



(51) International Patent Classification:

C12N 5/071 (2010.01) C12N 5/095 (2010.01)
C12N 5/074 (2010.01) A61K 35/34 (2015.01)
C12N 5/0789 (2010.01) A61K 35/44 (2015.01)
C12N 5/0797 (2010.01) A61L 27/54 (2006.01)

(21) International Application Number:

PCT/US2017/013001

(22) International Filing Date:

11 January 2017 (11.01.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/277,308 11 January 2016 (11.01.2016) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: ENGINEERED CARDIAC TISSUE AND RELATED METHODS

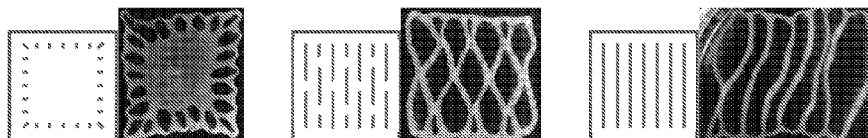


FIG. 1

(57) Abstract: An engineered cardiac tissue, a mold for forming an engineered cardiac tissue, and a method of forming an engineered cardiac tissue are provided. The engineered cardiac tissue includes a plurality of induced pluripotent stem cell-derived cardiac cells arranged in a configuration suitable for implantation onto the surface of a heart. The method of forming an engineered cardiac tissue includes providing a mold including an outer surface, pouring a cell solution onto the outer surface of the mold, and forming the engineered cardiac tissue from the cell solution, the engineered cardiac tissue having a geometry corresponding to a geometry of the mold. Methods of treating a myocardial infarction are further provided and include implanting an engineered cardiac tissue onto the heart of a subject.

ENGINEERED CARDIAC TISSUE AND RELATED METHODS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 62/277,308, filed January 11, 2016, the entire disclosure of which is incorporated herein by this reference.

TECHNICAL FIELD

[0002] The presently-disclosed subject matter relates to engineered cardiac tissues. In particular, the presently-disclosed subject matter relates to engineered cardiac tissues generated using induced pluripotent stem cell-derived cardiac cells.

BACKGROUND

[0003] Heart diseases are the leading cause of death worldwide. Even with a broad range of evidence-based therapies, the five-year survival rate of heart failure remains as low as approximately 50%. One reason for this low five-year survival rate is that, following cardiac injury, the damaged heart wall is replaced with scar tissue that is stiff and that cannot develop force to assist cardiac function. As cardiac function deteriorates, patients develop the symptoms of “heart failure,” the fundamental etiology of which is the result of massive loss or dysfunction of myocardial cells. This often leads to serious morbidity and mortality.

[0004] In this regard, multiple “cell therapies” are currently under investigation for cardiac repair. For example, tissue engineering technologies have emerged as robust modalities to realize cardiac regeneration due to the unique capacity to deliver numerous cardiac cells within an organized architecture onto the heart. To date, however, each of these cell therapies suffer from the major limitation that the “donor” cells die shortly after they are infused, injected, or implanted into animals or patients with damaged heart walls. Additionally, there are drawbacks to the current scale-up strategies which limit the formation of larger implantable tissue.

[0005] Accordingly, there remains a need for a cell therapy that provides increased cell survival and engraftment in implantable tissue.

SUMMARY

[0006] The presently-disclosed subject matter meets some or all of the above-identified needs, as will become evident to those of ordinary skill in the art after a study of information provided in this document.

[0007] This summary describes several embodiments of the presently-disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently-disclosed subject matter, whether listed in this summary or not. To avoid excessive repetition, this summary does not list or suggest all possible combinations of such features.

[0008] In some embodiments, the presently-disclosed subject matter includes an engineered cardiac tissue, comprising a plurality of induced pluripotent stem cell-derived cardiac cells arranged in a configuration suitable for implantation onto the surface of a heart. In one embodiment, the plurality of induced pluripotent stem cell-derived cardiac cells are arranged in a square, mesh, or parallel configuration. In a particular embodiment, the plurality of induced pluripotent stem cell-derived cardiac cells are arranged in a mesh configuration. In another embodiment, the plurality of induced pluripotent stem cell-derived cardiac cells are arranged in a configuration having a length of about 15 mm to about 40 mm and a width of about 15 mm to about 40 mm. In a further embodiment, the heart is a human heart.

[0009] In some embodiments, the plurality of induced pluripotent stem cell-derived cardiac cells are selected from the group consisting of cardiomyocytes, endothelial cells, mural cells, fibroblasts, and combinations thereof. In some embodiments, the engineered cardiac tissue comprises, by weight, between about 10 and 20% endothelial cells and/or between about 10 and 20% mural cells. In one embodiment, the concentration of cells facilitates *in vitro* expansion of vascular cells within the engineered cardiac tissue and subsequent *in vivo* vascular coupling between the engineered cardiac tissue and a recipient's myocardium. In another embodiment, the engineered cardiac tissue comprises an initial cell number of 6M or less. Additionally or

alternatively, in some embodiments, at least one of the induced pluripotent stem cells-derived cardiac cells is transfected with a light-sensitive ion channel, such as channelrhodopsin.

[0010] Also provided is a mold for forming an engineered cardiac tissue, the mold comprising a polymeric base material having an outer surface and a shape configured to produce an engineered cardiac tissue in a configuration suitable for implantation onto the surface of the heart. In one embodiment, a base material of the mold is polydimethylsiloxane. In another embodiment, the outer surface is a polydimethylsiloxane coating over a base material of the mold. In a further embodiment, the mold comprises at least one post arranged and disposed to form a pore in the engineered cardiac tissue. Additionally or alternatively, the mold comprises a plurality of posts arranged and disposed to form a mesh configuration in the engineered cardiac tissue.

[0011] Further provided is a method of forming an engineered cardiac tissue, the method comprising the steps of providing a mold including an outer surface, pouring a cell solution onto the outer surface of the mold, and forming the engineered cardiac tissue from the cell solution, the engineered cardiac tissue having a geometry corresponding to a geometry of the mold. In one embodiment, the cell solution comprises a mixture of cells and matrix. In another embodiment, forming the engineered cardiac tissue includes culturing the cell solution on the mold. In a further embodiment, the cell solution is cultured for about 14 days.

[0012] In some embodiments, the cell solution comprises a plurality of induced pluripotent stem cell-derived cardiac cells. In one embodiment, at least one of the induced pluripotent stem cell-derived cardiac cells is transfected with a light-sensitive ion channel. In another embodiment, the method further comprises optogenetic pacing of the engineered cardiac tissue.

[0013] Still further provided are methods for treating a myocardial infarction. In some embodiments, a therapeutic method is provided that comprises implanting an engineered cardiac tissue onto a heart of a subject. In some embodiments, the engineered cardiac tissue that is implanted comprises a plurality of induced pluripotent stem cell-derived cardiac cells arranged in a configuration suitable for implantation.

[0014] Further features and advantages of the presently-disclosed subject matter will become evident to those of ordinary skill in the art after a study of the description, figures, and non-limiting examples in this document.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **FIG. 1** shows images illustrating trays for forming square, mesh, or parallel cell configurations and a plurality of induced pluripotent stem cell-derived cardiac cells arranged in square, mesh, or parallel configurations according to the corresponding trays. The induced pluripotent stem cell-derived cardiac cells are shown at day 14.

[0016] **FIG. 2** shows images illustrating cell survival and functional properties of mesh large-format engineered cardiac tissues (LF-ECTs) with differing cell number and/or density of human induced pluripotent stem cell (hiPSC)-derived cardiac cells (6, 9, or 12M cells per construct).

