factor-reduced basement membrane matrix at 4 °C. Dilute 1:60 in ice-cold RPMI 1640. Coat the growth surface of the plates, using 1 mL per 9.5 cm^2. Incubate the plates at 37 °C for 30 min or at room temperature for 60 min. After coating, the plates can be stored at 4 °C for a couple of days before being used. Aspirate excess Matrigel right before use. ▲ **CRITICAL** Matrigel will rapidly begin to solidify near room temperature, so it is vital to work quickly, on ice and with cold tips.

PLL-g-PEG coating of cardiac tissue–formation platform

After autoclaving the bioreactors (dry cycle; 121 °C for at least 30 min, using saturated steam under at least 15 p.s.i. of pressure; and 30-min drying time), remove the pillars from the platform and add 100 µL of PLL-g-PEG to each well of the bioreactor platform. Incubate at room temperature for 1 h. Wash the wells twice with 100 µL of PBS. Insert the formed pillars back into the platform. The platforms should be prepared fresh each time.

Geltrex coating of glass-bottom dishes

Coat the center glass part of the 35-mm glass-bottom dishes with 70 µL of glass-dish-coating solution per dish. Incubate the dishes at 37 °C for 60 min and at room temperature for 60 min. Aspirate any excess fluid right before use.

Procedure

Thawing and expansion of cells

1 Thaw and expand both hiPS cells and NHDFs as described in options A and B, respectively.

(A) **Thawing and expansion of hiPS cells ● Timing 10 min plus 3 d for cell expansion before cardiac differentiation**

(i) Equilibrate an aliquot of the desired volume of hiPS cell medium in a 37 °C water bath before thawing cells. Add Y-27632 to the hiPS cell medium to obtain a final concentration of 5 µM.

(ii) Remove a cryovial of cells from the liquid nitrogen storage tank.
■ **PAUSE POINT** If needed, place the cryovial on dry ice for up to 10 min before thawing.

(iii) Immerse the cryovial in a 37 °C water bath, avoiding submerging the cap and keeping the tube stationary.
▲ **CRITICAL STEP** Use of a floating microcentrifuge tube rack is recommended.

(iv) Remove the cryovial from the water bath, spray with 70% ethanol, and place the cryovial in a biological safety cabinet.

(v) Gently transfer the cryovial contents to the tube containing the hiPS cell medium supplemented with Y-27632 at 37 °C.
▲ **CRITICAL STEP** Avoid repeated pipetting of the cell suspension.

(vi) Rinse the empty cryovial with 1 mL of 37 °C hiPS cell medium to recover any residual cells and transfer the cryovial contents to a 15-mL centrifuge tube containing the hiPS cell suspension. Invert the tube slowly to mix the cell suspension.

(vii) Remove a sample of cells to confirm viability and count the cells using a hemocytometer or an automated cell counter.

(viii) Dispense the cell suspension into a Matrigel-coated six-well plate. Distribute the cells by moving the plate with short side-to-side and back-and-forth motions.

(ix) Culture the hiPS cells in an incubator set to 37 °C, 5% CO_2, and 90% humidity.

(x) Feed the hiPS cells daily with 2 mL of prewarmed hiPS cell medium per 9.5-cm^2 growth surface. Passage them when they reach 60% confluence (as described in Step 2A) and start cardiac differentiation (Step 3 onward) when they are 85–90% confluent.
? TROUBLESHOOTING

(B) **Thawing and expansion of NHDFs ● Timing 10 min plus 3 d for expansion**

(i) Equilibrate an aliquot of the desired volume of fibroblast medium in a 37 °C water bath before thawing the cells.

(ii) Remove a cryovial with cells from the liquid nitrogen storage tank.
■ **PAUSE POINT** If needed, place the cryovial on dry ice for up to 10 min before thawing.

(iii) Immerse the cryovial in a 37 °C water bath; avoid submerging the cap and keep the tube stationary.
▲ CRITICAL STEP Use of a floating microcentrifuge tube rack is recommended.
(iv) Remove the cryovial from the water bath, spray it with 70% ethanol, and place it into a biological safety cabinet.
(v) Gently transfer the cryovial contents into the tube containing the 37 °C fibroblast medium.
▲ CRITICAL STEP Avoid repeated pipetting of the cell suspension.
(vi) Rinse the empty cryovial with 1 mL of 37 °C fibroblast medium to recover any residual cells and transfer the cryovial contents to the 15-mL centrifuge tube containing the fibroblast cell suspension. Invert the tube slowly to mix the cell suspension.
(vii) Remove a sample of cells to confirm viability and count the cells, using a hemocytometer or an automated cell counter.
(viii) Dispense the cell suspension into cell culture flasks. Distribute the cells by moving the flask with short side-to-side and back-and-forth motions.
(ix) Culture the NHDFs in an incubator set to 37 °C, 5% CO_2, and 90% humidity.
(x) Feed the cells daily with 2 mL of prewarmed fibroblast medium per 9.5-cm^2 growth surface. Passage them when they reach 80% confluence.
? TROUBLESHOOTING

Passaging of cells

2 Passage hiPS cells as described in option A and NHDFs as described in option B.

(A) **Passaging of hiPS cells** ● Timing **10 min**

(i) When the cells are 60% confluent, select the plates to be passaged. Wash with room-temperature PBS.
(ii) Incubate the cells with 1 mL of warm 0.5 mM EDTA per 9.5 cm^2 for 5 min at room temperature.
▲ CRITICAL STEP Monitor the cells under a microscope to determine whether cells are detaching from the cell culture plate.
(iii) Remove the EDTA.
(iv) Vigorously pipette up and down the surface of the wells with 1 mL of hiPS cell medium supplemented with Y-27632 to detach the cells.
(v) Transfer the cell suspension to a centrifuge tube containing hiPS cell medium supplemented with Y-26732.
(vi) Plate the cell suspension in Matrigel-coated plates at a ratio of 1:6–1:8 and incubate the cells in the TC incubator.
? TROUBLESHOOTING

(B) **Passaging of NHDFs** ● Timing **10 min**

(i) At 80% confluence, select the flasks to be passaged and transfer them to a biological safety cabinet.
(ii) Aspirate the medium from the flasks and add 7 mL of room-temperature PBS to wash the cells. Rotate the flask back and forth to fully wash the cells with PBS.
▲ CRITICAL STEP Completely wash the surface of the flask with PBS to remove residual FBS from the culture medium, as this will deactivate the trypsin treatment.
(iii) Aspirate the PBS and add 1 mL of warm 0.25% EDTA–trypsin per 9.5 cm^2. Incubate the cells for 5 min at 37 °C.
▲ CRITICAL STEP Monitor the cells under a microscope to observe whether cell dissociation has resulted in a detached single-cell suspension.
(iv) In the biological safety cabinet, gently transfer the cell suspension from the flask to a centrifuge tube with an equal amount of 37 °C fibroblast medium.
(v) Rinse the empty flask with fibroblast medium to recover any residual cells. Transfer the contents to the centrifuge tube containing the cell suspension. Invert the tube slowly to mix the cell suspension.
(vi) Centrifuge the cell suspension at 200*g* for 3 min at room temperature.
(vii) Carefully aspirate the supernatant, taking care not to disturb the cell pellet, and resuspend the cells in the desired volume of fibroblast medium.
(viii) Plate the cell suspension in new cell culture flasks at a ratio of 1:3–1:16.
? TROUBLESHOOTING

Cardiac differentiation ● Timing 12 d

▲ CRITICAL Culture the cells in an incubator set to 37 °C, 5% CO_2, and 90% humidity throughout the differentiation.

