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Cardiomyocyte electromechanical stimulation platform (CESP)

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CARDIOMYOCYTE ELECTROMECHANICAL STIMULATION PLATFORM (CESP)

by

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CARDIOMYOCYTE ELECTROMECHANICAL

STIMULATION PLATFORM (CESP)

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ABSTRACT

Developing robust biological models for accurately studying the physiology and behavior of the human heart has been a decades-long challenge in the field of tissue engineering. The lack of a reliable, accurate, and reproducible platform for analyzing the responses of human cardiac muscle to external stimuli has likely impeded the rate of biological discoveries and blurred the consistency of data across studies. To address the limitations of static 2D culture models, researchers have developed advanced 2D models using human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes, capable of providing electrical or mechanical stimulation. However, these models lack synchronous integration of these two important modes of stimuli with interdependent timing. The goal of this project was to develop, verify, and biologically characterize the Cardiomyocyte Electromechanical Stimulation Platform (CESP) capable of the integrated electrical pacing and mechanical stretching of 2D monolayers of hiPSC-cardiomyocytes *in vitro*. The platform provides the ability to run different electromechanical stimulation programs that recapitulate physiological and pathological cardiac behavior with the throughput needed to extract meaningful biological measurables to analyze transcriptional and morphological changes to stimuli.

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LIST OF ABBREVIATIONS

AC	Alternating Current
CAD	Computer-Aided Design
cDNA	Complimentary Deoxyribonucleic Acid
CES	Cardiomyocyte Electromechanical Stimulation
CESP	Cardiomyocyte Electromechanical Stimulation Platform
DC	Direct Current
DCM	Dilated Cardiomyopathy
DIC	Digital Image Correlation
ECM	Extracellular Matrix
FDM	Fused Deposition Modeling
EKG	Electrocardiogram
GFP	Green Fluorescent Protein
HCM	Hypertrophic Cardiomyopathy
hiPSC	Human Induced Pluripotent Stem Cell
hiPSC-CM	Human Induced Pluripotent Stem Cell-Derived Cardiomyocyte
IHC	Immunohistochemistry
MI	Myocardial Infarction
PDMS	Polydimethylsiloxane
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROI	Region of Interest

SLA	Stereolithography
SNR	Signal-to-Noise Ratio
TAC	Transverse Aortic Constriction
μTUG	Microfabricated Tissue Gauge

CURRENT STATE OF CARDIAC MODELS

Introduction

Cardiomyocytes, the parenchymal cell of cardiac muscle tissue, comprise around 70–85% of the heart by volume and work together as an engine that transduces electrical signals from the nervous system into mechanical force that pumps blood throughout the body to perfuse tissues with the oxygen and nutrients needed for survival.[1] While other stromal cell populations, such as fibroblasts and endothelial cells, are important for the structural integrity and maintenance of the heart itself, cardiomyocytes perform the organ's main function of cyclic force generation, allowing nerves in the chamber walls to coordinate the continuous contraction and relaxation of the heart to pump blood. In addition to receiving electrical signals from nerves, cardiomyocytes, like most cells, continuously transduce mechanical signals that they sense from their environment. Included in this mechanosensory stimuli is the stiffness of the extracellular matrix (ECM) or substrate proteins they adhere to and the structural stress on their cytoskeleton under tensive or compressive loads.

In different disease states, cardiomyocytes in a patient's cardiac tissue may receive significantly different electrical and mechanical signals than those in a healthy physiological state. In the case of pathological arrhythmias like atrial fibrillation (a-fib) and ventricular fibrillation (v-fib), cardiomyocytes are usually sent drastically different pacing signals by nerves in the heart, often containing pulses that vary in amplitude, frequency, and consistency of pace. Diseases like hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) can cause the cardiac tissue lining chambers of the

heart to thicken or thin, respectively, altering the tissue-level mechanical loading on cardiomyocytes to pathological levels during the cardiac cycle[2,3].

Using diagnostic tools like electrocardiograms (EKG) and echocardiograms (ultrasound), clinicians and researchers are able to observe the macroscopic organ-level performance of a patient's heart, allowing the monitoring of cardiac diseases for preventing and treating heart failure. However, so much about the physiology and behavior of cardiac disease at the microscopic cell and tissue levels still remains uncharacterized. By accurately recapitulating the stimuli received by healthy and disease-state cardiomyocytes in a laboratory setting, researchers are able to run biochemical assays to gain complex, valuable insight into the biological responses of cardiac muscle at the cellular level in different states. By continuing to map the signaling pathways and phenotypic responses to different stimuli, we are able to better identify biological targets for therapeutic development and expand the scope of effective treatments available for cardiac disease patients.

Animal Models (*in vivo*)

Despite the many ethical issues associated with animal testing, researchers often view *in vivo* animal models as the most physiologically accurate systems for observing responses to stimuli. The main advantage of animal models is that they naturally provide the full ensemble of biological stimuli received and cellular responses outputted by cardiac cells in an anatomically accurate organ-level assembly. To model cardiac electrical stimuli *in vivo*, researchers since the late 1990s have developed reproducible protocols for electrically pacing the hearts of animals, which has typically been

performed by implanting pacing leads onto different chambers of the heart during surgery.[4] Additionally, *in vivo* cardiac mechanical stimuli, such as supraphysiological strain on the heart due to cardiomyocyte hypertrophy, has been modeled in *in vivo* using genetic interventions, pharmacological agents, or physical methods like transverse aortic constriction (TAC), which recapitulate disease-like mechanical loading on the chamber walls.[5]

While *in vivo* animal models are commonly used for modeling cardiac disease due to the biological complexity and high reproducibility of electrical and mechanical stimulation protocols, substantial drawbacks to these models exist. First, a significant limitation of animal models is that they typically do not utilize any human cells or tissue. While researchers are able to draw certain correlations between the physiology of animals and humans, species-specific biological responses are highly prevalent and exist in any organ system, heavily limiting the translatability of any physiological response or biological discovery made in an animal to humans. Animal models often serve as precursor models to human trials in studies for therapeutics or medical devices, due to the system-wide administration and presence of immune responses, however, when trying to characterize gene and protein expression responses at the cellular level in a discovery experiment, it is significantly more physiologically relevant to measure human cells. In addition to species-specific responses, certain electromechanical stimuli that put the animal at risk are not able to be tested *in vivo* due to physical restrictions and ethical issues associated.

Human-Derived 2D Cultures (*in vitro*)

Since the development of protocols for producing human-derived induced pluripotent stem cells (hiPSCs) in the early 2000s, the use of static 2D culture models involving human cardiomyocytes has exploded. While in a relatively immature state following stem cell differentiation, hiPSC-derived cardiomyocytes (hiPSC-CMs) solve the species-specific cellular signaling limitation of *in vivo* animal models by allowing researchers to study human cardiac muscle cells *in vitro*. As the use of hiPSC-cardiomyocytes in cardiac tissue engineering has increased, companies have developed tools to reliably and programmably pace 2D monolayers of cardiomyocytes. Researchers have used these tools to develop and analyze the effects of pacing programs.[6] Additionally, researchers have developed custom tools for mechanically stretching 2D monolayers of hiPSC-cardiomyocytes seeded onto elastic substrates.[7] While these platforms have been effective at recapitulating electrical pacing and mechanical loading *in vitro*, they lack inclusion and synchronous integration of the two modes.

Human-Derived 3D Microtissues (*in vitro*)

While advances continue to be made, the construction of large-scale human organ models *in vitro* is not yet reliable or easily reproducible due to significant challenges like tissue death from a lack of vascularization and poor media diffusion through dense tissue. As a result, the need for a more practical and physiologically relevant *in vitro* cardiac model that utilizes human cells has prompted the exploration of 3D cardiac microtissues as suitable systems. Since the late 2000s, researchers have used devices like microfabricated tissue gauges (μTUGs) to analyze seeded microtissues by measuring

their exerted forces[8] and imaging their structural composition[9–11]. Over the past decade, researchers have expanded the functionality of these microtissue models to electrically pace and mechanically stretch cardiac muscle tissue, providing the ability to observe responses to two crucially important sources of stimuli that *in vivo* cardiac muscle receives.[12–13] While microtissues have successfully been used to measure some human cardiac phenomena *in vitro*, most notably force generation, the utility of these systems in biological discovery and disease modeling is currently limited by their small size and poor survival rate. Following seeding, 3D cardiac microtissues have a relatively low survival rate, which severely inhibits the throughput of experiments. For those that do survive, their small size, and thus low number of cardiomyocytes generating signals, make it extremely challenging to extract a sufficient yield of measurables needed for most biochemical assays, such as RNA for gene expression. This further inhibits the throughput of experiments and lowers the statistical confidence in obtained results.

PROJECT SIGNIFICANCE

Integrating Electrical Pacing & Mechanical Stretching

To address the significant limitations of current cardiac models, in partnership with my collaborators, I have developed an electromechanical stimulation platform capable of electrically pacing and mechanically stretching 2D monolayers of human iPSC-cardiomyocytes to model physiological and pathological cardiac behavior *in vitro*. By integrating electrical pacing and mechanical stretching, the CES platform provides a more physiologically relevant human-derived 2D culture model compared to static 2D cultures or platforms that are only able to pace or stretch without integration. Additionally, by integrating these two stimuli, the phase delay between pacing and stretching signals is able to be modulated, which was investigated in this project. By exhibiting 2D monolayers of hiPSC-cardiomyocytes, this platform significantly expands the continuous surface area of *in vitro* cardiac muscle subjected to stimulation, increasing the number of cardiomyocytes generating biological signals that can be measured with biochemical assays, such as RNA for gene expression. The monolayer model thus significantly improves the throughput and signal-to-noise ratio of biological measurements per experiment compared to a microtissue model. The platform has a simple, well-defined process for running integrated stimulation programs that simulate the electromechanical stimuli phase delay of healthy cardiac behavior or a pathological cardiac state. By enabling the reliable and accurate stimulation of hiPSC-cardiomyocyte monolayers, the CES platform seeks to accelerate the biological characterization of cardiac diseases, hopefully leading to faster treatment-dependent discoveries.