[0017] **FIGS. 3A-3D** show images illustrating histology of LF-ECT on the surface of the rat heart. (**FIG. 3A**) Cell nuclei visualized with DAPI (blue). (**FIG. 3B**) Human iPSCs visualized with human nuclear antigen (HNA-red). (**FIG. 3C**) Cardiomyocytes visualized with Troponin T (TNT-green). (**FIG. 3D**) Composite image showing cardiomyocytes surviving in the ECT and present in the adult rat heart (DAPI-blue, TNT-green). The dashed line shows the demarcation between the bottom of the LF-ECT and the surface of the adult rat heart.

[0018] **FIG. 4** shows images illustrating a comparison between a mesh LF-ECT and an extra-large format engineered cardiac tissue (XLF-ECT).

[0019] **FIGS. 5A-5I** show graphs and images illustrating the impact of initial geometry on LF-ECT maturation and cell survival. (**FIG. 5A**) Schematic diagram for generation of LF-ECTs composed of cardiomyocytes (CM), endothelial cells (EC), and mural cells (MC). Cells and matrix were poured into custom PDMS molds on day 0 and then matured *in vitro*. (**FIG. 5B**) Representative image of a LF-ECT mold constructed from 0.5 mm diameter PDMS sheets. Scale bar: 10 mm. (**FIG. 5C**) Representative initial LF-ECT geometries. PL7 mold (ME-ECT) included post lengths of 7 mm and post spacing of 2.5 mm. PL16 mold (ML-ECT) included post lengths of 16 mm and post spacing of 2.5 mm. PL0 mold (PS-ECT) included individual 1mm diameter loading posts located along the LF-ECT periphery. Representative images of LF-ECTs on day 0, day 14, and after release from the mold are shown. Scale bar: 10mm. (**FIG. 5D**) Representative image of a day 14 ME-ECT that shows the mature bundles and junctions and a day 14 PL-ECT that shows variation in bundle width. Scale bar: 2.5 mm. (**FIG. 5E**) Graph illustrating cross-sectional tissue area of ME-, ML-, and PS-ECTs. Cross-sectional tissue area

was increased in PS-ECT (n=8) versus ME- (n=10) and ML-ECTs (n=6) (**P<0.001 for each). **(FIG. 5F)** Graph illustrating bundle widths of ME- and ML-ECTs. Bundle widths were similar between ME- (n=10) and ML-ECTs (n=6). **(FIG. 5G)** Graph illustrating bundle width ratios of ME- and ML-ECTs. The ratio of maximum to minimum width of each bundle was calculated, and lower variance was noted in ME- versus ML-ECTs (n=36; **P<0.001). **(FIG. 5H)** Representative images for ME-, ML-, and PS-ECTs stained with EthD-III (red) for dead cells. Scale bar: 250µm. **(FIG. 5I)** Graph illustrating percentage of dead cells in ME-, ML-, and PS-ECTs at 14 days. ME-ECTs had a smaller percent of dead cells at day 14 versus ML- and PS-ECTs (n=20; *P<0.05, **P<0.001).

[0020] FIGS. 6A-6G show graphs and images illustrating the impact of initial geometry on LF-ECT cardiomyocyte alignment and contractile function. **FIGS. 6A-6C** show whole-mount cTnT-stained images of representative alignment analysis and graphs illustrating summary 3D alignment data for the adjacent image of **(FIG. 6A)** ME-ECT, **(FIG. 6B)** ML-ECT, and **(FIG. 6C)** PS-ECT. In the graphs, the construct long axis is identified by the red line. **(FIG. 6D)** Graph illustrating alignment concentration of ME-, ML-, and PS-ECTs. Note increased dispersion of CM orientation in PS-ECT 3D plot, quantified by reduced alignment concentration (κ), (n=4; *P<0.05 for PS versus ME). **(FIG. 6E)** Graph illustrating maximum capture rate of ME, ML, and PS. **(FIG. 6F)** Graph illustrating excitation thresholds of ME, ML, and PS. The maximum capture rate and excitation threshold in **FIGS. 6E and F** were similar between the three geometries [n=11 (ME), 11 (ML), and 8 (PS)]. **(FIG. 6G)** Graph illustrating active stress of ME, ML, and PS. Active stress was lower in PS-ECT (n=8) versus ME- and ML-ECTs (n=11; *P<0.05 for PS versus ME or ML).

[0021] FIGS. 7A-7E show graphs and images illustrating the impact of initial cell number and cell density on LF-ECT cell survival, maturation, and function for ME-ECTs. **(FIG. 7A)** Total cell number and volume from the standard 6M in 400µl were varied to 9M or 12M with increasing initial volumes or to 12M in the same initial volume (12MH, high density). **(FIG. 7B)** Graph illustrating changes in bundle width over 14 days. Increased initial LF-ECT construct volumes resulted in larger bundle widths at d14 (**P<0.001 for 9M and 12M versus 6M) [n=10 (6M), 7 (9M), 8 (12M), and 7 (12MH)]. **(FIG. 7C)** Representative 3D constructed tissue images for 6M, 9M, and 12M ME-ECTs stained with DAPI and cTnT; cardiac Troponin T. Scale bar: 200 µm. **(FIG. 7D)** Graph illustrating percentage of dead cells at day 14. 6M ME-ECTs had the

smallest percent of dead cells at day 14 versus 9M (**P<0.01) and versus 12M and 12MH (***P<0.001). N=20. (FIG. 7E) Graph showing active stress of various ECTs. Active stress was highest in 6M ME-ECT versus ECTs with greater initial cell numbers [n=11 (6M), 8 (9M), 8 (12M), and 8 (12MH); ***P<0.001 vs. 9M and vs. 12M, **P<0.01 vs. 12MH].

[0022] FIGS. 8A-8H show graphs illustrating the impact of culture duration on ME-ECT maturation. (FIG. 8A) Graph illustrating averaged normalized active force-time curves for ME-ECT cultured for either day14 (blue) or day28 (red) (n=6). Dashed lines highlight 50% and 90% relaxation time (RT). (FIG. 8B) Graph illustrating that prolonged in vitro culture for 28 days resulted in reduced relaxation time 50% (n=6; ***P<0.001). (FIG. 8C) Graph illustrating that prolonged in vitro culture for 28 days resulted in reduced relaxation time 90% (***P<0.001). (FIG. 8D) Graph illustrating that prolonged in vitro culture for 28 days resulted in increased maximum capture rate (n=6; ***P<0.001). (FIG. 8E) Graph illustrating that prolonged in vitro culture for 28 days resulted in shift from a negative to a positive force-frequency relationship (n=6; *P<0.05 to ***P<0.001). (FIG. 8F) Graph illustrating that prolonged in vitro culture for 28 days resulted in increased cardiomyocyte alignment concentration (κ), [n=4 (day14) and n=3 (day28); ***P<0.001]. (FIG. 8G), (FIG. 8H) Graphs illustrating comparison of gene expression analysis (qPCR) between 14-day and 28-day constructs (n=3; *P<0.05 to ***P<0.001). cTnT level was lower in 28-day constructs, and other genes were normalized to the value of cTnT expression.