3 When hiPS cells reach 85–90% confluence, start cardiac differentiation of selected plates by washing the hiPS cells with PBS at room temperature.

4 Change the medium to CDM freshly supplemented with 3 μM CHIR 99021. This is day 0 of differentiation.

5 On day 2 of differentiation, change the medium to CDM freshly supplemented with 2 μM Wnt-C59.

6 On day 4, change the medium to CDM with no additional supplements.

7 Change the medium every 2 d with 2 mL of prewarmed CDM per 9.5 cm^2 growth surface.

8 Beating cells should begin to appear at day 10; check that this occurs. On day 12 of differentiation, hiPS-CMs are ready for use to form cardiac tissues. Proceed to the next section.
? TROUBLESHOOTING

Fabrication of pillars ● Timing 16 h

9 Manufacture the pillars' supportive structure according to Box 1.

10 Clean the pillars' supportive structures in a 70% ethanol bath for 30 min, followed by a wet autoclave cycle in which the structures are immersed in a beaker of water and exposed to the autoclave liquid cycle (121 °C for at least 30 min, using saturated steam under at least 15 p.s.i. of pressure) to remove any unintentional surface contamination.

11 Prepare the PDMS by mixing the silicone elastomer base with the curing agent at a 10:1 ratio. Leave in the vacuum chamber for 20 min.
▲ CRITICAL STEP Ensure that the PDMS is fully mixed before use by stirring at least 50 times. For ease of use, a 1-mL glass aspiration tip can be used to stir the PMDS and should subsequently disposed of in the biological sharps container.

12 Assemble the pillar molds with the supportive structures and pour in the PDMS. Leave in the vacuum chamber for 20 min.

13 Centrifuge the molds at 300*g* for 5 min at room temperature.
▲ CRITICAL STEP Ensure that the PDMS is free of bubbles before putting in the oven. If needed, centrifuge one more time or increase the time in the vacuum chamber.

14 Cure in an oven overnight at 60–70 °C.
▲ CRITICAL STEP Ensure that the PDMS is completely cured before removing it from the oven by touching the exposed PDMS to ensure it is no longer sticky.

15 Open the molds; then trim and clean the pillars and their supportive structures.
? TROUBLESHOOTING

16 Wet-autoclave the PDMS pillars by immersing them in a beaker of water and running the autoclave liquid cycle (121 °C for at least 30 min, using saturated steam under at least 15 p.s.i. of pressure) to remove any unintentional surface contamination.

17 Assemble the cardiac tissue–formation platform by placing the pillars into the bioreactor wells, using the mating features for alignment.

18 Autoclave all components (121 °C for at least 30 min, using saturated steam under at least 15 p.s.i. of pressure, with a 30-min drying time).

Generation of human cardiac tissues ● Timing 2–4 h

19 Select and prepare the cells (both NHDFs, as described in option A, and hiPS cells, as described in option B) to be used, depending on the number of tissues being made. For each tissue, plan to use one well of a six-well plate of hiPS-CMs at 85–90% confluency.
▲ CRITICAL STEP Test the efficiency of your cardiac differentiations by flow cytometry by counting cardiac troponin-positive cells (as described in Step 44C) from the dissociated monolayer. If the cardiac differentiation is 85% efficient, the remaining population can be considered a part of the 25% supporting cell population. Therefore, to reach a final cell concentration containing 25% supporting cells from a cardiac differentiation with an efficiency of 85%, 15% NHDFs need to be added. For a cardiac differentiation efficiency of 75%, the NHDFs would not be added. We recommend a minimum cardiac differentiation efficiency of 80%, and therefore a minimum NHDF cell population of 5%.

(A) **Preparation of an NHDF cell suspension**

(i) After selecting the number of NHDF cells needed, transfer the flasks to the biological safety cabinet and aspirate the medium from the flasks. Add 7 mL of room-temperature PBS to wash the cells. Rotate the flask back and forth to fully wash the cells with PBS.
▲ **CRITICAL STEP** Completely wash the surface of the flask with PBS to remove residual FBS from the culture medium, as this will deactivate the trypsin treatment.

(ii) Aspirate the PBS and add 1 mL of warm 0.25% EDTA–trypsin per 9.5 cm^2. Incubate the cells for 5 min at 37 °C.
▲ **CRITICAL STEP** Monitor the cells under a microscope to observe whether cell dissociation has resulted in a detached single-cell suspension.

(iii) In the biological safety cabinet, gently transfer the cell suspension from the flask to a centrifuge tube with an equal amount of 37 °C fibroblast medium.

(iv) Rinse the empty flask with fibroblast medium to recover any residual cells. Transfer the contents to the centrifuge tube containing the cell suspension. Invert the tube slowly to mix the cell suspension.

(v) Centrifuge the cell suspension at 200*g* for 5 min at room temperature.

(vi) Carefully aspirate the supernatant, taking care not to disturb the cell pellet, and resuspend the cells in 1 mL of fibroblast medium.

(vii) Repeat Steps 19A(v, vi) once.

(viii) Remove a sample of cells to confirm viability and count the cells using a hemocytometer or an automated cell counter. Keep the cell suspension on ice until use for tissue formation.
■ **PAUSE POINT** Cells can be placed on ice for up to 30 min.

(B) **Preparation of a hiPS cell suspension**

(i) Incubate the plates of hiPS-CMs with 1 mL of warm fresh collagenase II solution per 9.5 cm^2 for 15 min at 37 °C.
▲ **CRITICAL STEP** Monitor the cells under a microscope to determine whether cell dissociation has resulted in a detached single-cell dissociation.

(ii) Vigorously pipette up and down the surface of the wells and add 1 mL of fresh collagenase II solution. Incubate for another 15 min at 37 °C.

(iii) Gently transfer the cell suspension from the culture wells to a centrifuge tube with cardiac medium supplemented with 10% FBS at 37 °C.