Survival & Throughput

The survival rate of hiPSC-cardiomyocyte monolayers following seeding is significantly higher than that of 3D cardiac microtissues. Without any extraordinary significant issues like bacterial contaminations, almost all iPSC-cardiomyocyte monolayers successfully spread to confluency or near-confluency following seeding without issue. On the other hand, while specific survival data of microtissues is not commonly reported, a project collaborator with microtissue seeding experience noted a rough survival yield of ~60%. Increasing from ~60% survival to 100% survival from 3D microtissues to 2D monolayers allows for a higher throughput of samples for electromechanical stimulation experiments and lowers the waste and cost of materials used.

SNR per Experiment & Statistical Confidence

While the exact number of hiPSC-cardiomyocytes used in 3D cardiac microtissue electromechanical stimulation models is often not discussed, many are limited to a size scale of several millimeters[13], with a project collaborator with microtissue experience seeding ~63,000 hiPSC-cardiomyocytes per microtissue. On the other hand, the substrates used in this project were seeded with ~400,000 hiPSC-cardiomyocytes each. Due to an increase in the number of hiPSC-cardiomyocytes stimulated per sample in these 2D monolayers compared to many 3D cardiac microtissues, the samples do not need to be pooled to collect sufficient cellular lysate and thus biological measurables like RNA for biochemical assays compared to microtissue models, allowing for an increase in the signal-to-noise ratio (SNR) of measured biological signals per experiment and thus

improving statistical confidence in results.

Project Specific Aims

The goal for this project was to develop and characterize an electromechanical stimulation platform utilizing 2D monolayers of hiPSC-cardiomyocytes capable of modeling human physiological and pathological cardiac behavior *in vitro*. This was divided into three distinct specific aims outlined below.

Aim 1. Develop an electromechanical stimulation platform capable of electrically pacing and mechanically stretching multiple 2D monolayers of cardiomyocytes in parallel.

Aim 2. Verify the platform's ability to integrate electrical pacing and mechanical stretching to mimic physiological and pathological cardiac muscle stimuli *in vitro*.

Aim 3. Characterize how physiological and pathological electromechanical stimulation programs change cardiomyocyte gene expression and morphology.

Figure 1 | Fully assembled CES platform. Complete assembly of the final Cardiomyocyte Electromechanical Stimulation Platform (CESP) ready to run experiments. The platform is capable of running integrated, synchronized electrical pacing and mechanical stretching programs to recapitulate cardiac stimuli *in vitro*.

PLATFORM DEVELOPMENT

Overview

The purpose of the first aim for this project was to develop an electromechanical stimulation platform capable of electrically pacing and mechanically stretching 2D monolayers of cardiomyocytes in parallel. The initial phase of this aim involved the design and fabrication of the CES platform, including all of its component parts and overall assembly. Without yet integrating the two modes of stimuli, the end objective for this aim was to separately pace and stretch multiple 2D monolayers of hiPSC-cardiomyocytes in parallel, verifying the platform's ability to reliably perform electromechanical stimulation of cell samples.

Stretching Components

Stretcher (Platform Base)

To provide a structural base for the platform and the excursion length needed for stretching, an aluminum-based rail-guided track containing a stage mounted onto a linear screw capable of moving 100 mm in total was used (Figure 2a). The linear track contains an input for a stepper motor, and the screw-driven moving stage converts the motor's rotational motion to the transverse linear motion needed to stretch cells (Figure 2b shows moving stage). Opposite the moving stage is an identical stationary stage that is anchored to the track (Figure 2c), ensuring the mechanical strain only comes from one side of the hiPSC-CM monolayers. The motor used is water resistant, helping prevent rusting and protecting it from damage in the humidity of the cell culture incubator, and it is rated with a maximum continuous current of 5 A and holding torque of 2 Nm, which met the

specifications needed for this project. The motor is controlled by a stepper motor driver (Figure 2e), which serves as the interface between the motor and the Arduino microcontroller that is programmed with the corresponding electromechanical stimulation program and dictates the movement of the motor. The motor is powered by a DC power supply (Figure 2g) that converts AC wall power to provide the 5 V needed to power the driver and Arduino. Two large heat sinks are taped to either side of the stepper motor to prevent overheating during long experiments in the 37ºC incubator. The assembly of these components comprises the mechanical stretcher that will perform stretching on the hiPSC-monolayers during electromechanical stimulation, while also providing a structural base for the rest of the CES platform. This mechanical stretcher was assembled by Rui Hu from the Bifano Lab at Boston University and repurposed for this project.

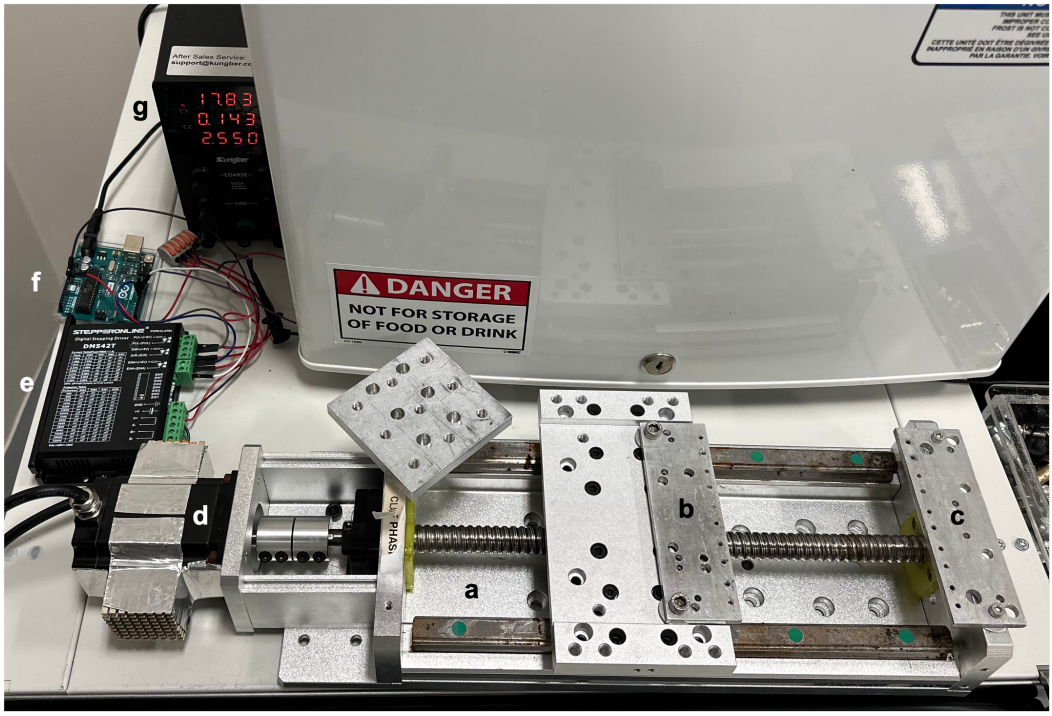

Figure 2 | Mechanical stretcher assembly. a, Linear track for stretching hiPSC-cardiomyocyte monolayers. **b**, Moving stage. **c**, Stationary stage. **d**, Stepper motor. **e**, Stepper motor driver. **f**, Arduino microcontroller. **g**, DC power supply.

Stretch Layers

To provide a durable, elastic substrate for stretching 2D monolayers of cells, hiPSC-cardiomyocytes were seeded onto 2 mm thin sheets of polydimethylsiloxane (PDMS) crosslinked at a 1:20 ratio between crosslinker and base (stretch layers, Figure 3a). PDMS is commonly used for cell interface devices in tissue engineering experiments as it is a biocompatible, sterilizable elastomer that can be surface treated to allow for the adsorption of ECM proteins and thus adherence of cells to the material. PDMS's viscoelastic mechanical properties allowed for recoverable elastic deformation during stretching.

The stretch layers were 32 mm long, 19 mm wide rectangles of 1:20 PDMS cut out of a circular layer casted in 150 mm diameter polystyrene Petri dishes using 36 ± 0.20 g of uncured liquid PDMS base and crosslinker, which resulted in layers that were approximately 2 mm thick towards the center of the dish. The PDMS silicone base and crosslinker curing agent were mixed in a speed mixer at 2,100–3,000 rpm (depending on the total mass) for three minutes and poured into the dishes. The dishes were kept as flat as possible while curing and the stretch layers were cut from the center to maximize the uniformity of thickness throughout. The layers were cured on hotplates at 70–80ºC for ~48 hours to minimize curing time without melting the dishes.

