SUPPORTING ONLINE MATERIAL

SUPPORTING TEXT

Engineered Microarchitecture of 2-Dimensional Myocardium

In the absence of extracellular gradients (chemical, mechanical, electrical or physical), cardiomyocytes cultured *in vitro* self-assembled into an isotropic 2D myocardium (Fig. 1A-C) with no preferential alignment of cell bodies. Examination of the sarcomeres, where the direction of contraction for each sarcomere was perpendicular to the Z-line (Fig. 1C), revealed that there was no preferential alignment and thus no net direction of contractile stress or strain. However, when cardiomyocytes were cultured on 20 μm wide, centimeter-long, micropatterned FN lines (Fig. 1D-F) the cells spontaneously aligned with a high aspect ratio and registration of sarcomeric Z-lines across their width (Fig. 1F). This anisotropic 2D myocardium, similar to published results (*1*), had uniaxial sarcomere alignment indicating a contractile direction along the length-wise axis of the cardiomyocytes and was scalable from the micrometer to centimeter length scales. In addition, this anisotropic architecture mimicked the laminar structure of the heart, where muscle fibers align in planar sheets wrap around the ventricular cavities (*2*). Because these two methods produced 2D myocardial syncytiums spanning centimeter length scales, spontaneous initiation of contraction anywhere within the 2D myocardium produced contraction of the entire MTF construct. While this was useful for autonomously actuated MTF devices, there were many cases where the temporal actuation of the MTF needed to be controlled. To achieve both temporal control and uniaxial cardiomyocyte alignment we utilized the 20 μm wide, centimeter-

long, micropatterned FN lines, but adsorbed a cell and protein resistant polyethylene glycol based polymer in between the lines. This produced an array of spatially separated muscle fibers (Fig. 1G-I) where each muscle fiber had uniaxial sarcomere alignment (Fig. 1I) but were electrically isolated from one another. Thus, extrasystoles could not spread to adjacent fibers because there was no electrical coupling between lines. Contraction of an entire MTF with a muscle fiber array occurred only when using external field stimulation electrodes and a voltage sufficient to activate all fibers. In total, by providing geometric cues encoded in the extracellular matrix and activating self-assembly both within, and among, cardiomyocytes, we controlled tissue microstructure to one of three types; isotropic, anisotropic 2D or arrays of muscle fibers.

Controlling Muscular Thin Film curvature

A unique aspect of the MTF technology was the ability to control whether the cardiomyocytes were on the convex or concave surface of curved constructs. Which surface cardiomyocytes were on, convex or concave, was controlled by the cure temperature of the PDMS, 22ºC (RT cure) or 65ºC (heat cure) respectively. The reason for the cure temperature dependence is unknown, but we hypothesize that PIPAAm, which is hydrophobic at >35ºC in a compacted and globular state (*3*), becomes physically entrapped in the elastomer network when the PDMS is cured at 65ºC. Once fabricated, cooling the MTF to 22ºC dissolves the PIPAAm releasing the MTF from the glass cover slip and leaving a thin hydrogel-like layer of entangled PIPAAm at the PDMS-water interface. The volume expansion caused by the hydrophilic swelling of the entrapped PIPAAm causes the MTF to bend resulting in cardiomyocytes on the concave surface. In

contrast, at 22ºC the PIPAAm is in an extended, hydrophilic state (*3*) and immiscible with the PDMS preventing incorporation during cure. In this case the exposed PDMS on the underside of the MTF is hydrophobic relative to the cardiomyocyte tissue layer, which can be loosely considered a hydrogel due to the high water content (*4*). As the PDMS side curves in to minimize contact with the water the cardiomyocyte side expands to maximize contact with the water causing the MTF to bend with cardiomyocytes on the convex surface.