[0023] FIGS. 9A-9G show graphs and images illustrating myocardial functional recovery and regional changes in wall motion after hiPSC-ME-ECTs cardiac implantation. (FIG. 9A) Schematic timeline of rat surgery. Echo: echocardiogram. Left anterior descending artery (LAD) ligation is performed (Week -1) then a ME-ECT matured *in vitro* for 14 days (or sham suture) is implanted in a nude male rat (Week 0). Echo is performed prior to LAD ligation on Week-1 (W-1), prior to surgery at Week0 (W0), then Week2 (W2) and Week4 (W4). (FIG. 9B) Image illustrating ME-ECT implanted onto the heart surface at infarction site (right). (FIG. 9C-FIG. 9E) Graphs illustrating results of B-mode echocardiogram [n=5 (Tx) and 5 (sham)]. (FIG. 9C) Left ventricular end diastolic area (LVAd; mm²), (FIG. 9D) ejection fraction (%), and (FIG. 9E) cardiac index, CI (mL/min/kg) (baseline before LAD ligation (W-1), before treatment (W0), and at week 2 (W2) and week 4(W4, *P<0.05 implant versus sham at W4). (FIG. 9F) Graph illustrating radial strain of sham (blue, n=5) and implant (red, n=5) at W0 (dotted line) and W4

(solid line). Comparison between W0 and W4 averaged composite long axis radial strains for implant and sham treatment shows an increase in posterior apex and posterior mid region shortening at W4 after ME-ECT implantation (** $P < 0.01$ versus W4 Sham). (**FIG. 9G**) Graph illustrating longitudinal strain of sham (blue, $n=5$) and implant (red, $n=5$) at W0 (dotted line) and W4 (solid line). Comparison between W0 and W4 averaged composite long axis longitudinal strains for implant and sham treatments shows an increase in anterior apex shortening at W4 after ME-ECT implantation (* $P < 0.05$ versus W4 Sham and ($\dagger P < 0.05$ versus W0 Implant). There is also a compensatory increase in anterior mid ($\ddagger P < 0.01$ versus Sham at W0) and anterior base shortening ($\S P < 0.05$ versus Sham at W0) consistent with adaptive remodeling.

[0024] FIGS. 10A-10G show graphs and images illustrating morphometric analysis of LV remodeling after hiPSC-ME-ECTs cardiac implantation. (**FIG. 10A-FIG. 10B**) Images illustrating representative Masson's trichrome staining of (**FIG. 10A**) sham treated and (**FIG. 10B**) ME-ECT implanted rat hearts at W4. Scale bar: 2mm. (**FIG. 10C**) Graph illustrating scar area (% of LV). (**FIG. 10D**) Graph illustrating risk area (% of LV). (**FIG. 10E**) Graph illustrating viable myocardium (% of risk area). (**FIG. 10F**) Graph illustrating LV wall thickness at the infarct (mm). (**FIG. 10G**) Graph illustrating expansion index after ME-ECT implantation (* $P < 0.05$, N.S., not significant). Expansion index = (endocardial circumference/epicardial circumference) $\times$ (non-infarcted region wall thickness/risk region wall thickness). Multiple indices support reduced scar and increased viable myocardium after hiPSC-ME-ECT implantation [$n=5$ (Tx) and 5 (sham)].

[0025] FIGS. 11A-11K show images illustrating myocardial regeneration and post-implantation *in vivo* reorganization of hiPSC- ME-ECT. (**FIG. 11A**) Image showing representative left ventricular histology 4 weeks after implantation of hiPSC-ME-ECT. Scale bar: 1 mm. Red dotted line indicates engrafted area and yellow dotted line represents region seen under higher magnification (**FIG. 11B-FIG. 11E**, Scale bar: 50 μm). (**FIG. 11B**) Image showing higher magnification identifying all nuclei via DAPI, nuclear stain. (**FIG. 11C**) Image showing lineage-specific stain cTnT; cardiac Troponin T. (**FIG. 11D**) Image showing h-iPSC-derived cells identified by HNA, human nuclear antigen. (**FIG. 11E**) Merged image. (**FIG. 11F**) Image showing region indicated by orange dotted line in (**FIG. 11E**) under higher magnification in triple immunostaining showing sarcomeric structure in hiPSC-derived cardiomyocytes. Scale bar: 10 μm . (**FIG. 11G**) Image showing colocation of CM and mural

cells. All nuclei, DAPI; mural cells, NG2 proteoglycan; CM, cTnT. Some cells stained positive for both cTnT and NG2. Scale bar: 50 μ m. **(FIG. 11H)** Image showing region indicated by yellow dotted line in **(FIG. 11G)** seen under higher magnification (Scale bar: 10 μ m). **(FIG. 11I)** Triple immunostaining image showing hiPSC-derived cells by HNA, endothelial cells identified by von Willebrand factor, vWF, and nuclei by DAPI. Scale bar: 100 μ m. **(FIG. 11J)** Image showing region indicated by yellow dotted line in **(FIG. 11I)** seen under higher magnification (Scale bar: 20 μ m). **(FIG. 11K)** Representative image of extra large-format ME-ECT intended for large animal preclinical trials after release from the PL7 mold. Scale bar: 10 mm.

[0026] FIG. 12 shows a schematic illustrating formation of light sensitive ECTs and ECT analysis.

[0027] FIGS. 13A-13B show hiPSC-derived ECT **(FIG. 13A)** before and **(FIG. 13B)** after transfection with ChIEF light sensitive ion channel expressing tdTomato.

[0028] FIGS. 14A-14B show graphs illustrating patch clamp results during optical stimulation of transfected hiPSC ECTs. **(FIG. 14A)** Graph illustrating patch clamp response to 1 ms duration light pulses. **(FIG. 14B)** Graphs illustrating response to 1 ms duration pulses at 1 Hz (top) and 10 Hz (bottom).

[0029] FIG. 15 shows graphs illustrating contractile stress of ECTs at intrinsic beat rate (top) and optically paced at 5 Hz (bottom). The FFT power spectra (right) show the dominant beat frequency.

[0030] FIG. 16 shows a graph illustrating mean active stress of a control and a paced ECT.

[0031] FIGS. 17A-17C show graphs illustrating that chronic optical pacing improves ECT functional maturation. **(FIG. 17A)** Graph illustrating that intrinsic and maximal capture rates (MCR) are higher in chronically paced ECTs at day 14. **(FIG. 17B)** Graph illustrating that the force-frequency relationship is improved after chronic pacing. **(FIG. 17C)** Graph illustrating that beat-to-beat hysteresis is reduced in paced ECTs. * $p < 0.05$, $n = 4$ control, $n = 5$ paced.

[0032] FIGS. 18A-18D show images illustrating immunohistochemistry of control and optically paced ECTs. **(FIG. 18A)** Image showing control ECT. **(FIG. 18B)** Image showing optically paced ECT. **(FIG. 18C)** Image showing portion of control ECT under high magnification. **(FIG. 18D)** Image showing portion of optically paced ECT under high

magnification. DAPI (blue), cTnT (green), and tdTomato (red). Overlap of cTnT and tdT identifies transfected CMs.

[0033] FIGS. 19A-19B include schematic diagrams and graphs showing protocols to differentiate cardiovascular cell populations from human iPS cells. **(FIG. 19A)** Schematic diagrams of protocols used to induce cardiac lineage cells (CM+EC protocol, n=53, and MC protocol, n=53). CM, cardiomyocyte; EC, endothelial cell; MC, vascular mural cell; iPSC, induced pluripotent stem cell; MG, Matrigel; ActA, Activin A; Wnt3a (Wnt antagonist), BMP4, Bone morphogenetic protein 4; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial cell growth factor; FBS, fetal bovine serum. **(FIG. 19B)** Population of cTnT⁺ CMs, VE-cadherin (CD144)⁺ ECs and PDGFR β (CD140b)⁺ MCs for ECT preparation quantified by flow cytometry (n=107). cTnT, cardiac troponin-T; VE-cadherin, vascular endothelial-cadherin; PDGFR β , platelet-derived growth factor receptor beta

[0034] FIG. 20 includes schematic diagrams and images showing initial LF-ECT geometries considered to investigate the effect of construct geometry on tissue maturation and function. Upper row includes a range of mold templates: PL-ECT (peripheral posts only) and a range molds that vary the post length (PL 4, 7, 16 mm) and post spacing (PS 4 or 2.5 mm). Lower row shows various representative LF-ECTs at d14 in their respective molds. Scale bar: 10 mm.

[0035] FIGS. 21A-21C are images showing contractile force measurement. **(FIG. 21A)** Representative ME-ECT segment, which was cut off at the red dotted line, used to determine in vitro force-length and force-frequency relations. Note that the isolated ME-ECT construct includes junctions and remnants of adjacent bundles. ECTs are attached to the muscle testing system using 10-0 nylon suture. **(FIGS. 21B-21C)** Similar views are provided for an ML-ECT and a PS-ECT construct.