Stretch Layer-Device Assemblies

To provide the culture well needed to seed hiPSC-cardiomyocytes onto the stretch layers, 4 mm thick frames casted from 1:20 PDMS (stretch devices, Figure 3c) containing a 15 mm x 15 mm square culture well with 1 mm rounded corners were glued onto the

layers during fabrication. The stretch device walls provided a containment well to prevent the leakage of liquids during surface treatment, protein adsorption, and cell adhesion to the layers, delineating a surface area of ~225 mm^2 at the center of the stretch layers for confluent monolayers of hiPSC-cardiomyocytes to form following seeding. Following casting, lines on the lateral sides of the devices marked by the mold were used to cut the devices to the desired 32 mm x 19 mm specifications. Any excess PDMS in the well areas was cut out using a custom tool to ensure hiPSC-cardiomyocytes were only seeded onto the stretch layers. The stretch devices were lightly glued to the stretch layers with two drops of uncured 1:20 PDMS on either side of the well to provide a sufficiently strong seal to prevent leakage while also allowing the devices to be removed prior to use in experiments. The stretch layer-device assemblies were placed in an oven in glass Petri dishes at 100ºC to cure for ~24 hours and then removed and allowed to cool for 10 minutes. The stretch devices also served as a template for shaping the stretch layers and cutting holes in their corners during fabrication. A biopsy punch was used to cut 4 mm diameter holes through the 4.2 mm holes in the stretch devices of the layers, providing insertion points for the adapters that stretched the layers. Any excess layer material outside of the stretch device template was cut off using a razor blade to ensure the layers were 32 mm x 19 mm. The final stretch-layer device assemblies were placed in groups of four in Petri dishes with extra depth for holding large volumes of stimulation media added before experiments later. The stretch devices were casted in molds (Figure 3b) that were designed by Sudong Kim from the Chen Lab at Boston University and repurposed for this project.

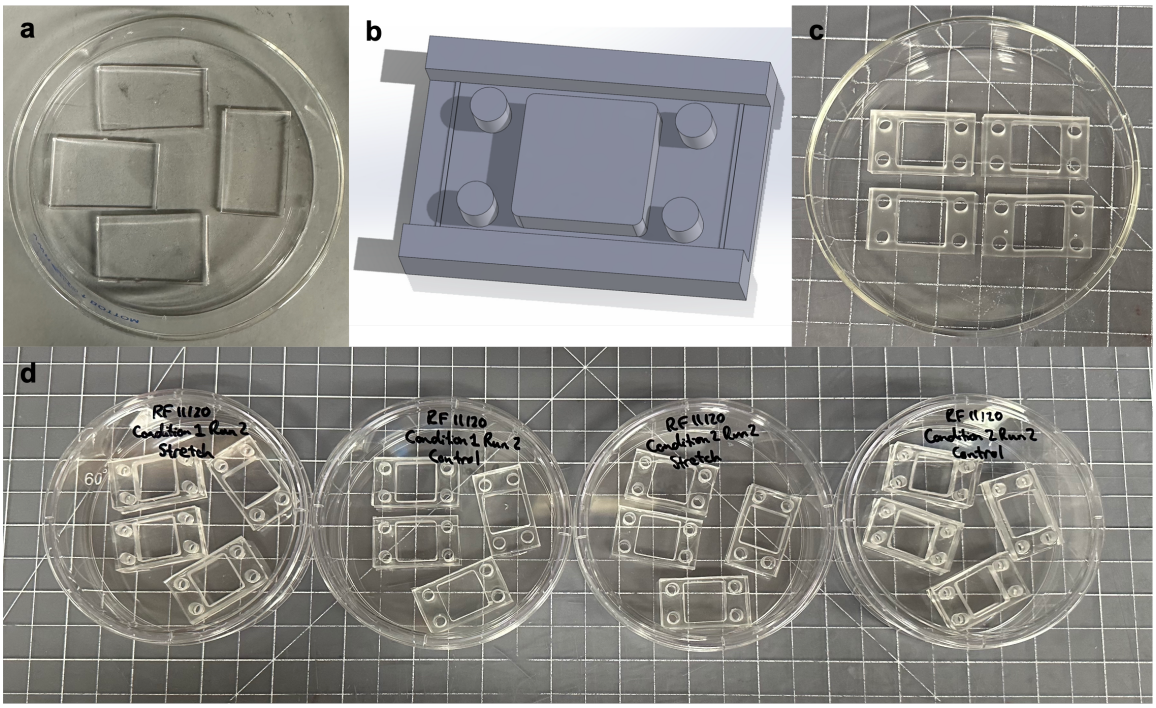

Figure 3 | Stretch layer fabrication. a, Four stretch layers cut from casted 1:20 PDMS. **b**, CAD model of mold used to cast stretch devices. **c**, Four casted stretch devices. **d**, Four sets of four complete stretch layer-device assemblies prepared for an experimental run of two conditions.

Stretch Adapters

To transfer the force from the motor, the layers were pulled apart by two opposite-facing adapters with 18.5 mm long posts inserted into holes in the stretch layers (stretch adapters, Figure 4). The plates of the long and short adapters were screwed into the moving and stationary stages respectively on the stretcher's linear track with M4 screws held in place with washers. Two posts from each adapter were inserted into either side of the stretch layers at 3 mm wide notches 14.5 mm down the posts. The post notches were designed to keep the stretch layers at a fixed height by limiting sliding and preventing the layers from falling off of the posts during stretching. After the posts were inserted, the stretch layers were placed into wells containing culture media for

electromechanical stimulation. The adapters were designed with the optimal dimensions to fit over the IonOptix C-Dish used to pace the hiPSC-cardiomyocytes (see Pacing Components below) with the posts extending down through openings in the C-Dish and into the seeded stretch layers submerged in culture media below. The full procedure of setting up stimulation experiments is outlined in the Stimulation Experimental Process section.

The stretch adapters were 3D printed from photopolymerizable resin via stereolithography (SLA) from computer-aided design (CAD) models designed in SolidWorks. Following printing, the adapters were manually washed with IPA and then washed in an automated washer for 15 minutes and dried overnight to remove any excess uncured resin from the molds. Next, the molds were cured in UV light at 60ºC for 30 minutes and then left in a 60ºC oven overnight to finalize curing. All parts designed in this project that were 3D printed via SLA were manufactured using this same process. Following curing, all casting surfaces of molds used in this project were silanized for 14–18 hours in a vacuum chamber with one drop of 1H,1H,2H,2H-Perfluorooctyltriethoxysilane to activate the surfaces and allow casted PDMS to be removed without ripping.

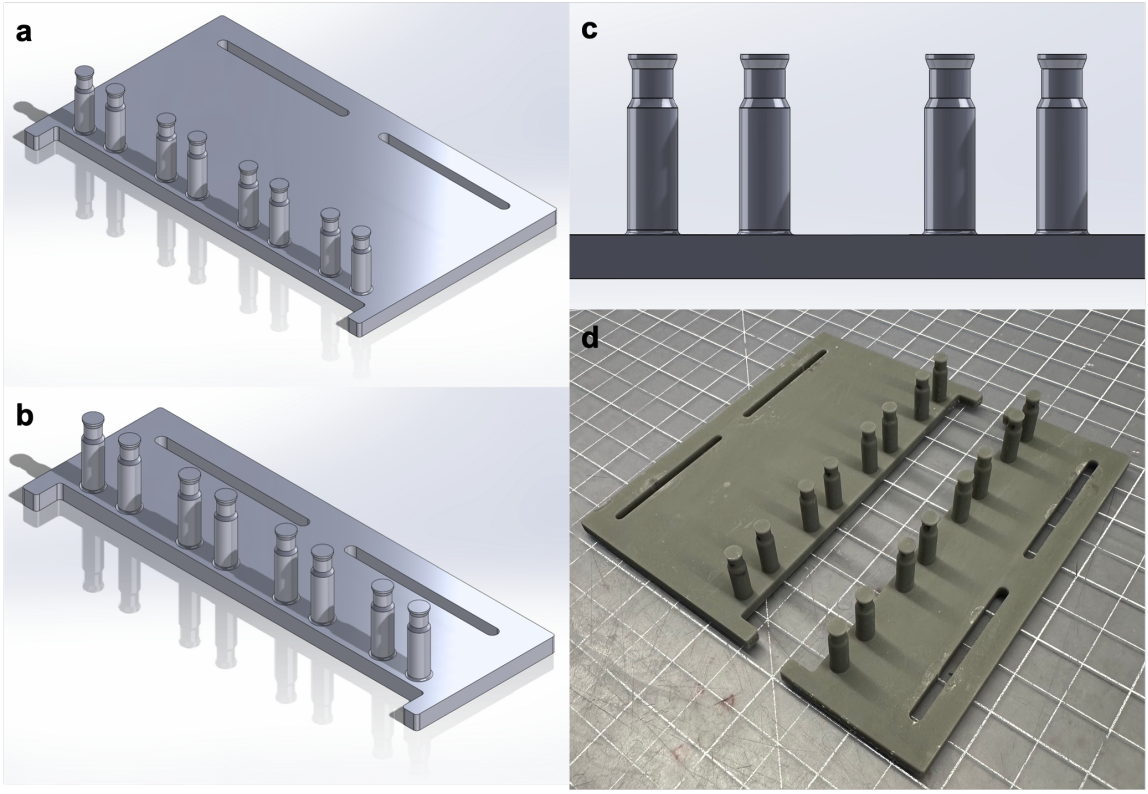

Figure 4 | Stretch adapter design. a, Long stretch adapter CAD model. **b**, Short stretch adapter CAD model. **c**, Side view of stretch adapter posts. **d**, Long (left) and short (right) stretch adapters.

Mechanical Stretch Test

As the stepper motor moves the moving stage away from the stationary stage, the long adapter pulls four stretch layers in parallel, equally stretching them to the programmed strain. Next, the stepper motor moves the moving stage the same distance back towards the stationary stage, causing the layers to contract back to their original length. For the electromechanical stimulation programs run in this project, a 30% cyclic strain at a frequency of 1.5 Hz on the stretch layers was used to recapitulate cardiac mechanical loading on the hiPSC-cardiomyocyte monolayers. To test the ability of the stepper motor and adapter posts to withstand cyclic stretching of four layers in parallel to

30% strain at a frequency of 1.5 Hz, a mechanical stretch test was performed with the layers submerged in water to simulate culture media (Figure 5). Cyclic stretching of four parallel stretch layers ~2 mm thick to 30% strain at 1.5 Hz for 10 minutes was successfully performed using the CES platform. This verified that the mechanical stretching setup used in the platform was capable of handling the deformation intensity and frequency required for the electromechanical experiments performed for this project.