MATERIALS AND METHODS

Substrate fabrication

PDMS thin film substrates were fabricated via a multi-step spin coating process. Glass cover slips (25 mm diameter) were cleaned by sonicating for 60 minutes in 95% ethanol and air dried. Next, poly(N-isopropylacrylamide) (PIPAAm, Polysciences) was dissolved at 10 wt% in 99.4% 1-butanol (w/v) and spin coated onto the glass cover slips for 1 minute at 6,000 RPM. Sylgard 184 (Dow Corning) polydimethylsiloxane (PDMS) elastomer was mixed at a 10:1 base to curing agent ratio and spin coated coat on top of the PIPAAm coated glass cover slip at 5,000 RPM for 2 minutes. Once mixed the PDMS prepolymer slowly increased in viscosity reaching gelation at ~8 hours. Thicker PDMS layers were formed by spin coating higher viscosity PDMS prepolymer between 0 and 6 hours post mixing (see figure S1 for calibration curve) allowing films from 10 and 100 μm thick to be formed. PDMS coated cover slips were then cured either at room temperature (~22ºC) for 48 hours or at 65ºC for 4 hours. The different cure temperatures

were used to control the curvature of the PDMS film when it was released from the cover slip upon dissolution of the PIPAAm layer (described subsequently).

PDMS surface functionalization

The PDMS thin films were coated with either an isotropic or anisotropic layer of fibronectin (FN, Sigma). In either case, immediately prior to FN treatment the PDMS coated cover slips were UV ozone treated (Model No. 342, Jetlight Company, Inc.) for 8 minutes to sterilize the surface and increase hydrophilicity. All subsequent processing was performed in a biohood under sterile conditions. Isotropic FN was deposited by placing a 1 mL lens of 25 μg/mL FN in sterile deionized (DI) water on the PDMS and incubating for 15 minutes. It was essential that water did not contact the periphery of the cover slip during this or any subsequent step because it would seep under the PDMS and prematurely dissolve the PIPAAm. Following incubation, excess FN was removed by washing 3 times with DI water and then air dried prior to cardiomyocyte seeding within 3 hours.

Anisotropic patterning of FN was performed using microcontact printing (μCP). The basic μCP technique is well established and allows the rapid patterning of biomolecules on a variety of planar substrates using PDMS stamps (*5, 6*). The variation employed here used a PDMS stamps to pattern FN on the PDMS coated glass cover slips to in order to form anisotropic 2D myocardium, based on (*1*) . PDMS stamps were fabricated with 20 μm wide, 2 μm tall ridges separated by 20 μm spacing using established methods. Briefly, silicon wafers were spin coated with SU-8 photoresist (Microchem) and exposed

to UV light through a photomask selectively cross-linking regions of the photoresist. The photoresist was then developed removing unexposed regions and a negative of the patterned photoresist wafer was formed by casting PDMS prepolymer against it. Prior to each use, the PDMS stamps were sonicated in 50% ethanol for 30 minutes to sterilize and remove surface contaminants. Once dried, the PDMS stamp was inked with a 250 μL droplet of 50 μg/mL FN in DI water and incubated for 1 hour. The stamp was then rinsed twice in DI water to remove excess protein and dried under a stream of compressed air. FN was transferred from the stamp to the PDMS thin film by making conformal contact for 1 minute. Upon stamp removal, a 1 mL lens of either 1% Pluronics F127 (BASF Group) or 2.5 μg/mL FN in DI water was incubated on the PDMS surface for 15 minutes. Following incubation, the PDMS film was washed 3 times with DI water, air dried and then seeded with cardiomyocytes according to the described protocol.

Cardiomyocyte harvest, seeding and culture

Neonatal rat ventricular cardiomyocytes were isolated from 2-day old neonatal Sprague-Dawley. All procedures were approved by Harvard animal care and use committee. Reagents were obtained from Sigma unless otherwise indicated. Ventricles were surgically isolated and homogenized by washing in Hanks balanced salt solution followed by digestion with trypsin and collagenase with agitation overnight at 4°C. Subsequently, cells were re-suspended in M199 culture medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 3.5 g/L glucose, 2mM L-glutamine, 2 mg/L vitamin B-12, and 50 U/ml penicillin and seeded at a density of 1

million cells per cover slip. Samples were incubated under standard conditions at 37°C and 5% CO_2. Streptomycin was not added to the media in order to prevent interference with stretch activated ion channels (*7*). After 24 hours incubation the cover slips were washed 3 times with PBS to remove non-adherent cells and covered with media. After an additional 24 hours the media was exchanged with maintenance media (M199 media supplemented as above but with 2% FBS) to minimize growth of fibroblasts inevitably present in the primary harvest cardiomyocyte population. Subsequently, media was exchanged with maintenance media every 48 hours until use, typically 3 to 5 days, but never longer than 6 days post seeding.