[0036] FIGS. 22A-22C are graphs showing force-frequency relationship of ME-ECTs. **(FIG. 22A)** Representative waveforms of active stress at different pacing frequency in 14-day and 28-day constructs. **(FIG. 22B)** The actual value of active stress at each beating rate (n=6; **p<0.01; ***p<0.001 vs baseline at 1.5Hz in D14). **(FIG. 22C)** 28-day constructs showed frequency-dependent acceleration of relaxation (n=6; *p<0.05; **p<0.01** vs baseline at 1.5Hz in D28).

[0037] FIGS. 23A-23E are images and a graph showing cardiomyocyte and endothelial cell populations in LFECT bundles. **(FIG. 23A-23B)** Representative cross-sectional images of DAPI (blue) and cTnT (cardiac Troponin T, green) in 14-day (FIG. 23A) and 28-day (FIG. 23B) LF-ECT bundles. The inset in (FIG. 23A) shows the nuclei classification for the region marked by the dashed yellow box. cTnT positive nuclei are outlined in orange and negative nuclei are outlined in grey. **(FIG. 23C-23D)** Representative cross-sectional images of DAPI (blue) and CD31 (red) in 14-day (FIG. 23C) and 28-day (FIG. 23D) LF-ECT bundles. The inset in (FIG. 23C) shows the nuclei classification for the region marked by the dashed yellow box. CD31 positive nuclei are outlined in pink and negative nuclei are outlined in grey. Scale bars: 250 μm , 50 μm inset. **(FIG. 23E)** The percent positive cTnT and CD31 cell fractions in day 14 and day 28 LF-ECTs (n=4).

[0038] FIGS. 24A-24B are images showing ME-ECT survival and vascular coupling. **(FIG. 24A)** Representative left ventricular histology 4 weeks after implantation of hiPSC-ME-ECT onto non-infarcted heart. Grey dotted line indicates the transition from implanted ME-ECT to recipient myocardium. DAPI, nuclear stain; HNA, human nuclear antigen; cTnT, cardiac Troponin T; merged image. Scale bar: 250 μm . **(FIG. 24B)** Higher magnification within ME-ECT showing evidence for perfusion by injected lectin (red arrows). DAPI, nuclear stain; lectin, intravascular perfusion; HNA, human nuclear antigen; merged image. Scale bar: 125 μm .

DESCRIPTION OF EXEMPLARY EMBODIMENTS

[0039] The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

[0040] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure

herein, unless noted otherwise, are incorporated by reference in their entirety. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

[0041] Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are now described.

[0042] Following long-standing patent law convention, the terms “a”, “an”, and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a cell” includes a plurality of such cells, and so forth.

[0043] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

[0044] As used herein, the term “about,” when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed method.

[0045] As used herein, ranges can be expressed as from “about” one particular value, and/or to “about” another particular value. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

[0046] The presently-disclosed subject matter is based, at least in part, on the development of implantable tissues that can repair and regenerate damaged heart muscle. In particular, the presently-disclosed subject matter is based on the development of replacement heart muscle in culture in formats large enough to support the survival and function of replacement heart cells after implantation.

[0047] In some embodiments, an engineered cardiac tissue (ECT) is provided. In some embodiments, the ECT is comprised of induced pluripotent stem cells (iPSCs) and/or human induced pluripotent stem cells (hiPSCs), including, but not limited to, hiPSC-derived cardiac cells that contain multiple cell lineages (cardiomyocytes, endothelial cells, mural cells, fibroblasts). For example, in one embodiment, the ECT includes a ECT composed of cardiovascular (CV) lineages derived from hiPSCs (hiPSC-ECTs), including hiPSC-derived cardiomyocytes (CM), endothelial (EC), and vascular mural cells (MC). Other CV lineage compositions include, but are not limited to, CM and EC, and CM and MC. In another embodiment, the ECT forms an implantable tissue including iPSC-derived cardiac cells arranged in a configuration suitable for implantation onto the surface of a heart. In a further embodiment, the implantable ECT provides the capacity to deliver numerous cardiac cells with an organized architecture into the heart.

[0048] The type and/or amount of cardiac cells incorporated in the ECT may be varied and/or adjusted depending upon the desired composition of the implantable tissue. For example, in some embodiments, the concentration of cardiomyocytes (CM) is in the range of 40 to 60% of total cells. In some embodiments, non-cardiomyocytes including endothelial cells (ECs) and mural cells (MCs) are mixed to adjust the final EC concentrations to represent between 10 and 20% of the total cells in the ECT and MC concentrations to represent between 10 and 20% of the total cells in the ECT. In another embodiment, this concentration facilitates the *in vitro* expansion of vascular cells within the ECT and subsequent *in vivo* vascular coupling between the ECT and a recipient's myocardium. In a further embodiment, the ECT including CM, EC, and MC provides the higher beat rates, faster relaxation, lower pacing threshold, greater force at higher heart rate (HR), and/or greater sarcomere maturation as compared to ECTs including CM and EC or CM and MC. The individual cell types may be generated through any suitable CV cell differentiation protocol, including, but not limited to, those described in the Examples below.

[0049] The ECT includes any suitable size, shape, and/or geometry for implantation. Suitable geometries include, but are not limited to, square, rectangular, circular, oval, triangular, polygonal, irregular, any other suitable geometry for implantation, or a combination thereof. For example, in one embodiment, the ECT includes a 1 mm x 15 mm linear geometry. In another embodiment, the size of the ECT is increased to form large-format (LF)-ECTs suitable for implantation in larger animals, such as humans and other animals with human-sized hearts, with dimensions beginning at 15 mm x 15 mm and including ECTs with larger dimensions. The LF-ECT includes any ECT having an increased size, such as, but not limited to, an ECT having a size equal or greater than 15 mm x 15 mm, such as, but not limited to, a size of between 30 mm x 30 mm and 40 mm x 40 mm. Although discussed above with regard to square geometries, as will be appreciated by those of ordinary skill in the art, the ECTs are not so limited and may include any other suitable size, shape, and/or geometry, including 3D geometry. More specifically, in certain embodiments, the size, shape, and/or geometry of the ECT, including the 3D geometry, is selected based upon the desired placement and/or use.

[0050] In some embodiments, the size, shape, and/or geometry of the ECT is provided by a mold. For example, in one embodiment, a cell solution or cell/matrix mixture is poured onto the mold and cultured to form the ECT with a size, shape, and/or geometry corresponding to that of the mold. Accordingly, the mold includes any suitable size, shape, and/or geometry for forming the desired shape of the ECT, and may be modified to provide the desired size, shape, and/or geometry of the ECT. In another embodiment, the mold includes any suitable material and/or coating for forming the ECT thereon. Suitable materials include, but are not limited to, a polydimethylsiloxane (PDMS) and/or a PDMS coating or, in some embodiments, a 3D printed and curable material such as a light-curable, biocompatible, FDA approved cyanoacrylate, Loctite® 3211 (Henkel Adhesives North America).

[0051] Additionally or alternatively, the ECT may be porous. The porous ECT includes any suitable porosity based upon the intended use. In one embodiment, the porosity in the ECT is formed by posts in the mold. In another embodiment, the mold includes one or more internal posts which form pores in the ECT as the cells grow around the posts. The posts include any suitable geometry, including, but not limited to, square, rectangular, circular, oval, triangular, polygonal, irregular, any other suitable geometry for forming the desired pore(s), or a combination thereof. For example, as illustrated in **FIG. 1**, the mold and/or the posts therein

may be arranged to form ECTs including a plurality of induced pluripotent stem cells-derived cardiac cells arranged in a square, mesh, or parallel configuration. More specifically, in a further embodiment, a mesh (ME) ECT is formed with a mold having rectangular internal staggered posts (**FIG. 1**, middle image). As will be appreciated by those skilled in the art, the posts are not limited to a staggered arrangement, and may include any other orientation or arrangement for forming a desired porosity/ECT geometry. Other orientations include, but are not limited to, a perimeter of posts forming a square ECT (**FIG. 1**, left image) or long parallel posts arranged and disposed to form multiple linear bundles (ML) (**FIG. 1**, right image). Alternatively, the mold may be devoid of posts to form a plain sheet (PS) ECT.