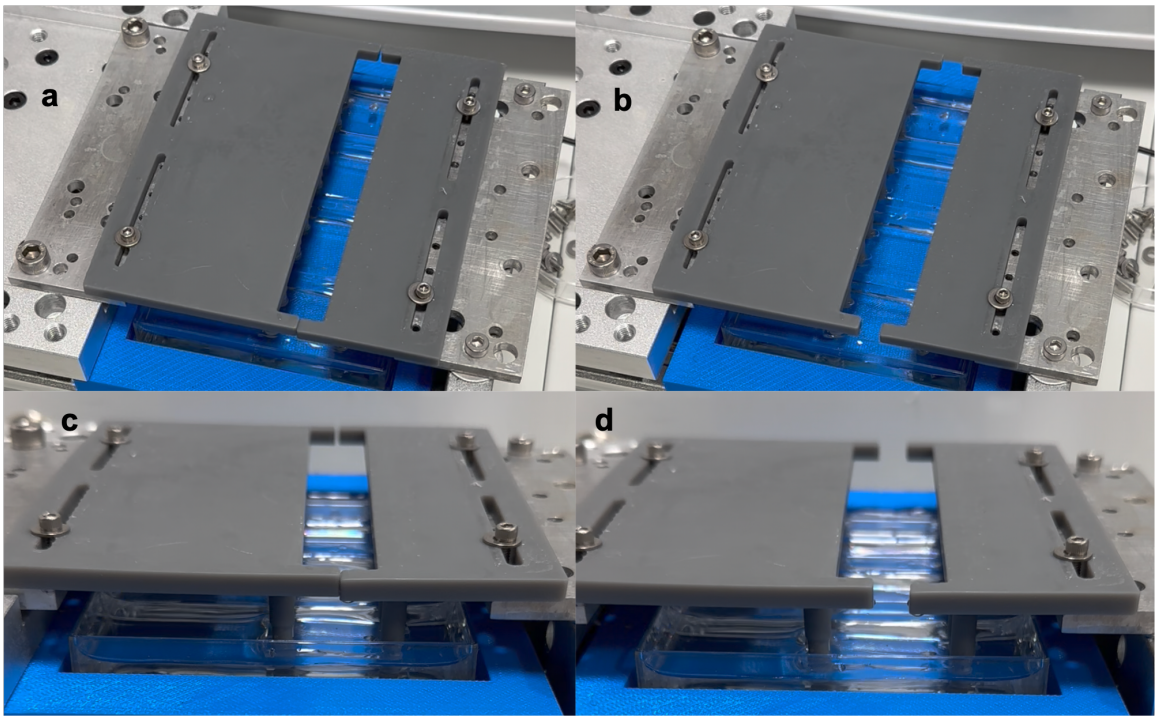

Figure 5 | Cyclic strain mechanical stretch test. **a**,**c**, Top, side views of four stretch layers at 0% strain. **b**,**d**, Top, side views of the layers stretched to 30% strain.

Pacing Components

C-Dish & C-Pace CM

To transfer the electric current needed to pace hiPSC-cardiomyocytes monolayers adhered to the stretch layers in the culture wells, an IonOptix C-Dish containing four pairs of carbon electrodes was used. The C-Dish is placed into the 4-well dish, each

containing a stretch layer attached to the stretch adapters and submerged in culture media, with one electrode on either side of the layer, allowing the electric field emitted by the C-Dish to transport ions in the RPMI+ culture media back and forth uniaxially through the cardiomyocyte cell membranes from one side of the monolayer to the other. This causes the hiPSC-CM monolayer to beat synchronously at the programmed frequency as one unified layer, instead of the spontaneous, nonuniform beating often containing multiple pacemaker nodes that occurs in monolayers without pacing. The C-Dish is connected via a ribbon cable to an IonOptix C-Pace EM cell pacer, which generates and transmits the inputted pacing protocol through the electrodes in the media. The C-Pace EM generates biphasic pulse waveforms that are used to pace and transmits them into the culture wells via electrodes, which includes a positive voltage pulse that initiates pacing throughout the monolayer, following by a negative voltage pulse of equal magnitude for bringing ions back to the other side of the well to pace again. The C-Pace EM was capable of transmitting biphasic pulses at -40–40 V amplitudes, 0.01–99 Hz frequencies, and 0.4–24 ms durations.

Electrical Pace Test

For the electromechanical stimulation programs run in this project, a pacing frequency of 1.5 Hz was used. To find the minimum voltage (amplitude) and pulse duration needed to properly pace multiple confluent monolayers of hiPSC-cardiomyocytes adhered to stretch layers at 1.5 Hz using the CES platform, a pace test was performed (Figure 6). Four samples seeded with hiPSC-cardiomyocytes were used for this test: three stretch layers with their devices removed and only the confluent

monolayer adhered and one stretch layer-device assembly to serve as a control. A hypothesis was made that the walls of the stretch device would inhibit pacing in the hiPSC-cardiomyocyte monolayer, acting as a barrier that blocked the transmitted voltage from reaching the monolayer at the base of the well. Simultaneous pacing at 1.5 Hz was successfully achieved in the three stretch layers with removed devices at a minimum voltage of 20 V and pulse duration of 6 ms using the CES platform. Beating in the four monolayers was recorded using brightfield microscopy through a 20x objective before and after electrical stimulation. The natural beating frequencies before pacing for the three experimental layers and control device were observed to be 0.65 Hz, 0.57 Hz, 0.52 Hz, and 0.47, respectively. After initiating pacing at 1.5 Hz frequency, 20 V amplitude, and 6 ms, duration, the beating of the three experimental layers increased to 1.48 Hz, 1.5 Hz, and 1.5 Hz, respectively, while the control device's beating decreased to 0.08 Hz, indicating it was not pacing and supporting the hypothesis. From reviewing video footage of the first layer, it was observed to be beating at 89 bpm or 1.48 Hz, which was close enough to the desired 90 bpm for 1.5 Hz to be considered pacing. The decrease in the control device's beating frequency was likely due to being out of the incubator environment while running the pace test. These minimum parameters needed for pacing were chosen for the electromechanical stimulation programs used in this project to minimize intensity of the pacing signals and thus potential damage to the hiPSC-cardiomyocytes. This verified that the pacing setup in the CES platform was capable of successfully pacing multiple monolayers of hiPSC-cardiomyocytes seeded on stretch layers simultaneously. The results of this pace test are summarized in Table 1 below.

Figure 6 | Electrical pace test. The top four wells contain paced stretch layers with their stretch devices removed after fabrication, while the bottom well contains a still assembled stretch layer-device assembly as an unpaced negative control. Pacing was achieved in the three experimental layers at a 1.5 Hz frequency, 20 V amplitude, and 6 ms pulse duration.

Sample	Natural Beating	Pacing at 1.5 Hz, 20 V, 6 ms
Stretch Layer 1	39 bpm = 0.65 Hz	89 bpm = 1.46 Hz
Stretch Layer 2	34 bpm = 0.57 Hz	90 bpm = 1.50 Hz
Stretch Layer 3	31 bpm = 0.52 Hz	90 bpm = 1.50 Hz
Control	28 bpm = 0.47 Hz	5 bpm = 0.08 Hz

Table 1 | Pace test results. The three experimental layers exhibited pacing, while the control stretch layer-device assembly did not.

ENGINEERING VERIFICATION

Overview

The purpose of the second aim for this project was to verify the platform's ability to integrate electrical pacing and mechanical stretching to mimic physiological and pathological cardiac muscle stimuli *in vitro*. The first phase of this aim involved defining electromechanical stimulation programs with stretching and pacing regimens that recapitulate cardiac stimuli while differentiating between healthy physiological stimuli and disease-like pathological stimuli. Both electromechanical stimulation programs were then programmed into the CES platform's Arduino microcontroller by writing program code containing algorithms to execute the integrated stretch-pace programs. The second phase of this aim involved analyzing the subjected mechanical strain exerted on the stretch layers by the stepper motor to verify the displacement throughout and strain in the stretch layers and thus the hiPSC-cardiomyocyte monolayers. Finally, the last part of this aim involved measuring the electrical pacing signals outputted by the C-Pace EM pacer to verify the signals received by the hiPSC-cardiomyocyte monolayers. This section characterizes the engineering performance of the CES platform and outlines its scope of capability utilized in this project.

Electromechanical Stimulation

Stretching Protocol

To recapitulate the pathological mechanical loading and unloading of cardiomyocytes in the left ventricular chamber wall due to excessive fibrosis during the cardiac cycle, a cyclic strain protocol of stretching the hiPSC-cardiomyocyte monolayers

to 30% strain followed by contraction back to 0% strain was chosen.[14] In diastole, the left ventricle is filled with oxygenated blood it receives through the mitral valve from the left atrium. Throughout this phase, the myocardial tissue in the chamber wall is stretched as the chamber expands to fill with blood while cardiomyocytes are relaxed. This causes cardiomyocytes within the tissue to experience an increase in the mechanical loading force they need to contract against to eject blood. To recapitulate this *in vitro*, the CES platform uniaxially stretches hiPSC-cardiomyocyte monolayers to 30% strain. In systole, the left ventricle ejects the oxygenated blood into the aorta to perfuse tissues throughout the body. Throughout this phase, the left ventricular myocardium contracts back to its resting length as blood is ejected due to increased ventricular pressure from cardiomyocyte contraction. This causes cardiomyocytes to experience a decrease in the mechanical loading they are subjected to at the tissue level while they contract. To recapitulate this *in vitro*, the CES platform uniaxially contracts hiPSC-cardiomyocyte monolayers back to their original resting length at 0% strain. Therefore, for both electromechanical stimulation programs run in this project, the tissue-level force of mechanical loading exerted on cardiomyocytes in the heart chambers was modeled via mechanical stimulation.[15]

Pacing Protocol

To recapitulate the electrical pacing of cardiomyocytes in the chamber walls by the sinoatrial pacemaker node in the heart's nervous system during the cardiac cycle, a pacing protocol of 1.5 Hz was selected. Normal physiological beating of the heart typically occurs at 60–100 bpm[16], corresponding to pacing at 1–1.67 Hz. Since most

hiPSC-cardiomyocyte monolayers in culture usually have a natural beating frequency of 0.5–1 Hz, a frequency of 1.5 Hz was chosen so that the pacing signals were fast enough to be physiologically relevant while slow enough to not exceed the monolayers' natural beating too much to the point where the cells could not follow the pacing. Due to achieving pacing from pulses of 20 V at 6 ms durations in the pace test (Figure 6), the pacing parameters for both electromechanical stimulation programs in this project were 1.5 Hz frequency, 20 V amplitude, and 6 ms duration.