Shaping and release of MTF constructs

MTFs were cut into specific shapes and released from the cover slip once the cardiomyocytes had formed the appropriate 2D myocardium microstructure. Cover slips were removed from the incubator and placed into a Petri dish filled with 37ºC normal Tyrode's solution. The Tyrode's solution was an extracellular mammalian electrolyte (consisting of 1.192 g of HEPES, 0.901 g of glucose, 0.265 g of $CaCl_2$, 0.203 g of $MgCl_2$, 0.403 g of KCl, 7.889 g of NaCl and 0.040 g of NaH_2PO_4 per liter of deionized water, reagents from Sigma) and was used to support *in vitro* contractility assays. The Petri dish was placed on a stereomicroscope with darkfield illumination (Model MZ6 with darkfield base, Leica) and shapes were formed by using a surgical scalpel or straight-blade razor to cut through the cardiomyocyte and PDMS thin film layers. The alignment of tissue microstructure relative to the film shape was critical to achieving different MTF

functionality (as described in the main text). Stencils printed onto transparency films were used as guides to repeatedly cut out similar shapes. Cutting the PDMS film allowed the Tyrode's solution access to the underlying PIPAAm, such that as the PIPAAm cooled below 35ºC, it transitioned from a hydrophobic to a hydrophilic state and began to dissolve. The dissolution of PIPAAm was a diffusion limited process initiating at the gaps in the PDMS films at the periphery of the cover slip and borders of the cutout shapes. Once the PIPAAm dissolved, the contractions of the cardiomyocytes pulled the MTF free from the cover slip and into solution. Alternately, MTFs were gently pulled off of the PIPAAm surface prior to its dissolution. The conformations of MTFs once released from the cover slip were dependent on the 2D myocardium microstructure, overall shape and cure conditions (i.e. the curvature), and were specific for each of the MTF constructs and actuators. In addition, MTF conformations were, selectively, further manipulated using tweezers to bend the film and press together the PDMS (non-cardiomyocyte) sides of the MTF (Fig. 3A), or to join two or more MTFs together. This was possible because the hydrophobic PDMS readily adhered to itself in aqueous buffer providing a strong yet reversible bond (i.e. the PDMS surfaces could be manually peeled apart again if needed).

Experimental testing procedure, data capture and image analysis

Experiments on live MTF constructs were conducted at room temperature (~22°C) in Tyrode's solution (exchanged every 30 minutes). Video microscopy of MTFs was accomplished with a stereomicroscope coupled to a Sony DCS-V3 digital camera capable of video (640x480 pixels, 25 fps) or still (3072x2304 pixels) imaging modes. Contraction of MTFs was due to either intrinsic contractions or paced via external field stimulation. External pacing used parallel platinum wire electrodes spaced ~1 cm apart and lowered directly into the Petri dish. An external field stimulator (Myopacer, IonOptix Corp.) was used to apply an ~10 V, 10 msec duration square wave between the electrodes at pacing rates from 0.1 to 5 Hz for durations of up to 2 minutes.

Analysis of MTF motion was performed in a post-processing step by tracking the frame-to-frame displacement with image processing software. Video clips were converted from MPEG to uncompressed AVI and opened in ImageJ (National Institutes of Health) (*8*) as image stacks. The conversion factor from pixels to micrometers was calculated for each video clip using the millimeter ruler included in the field of view for calibration. The movements of the myopod and swimmers were tracked manually by choosing a reference point on the MTF and using the ImageJ *Manual Tracking* plug-in to record the position in each frame. Displacements were then calculated as the difference in position between frames and combined to determine the paths. This same method was also used to track the edges of the MTF squares (Fig. S3), the right tip of the helical actuator (Fig. 2C), and the ends of the gripper (Fig. 2E).