(iv) Rinse the empty wells with cardiac medium supplemented with 10% FBS to recover any residual cells. Transfer to the centrifuge tube containing the cell suspension. Invert the tube slowly to mix the cell suspension.

(v) Centrifuge the cell suspension at 200*g* for 3 min at room temperature.

(vi) Carefully aspirate the supernatant, taking care not to disturb the cell pellet, and resuspend the cells in cardiac medium supplemented with 10% FBS.
▲ **CRITICAL STEP** Ensure that the supernatant is fully aspirated from the cell pellet before adding fibrinogen hydrogel because residual supernatant will dilute the final hydrogel concentration.

(vii) Repeat Step 19B(v, vi).

(viii) Take a sample of cells to confirm viability, and count the cells using a hemocytometer or an automated cell counter. Place the cell suspension on ice until tissue formation.
■ **PAUSE POINT** The cells can be kept on ice for a maximum of 30 min.

20 After being autoclaved, according to the 'Fabrication of pillars' section (Steps 9–18), put one cardiac tissue–formation platform inside a cell culture dish. Remove autoclaved pillars from the platform and add 200 µL of PLL-g-PEG to each well. Incubate at room temperature for 1 h. Wash the wells with 200 µL of PBS twice. Place the pillars back into the platform.

21 Mix the required amounts of the hiPS-CM and NHDF cell suspensions to create an overall cell number equal to 1 million cells per tissue being formed (include calculations for the cells and hydrogel of an extra cardiac tissue to account for pipetting losses). Ensure a ratio of 75% hiPS-CMs to 25% NHDFs (adjusted according to the percentage of cardiac troponin-positive cells, as discussed in Step 19).

22 Centrifuge at 200*g* for 5 min at room temperature.

23 Add 8 µL of thrombin to the bottom of each cardiac tissue formation well.

24 Resuspend the cells in 84 µL of fibrinogen per tissue by gently pipetting the fibrinogen and swirling the pipette tip to thoroughly mix the cells and the fibrinogen.

▲ CRITICAL STEP Before resuspending the cells, test the fibrinogen and thrombin by making a hydrogel without cells.

▲ CRITICAL STEP Fibrinogen should be used at room temperature.

▲ CRITICAL STEP Calculate the extra volume of one cardiac tissue to compensate for hydrogel loss during pipetting.

25 Add 84 μL of the hydrogel to each well, making sure to change pipette tips after each addition.

26 Add 8 μL of thrombin to the top of each well.

▲ CRITICAL STEP Avoid air bubble formation when pipetting. If bubbles form, use a sterile needle to pop them or gently aspirate them with a small pipette tip.

▲ CRITICAL STEP Change tips every time thrombin is used.

27 Place the culture plates into a 37 °C, 5% CO_2, and 90% humidity incubator for 20 min.

28 Add 25 mL of cardiac medium supplemented with 0.02 mg/mL aprotinin to the wells.

29 Keep culture plates in a 37 °C, 5% CO_2, and 90% humidity incubator and change the cardiac medium supplemented with 0.02 mg/mL aprotinin every 2 d for 7 d.

? TROUBLESHOOTING

Maturation of human cardiac tissues ● Timing 21 d

30 On day 7, check to see whether the cardiac tissues have compacted within the cardiac tissue–formation platform. Use a sterile needle to remove any cardiac tissue that is attached to the sides of the wells in the tissue-formation platform wells.

31 Using sterile forceps, gently remove the cardiac tissues from the tissue-formation platform by gently wiggling each pillar's supportive structure until the tissue is easy to remove via handling of the pillar's supportive structure. Transfer the cardiac tissues to the cardiac maturation platform, designed with mating features to enable the alignment of 12 tissues per maturation platform.

▲ CRITICAL STEP A second forceps can be used to stabilize the platforms during tissue removal and transfer.

32 Connect one alligator test lead to one platinum wire of the cardiac maturation platform and another alligator test lead to the other platinum wire of the cardiac maturation platform.

▲ CRITICAL STEP Secure the test leads with tape outside the incubator so that they do not move when the incubator door is open or closed.

33 Connect both alligator test leads to an electrical stimulator (Box 2).

▲ CRITICAL STEP Make sure the stimulator is turned off when performing this step.

34 Start stimulation with 2-ms monophasic square repeating pulses, delivered at 2 Hz and an amplitude of 4.5 V/cm. Place the platform and stimulator in the TC incubator.

35 Increase the stimulation frequency daily by 0.33 Hz (from 2 Hz at day 0 of stimulation to 6 Hz on day 12. Culture cardiac tissues in a 37 °C, 5% CO_2, and 90% humidity incubator and replace 50% of the culture medium every 2 days.

36 From day 12 to day 15 of stimulation, keep the stimulation at 6 Hz. Continue to culture cardiac tissues in a 37 °C, 5% CO_2, and 90% humidity incubator and replace 50% of the culture medium every 2 d.

37 At day 15, drop the stimulation to 2 Hz and keep it at that level until day 21 of stimulation. Continue to culture cardiac tissues in a 37 °C, 5% CO_2, and 90% humidity incubator and replace 50% of the culture medium every 2 d.

Characterization of human adult-like cardiac tissues

38 From day 22 of culture onward, cells can be characterized. Follow option A for video analysis of contractility, option B to assess the excitation threshold (ET) of the tissues, option C to assess the maximum capture rate (MCR) of the tissues, option D to measure the contractile force within a Muscle Strip Myograph, option E for calcium imaging, option F for histological analysis, and option G for immunostaining of tissue.

(A) **Video analysis of contractility** ● Timing **1 min per tissue**

(i) Assess contractility by taking 20-s videos at 100 frames per s (f.p.s.) of the cardiac tissue beating spontaneously. Process results as described in Box 3.

(B) **ET assessment** ● Timing **5 min per tissue**

(i) To assess ET, transfer cardiac tissues in the cardiac maturation platform to a microscope and initiate electrical stimulation at 1.0 Hz, 2-ms pulse, and 4.5 V/cm voltage. Wait 1 min to allow the tissue to equilibrate to the applied electrical stimulation parameters. If the

tissue beats synchronously with an electrical stimulus of 1 Hz, go to Step 38B(ii). If the tissue is not beating synchronously with electrical stimulus of 1 Hz, go to Step 38B(iii).

(ii) Decrease the stimulation voltage by 0.1 V until the tissue stops beating in sync with the applied electrical stimuli. Note this value as the ET.

(iii) Increase the stimulation voltage by 0.1 V until the tissue starts to beat in sync with the applied electrical stimuli. Note this value as the ET.

▲ **CRITICAL STEP** If the ET cannot be determined at 1 Hz because of the presence of spontaneous beating, increase the stimulation rate to 1.5 Hz or 2 Hz and repeat the process at this higher stimulation rate.