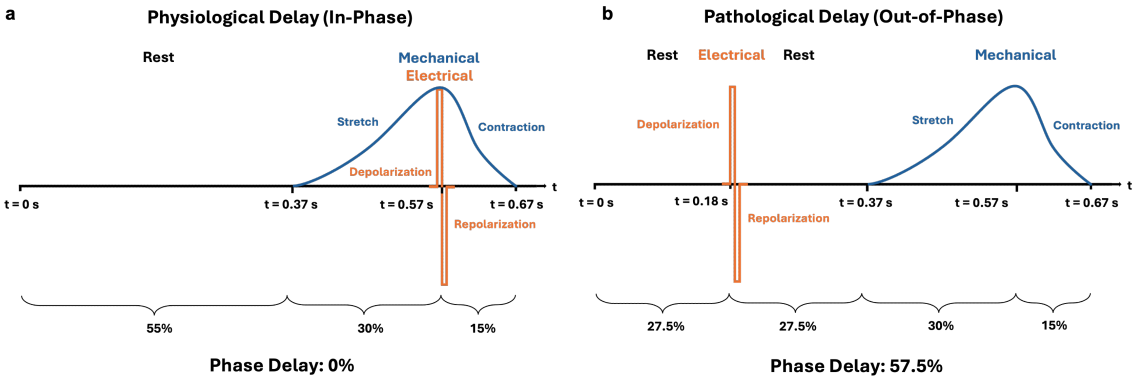

Figure 7 | Electromechanical stimulation programs. a, In-phase electromechanical stimulation program recapitulating healthy stimuli with hiPSC-cardiomyocytes pacing at maximum stretch. **b**, Out-of-phase electromechanical stimulation program recapitulating pathological stimuli with hiPSC-cardiomyocytes paced 27.5% of the cycle duration before stretching begins.

Physiological Delay (In-Phase) Program

To model physiologically healthy cardiac stimuli *in vitro*, an electromechanical stimulation program aligning electrical pacing and mechanical stretching in phase was developed (Figure 7a). Cardiomyocytes in the heart's myocardial tissue contract to eject blood from the ventricles due to transduction of electrical pacing signals sent from nerve

endings within the heart's intrinsic nervous system when the mechanical loading on these cells from chamber expansion is the highest. This is because blood is ejected when the volume in ventricles is at its highest, causing the chamber wall to expand and strain cardiomyocytes. Therefore, the healthy physiological delay program used in this project included the electrical pacing signal being sent from the C-Pace EM as the stretch layers seeded with hiPSC-cardiomyocytes were maximally strained (Figure 8).

Figure 8 | Physiological delay (in-phase) program. The green and red indicator lights are illuminated when the stretch adapters are maximally separated, indicating pacing at maximum (30%) hiPSC-cardiomyocyte monolayer strain. **a**, Front view. **b**, Side view.

Pathological Delay (Out-of-Phase) Program

To model pathological disease-state cardiac stimuli *in vitro*, an electromechanical stimulation program offsetting electrical pacing and mechanical stretching by the maximum phase delay was developed (Figure 7b). In many cardiac diseases, such as HCM or myocardial infarctions (MIs), excessive fibrosis within the myocardium can lead to a stiffening of the chamber walls, causing cardiomyocytes to experience

supraphysiological strain prior to contraction.[14] Additionally, many diseases involving pathological pacing, like cardiac arrhythmias, involve irregularities within the pacing signal that lead to suboptimal blood pumping and thus perfusion by the heart.[17] Disease-like cardiac states and electromechanical stimuli could be modeled by varying the extent of mechanical strain on hiPSC-cardiomyocyte monolayers or varying different aspects of the pacing signal waveforms like frequency, amplitude, duration, and pulse variability. For this project, pathological cardiac electromechanical stimuli were modeled and differentiated from the physiological program by altering the phase delay between pacing and stretching. The pathological delay (out-of-phase) program in this project involved the electrical pacing signal being sent by the C-Pace EM pacer halfway through the resting phase, which offsets it from the point of maximum strain as much as possible each cycle with a 57.5% phase delay. The goal for this was to model a delay between the transduction of electrical pacing signals by cardiomyocytes in the myocardium and the generation of force to pump blood, as exhibited in my cardiac diseases.[18]

Figure 9 | Pathological delay (out-of-phase) program. The green and red indicator lights are illuminated when the stretch adapters are touching, indicating pacing during the rest phase at 0% hiPSC-cardiomyocyte monolayer strain. **a**, Front view. **b**, Side view.

Mechanical Strain Analysis

To assess the strain throughout the PDMS stretch layers during mechanical stimulation, digital image correlation (DIC) was performed. Graphite powder was sprinkled onto a stretch layer ~2 mm in thickness and a light was shined through the gap in the stretch adapters using a white background behind the layers. A video of the layer stretching to 35% strain was recorded from above. Next, images were processed from this video at 60 frames per second using MATLAB code, and the images were uploaded and analyzed using the open-source DIC program Ncorr in MATLAB. Using this software, u-axis displacement analysis along the x-axis in the video was performed using a dot located towards the center of the layer, allowing tracking of displacement from this dot during stretching and contraction for each section of the material. At maximum stretch, the displacement within the stretch layer was found to be symmetrical, with the largest

displacement being on both lateral sides of the device, generally further away from the reference point selected. This displacement analysis indicated that there was approximately uniform strain being applied throughout the stretch layer and thus hiPSC-cardiomyocyte monolayers stimulated. The software calculated the maximum, median, and minimum strains in the region of interest (ROI) to be -4.4407 mm, -2.9395 mm, and -1.1497 mm, respectively. The strain profile analysis in Ncorr did not compute, but approximately uniform strain was visually determined using the generated heat map.[19]

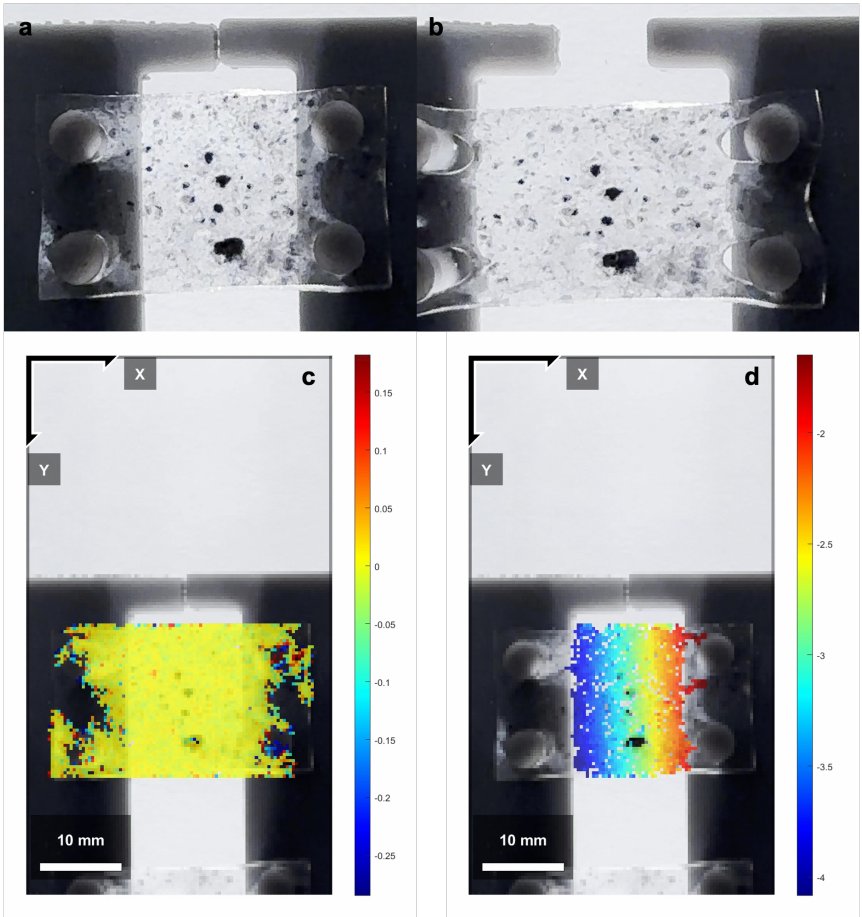

Figure 10 | Displacement profile analysis of stretch layer with digital image correlation. Graphite speckles sprinkled onto a 2 mm thick stretch layer to track displacement throughout the layer at 35% cyclic strain. **a–b**, Overhead images of the layer at 0% and 35% strain, respectively. **c–d**, X-axis displacement measurements corresponding to the frames in a and b.