The forces generated by the 2D myocardium during the systolic bending of specific MTF constructs was determined by using the radius of curvature of the MTF to calculate stress based on a modified Stoney's equation. Specifically, MTF constructs were viewed on-edge such that the radius of curvature could be calculated directly from the video images. The grayscale video clips were converted to a binary skeleton to create a 1 pixel wide representation of the MTF construct. These skeletonized images were loaded into MATLAB (Mathworks, Inc.) and analyzed using custom written code that fit a circle to the skeleton image, thus finding the radius of curvature. The radius of curvature was converted to stress using a modified Stoney's equation (9). The standard form of Stoney's equation requires that the coating be less than 1% the thickness of the substrate. The thickness of the 2D myocardium (~ 5 μm based on laser scanning confocal images) was 10% to 30% the thickness of the PDMS film requiring a correction factor to be applied to account for this deviation. The error was reduced to less than 1% using the modified Stoney's equation introduced by Atkinson (*10, 11*);

$$\sigma = \frac{Et^3}{6R(1-\nu)h^2(1+t/h)}$$

where σ is cardiomyocyte contractile stress, E is elastic modulus of the PDMS, t is PDMS thickness, R is MTF radius of curvature, h is 2D myocardium thickness and υ is Poisson's ratio of the PDMS. For this system, PDMS elastic modulus is E ~ 1.5 MPa (*12*), υ = 0.49 (*13*), h ~ 5 μm (based on confocal and AFM data), t is typically 30 μm (measured using a stylus profilometer) and R is determined by the MATLAB code for each frame of the video clip. This resulted in stress versus time data allowing contraction

frequency, diastolic stress, peak systolic stress and individual contraction rate and duration to be calculated.

Immunofluorescent staining and imaging

Tissue microstructure was determined by immunofluorescence to visualize the cell, cytoskeletal and sarcomere orientation. Reagents were obtained from Sigma unless otherwise indicated. Cardiomyocytes were cultured on PDMS coated cover slips with the three different surface chemistries (isotropic FN, anisotropic FN and FN lines separated by nonadhesive regions blocked with Pluronics) for a period of 4 days forming complete 2D muscle tissues. Samples were then removed from the incubator, washed 3 times with PBS at 37°C and fixed for 15 minutes in 4% paraformaldehyde and 2.5% TritonX-100 in PBS at 37°C. Following fixation samples were washed 3 times with PBS and stained with a 1:200 dilution of mouse anti-sarcomeric α-actinin monoclonal primary antibody in PBS for 1 hour. Samples were then washed 3 times with PBS and concurrently stained with 1:200 dilutions of DAPI, phalloidin conjugated to Alexa-Fluor 488 (Invitrogen) and goat anti-mouse conjugated to rhodamine secondary antibody (Sigma) in PBS for 1 hour at room temperature. Samples were then washed 3 times with PBS, mounted to glass slides and imaged on an inverted light microscope (Model DMI 6000B, Leica) in epifluorescense using a Coolsnap HQ digital camera (Roper Scientific). Image post-processing was performed with ImageJ software to enhance contrast-brightness and merge color channels.