(C) **MCR assessment** ● **Timing 5 min per tissue**

(i) Set the voltage of the electrical stimulator to twice that of the ET determined in Step 38B.

(ii) Increase the rate of electrical stimulation by 0.1 Hz every 2 s until the tissue can no longer synchronously beat in response to the applied stimulus frequency. Record this value as the MCR.

▲ **CRITICAL STEP** The tissue should continue to increase in beat frequency until the MCR, at which point it may miss every other beat or show a more sporadic beating pattern in which the tissue is responding to only some of the applied stimuli.

(D) **Muscle Strip Myograph measurement of contractile force** ● **Timing 2–4 h**

(i) Set up the Muscle Strip Myograph System 1 h before transferring cardiac tissues to the instrument, by opening the controlling software (LabChart v.8) and filling each chamber of the instrument with 6 mL of modified Tyrode's solution and setting the temperature to 37 °C.

(ii) On the instrument, follow the directions to calibrate each chamber with the 2-g weight.

▲ **CRITICAL STEP** Ensure the chambers have stabilized at 37 °C before performing to this step.

(iii) On the computer, also calibrate the corresponding chamber's force by selecting the trace containing the force measurement from Step 38D(ii). On the top right of each channel, select the drop-down menu and select 'unit calibration'. Select the baseline part of the force trace and set this to '0'. Select the part of the trace after the 2 g weight is added and set this to '9.81'. Set the units to 'mN'.

(iv) Turn on the gas supply (95% O_2 and 5% CO_2) and open the bubbler so a slow bubbling of the gas can be seen within each chamber. Let this equilibrate for 15 min.

(v) Load engineered cardiac tissues into the organ bath chamber by moving the holder closer to the force transducer and by turning the dial attached to each organ bath chamber until the attachment hooks are spaced to align with the inside edge of the PDMS pillar within the cardiac tissue (Supplementary Fig. 2a).

(vi) Place the tissue in the chamber and use forceps to hold the supportive structure of the pillar's cardiac tissue; then gently move the tissue toward the hooks until the tissue is pierced fully on each inside edge of the PDMS pillar (Supplementary Fig. 2b).

(vii) Using another set of forceps, hold the cardiac tissue in place by gently applying pressure with the forceps on the area of the tissue between the two hooks, while using the other forceps (which is already holding the cardiac tissue pillar's supportive structure) to pull the PDMS pillar away from the tissue (Supplementary Fig. 2c).

(viii) Ensure that the cardiac tissue is fully mounted on both hooks and use forceps to push the tissue further onto each hook as needed (Supplementary Fig. 2d). Let the tissue equilibrate for 15 min.

▲ **CRITICAL STEP** Use caution to avoid touching the force transducer while doing this.

(ix) Turn the stimulator on to a frequency of 2 Hz, 5-ms pulse, and 80–100 mA and let the tissue equilibrate for another 15 min.

(x) Optimize the length for maximal force generation (Frank–Starling response) by adjusting the length of the tissue at stepwise increments to determine the maximal length at which the tissue exerts the maximal force (Supplementary Fig. 2e,f). Record the initial length of the tissue.

(xi) Turn the dial that controls the position of the tissue holder to 0.1 mm and let the tissue force equilibrate for a minimum of 1 min. Take note of the new length.

(xii) Repeat Step 38D(xi) until subsequent increases in length no longer increase the force generated. At this point, stop increasing the length and leave the tissue at this maximal length for the remainder of the experiment. Record the final length of the tissue used to perform the remainder of the steps.

(xiii) Determine the CICR by continuously adding 0.2- or 0.4-mM increments of $CaCl_2$ to the chamber until a maximum of 2.8 mM is reached.
▲ **CRITICAL STEP** Use the note function within the LabChart v.8 software to make notes of each calcium concentration upon adding more $CaCl_2$ to the chamber.

(xiv) Obtain the FFR by increasing the frequency of stimulation by 1 Hz every 30–60 s until 6 Hz is reached.
? TROUBLESHOOTING

(xv) After reaching 6 Hz following the FFR, turn the stimulation off and wait an additional 10, 30, or 60 s. After these times, turn the stimulation back on to a frequency of 1 Hz. This first beat will detail the post-rest potentiation (PRP).

(xvi) To assess the response to cardiac drugs, record the baseline force for 1 min by making a note in the LabChart v.8 software and continuing to record the experiment.

(xvii) Use a concentrated stock solution of the drug to sequentially add the drug directly to the tissue bath with a pipette. Record the addition of the drug and the concentration in the LabChart v.8 software.

(xviii) Wait 5–15 min between drug additions, depending on the time needed to elicit a response, to record the full response before adding the next dose. Record each serial addition of the drug and the new concentration in the LabChart v.8 software.

(xix) Upon the conclusion of the experiment, use calipers to measure the cross-sectional area (width and thickness) in the middle of the tissue.
▲ **CRITICAL STEP** Record these measurements for use in determining the cross-sectional area when analyzing the force data.

(xx) Calculate twitch forces with the LabChart v.8 software as the average of the difference between cyclic peak maximum and minimum force, normalized to the cross-sectional area.

(E) **Calcium imaging ● Timing 1–3 h**

(i) Prepare 1× Fluo-4 Direct calcium assay reagent solution by mixing an equal amount of 2× Fluo-4 Direct calcium assay reagent and cardiac medium.

(ii) Aspirate the medium from the cardiac tissues within the maturation platform and add 25 mL of the 1× Fluo-4 Direct calcium assay reagent.

(iii) Incubate plates at 37 °C for 35 min.

(iv) Wash out the 1× Fluo-4 Direct calcium assay reagent solution and replace it with 25 mL of prewarmed blebbistatin solution.

(v) Transfer the tissue to a fluorescence microscope with filters appropriate for excitation at 494 nm and emission at 516 nm.

(vi) Connect tissue maturation platform to an electrical stimulator and turn it on to a frequency of 1 Hz, 2-ms pulse, and 3-V voltage.

(vii) Capture the calcium baseline transient by recording a 20-s video at 100 f.p.s.

(viii) After recording the baseline calcium measurements, perform the intended dose–response study by adding increasing drug dosages via a syringe connected to sterile tubing that is inserted into the medium of the cardiac maturation platform. Take a video immediately after adding the drug and again 10 min after each drug addition.
▲ **CRITICAL STEP** Electrical pacing is critical when analyzing the changes in calcium handling in response to drugs that may also affect beat frequency, because the beating frequency directly influences calcium dynamics. Thus, for drugs such as isoproterenol, electrical pacing should be applied throughout the dose–response study.

(F) **Imaging of histological sections of adult-like cardiac tissues ● Timing 48 h**

(i) Fix mature cardiac tissues with 4% paraformaldehyde or 10% neutral buffered formalin overnight with gentle tumbling at 4 °C.