Pacing Signal Analysis

Pacing signals sent by the C-Pace EM for both stimulation programs were analyzed using an oscilloscope to verify their pulse waveforms, measuring frequency and duration. An oscilloscope was connected to the C-Pace EM pacer and used to measure the signal transmitted to each of the four wells in the C-Dish for both the physiological and pathological delay programs utilized. The pacing signals generated for all four channels for both programs were found to have the sufficiently same waveform and all were measured to have ~1.4 Hz frequency and ~6 ms positive pulse duration. The voltage was lowered to 5 V for this measurement as the oscilloscope was unable to measure voltages at 20 V. The negative pulse durations were measured at ~700 ms since they asymptote off to zero. More investigation into why this was occurring would be needed, but since the negative voltage was still counteracting the positive voltage, it was determined to be sufficient for this project. Although the frequency for these waveforms was measured at ~1.4 Hz, it was determined to be sufficiently close to 1.5 Hz for use in this project. The values of the measurements taken for each of these channels are summarized in Table 2 below.

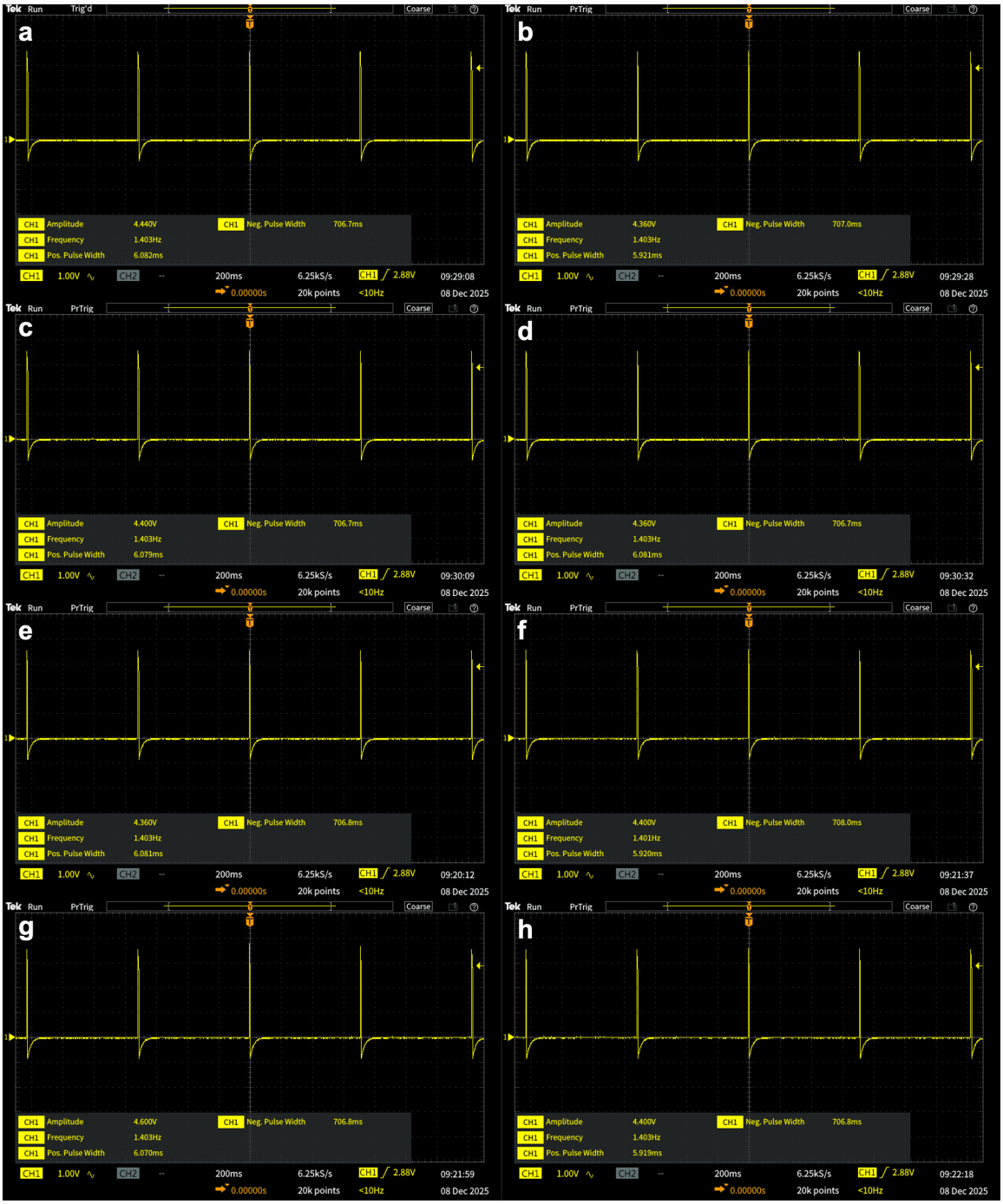

Figure 11 | Pacing signal waveforms. Screenshots display amplitude, frequency, positive pulse duration, and negative pulse duration. **a–d**, Physiological in-phase electromechanical stimulation pacing pulses for wells 1–4, respectively. **e–h**, Pathological out-of-phase electromechanical stimulation pacing pulses for wells 1–4, respectively.

Program, Well	Frequency (Hz)	Positive Pulse Duration (ms)	Negative Pulse Duration (ms)
Physiological, 1	1.403	6.082	706.7
Physiological, 2	1.403	5.921	707.0
Physiological, 3	1.403	6.079	706.7
Physiological, 4	1.403	6.081	706.7
Pathological, 1	1.403	6.081	706.8
Pathological, 2	1.401	5.920	708.0
Pathological, 3	1.403	6.070	706.8
Pathological, 4	1.403	5.919	706.8

Table 2. Pacing program parameters. Measured pacing signal values for each of the four channels in the C-Dish for both electromechanical stimulation programs used.

BIOLOGICAL CHARACTERIZATION

Overview

The purpose of the third aim for this project was to use the developed and verified CES platform to run electromechanical stimulation experiments with hiPSC-cardiomyocyte monolayers to characterize their biological responses to the physiological delay and pathological delay programs. Gene expression of cardiac stress markers with quantitative polymerase chain reaction (qPCR) was performed to analyze the cellular level stress responses to each program, looking at the expression of specific genes in human cardiomyocytes known to be upregulated or downregulated under stress. Fluorescence imaging of hiPSC-cardiomyocyte morphology was performed using immunohistochemistry (IHC) to analyze changes to cardiac anatomy, sarcomere alignment, and spatial expression of cardiac stress markers.

Stimulation Experimental Process

hiPSC to Cardiomyocyte Differentiation

To produce the hiPSC-cardiomyocytes needed to run experiments, human induced pluripotent stem cells (hiPSCs) were differentiated into cardiomyocytes. The hiPSCs used in this project were of the wild-type titin-green fluorescent protein (WT TTN-GFP) cell line, which is explained in the Morphological Changes section below. On Day 1, RPMI 1640 cardiomyocyte culture media containing the B-27 supplement without insulin (RPMI-) was added to 6-well plates containing confluent monolayers of hiPSCs. This media contained CHIR99021, which is used to activate the Wnt signaling pathway in the iPSCs to initiate differentiation[20]. On Day 2, media was changed on the cells with new

RPMI- not containing CHIR99021, in order to deactivate Wnt pathway activation. On Day 4, media was changed on the cells with CDM3, a cheaper but sufficient RPMI alternative, containing IWP-4, which is used to selectively inhibit Wnt signaling[20], and BMS-453. On Day 6, IWP-4 was removed by changing media containing only CDM3 and BMS-453. On Day 8, BMS-453 was removed by changing media with CDM3. On Day 9, the differentiated hiPSC-cardiomyocytes typically begin beating, and the following day lactate selection is started. While cardiomyocytes are able to efficiently metabolize lactate as an energy source in the absence of glucose, impure cells that did not fully differentiate into cardiomyocytes are not able to and thus will not survive lactate selection, resulting in a monolayer containing almost all cardiomyocytes following this process.[21] After lactate selection was completed, the monolayers of differentiated hiPSC-cardiomyocytes were lifted from their wells and frozen in cryovials to be used later in experiments. The entire differentiation process was performed inside a sterile level 2 biosafety cabinet using aseptic cell culture technique.

Preparing Stretch Layers for Seeding

To produce the substrates needed to seed hiPSC-cardiomyocyte monolayers for each experimental run, 16 stretch layer-device assemblies were fabricated using the protocol outlined in the Platform Development section. Each experimental run was defined as a pair of the physiological delay (in-phase) and pathological delay (out-of-phase) conditions, with each condition needing 8 stretch layer-device assemblies with four to be stretched and paced by the corresponding program and four serving as unstimulated controls. After producing the 16 stretch-layer device assemblies needed for

an experimental run, the stretch layers were prepared for cell seeding. First, the layers were surface treated with polydopamine to functionalize the monolayer's surface area for protein adsorption. This consisted of removing dust from the stretch layer well with tape and then adding a solution of dopamine hydrochloride dissolved in 1x TrisHCl for 1 hour. Next, the stretch layers were washed 3x with PBS and sterilized under UV light for 15 minutes. Afterwards, ECM proteins were adsorbed onto the surface of the stretch layer wells by coating them with Matrigel, a proprietary compound containing mostly laminin and several other commonly occurring ECM proteins, dissolved in DMEM/F-12 culture media. The layers were then left to incubate for several hours to allow the Matrigel proteins to adsorb onto their surfaces.