[0052] In some embodiments, the size, shape, and/or geometry of the mold, the cellular composition of the ECT, and/or the cellular density of the ECT is selected to promote *in vitro* pre-implant cell survival and/or satisfactory engraftment after *in vivo* implantation onto animal hearts. For example, in one embodiment, as discussed in the Examples below, ME-ECTs displayed the lowest dead cell ratio ($p < 0.001$) and matured into 0.5 mm diameter myofiber bundles with greater 3D cell alignment and higher active stress than PS-ECTs. In another embodiment, increased initial ECT cell number beyond 6M per construct resulted in reduced cell survival and lower active stress, while 6M-ME-ECTs engrafted, displayed evidence for host vascular coupling, and recovered myocardial structure and function with reduced scar area. In a further embodiment, the varied 3D geometries and/or cell densities facilitate scale-up of the ECT for large animal implantation (e.g., formation of large-format ECTs).

[0053] In certain embodiments, ECT provides and/or facilitates cardiac regeneration. Without wishing to be bound by theory, it is believed that the coexistence of multiple vascular lineages with CMs within the 3D ECT composition promote structural and electrophysiological tissue maturation. For example, in one embodiment, the hiPSC-ECTs provide improvement of cardiac function, regenerate myocardium, and/or increase/enhance angiogenesis. In another embodiment, the ECT disclosed herein treats, reduces, and/or ameliorates left ventricular dysfunction, including that caused by ischemic and dilated cardiomyopathy accompanying heart failure. Other uses of the hiPSC LF-ECTs include, but are not limited to, as a bioengineered implantable cell/tissue product for severe heart disease (cardiomyopathies, congenital, etc.), as a product for drug screening (cardiotoxicity, arrhythmia, etc.), and/or as an *in vitro* microenvironment to investigate cell-cell interactions and drug-cell interactions relevant to tissue

engineering or to investigating human disease processes. Additionally or alternatively, instead of “normal” human iPSCs, cells from patients with unique genetic human diseases can also be used to generate “disease model” hiPSC LF-ECTs.

[0054] In some embodiments of the presently-disclosed subject matter, therapeutic methods are further provided that make use of the engineered cardiac tissues described herein. For example, in some embodiments of the presently-disclosed subject matter a method of treating a myocardial infarction is provided that comprises implanting an engineered cardiac tissue onto a heart of a subject in need thereof. In some embodiments of the therapeutic methods, and as described herein above, the engineered cardiac tissue comprises a plurality of induced pluripotent stem cell-derived cardiac cells arranged in a configuration suitable for implantation onto the surface of the heart. In some embodiments of the therapeutic methods, the treatment need not be limited to myocardial infarction, but may be further expanded to the treatment of heart failure, including, but not limited to, the treatment of heart failure characterized by left ventricular dysfunction, including, as indicated above, that caused by ischemic and dilated cardiomyopathy.

[0055] As used herein, the terms “treatment” or “treating” relate to any treatment of a condition of interest (e.g., a heart failure), including but not limited to prophylactic treatment and therapeutic treatment. As such, the terms “treatment” or “treating” include, but are not limited to: preventing a condition of interest or the development of a condition of interest; inhibiting the progression of a condition of interest; arresting or preventing the further development of a condition of interest; reducing the severity of a condition of interest; ameliorating or relieving symptoms associated with a condition of interest; and causing a regression of a condition of interest or one or more of the symptoms associated with a condition of interest.

[0056] With further respect to the engineered cardiac tissues described herein, in some embodiments, the engineered cardiac tissues possess a number of distinct advantages of current technology, including the possibility that: hiPSCs may bring much higher efficiency of cardiomyocyte production compared to any other stem cell types used in clinical researches (cardiac progenitor cells, mesenchymal stem cells, skeletal myoblasts etc.) which is needed to realize cardiac regeneration beyond paracrine effects reported in previous stem cell-based clinical reports; hiPSCs may bring diverse cardiovascular cell populations which is also needed

to regenerate vascularized myocardium with sufficient stiffness; a 3-D construct using biomaterials brings much higher efficiency of survival after implantation which is advantageous for cardiac regenerative therapy; the formulation of the inclusion of multiple distinct iPSC lineages (cardiomyocyte, endothelial cell, mural cell) within a 3D engineered tissue provides more rapid maturation and increased force production which is important for clinical translation; and the formulation of the construct geometry provides better cell survival prior to and after implantation.

[0057] In some embodiments, the ECT according to one or more of the embodiments disclosed herein includes at least one iPSC transfected with a light-sensitive ion channel. For example, in one embodiment, the ECT includes at least one iPSC-derived cardiomyocyte transfected with a light-sensitive ion channel. In another embodiment, the light-sensitive ion channel includes a channelrhodopsin (ChR). In a further embodiment, the light-sensitive ion channel includes a ChIEF light sensitive ion channel expression tdTomato. In certain embodiments, the iPSC virally transfected with the light-sensitive ion channel.

[0058] Also provided herein is a method for increasing and/or enhancing ECT structural and/or functional maturation. In one embodiment, the method includes optogenetic pacing (OP). In another embodiment, the method includes viral transfection of light-sensitive ion channels, such as channelrhodopsin (ChR), as a noninvasive, novel stimulation method for excitable tissues. In a further embodiment, ChR transfected iPSC-derived cardiomyocytes within 3D ECTs are paced above the intrinsic rate with a pulsed LED. In certain embodiments, OP increases and/or improves electrophysiological properties (e.g., maximum capture rate and beat hysteresis) of ECTs.

[0059] Furthermore, with respect to the possibility of tumor formation related to the use of iPSCs, such a possibility is avoided by the presently-disclosed subject matter by making use of several methods, including: 1) using genomic integration-free human iPSC lines which is established with episomal plasmid vectors; and 2) depletion of undifferentiated iPSCs from bulk assembly of differentiated cell population by, for example, equipping a magnetic-activated cell sorter system for research use (AutoMACS, Miltenyi) and to deplete the cells using surface markers specific for undifferentiated iPSCs (Tra-1-60 / Tra-1-81).

[0060] As used herein, the term “subject” includes both human and animal subjects. Thus, veterinary therapeutic uses are provided in accordance with the presently disclosed subject matter. As such, the presently-disclosed subject matter provides for the treatment of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; and horses. Also provided is the treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, i.e., poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economic importance to humans. Thus, also provided is the treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses (including race horses), poultry, and the like.

[0061] The practice of the presently-disclosed subject matter can employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning A Laboratory Manual* (1989), 2nd Ed., ed. by Sambrook, Fritsch and Maniatis, eds., Cold Spring Harbor Laboratory Press, Chapters 16 and 17; U.S. Pat. No. 4,683,195; *DNA Cloning*, Volumes I and II, Glover, ed., 1985; *Oligonucleotide Synthesis*, M. J. Gait, ed., 1984; *Nucleic Acid Hybridization*, D. Hames & S. J. Higgins, eds., 1984; *Transcription and Translation*, B. D. Hames & S. J. Higgins, eds., 1984; *Culture Of Animal Cells*, R. I. Freshney, Alan R. Liss, Inc., 1987; *Immobilized Cells And Enzymes*, IRL Press, 1986; Perbal (1984), *A Practical Guide To Molecular Cloning*; See *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells*, J. H. Miller and M. P. Calos, eds., Cold Spring Harbor Laboratory, 1987; *Methods In Enzymology*, Vols. 154 and 155, Wu et al., eds., Academic Press Inc., N.Y.; *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987; *Handbook Of Experimental Immunology*, Volumes I-IV, D. M. Weir and C. C. Blackwell, eds., 1986.