(ii) Wash with PBS three times for 15 min each.
■ **PAUSE POINT** The samples can be left in PBS at 4 °C until they are sent for histological processing.

(iii) Outsource paraffin embedding and sectioning to appropriate specialized core(s).
▲ **CRITICAL STEP** To ensure tissues stay intact during sectioning, request 10-μm-thick sections.
▲ **CRITICAL STEP** To evaluate the presence of T-tubules, axial and longitudinal cross-sections should be examined.

(iv) Dewax sectioned paraffin-embedded cardiac tissues by immersing the slide in fresh CitriSolv twice for 10 min each time with agitation about once a minute.

(v) Rehydrate the slides with a serial exposure to solutions with lower concentrations of ethanol (100%, 90%, 70%, water). Wash twice with each solution for 3 min and agitate every 10–20 s.

(vi) Perform antigen retrieval by immersing slides in Dako target retrieval solution in a container in a 95 °C water bath for 30 min.

(vii) Let the slides cool at room temperature for 20 min and then place them under running cold water for 5 min.

(viii) Wash the slides three times with PBS for 5 min each time.

■ PAUSE POINT The samples can be left in PBS for 24 h at 4 °C.

(ix) When staining for T-tubules, immerse the entire slide in WGA at a concentration of 1 μg/mL in PBS at 4 °C for 15 min. Skip this step and proceed directly to the next step if you are not staining for T-tubules. Gently wash the tissues three times for 15 min each with PBS.

(x) Permeabilize with 0.25% Triton X-100 in PBS for 15 min at room temperature.

(xi) Wash the slides three times with PBS for 5 min each time.

(xii) Remove the slides from the rack, wipe around the section of the tissue to create an 'island' and pipette 100–200 μL of PBS onto each section to prevent it from drying out.

(xiii) Use a hydrophobic pen to draw a ring or rectangle around each tissue and shake off the PBS.

(xiv) Wash three times with PBS for 5 min each.

(xv) Add 100–200 μL of blocking buffer to the tissues and incubate for 60 min at room temperature.

(xvi) Incubate with primary antibody diluted in blocking buffer for 60 min at room temperature or overnight at 4 °C.

(xvii) Shake off the primary antibody and wash three times with 100–200 μL of PBS for 5 min.

(xviii) Incubate with 100–200 μL of secondary antibody diluted to 2 μg/mL in blocking buffer for 60 min.

(xix) Shake off the secondary antibody and wash three times with 100–200 μL of PBS for 5 min.

▲ CRITICAL STEP Check under a fluorescence microscope to see whether the staining worked. If the expected staining is not seen, repeat antibody incubation for longer time periods.

(xx) Place one drop of Vectashield with DAPI on the short end of the glass slide and use forceps to slowly lower a coverslip at an angle, starting from the side of the coverslip with the Vectashield and working toward the other side.

▲ CRITICAL STEP Avoid the formation of air bubbles. If an air bubble is seen, try to reorient the slide without lifting the coverslip, so that the air bubble is not located over the tissue region.

■ PAUSE POINT Store in a refrigerator for up to 2 weeks. The slides can be stored at −20 °C for longer.

(xxi) Image tissues with a virtual slide scanner or according to the microscope's user manual.

▲ CRITICAL STEP The use of a virtual slide scanner provides ideal resolution for imaging T-tubules. We found that imaging at either 20× or 40× was sufficient. In addition, a virtual z-stack should be acquired, and combined into a flattened image afterward, to encompass the entire tissue section.

? TROUBLESHOOTING

(G) **Immunostaining of whole adult-like cardiac tissue ● Timing 62–116 h**

(i) Fix the tissues with 4% PFA.

(ii) Gently wash the fixed tissues with PBS three times for 5 min each.

(iii) When staining for T-tubules, add WGA at a concentration of 1 μg/mL in PBS, covering the entire tissue. Place it on a shaker under low agitation at 4 °C for 30 min. Skip this step and proceed directly to Step 38G(iv) if you are not staining for T-tubules. Gently wash tissues with PBS three times for 15 min each.

(iv) If staining structural proteins, add 0.25% Triton X-100, covering the tissues completely, and place them on the shaker under low agitation at 4 °C for 15 min. Otherwise, proceed to next step.

(v) Add 0.5% Triton X-100 so that it completely covers the tissues and place them on the shaker under low agitation at 4 °C for 15 min.

(vi) Dip each tissue in 1% Tween 20—so that it completely covers the tissue—five times for 15 s each at room temperature.

(vii) Wash each tissue immediately by dipping it into PBS five times for 5 s each.

(viii) For surface-marker stains/gap junctions, dip the tissue in 1% Tween 20—so that it completely covers the tissues—for 15 min at 4 °C. Otherwise, proceed to the next step.

(ix) Wash the tissue immediately by dipping it into PBS five times for 5 s each.

(x) Gently wash the tissues with PBS three times at 4 °C for 5 min each on a shaker under low agitation.

(xi) Add quenching solution so that it completely covers the tissues and place them on a shaker under low agitation at 4 °C for 30 min.

(xii) Add blocking buffer so that it completely covers the tissues and place them on a shaker under low agitation at 4 °C for 2 h.

PAUSE POINT The tissues can be left overnight on the shaker at low agitation at 4 °C.

(xiii) Add primary antibodies at the appropriate dilution in 0.2% BSA so that the tissue is completely covered. Place the tissues on a shaker under low agitation at 4 °C for 12–48 h. For a list of standardized antibody dilutions, see 'Reagents' section.

CRITICAL STEP If using multiple primary antibodies, ensure that they are from different species and that there is no cross-reactivity.

CRITICAL STEP 0.1% Tween 20 can be used during primary antibody incubation to increase binding for structural proteins.

(xiv) Gently wash the tissues with PBS three times for 5 min each.

(xv) Continue to wash the tissues in PBS with 0.2% BSA on a shaker under low agitation at 4 °C for 6–12 h.

(xvi) Add secondary antibodies at the appropriate dilution in 0.2% BSA so that it completely covers the tissues and place them on a shaker under low agitation at 4 °C for 12–48 h.

CRITICAL STEP If using multiple secondary antibodies, ensure that the species reactivity corresponds with the intended primary antibody and that there is no cross-reactivity.

(xvii) Gently wash the tissues with PBS three times for 5 min each.

(xviii) Continue to wash the tissues in PBS with 0.2% BSA on a shaker under low agitation at 4 °C for 6–12 h.

(xix) Add DAPI stain at a dilution of 1:1000 in PBS so that it completely covers the tissues and place them on the shaker under low agitation at 4 °C for 30 min.

(xx) Gently wash the tissues with PBS three times for 15 min each.