Seeding Stretch Layers

Concurrent with fabricating the stretch layer-device assemblies needed for each experimental run, previously frozen hiPSC-cardiomyocytes were thawed onto 6-well plates to restart their beating and biological activity. The day following seeding, media was changed on the cells and then the following day stretch-layers were prepared for cell seeding. On the third day following thawing, hiPSC-cardiomyocytes were seeding onto the culture wells inside the stretch-layer device assemblies. First, hiPSC-CM monolayers on the 6-well plates were lifted using a re-plating protocol with DNAse dissolved in 0.25% trypsin-EDTA used to break the hiPSC-cardiomyocyte myosin adhesions to Matrigel proteins previously adsorbed onto the wells. The hiPSC-CMs were then spun down into a pellet in a centrifuge at 1,000 rpm for 4 minutes, resuspended in RPMI+ media, and cellular density was manually counted using a hemocytometer in brightfield

microscopy to determine the volume of cells and dilution media needed to reach the desired seeding density of 400,000 hiPSC-cardiomyocytes in 500 µL of RPMI+ media per stretch layer well. Finally, hiPSC-cardiomyocytes were seeded at this density into each of the 16 stretch layer-device assemblies in RPMI+ media containing the ROCK inhibitor Y-27632. These newly seeded stretch layer-device assemblies were incubated overnight. The following day, media was changed again with RPMI+ to remove Y-27632.

Running Stimulation Experiments

In the day following the removal of Y-27632 from the culture media, the stretch devices were removed from the stretch layers inside the deep dishes, and the full volume of culture media was added on top of the layers. For each experiment, four layers were submerged in individual wells of a 4-well plate that fits the C-Dish used for pacing. It was determined that 12 mL was enough RPMI+ culture media per well to maximally submerge the monolayers in media to ensure proper pacing while preventing spilling during handling. Therefore, 48 mL of total RPMI+ media containing 1% penicillin/streptomycin (Pen/Strep) antibiotics to defend against bacterial contamination was added onto all four stretch layers in the deep dishes for one day to allow the monolayers to acclimate to the significantly larger volume of media than the 500 µL per layer using for seeding. For the four control devices, this step was not performed and new media of 500 µL RPMI+ with 1% Pen/Step was changed onto the layers inside their assemblies. The following day, the stretch layers were ready to run an experiment.

To prepare the platform for running an electromechanical stimulation experiment, the seeded stretch layers needed to be introduced into the platform inside of the sterile biosafety cabinet. First, the stretch adapters were set facing each other with the short adapter on the left-hand side and the long adapter on the right-hand side, resembling the reverse of the setup in the platform. Double-sided tape is added to each side of the C-Dish frame to which a custom-cut piece of parafilm is added to the frame of the C-Dish containing holes for the posts of the stretch adapters and sized to properly fit the C-Dish. A custom parafilm cutting template CAD model was designed in SolidWorks and 3D printed via fused deposition modeling (FDM) for this use. Parafilm was used as a barrier between the open wells containing the hiPSC-CM monolayers submerged in media and the external environment, helping to prevent media evaporation during experiments and bacterial contamination while handling outside. Next, the C-Dish was flipped over, and the adapter posts were inserted through their corresponding holes in the parafilm and the C-Dish frame, pushing the parafilm down below the notches in the adapter posts to provide space for the stretch layers. After this step, 12 mL of the total 48 mL of RPMI+ culture media in the deep dishes was transferred to each of the wells using a serological pipette to prepare the 4-well plate for the stretch layers. Finally, each stretch layer was removed from the deep dish and inserted onto the stretch adapter posts. The entire adapter-C-Dish assembly containing all four experimental stretch layers was then flipped over and placed inside of the 4-well plate containing media.

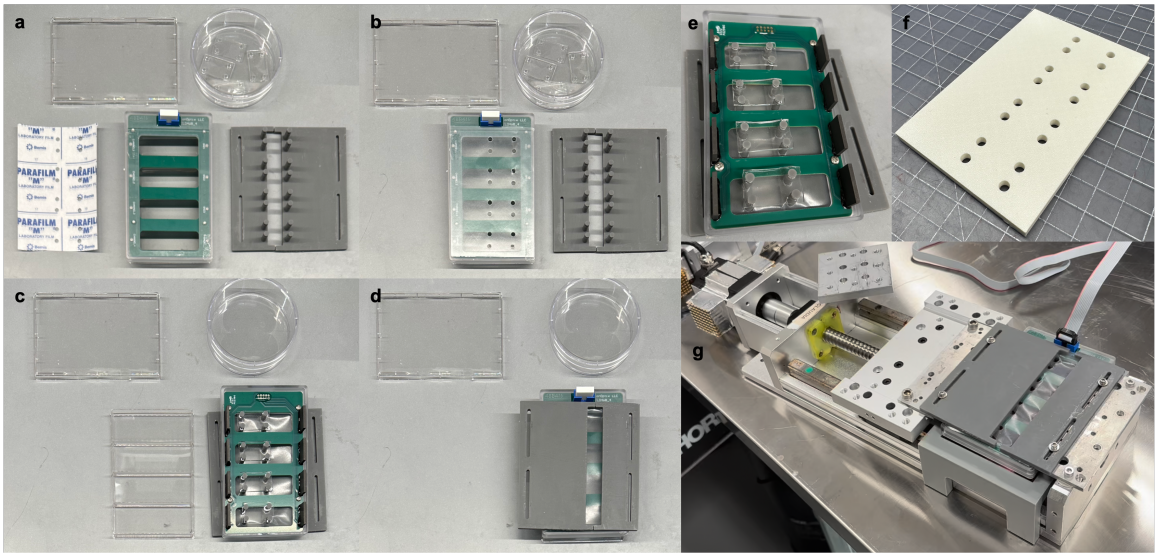

Figure 12 | Stimulation experimental setup. a, Initial layout of materials needed to set up stimulation experiment, including 4-well plate, C-Dish, deep dish containing four stretch layers, parafilm, and stretch adapters. **b**, Parafilm taped onto C-Dish. **c**, Stretch adapter posts inserted through C-Dish, parafilm, and stretch layers. **d**, Adapter-C-Dish assembly with stretch layers turned over and place into 4-well plate. **e**, Close-up view of adapter-C-Dish assembly with stretch layers. **f**, Parafilm cutting template. **g**, Fully assembled CES platform running a stimulation experiment before being placed in an incubator.

To set up the stretcher for stimulation, the adapter-C-Dish assembly containing the seeded stretch layers was placed inside of a 3D-printed well plate holder that is customized to fit in the track of the mechanical stretcher. After placing this in the stretcher, the stretch adapters were screwed into the platform, lining up the frame of the long adapter on the left-hand side with that of the short adapter on the right-hand side. The CES platform was then fully assembled to begin an electromechanical stimulation experiment. To start an experiment, the chosen electromechanical stimulation program code was first uploaded to the Arduino microcontroller. A BNC cable connects the pacing output pin on the Arduino to the pulse input on the C-Pace EM to receive the pacing signals from the code. A ribbon cable connects the C-Dish to the corresponding

channel on the C-Pace EM set to pace 4 wells, and the C-Pace EM is set to 20 V, 6 ms duration, and "TTL Lock" to be controlled by the Arduino. The C-Pace EM channel was then set to "Enabled," the DC power supply was turned on, and the platform was plugged in to begin the electromechanical stimulation experiment. Immediately, the platform begins simultaneous, integrated stretching and pacing of the stretch layers seeded with 2D monolayers of hiPSC-cardiomyocytes. The entire platform was then lightly sprayed with 70% ethanol and placed inside a cell culture incubator for 16 hours of stimulation.

For this project, a pilot run of the physiological delay (in-phase) program was first run to assess performance of the platform. Following 16 hours of stimulation, the experiment was stopped, the CES platform was removed from the incubator, the stretch adapters were unscrewed, and the entire adapter-C-Dish assembly was brought back into the biosafety cabinet for sample processing. The four experimental layers were each placed in separate smaller diameter Petri dishes for data collection processing later. Next, the areas around the hiPSC-CM monolayers on the stretch layers were dried using Kimwipes and stretch devices were added back onto the stretch layers with double-side tape to prevent leakage of liquid during processing. 500 μL of media from each of the layer's corresponding experiment wells was added onto these devices for transport. The control devices were also removed from the incubator for processing at this time.

Following the pilot experiment of the physiological delay (in-phase) program, a second run of this program was completed as part of the pair of conditions to be analyzed in this project. After this experiment, a minor bug was corrected in the program's code to prevent multiple pacing signals from being sent in the same cycle. Finally, an

experimental run of the pathological delay (out-of-phase) program was completed. All experiments were run in a cell culture incubator for 16 hours. For each of the three experiments run, two of the stretch layers ripped at the corners during stimulation and were pooled to form one sample but were not processed. One of the other experimental layers was used for gene expression analysis and the other was used for morphological analysis. This is likely due to the stretch layers fabricated being slightly less than 2 mm thick and should be resolved in the future by using layers that are ≥2 mm in thickness.

Gene Expression Analysis

Following electromechanical stimulation, one experimental and one control sample for each experiment were processed. First, hiPSC-cardiomyocyte monolayers were lysed by adding phenol-based TRIzol to the culture wells and scratching the layers with a micropipette tip. This step breaks all of the stimulated hiPSC-CM's cellular membranes, leaking out their cellular content to be collected. Following centrifugation to separate layers, the lysate for each sample was extracted and stored in tubes. Following lysate collection, RNA was extracted from each sample's lysate using the Qiagen RNA extraction kit, adding proprietary buffers and filtering the lysate samples until the cellular RNA was extracted and dissolved in 30 μL of water per sample. The RNA concentration of each sample was measured using a NanoDrop microvolume spectrophotometer to determine the necessary dilution volumes for qPCR. Next, cDNA was synthesized from each sample's RNA by adding a qScript cDNA SuperMix and using a heating and cooling schedule of 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC, and cooling down to 4ºC in a thermal cycler. Finally, a 384-well plate array for eight genes

containing six cardiac stress marker genes and two housekeeping reference genes was prepared for qPCR by combining the cDNA samples with gene-specific primers, the fluorescent dye SYBR Green, and nuclease free water for dilution. The 384-well plate was run in a qPCR instrument, and the Cp values were measured and used to calculate $\log_2$-fold expression changes for each experimental sample compared to its corresponding control (Figure 13). The expression of known cardiac stress markers – ANKRD1, FLNC, NPPA, NPPB, DES, and CSRP3 – that have been found to be upregulated or downregulated in various different cardiac disease states were measured[22–26]. Upon analyzing these results, more data is needed to elucidate trends across the genes used for these electromechanical stimulation programs. However, what is extremely significant about these results is that they support the CES platform's ability to yield sufficient extractable RNA for measuring gene expression on individual samples of hiPSC-cardiomyocytes. This supports the throughput of the platform and emphasizes its utility for running biochemical assays on electromechanically stimulated hiPSC-cardiomyocytes.