[0062] The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples.

EXAMPLES

[0063] EXAMPLE 1

[0064] The production of engineered cardiac tissues was undertaken and the resulting structures were called “engineered cardiac tissues” or “ECTs”. The unique composition that was produced and utilized had the following features: 1) use of a cellular composition of human iPSC-derived cardiac cells that contain multiple cell lineages (cardiomyocytes, endothelial cells, mural cells, fibroblasts); and 2) revision of single strand, linear ECTs, which are 1 mm in diameter by 15 mm in length, into “large format – LF” porous structures that were 15 mm by 15 mm in diameter. That larger format engineered tissue was more suitable for implantation onto the surface of the human heart. In this regard, during the course of development, the research explored multiple variations on the design of the LF-ECT by varying the size and shape of the architecture within the ECT (**FIG. 1**). In some instances, it was found that, after removal from the indicated tray on day 10, the Mesh LF-ECT (**FIG. 1**, middle image) had beneficial structural and functional properties to generate working replacement heart muscle.

[0065] During the development, it was found that the features of the LF-ECT included optimal cell survival, accelerated maturation measured by gel compaction at day 14, increased force generation at day 14, and increased survival of cardiomyocytes on the surface of the LF-ECT structures. Additional experiments were also completed to determine the optimal cell number and density of hiPSC-derived cardiac cells in the Mesh LF-ECT (6, 9, or 12M cells per construct) and it was found that, in some instance, 6M cells per construct provided optimal cell survival and functional properties, as shown in **FIG. 2**.

[0066] Data further showed the survival of implanted Mesh LF-ECT onto the surface of immune tolerant (nude) rats with evidence of the survival of donor LF-ECT hiPSC-cardiomyocytes and vascular cells on the surface of the recipient hearts at 4 weeks, as shown in **FIG. 3**.

[0067] A larger version of the LF-ECT designed to be approximately 40 mm in diameter was also generated for implantation onto the human heart. This XLF-ECT has a similar geometric design and cellular composition to the Mesh LF-ECT, as shown in **FIG. 4**.

[0068] During the experiments, it was further confirmed that the hiPSC LF-ECTs including vascular cells along with cardiomyocytes possess higher electrical potential which can capture higher beating rate with lower excitation threshold.

[0069] **EXAMPLE 2**

[0070] Material and Methods

[0071] *Human iPSC Culture and Differentiation.*

[0072] Human iPSCs [4-factor (Oct3/4, Sox2, Klf4 and c-Myc) line: 201B6 and culture methods were used. In brief, these cells were adapted and maintained on thin-coat Matrigel (growth factor reduced, 1:60 dilution; BD Biosciences, San Jose, USA) in mouse embryonic fibroblast conditioned medium (MEF-CM) supplemented with 4 ng/mL human basic fibroblast growth factor (hbFGF; WAKO, Osaka, Japan). Cells were passaged every four or five days using CTK solution [0.1% collagenase IV, 0.25% Trypsin, 20% knockout serum replacement (KSR), and 1 mM CaCl₂ in phosphate buffered saline (PBS)].

[0073] Cardiovascular (CV) cell differentiation was induced as previously reported (**FIGS. 19A-19B**). Cells were detached following a 4 to 7 minutes incubation with Versene (0.48 mM EDTA solution; Life Technologies, Carlsbad, USA) treatment and seeded onto Matrigel-coated plates at a density of 1,000 cells/mm² in MEF-CM with 4 ng/mL bFGF for 2 to 3 days before induction. Cells were covered with Matrigel (1:60 dilution) on the day before induction. To induce CV cell population, MEF-CM was replaced with RPMI+B27 medium (RPMI1640; Life Technologies, 2mM L-glutamine; Life Technologies, 1× B27 supplement without insulin; Life Technologies) supplemented with 100 ng/mL of Activin A (R&D, Minneapolis, USA) and 100 ng/mL of Wnt3a (R&D) for 24 hours (differentiation day 0; d0), followed by 10 ng/mL human bone morphogenetic protein 4 (BMP4; R&D) and 10 ng/mL hbFGF (d1) for 2 or 4 days without culture medium change. For induction of CM and EC (CM+EC protocol), the culture medium was replaced at d5 with RPMI+B27 supplemented with VEGF165 (Miltenyi, Bergisch Gladbach, Germany), and culture medium was refreshed every other day. Beating cells appeared at d11 to

13. For induction of MC (MC protocol), the culture medium was replaced at d3 with RPMI+10% FBS medium [RPMI1640, 2mM L-glutamine, 10% fetal bovine serum (FBS)], and culture medium was refreshed every other day (**FIGS. 19A-19B**).

[0074] *Flow Cytometry*

[0075] HiPSC-derived CV cells were dissociated by incubation with Accumax (Innovative Cell Technologies, San Diego, CA) and stained with one or a combination of the following surface markers: anti-VCAM1 conjugated with allophyco-cyanin (APC), clone STA, 1:200 (BioLegend, San Diego, CA); anti-PDGFR β conjugated with phycoerythrin (PE), clone 28d4, 1:100 (BD, Franklin Lakes, NJ); anti-VE-cadherin conjugated with fluorescein isothiocyanate (FITC), clone 55-7h1, 1:100 (BD); anti-TRA-1-60 conjugated with FITC, clone TRA-1-60, 1:20 (BD). To eliminate dead cells, cells were stained with the LIVE/DEAD fixable Aqua dead cell staining kit (Life Technologies). For cell surface markers, staining was carried out in PBS with 5% FBS. For intracellular proteins, staining was carried out on cells fixed with 4% paraformaldehyde (PFA) in PBS. Cells were stained with the anti-cardiac isoform of Troponin T (cTnT) (clone 13211, Thermo Fisher Scientific, Waltham, MA) labeled with Alexa-488 using Zenon technology (Life Technologies) (1:50). The staining was performed in PBS with 5% FBS and 0.75% Saponin (Sigma, St. Louis, MO). Stained cells were analyzed on an LSRII flow cytometer (BD). Data were collected from at least 5,000 events. Data were analyzed with DIVA software version 6.1.3 (BD).

Group	n	CM (%)	EC (%)	MC (%)	UD (%)
6M	12	45.3 $\pm$ 1.6	19.1 $\pm$ 1.2	11.2 $\pm$ 0.6	5.1 $\pm$ 0.3
9M	7	44.3 $\pm$ 1.2	17.3 $\pm$ 1.6	11.5 $\pm$ 0.8	5.1 $\pm$ 0.4
12M	12	43.5 $\pm$ 1.7	17.6 $\pm$ 1.9	12.3 $\pm$ 1.8	5.4 $\pm$ 0.3
12MH	6	43.9 $\pm$ 1.5	20.7 $\pm$ 2.2	11.3 $\pm$ 0.8	4.6 $\pm$ 0.4

Table 1: Initial cell compositions to investigate cell number and density. Percentage of cTnT, cardiac troponin-T (cTnT)⁺ cardiomyocytes (CM), vascular endothelial (VE)-cadherin

(CD144)⁺ endothelial cells (EC), platelet-derived growth factor receptor beta (PDGFR β ; CD140b)⁺ vascular mural cells (MC), TRA-1-60⁺ undifferentiated iPS cells (UD) prior to generating ECTs quantified by flowcytometry. Data are expressed as the mean $\pm$ SEM.