(xxi) Prepare the tissues for confocal imaging (Supplementary Fig. 3) and take images according to the confocal microscope user guide.

Papain digestion of mature engineered cardiac tissues for single-cell analysis ● Timing 1 h

CRITICAL Single cells are needed to perform certain analyses, such as electrophysiology studies, single-cell sequencing, or metabolism studies using Seahorse assays. We found that common methods of digesting cells (i.e., collagenase II or trypsin–EDTA solutions) were too harsh and did not produce a completely dissociated, single-cell suspension when applied to adult-like cardiac tissues. We therefore developed a papain-based dissociation solution capable of dissociating the adult-like tissues discussed in this protocol into a single-cell suspension.

CRITICAL Note that, depending on which downstream assays you plan to perform, you may need to undertake some of the steps for the downstream assay during papain digestion. See Step 44 for details.

39 Prepare the papain-based cardiac tissue dissociation solution freshly. Incubate the dissociation solution in a 37 °C water bath for 30 min to activate the papain. Add DNase solution to the papain solution before use.

40 Wash cardiac tissues with PBS twice. Next, incubate the tissues in the papain-based cardiac tissue dissociation solution containing DNase for 15 min at 37 °C (one cardiac tissue in 10 mL of papain solution in a 15-mL tube). Gently shake the tube every 2 min. After 15 min incubation, gently pipette the tissues with 10 mL of serological plastic pipette ten times.

CRITICAL STEP Fifteen minutes incubation of the cardiac tissue in papain solution was used to achieve a mild dissociation of the tissue and enhance the recovery of the cells after the dissociation. The tissue might not be completely dissociated into single cells under this condition.

CRITICAL STEP It may also be necessary to undertake preparations for downstream assays during this step. Consult the procedure for the downstream assay you plan to complete next for details (these can be found in Step 44).

41 Terminate the papain activity using serum-containing post-papain digestion cardiomyocyte culture medium (5 mL of DMEM supplemented with 10% FBS for 10 mL of papain solution).

42 Centrifuge the cell suspension at 300*g* for 5 min at room temperature, aspirate all of the solution, wash one more time with cardiac medium and add fresh culture medium into the tube.

43 Count the cells and plate them for the desired single-cell experiment (see Step 44 for some possible assays).

Characterization of cells using single-cell techniques

44 If you have followed Steps 39–43, you can proceed to electrophysiological analysis (option A), metabolic flux assay (option B), flow cytometry (option C).

(A) **Electrophysiological analysis of single-cell cardiomyocytes from adult-like cardiac tissues**

● **Timing 3 d**

(i) During dissociation (Steps 39–43), incubate the center glass part of the 35-mm glass-bottom dishes with 70 µL of glass-dish-coating solution (containing Geltrex) per dish. Incubate for 1 h at 37 °C and 1 h at room temperature. Aspirate the solution immediately before plating cardiomyocytes.

(ii) Resuspend the dissociated cells with the post-papain digestion cardiomyocyte culture medium (2–6 mL of medium per tissue, depending on the original size). Plate the dissociated single cardiomyocytes on the center glass part of the Geltrex-coated dishes (7-mm diameter; the center part can hold ~70 µL solution).

▲ **CRITICAL STEP** Low cell confluency (~2–5%) is required to efficiently use multiple electrophysiological recording conditions.

(iii) Put the dishes into a cell culture incubator (37 °C, 5% CO_2, 90% humidity) to allow the dissociated cells to recover. A dish containing sterile water without a cover can be placed next to the two dishes containing cells in a 100-mm dish to retain humidity and prevent the medium from drying out.

(iv) Twenty-four hours after cardiomyocyte plating, add 2 mL of post-papain digestion cardiomyocyte culture medium to the dishes.

(v) Two or three days after cardiomyocyte plating, wash the dishes with patch-clamp external recording solution (action potential or IK1) once at 37 °C and add the recording solution (~2 mL/dish at 37 °C).

▲ **CRITICAL STEP** On the first day after dissociation, the dissociated myocytes are not fully recovered. More than 4 d after myocyte dissociation, the cells (without any spontaneous contraction) will no longer be healthy enough for electrophysiological recordings. Therefore, the whole-cell patch-clamp recordings should be completed in 2–3 d after the cell dissociation and plating.

(vi) Transfer the dish to the electrophysiological recording setup (Supplementary Fig. 4) and use the microscope to find single cardiomyocytes with no spontaneous beating in the dish.

(vii) Fill the glass pipette with the action potential recording solution to record action potential, and install the pipette into the electrode connected to the manipulator holder. Move the pipette in the external solution with the manipulator controller (speed 0 or 1).

(viii) Examine pipette resistance (smaller, ~8 MΩ, is better)

▲ **CRITICAL STEP** Using a larger pipette with 2–6 MΩ resistance often results in a failure to seal and breaking through and/or leakage from the cardiomyocytes.

(ix) Place the pipette tip on top of the target cell slowly (speed 4).

▲ **CRITICAL STEP** Confirm that there are no bubbles inside the glass pipette. If any bubbles or cell debris are observed, use a 10-mL syringe to remove the bubbles/debris (after moving the pipette far from the target cell).

(x) Move the pipette down slowly (speed 6, down to ~30 µm above the cell) and increase the magnitude of the microscope objectives.

(xi) Seal the cell with a glass pipette (gentle pressure).

(xii) Seal a thicker and elastic region of the target cell (but not close to the nucleus). Hold the potential at −60 mV after sealing is successful (>3 GΩ).

(xiii) Apply a small amount of pressure multiple times, using mouth suction (i.e., sharp pulses), to the electrode/pipette in order to break through the plasma membrane of the sealed cardiomyocyte. Immediately after breaking through, the cell capacitance should be measured in whole-cell clamp mode. Then switch the mode to current-clamp to make sure that there are no spontaneous action potentials in the patched cardiomyocytes.

(xiv) Start action potential recordings. For I_{K1} current recording in human pluripotent stem cell–derived cardiomyocytes, the external I_{K1} recording solution and internal I_{K1} recording solution are used as the external and internal solutions, respectively. $BaCl_2$ (0.5 mM) is used for measuring Ba^{2+}-sensitive current to dissect I_{K1} current in the cardiomyocytes. Use 2-s-long voltage-clamp, applied from −130 to +10 mV (holding at −40 mV, 0.1 Hz, 2-s voltage pulse). In our experiments, the I_{K1} reversal potential (Ba^{2+}-sensitive current) had a negative slope conductance consistent with inward rectification. The current–voltage plot can be analyzed before and after the addition of 0.5 mM $BaCl_2$ for 2 min.