SUPPORTING FIGURES

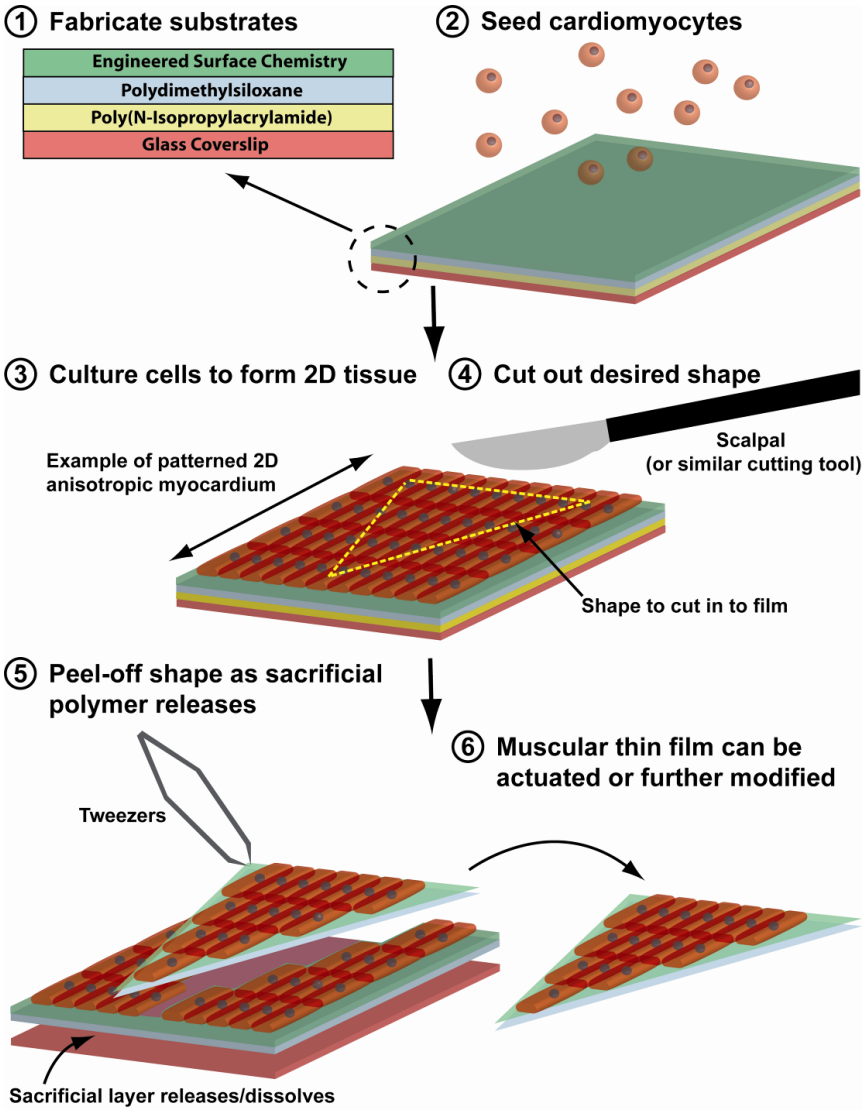

Figure S1. Schematic of MTF fabrication process. (1) The substrates were fabricated as a glass cover slip spin coated with PIPAAm that provided temporary adhesion to a PDMS top layer. The PDMS was then patterned with FN to elicit cell adhesion and growth. (2) Substrates were placed in culture with a cell suspension to allow cardiomyocytes to settle and adhere to the surface. (3) MTFs were cultured in an

incubator until the cardiomyocytes formed a 2D myocardium. (4) A shape was cut in the myocardium/PDMS film using a scalpel. (5) The PIPAAm was dissolved by lowering the bath temperature below 35º C, thus releasing the MTF. The cutout shape floated free or was gently peeled off with tweezers. (6) The free-standing MTF was then used directly or modified further by folding into a 3D conformation.