[0076] *Tissue Mold Fabrication*

[0077] Tissue molds were fabricated from polydimethylsiloxane (PDMS) (**FIG. 5B**). A thin (0.5 mm) layer of PDMS (Sylgard 184, Dow Corning) was cast by mixing the prepolymer and cross-linking solution at a ratio of 10:1 and allowed to cure at 80°C for three hours. The sheet was cut and bonded with silicone adhesive to fabricate tissue trays according to design drawings. Tissue molds were 21 mm x 20.5 mm in outer diameter and had 0.5 mm wide and 2.5 mm high PDMS rectangular posts with three different patterns: PL7, PL16, and PL0 (**FIG. 5C**). PL7 had 7 mm long posts at a staggered position and PL16 had parallel 16 mm long posts. PL0 had 1 mm long pins at the periphery and no posts in the middle. Horizontal post spacing between two lines of posts was 2.5 mm (**FIG. 5B**). For the generation of an extra-large format ECT (XLF-ECT, **FIG. 11K**), a 39 mm x 40.5 mm mold with PL7 patterned posts was also fabricated. Molds were autoclaved and coated with 1% Pluronic F127 [Pluronic® F-127 10% Solution (Molecular Probes) diluted with PBS] for one hour. Before tissue moulding, Pluronic F127 was removed, and the mold was rinsed with PBS sufficiently.

[0078] *LF-ECT fabrication*

[0079] Differentiated cells from CM+EC and MC protocols were combined so that the final concentration of MCs became 10 to 20%. Combined cells were mixed with acid-soluble rat-tail collagen type I (Sigma) and matrix factors (Matrigel; BD Biosciences). Cell/matrix mixture was performed as follows. For a 6M construct fabrication: (1) Six million cells were suspended in a culture medium (high glucose-modified Dulbecco's essential medium; Life Technologies) containing 20% fetal bovine serum (Life Technologies); (2) Acid-soluble collagen type I solution (pH 3) was neutralised with alkali buffer (0.2M NaHCO₃, 0.2M HEPES, and 0.1M NaOH) on ice; (3) Matrigel (15% of total volume) was added to the neutralised collagen solution; and (4) Cell suspension and matrix solution were mixed. The final concentration of collagen type I was 0.67 mg/mL in a total volume of 400 μ L (**FIG. 5A**).

[0080] The cell/matrix mixture was poured onto the Pluronic F127 coated PDMS tissue mold, which was placed in a usual six-well culture plate, and polymerised in a standard CO₂ incubator (37°C, 5% CO₂) for 60 minutes. When the tissue was formed, the tissue mold was soaked with pre-culture medium [α minimum essential medium (α MEM; Life Technologies) supplemented with 10% FBS, 5 $\times$ 10⁻⁵M 2-mercaptoethanol (Sigma) and 100U/mL Penicillin-Streptomycin (Life Technologies)]. Constructed ECTs were cultured for 14 days with medium change every day and analyzed at day14. A gel solution of 9 million cells, 600 μ L or 12 million cells, 800 μ L was prepared for the fabrication of 9M or 12M construct. Furthermore, a 12 million cell, 400 μ L cell/matrix mixture, which has two times higher cell density than others, was prepared for 12MH construct (**FIG. 7A**). For an XLF-ECT, a cell/matrix mixture of 24 million cells in 1600 μ L matrix was used.

[0081] *Live/Dead Assay*

[0082] ECTs were incubated with staining solution (50 mL/L Ethidium Homodimer III and 50 mL/L Hoechst 33342, PromoCell GmbH, Heidelberg, Germany) in a pH-adjusted buffer for 60 minutes at room temperature and protected from light. Fluorescent images were obtained using an Olympus DP72 optical microscope (Olympus, Tokyo, Japan).

[0083] *Contractile Force Measurement*

[0084] For contractile force measurements, a strip with the length of approximately 15 mm was cut off from an ECT (**FIGS. 21A-21C**), comparable to the length of linear ECTs. As previously described, the strip was preserved in cold (25°C) Tyrode solution containing (in mM) 119.8 NaCl, 5.4 KCl, 2.5 CaCl₂, 1.05 MgCl₂, 22.6 NaHCO₃, 0.42 NaH₂PO₄, 0.05 Na₂EDTA, 0.28 ascorbic acid, 5.0 glucose, and 30 2,3-butanedione monoxime (BDM) gassed with 95% O₂ and 5% CO₂. One end of the specimen was gently attached to a force transducer (model 403A, Aurora Scientific, Ontario, Canada) and the other end to a high-speed length controller (model 322C, Aurora Scientific) mounted on a micromanipulator using 10-0 nylon threads. The perfusion chamber containing the construct was then filled with BDM-free warmed Tyrode solution (37°C, 1 ml total volume). During a 20 minute equilibration period, the construct was field-stimulated (2Hz / 5V). The segment length of the tissue was gradually increased until total force reached maximum (*L_{max}*). According to the 3D reconstructed confocal images of whole tissues, each bundle of an ME or ML-ECT could be assumed to have an elliptical cylindrical

shape. The minor to major axis ratio of the representative cross-section was 0.65 in average, and there was no significant difference among all groups. The cross-sectional area (CSA, mm²) was estimated from the mean bundle width while strained on the tissue mold and the axis ratio. Additional measurements included 1) active force under 1.5-4.5 Hz and 5 V pacing at slack length to *L_{max}*, and 2) maximum capture rate (without capturing failure for 10 seconds) under 5 V at *L_{max}*. Active stress (mN/mm²) was calculated by dividing the value of active force by CSA.

[0085] *Histology*

[0086] For whole mount tissue staining, ECTs were fixed in 4% PFA for 30 minutes at room temperature (RT) and washed in 1% Triton-X-100/PBS for 1 hour at RT, followed by blocking with 1% Triton-X-100/PBS +10% FBS for 1 hour at RT. ECTs were incubated with primary antibody Troponin T (ab45932) 1:300 or CD31 in 1% Triton-X-100/PBS+ 10% FBS+0.02% sodium azide overnight at 4°C. On the second day, after washed with 1% Triton-X-100/PBS+ 10% FBS, ECTs were incubated with donkey anti-rabbit IgG secondary antibody then Alexa Fluor 488 conjugated overnight at 4°C. After stained with DAPI for 30 minutes, ECTs were incubated with 100% glycerol overnight, 75% glycerol 2 hours, clearing solution 2 hours and changed to new clearing solution overnight. All the clearing procedures were performed at room temperature in the dark.

[0087] Hearts were arrested in diastole using 200 µl of chilled 3M KCl and 0.1 M CdCl₂ solution followed by 4% PFA (perfusion fixation), embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan) and frozen. Sequential sections from hearts (8 µm thickness) were prepared and stained with Masson-trichrome. For immunofluorescence staining, sections were treated with 0.01 mol/L sodium citrate buffer (pH 6.0) at 100°C for 15 minutes and incubated with primary antibodies overnight at 4°C; Alexa Fluor 488/594 conjugated donkey anti-mouse, donkey anti-rabbit and donkey anti-rat (1:100 to 1:400, Life Technologies) were used as secondary antibodies. Engrafted human cells in ECTs were detected by human nuclear antibody (HNA) (mouse monoclonal, clone 235-1; Millipore, Billerica, MA; 1:300). Anti-cTnT antibody (rabbit polyclonal; Abcam, Cambridge, UK; 1:400 or mouse monoclonal; clone 13211, Thermo Fisher Scientific; 1:400) was used for double staining with cTnT and HNA. The following antibodies were used for primary antibodies: anti-von Willebrand factor (vWF) (DAKO, Carpinteria, CA;

1:50); ant-NG2 (rabbit polyclonal; Abcam, Cambridge, UK; 1:100). Nuclei were visualised with DAPI (4, 6 diamidino -2-phenylindole; Life Technologies). For visualisation of perfused vasculature within ECTs after implantation, 10 µg/g of DyLight 488 Labelled Lycopersicon Esculentum (Tomato) Lectin (Vector Laboratories, Burlingame, CA) was administrated from right jugular vein under general anaesthesia and mandatory respiration. After 20 min of systemic perfusion, the rat was euthanised. The heart was freshly harvested and embedded in OCT compound and frozen for sectioning. All immunostained sections were photographed with Nikon ECLIPSE Ti Confocal System (Nikon, Tokyo, Japan) attached to a Nikon Ti-E inverted microscope platform; images were captured at 1024×1024 pixel density with 1.4 NA using Nikon NIS Elements AR software (Nikon) to save as 12-bit raw files for further processing.