(B) **Metabolic functionality using the Seahorse XF96/XFe96 assay** ● **Timing 3 d**

(i) Prepare Seahorse XF96/XFe96 cell culture microplates for cell attachment by coating each well with 200 µL of Matrigel diluted 1:40 in cold RPMI 1640 medium (i.e., 30 µL of Matrigel in 1.2 mL of cold RPMI 1640 medium). Place the Matrigel-coated plate into a 37 °C incubator for 1 h.

▲ **CRITICAL STEP** This must be carried out at least 1 d before running the Seahorse assay.

(ii) After dissociation of the cardiac tissues, transfer the cells to a 15-mL Falcon centrifuge tube and spin down at 1,000*g* for 5 min at room temperature.

(iii) Wash the cells by resuspension in 10 mL of post-papain digestion cardiomyocyte culture medium and spin down at 1,000*g* for 5 min at room temperature.

(iv) Resuspend the cells in 15 mL of post-papain digestion cardiomyocyte culture medium and add the cells to a 150-cm^2 TC-treated flask to enrich the cell population for mature hiPS-CMs. Place the cells into a 37 °C incubator for 30 min.

(v) Check the flask containing the dissociated cells under a bright-field microscope to see that fibroblasts have attached to the bottom. If no cell attachment is seen, culture cells for an additional 15 min.

▲ **CRITICAL STEP** Do not exceed 1 h of culture total in the TC-treated flask.

(vi) Remove the supernatant from the TC-treated flask, which will now be enriched for mature hiPS-CMs. Spin the cells down at 100*g* for 5 min at room temperature.

(vii) Resuspend the cells in 1 mL of post-papain digestion cardiomyocyte culture medium and count the cells with a hemocytometer.

▲ **CRITICAL STEP** Single cells are needed to promote cell seeding of evenly cultured monolayers that do not contain cell clumps, which interfere with the proper data acquisition.

(viii) Dilute the cells with post-papain digestion cardiomyocyte culture medium to achieve a final concentration of 225,000 cells/mL.

(ix) Remove the Matrigel-coated XF96 cell culture microplate from the incubator and aspirate excess media from each well.

(x) Seed 200 µL of the cell-containing medium (Step 44B(viii)) to plate 45,000 hiPS-CMs into each well.

(xi) Tilt the plate to the left and right, front and back, and toward each corner to ensure even seeding of the cells within the plate.

(xii) Place the XF96 cell culture microplate containing cells into the incubator and allow the cells to attach for 24 h.

(xiii) To hydrate the Seahorse XF96/XFe96 sensor cartridge, open the Seahorse XFe96 FluxPak in a sterile biosafety hood and remove the contents. Take the sensor cartridge off and place it upside down next to the utility plate. Add 200 µL of Seahorse XF calibrant to each well of the utility plate, regardless of whether or not sample will be run in that well. Replace the lid and seal the plate by wrapping it with Parafilm around the edges. Place the plate in a non-CO_2 37 °C incubator overnight for at least 1 d before running the Seahorse assay.

▲ **CRITICAL STEP** Add Seahorse XF calibrant to every well of the plate, regardless of whether sample will be run in that well.

▲ **CRITICAL STEP** A non-CO_2 37 °C incubator must be used.

(xiv) On the day of the planned Seahorse assay, prepare the XF assay medium.

(xv) Look at the cells plated in the XF96 cell culture plate under a bright-field microscope to confirm that the cells have adhered to the bottom of the well and that they look healthy.

(xvi) Remove 180 µL from each well containing cells (leaving 20 µL remaining in the well) and rinse with 200 µL of XF assay medium.

(xvii) Remove all medium from each well containing cells and replace it with 175 µL of XF assay medium. Place the plate into a 37 °C incubator without CO_2 for 1 h before the assay.

▲ **CRITICAL STEP** A non-CO_2 37 °C incubator must be used.

(xviii) Retrieve the sensor cartridge from the 37 °C incubator without CO_2 and load the XFe96 sensor cartridge by adding 20 µL of oligomycin to port A, 22 µL of FCCP to port B, and 25 µL of rotenone–antimycin A to port C.

(xix) Run the assay according to the Seahorse instruction manual, choosing the following options in the Seahorse machine: group definitions = injection strategy 1 and assay medium 1; the instrument protocol = three injections; the plate map = specify groups according to the experimental design.

(C) **Flow cytometry of single cells isolated from mature cardiac tissues** ● **Timing 1–3 h**

(i) Spin papain-dissociated cells down into a pellet in 1.5-mL Eppendorf tubes at 300*g* for 5 min at room temperature.

(ii) Resuspend the cells in 250 µL of BD Cytofix reagent; mix well and keep at 4 °C for 15 min.

(iii) Wash the cells twice with 1:10 Perm Wash buffer (diluted from the 10× Cytoperm reagent in deionized water).

(iv) Use a centrifuge to spin the cells down again at 300*g* for 5 min at room temperature.

(v) Resuspend the cells in 100 µL of Perm Wash buffer.

(vi) Add the appropriate amount of primary antibody.

(vii) Incubate for 20 min at 4 °C in the dark.

(viii) Wash twice with 1 mL of Perm Wash buffer.

(ix) If conducting double staining, repeat Steps 44C(vi–viii) with the secondary antibody.

(x) Resuspend in 500 µL of stain buffer (0.1% BSA).

(xi) Pass the cells through a filter-cap flow tube and proceed to flow cytometry for analysis. We undertook flow cytometry and analysis with a BD FACSCanto II flow cytometer. A minimum of 10,000 events should be collected for each run. We analyzed the data in FlowJo, with every stained sample being compared to an unstained control that otherwise went through the same fix/perm process. For example gating and flow cytometry results for early-stage hiPS-CMs, see Supplementary Fig. 5.

▲ **CRITICAL STEP** We recommend use of a flow cytometry expert or core center. Proper training and equipment are required.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
1A(x)	Poor attachment of hiPS cells	hiPS cell medium was not supplemented with Y-27632	Supplement the medium with Y-27632
		Imperfect coating	Plate the cells in appropriately coated plates
	Spontaneous differentiation of hiPS cells	Cultures were too sparse or too dense	Adjust the passage ratio
	hiPS cells are proliferating very slowly	hiPS cells will show their normal growth behavior only after being passaged several times	Wait for some passages to occur
1B(x)	NHDFs are proliferating very slowly	Cultures were too sparse	Adjust the passage ratio
2A(vi), 2B(viii)	Many dead cells	EDTA incubation lasted too long	Monitor the cells under a microscope and stop dissociation at the right time
8	No spontaneously contracting cells on day 12	Poor-quality hiPS cell culture	See above problems because the problem, and hence solutions, are probably related to problems during the expansion of hiPS cells
		Small molecules were not added or were incorrectly added in the CDM medium	Supplement the CDM medium with the right molecules at the right time
		Small molecules might be old	Prepare new stocks of small molecules
15	Bubbles in PDMS pillars	PDMS was left to sit too long before pouring into the mold and therefore was not as	Pour PDMS immediately after mixing; immediately transfer the mold with PDMS to the