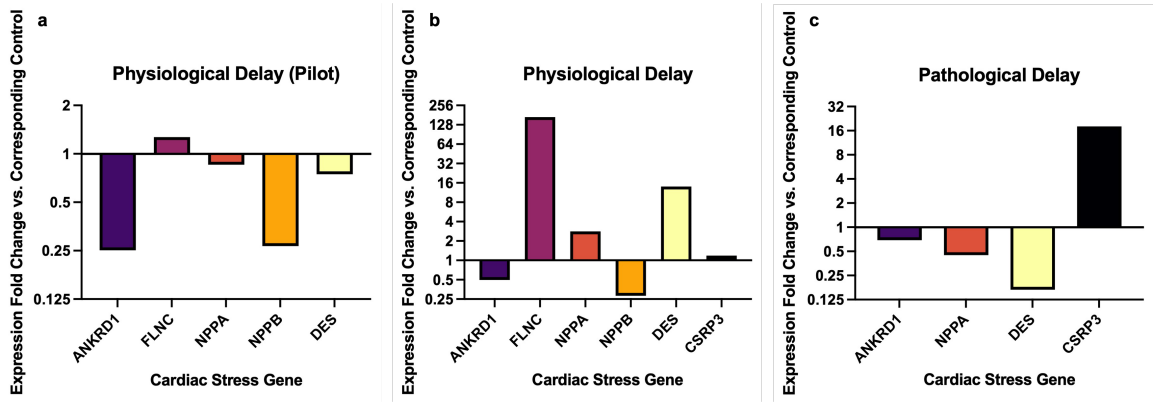

Figure 13 | Log_2-fold change of cardiac stress marker gene expression. Each sample's gene expression fold changes for ANKRD1, FLNC, NPPA, NPPB, DES, and CSRP3 are based on their control sample's expression value, normalizing the changes across samples. **a**, Physiological delay (in-phase) pilot experimental sample. **b**, Physiological delay (in-phase) experimental sample. **c**, Pathological delay (out-of-phase) experimental sample.

Morphological Analysis

In addition to gene expression analysis, following electromechanical stimulation, experimental and control samples from each experiment were processed using immunohistochemistry and fluorescently imaged to analyze morphological changes to the hiPSC-cardiomyocyte monolayers. First, KCl was added to these samples to relax the hiPSC-cardiomyocytes in the monolayer before they were fixed in 4% paraformaldehyde. Following fixing, these samples were stained with primary and secondary antibodies that allowed for the targeting of fluorescent markers to different biological components within each monolayer. For this project, the four fluorescent markers used to image hiPSC-cardiomyocyte monolayer morphology were DAPI, TTN-GFP, CSRP3, and FLNC. DAPI was used to stain cell nuclei and was imaged using the blue channel at a wavelength of 411 nm, gain of 10, and power of 9. Titin proteins within the sarcomeres of the hiPSC-cardiomyocytes were already tagged with GFP and imaged using the green

channel at a wavelength of 488 nm, gain of 30, and power of 15. CSRP3 proteins were stained with corresponding antibodies and imaged using the yellow channel at a wavelength of 568 nm, gain of 10, and power of 9. Finally, FLNC proteins were stained with corresponding antibodies and imaged using the red channel at a wavelength of 647 nm, gain of 30, and power of 15. Upon analyzing these results, more data is needed to elucidate trends across the protein images for these electromechanical stimulation programs. However, what is extremely significant about these results is that they support the CES platform's ability to image morphological changes on individual samples of hiPSC-cardiomyocytes. This supports the throughput of the platform and emphasizes its utility for fluorescently imaging electromechanically stimulated hiPSC-cardiomyocytes.

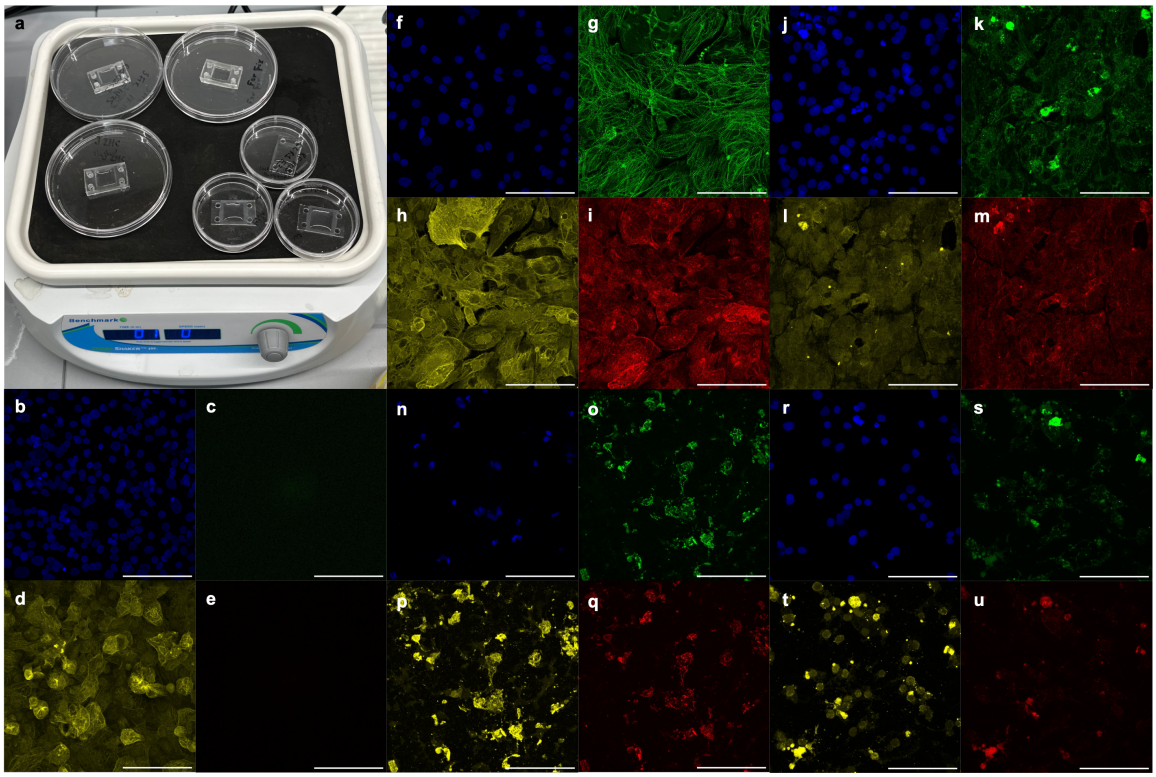

Figure 14 | Fluorescence imaging of post-stimulation hiPSC-cardiomyocyte morphology. Monolayer images following immunohistochemistry. **a**, Representative image showing experimental process of washing devices with PBS while permeabilizing cellular membranes and staining with primary and secondary antibodies for fluorescence imaging. Blue indicates cellular nuclei stained with DAPI, green indicates GFP-tagged titin in hiPSC-CM sarcomeres, yellow indicates CSRP3 and red indicates FLNC. **b–e**, Physiological delay pilot experimental sample. **f–i**, Physiological delay control sample. **j–m**, Physiological delay experimental sample. **n–q**, Pathological delay control sample. **r–u**, Pathological delay experimental sample.

DISCUSSION

By integrating the programmable stretching and pacing of cardiomyocyte 2D monolayers, the CES platform provides a robust and reliable method for investigating the behavior of cardiac muscle cells exposed to varying electrical and mechanical stimuli and additional functionality compared to other dynamic 2D culture models. By utilizing a 2D monolayer model instead of 3D cardiac microtissues for electromechanical stimulation, the survival rate of samples and number of hiPSC-cardiomyocytes generating measurable biological signals increases. Therefore, the throughput of running biochemical assays for analyzing cardiac muscle biological responses at the cellular level *in vitro* using this platform increases. Additionally, due to this throughput increase, less pooling of samples is required to acquire sufficient biological measurables per experiment, such as RNA for gene expression, increasing the SNR per experiment and thus statistical results obtained from electromechanical stimulation experiments. The CES platform's design has the capacity to be scaled even larger in the future with the addition of more C-Dishes, further increasing the throughput of the platform.

APPENDIX

Materials

Linear Track – Heechoo CNC Linear Rail Guide 100mm Ballscrew 1605

Stepper Motor – P Series IP65 Waterproof Nema 23 Stepper Motor

Stepper Motor Driver – StepperOnline DM542T CNC Stepper Motor Driver

Microcontroller – Arduino Uno R3

Power Supply – Kungber DC Power Supply

PDMS Base – Dow SYLGARD 184 Silicone Elastomer

PDMS Crosslinker – Dow SYLGARD 184 Silicone Elastomer Curing Agent

SLA 3D Printer – Formlabs Form 3+

SLA Material – Formlabs Grey V4 Resin

Silane – Sigma Aldrich 1H,1H,2H,2H-Perfluorooctyltriethoxysilane

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CURRICULUM VITAE