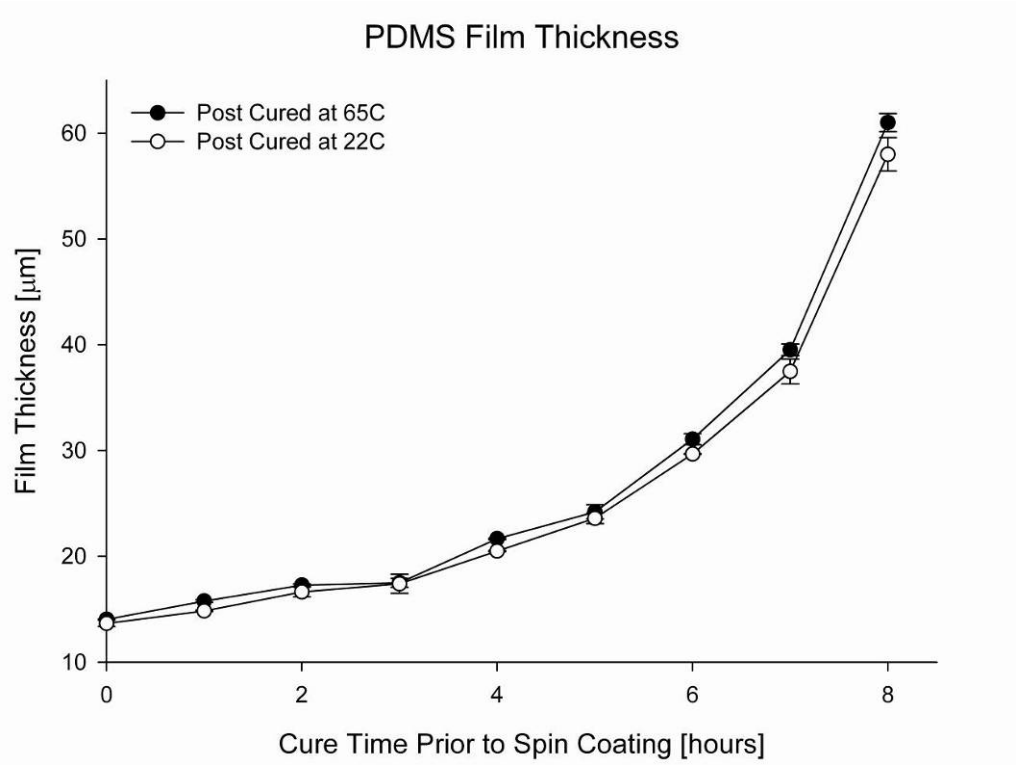

Figure S2. Calibration curve for PDMS film thickness. After mixing the PDMS prepolymer, its viscosity increased as the cross-link density increases. The change in viscosity between mixing (0 hours) and gelation (9 hours) was utilized to spin coat different thickness PDMS films. Following spin coating, the PDMS films were either cured at room temperature (~22ºC) or at 65ºC. Error bars indicate standard deviation.