Table continued

Table 2 (continued)

Step	Problem	Possible reason	Solution
		viscous; vacuum pump was not used to pull bubbles out; centrifuge speed was not set high enough to push bubbles out	vacuum chamber to de-bubble (can increase vacuum time to 30 min); centrifuge the PDMS in pillar-formation molds at 400*g* for 5 min at room temperature
29	Tissues did not form	Fibrinogen, thrombin, or aprotinin stocks may not be fresh. If the tissue did not gel properly from the beginning, the problem is probably the fibrinogen or thrombin quality. If the tissue did gel properly but subsequently fell apart, it is probably the aprotinin quality	Make the fibrinogen and thrombin fresh, and test that the two gel when mixed together by combining them in a 5:1 ratio in a Petri dish, confirming that they gel by touching the hydrogel with a pipette tip. If the problem is the aprotinin quality, make a fresh stock. If this does not solve the problem, increase the aprotinin concentration to 6.6 mg/mL or decrease the amount of fibroblasts added to the coculture (be cognizant of the fibroblasts already present in the hiPS-CM differentiation culture)
	Tissues are not beating well	Cardiac differentiation efficiency may have been too low and/or the cell digestion was too harsh	Only use hiPS-CM differentiations with an efficiency ≥80%. Limit the time in collagenase during digestion. If cells are difficult to dissociate, use the cells a day or two early, when they will be easier to digest
	Medium turns yellow quickly	Tissue is too metabolically active to be supported by surrounding medium	Ensure that tissues are cultured in a large bath of medium. Ensure that the medium has fatty acids (B-27 Supplement) and is maintained in an environment with 5% CO_2
38D(xiv)	Force not increasing at higher stimulation frequencies	Tissue may not be stretched to allow optimal force generation. Or, the organ bath solution has not been refreshed and thereby has become toxic to the tissue	Use the Frank–Starling force–length relationship to continuously increase the tissue length within the Muscle Strip Myograph System until the force output is stable. Then retry the force–frequency response. If the organ bath solution is bubbling or has not been exchanged recently, replace it with fresh solution and ensure it is bubbled properly
38F(xxi)	High background in immunostained samples	Inadequate washing steps	Increase the time of the washing steps (can do 24-h washes on a shaker at 4 °C) and add a gentle detergent (0.01% Triton X-100) to facilitate removal of excess antibody
Box 3, step 9	Too many peaks are found		Change the 'peakDistance' value (line 205 of code) to a higher value
	Not enough peaks are found		Change the 'peakDistance' value (line 205 of code) to a lower value

Timing

Step 1A, thawing and expansion of hiPS cells: 10 min plus 3 d for cell expansion
Step 1B, thawing and expansion of NHDFs: 10 min plus 3 d for cell expansion
Step 2A, passaging of hiPS cells: 10 min
Step 2B, passaging of NHDFs: 10 min
Steps 3–8, cardiac differentiation: 12 d
Steps 9–18, fabrication of pillars: 16 h
Steps 19–29, generation of human cardiac tissues: 2–4 h
Steps 30–37, maturation of human cardiac tissues: 21 d
Step 38A, video analysis of contractility: 1 min per tissue
Step 38B, ET assessment: 5 min per tissue
Step 38C, MCR assessment: 5 min per tissue
Step 38D, Muscle Strip Myograph System measurements of contractile force: 2–4 h
Step 38E, calcium imaging: 1–3 h
Step 38F, imaging of histological sections of adult-like cardiac tissues: 48 h
Step 38G, immunostaining of whole adult-like cardiac tissues: 62–116 h
Steps 39–43, papain digestion of mature engineered cardiac tissues for single-cell analysis: 1 h
Step 44A, electrophysiological analysis of single-cell cardiomyocytes from adult-like cardiac tissues: 3 d

Step 44B, metabolic functionality using the Seahorse XF96/XFe96 assay: 3 d
Step 44C, flow cytometry analysis of single cells isolated from matured cardiac tissues: 1–3 h
Box 2, electromechanical stimulation of cardiac tissues: 21 days
Box 3, assessment of contractile motion within engineered cardiac tissues: 1 min/video

Anticipated results

In our protocol, we demonstrate the ability to generate cardiac tissues with electrophysiological activity, cell structure, and mechanical activity closer to those seen in the adult myocardium than the fetal myocardium[32]. Similar results should be obtained if the cells are used during the period of high plasticity, directly following cardiac differentiation, and tissues are properly formed and subsequently exposed to electromechanical stimulation at a continuously increasing intensity for a period of 2 weeks. This protocol facilitates the development of cardiomyocytes of enhanced maturity, enabling more physiological results from standard assays (of immunohistochemistry, electrophysiology, metabolic function, calcium handling, contractility) and new assays that were previously not possible to carry out because of the immaturity of hiPS-CMs (assays of force–frequency relationship and chronotropic, lusitropic, and ionotropic drug responses) (Supplementary Fig. 6). Different types of evaluations can also be carried out on tissues obtained during this protocol, including quantification of the tissue's developmental stage (maturity), and recapitulation of disease phenotypes (i.e., the effects of genetic mutations that can be modeled only if healthy controls display a positive force–frequency relationship).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Source data for the quantitative data shown in all figure panels are available without restrictions and can be accessed at https://doi.org/10.6084/m9.figshare.5765559. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The GTEx data used for the analyses described in this article were obtained from the dbGaP Portal (https://www.ncbi.nlm.nih.gov/gap/) using the following dbGaP accession number: phs000424.v7.p2.

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Author contributions

K.R.-B., K.Y., and G.V.-N. designed the methodological approach; K.R.-B., D.T., K.Y., and G.V.-N. designed the single-pillar molds and cardiac tissue formation and maturation platforms; K.R.-B., D.T., and L.S. cultured cell lines; K.R.-B., D.T., T.C., and S.M. formed cardiac tissues and performed maturity assessment experiments; L.S., K.M., and M.Y. conducted single-cell dissociation and electrophysiology readings; H.M.W. conducted flow cytometry analysis; A.V. and E.C.R. conducted the analysis of gene expression; K.R.-B., D.T., K.Y., M.Y., and G.V.-N interpreted the data and wrote the manuscript.

Competing interests

G.V.-N. and K.R.-B. are co-founders of TARA Biosystems, a Columbia University spin-off that is commercializing the use of bioengineered human cardiac tissues for drug development.

Additional information

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Radisic, M. et al. *Proc. Natl Acad. Sci. USA* **101**, 18129–18134 (2004): https://doi.org/10.1073/pnas.0407817101
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Corresponding author(s): Gordana Vunjak-Novakovic

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