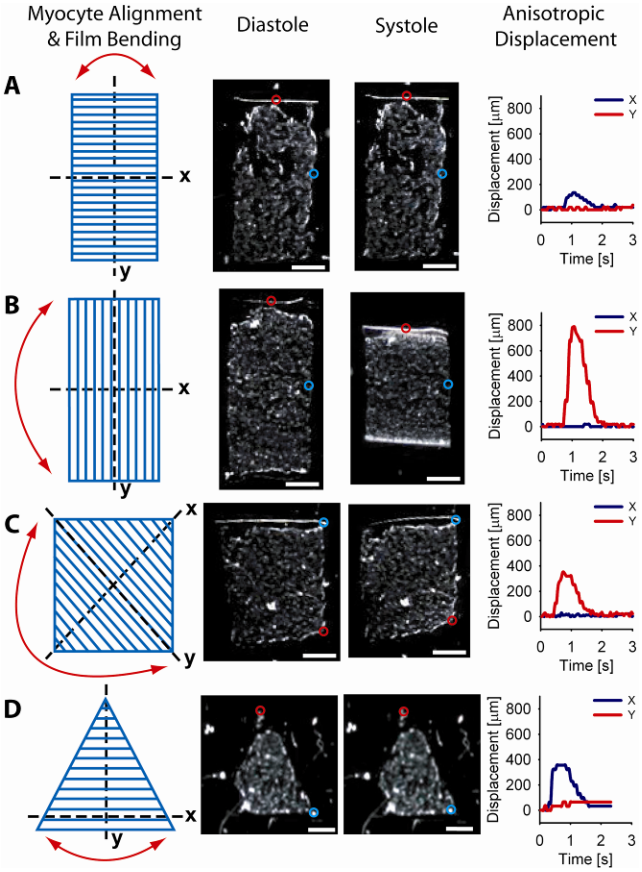

Figure S3. MTFs were fabricated with the direction of cardiomyocyte alignment varied along different symmetry axes of simple geometric shapes. For each 2D shape a schematic is shown with cardiomyocyte alignment indicated by blue lines and the points that bend out of plane during contraction indicated by red arrows. Next to the schematics are two video stills showing the construct in a relaxed state at time 0.00 s and in a contracted ~0.25 s later. Positions tracked for each construct type are indicated by blue circles for the *x* axis and red circles for the *y* axis and plotted for a single contraction in the subsequent graph. (A) Cardiomyocytes aligned along the width of a rectangle showed a small, uni-axial displacement along the width only. (B) Cardiomyocytes

aligned along the length of a rectangle showed a larger, uni-axial displacement along the length only. (C) Cardiomyocytes aligned along the one diagonal of a square showed an intermediate, uni-axial displacement along that diagonal only. (D) Cardiomyocytes aligned along the base of a triangle showed a similar intermediate, uni-axial displacement along the base only. Scale bars are 1 mm.

SUPPORTING MOVIES

Movie S1: MTF rectangles

Each MTF rectangle had anisotropic 2D myocardium aligned along a different axis; the width (left), the length (middle) and the diagonal (right). Contractile motion was constrained entirely to the direction of myocardial alignment with no perceptible contraction along the orthogonal axis.

Movie S2: MTF coiled strip

MTFs in specific conformations exhibited self exciting behavior due to mechanical deformation that initiated successive contractions. This was demonstrated by a rectangle strip with myocardium aligned along the length on the concave surface. This construct curled in on itself many times changing diameter by over 100% and entering periods of regular contractions. Examining the diameter of the coiled strip as a function of time helped to visualize the difference in the rate of systole versus diastole when plotted in 3-dimensions.

Movie S3: MTF helical actuator

The spontaneous 3-dimensional roll-up of a rectangle MTF into a helical actuator was dictated by the axis of myocardial alignment relative to the length of the rectangle. Myocardium on the convex surface of the actuator caused it to lengthen (unwind, helical pitch decreased) during systole. Patterning the cardiomyocytes into an array of 1D strips

electrically isolated them, requiring an externally applied electric field to initiate coordinated contraction of the entire MTF.

Movie S4: MTF grippers

Myocardium on the concave surface of a thin rectangular strip resulted in touching of the rectangle ends during contraction forming a gripper. The open/closed state of the gripper was controlled by the pacing rate of the externally applied electric field reaching a "closed" state at 5.0 Hz pacing.

Movie S5: Myopod

Basic locomotion was achieved by controlling the shape of MTFs constructs. The 3-manually created 3-dimensional conformation altered the direction of contraction of the isotropic MTF resulting in a simple walking uniped construct termed a "myopod" that migrated along the bottom of a Petri dish under paced contractions at 1.0 Hz. Originally an isosceles triangle, tweezers were used to fold the tip of the triangle underneath half way along the triangle height. This formed an angled footpad that slid in only one direction maintaining directed motion under the propulsive force from the cyclically contracting rear 'leg.'

Movie S6: Isotropic and anisotropic MTF swimmers

Comparing the motility of isotropic and anisotropic triangle MTF swimmers at 0.5 Hz external pacing demonstrated that anisotropic 2D myocardium aligned along the height was required for directed locomotion.

Movie S7: Maximizing MTF swimming velocity

A triangle MTF swimmer was used to visualize the change in swimming hydrodynamics as a function of contraction rate. At 1.0 Hz pacing the swimming velocity was maximized as the triangle swimmer coasted for a short distance before contracting again. Increasing the contraction rate to 2.0 Hz prevented the triangle swimmer from fully relaxing thus limiting the power stroke and preventing the swimmer from reaching its maximum speed.

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