[0088] *Cardiomyocyte Alignment Analysis*

[0089] Alignment of CM in ECT bundles was quantified from 3D confocal stacks of whole-mount cTnT immunostained samples. For each ECT group (ME, PL, PS, 6M ME, 9M ME, 12M ME, 12MH ME), 4 to 6 images were analyzed, where the mean image size was 1.3 x 1.7 x 0.45 mm. Attenuation correction was applied followed by a low pass Gaussian filter (standard deviation of 3 µm) prior to any analysis (ImageJ, NIH, Bethesda, MD). The cTnT and DAPI fields were merged in to a single grayscale image and manually segmented the whole ECT bundle with Seg3D (Salt Lake City, UT). The centerline (medial axis) of the ECT bundle was extracted from the distance transform of the segmented image and used to form an ECT-based coordinate system, similar to cylindrical coordinates. Local CM orientations were calculated from the intensity gradient of the cTnT image and mean orientation and alignment were quantified using spherical statistics. All subsequent analysis was implemented in Matlab (Mathworks, Natick, MA).

[0090] For a given cTnT image I , the intensity gradient vector at each voxel, $\mathbf{G} = [G_x, G_y, G_z]^T$, was calculated by convolution with kernel h . We used a σ value of 5 µm for this study. The kernel size (s) was chosen such that the minimum value of h was 0.01, so that $-s \leq x, y, z \leq s$.

$$G_x = h_x * I, \quad G_y = h_y * I, \quad G_z = h_z * I \quad (1)$$

$$h_i(x, y, z) = \frac{2i}{\sigma^2} \exp\left(-\left(x^2 + y^2 + z^2\right)/\sigma^2\right), \quad i \in \{x, y, z\} \quad (2)$$

[0091] To calculate local CM orientation, the image was divided into subregions of size $m \times m$. All of the gradient vectors within a subregion ($\mathbf{G}^m$) were collected and treated as a girdle distribution. A girdle distribution of axes is concentrated around a great circle; it is an equatorial distribution. The polar axis of a girdle distribution is the vector normal to the equatorial plane. As image gradient vectors are normal to the surfaces of objects, the polar axis is thus the dominant orientation of objects within the local window. The polar axis is the eigenvector associated with the smallest eigenvalue of the orientation matrix $\mathbf{T}^{12}$.

$$\mathbf{T} = \begin{bmatrix} \sum \mathbf{G}_x^m \mathbf{G}_x^m & \sum \mathbf{G}_x^m \mathbf{G}_y^m & \sum \mathbf{G}_x^m \mathbf{G}_z^m \\ \sum \mathbf{G}_y^m \mathbf{G}_x^m & \sum \mathbf{G}_y^m \mathbf{G}_y^m & \sum \mathbf{G}_y^m \mathbf{G}_z^m \\ \sum \mathbf{G}_z^m \mathbf{G}_x^m & \sum \mathbf{G}_z^m \mathbf{G}_y^m & \sum \mathbf{G}_z^m \mathbf{G}_z^m \end{bmatrix} \quad (3)$$

[0092] The selection of the window size m defines “local”. Determining the window size requires some information about the size and shape of CM. A minimum window size was first defined, 30 μm in this study, based on the approximate average width of CM. The image was divided into subregions of this size and any subregion where the overall mean intensity was less than 5 times the global mean intensity of the entire image was rejected. For each of the remaining initial subregions, the orientation matrix eigenvalues ($\lambda_1 \leq \lambda_2 \leq \lambda_3$) and corresponding eigenvectors ($\mathbf{e}_1, \mathbf{e}_2, \mathbf{e}_3$) for window sizes between 30 μm and one-fourth the smallest image dimension, where the window center remained fixed and we increased the window size in 10 μm increments, were computed. The shape parameter (γ) was computed from the eigenvalues and selected the window size associated with the smallest γ value. Small γ values indicate blob or rod shaped objects (likely CM) as opposed to plate-like objects. The dominant local CM orientation for the window was then defined as the polar axis of the gradient vectors. For ME and ML ECTs, local CM orientations were converted from Cartesian to ECT coordinates based on the bundle centerline. The ECT coordinate was used for all further analysis.

$$\gamma = \frac{\ln(\lambda_3/\lambda_2)}{\ln(\lambda_2/\lambda_1)} \quad (4)$$

[0093] Once all of the local CM orientations were computed, the set was treated as a Watson bipolar distribution. A Watson bipolar distribution is used for axial data, where the positive and

negative directions of a vector are considered the same. The principal axis was computed as the eigenvector associated with the largest eigenvalue of the orientation matrix $\mathbf{T}$. The alignment of CM was defined as the concentration of the distribution (κ), computed from eq. (5), where λ_3 is the largest eigenvalue of $\mathbf{T}$ and N is the total number of orientations. A larger κ value indicates that the orientations are more concentrated along the principal axis. Concentrations were compared across ECT types using ANOVA to identify significant differences in CM alignment.

$$\lambda_3/N = \int_0^1 x^2 \exp(\kappa x^2) dx / \int_0^1 \exp(\kappa x^2) dx \quad (5)$$

[0094] *ECT cellular quantification*

[0095] The CM or EC percentage in ECT bundles was quantified from 40X confocal images of DAPI and cTnT or CD31 stained tissue samples. We preprocessed DAPI images with a rolling ball background subtraction and cTnT/CD31 images with contrast-limited adaptive histogram equalization. DAPI and cTnT/CD31 images were then segmented using the Chan-Vese level set method. After DAPI segmentation, nuclei were separated using a watershed and vertex graph algorithm. For each nucleus, CM or EC identity were determined based on the overlap between the nucleus boundary and the segmented cTnT image.

[0096] *RNA extraction and quantitative reverse-transcription polymerase chain reaction (qPCR).*

[0097] As previously described, fresh ECT samples were homogenized by an Omnitip Tissue homogenizer (USA Scientific, Ocala, USA; Cat. No. 6615-7273). Total RNAs were isolated using RNeasy Mini Kit (Qiagen, Valencia, USA; Cat. No. 74104) according to the manufacturer's instructions. RNA quality and quantity were measured using the NanoDrop ND-2000 (Thermo Fisher Scientific). Reverse transcription was performed with the SuperScript VILO cDNA synthesis system (Invitrogen). TaqMan Gene Expression Assays (Best coverage and dose not detect gDNA) ordered from Thermo Fisher Scientific. qPCR was performed with StepOnePlus Real-time PCR system (Applied Biosystems). All qPCR were performed with biological triplicates for each group and 18S rRNA as endogenous control.

[0098] *Animal Model Preparation and ECT Implantation*

[0099] All animal surgeries were performed following protocols approved by the University of Louisville Institutional Animal Care and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute for Laboratory Animal Research, U.S.A. (8th ed., 2011). Male athymic nude rats (NTac: NIH-Foxn1^{tmu}, Taconic Biosciences, Hudson, USA) weighing 270-340g were used as recipients for surgeries.

[00100] One-half of ME-ECT was implanted in a normal nude rat to confirm the engraftment of the ECT on a rat heart through left thoracotomy. An ME-ECT was then implanted in a myocardial infarction (MI) model rat (**FIG. 9A**). MI was induced by permanent left anterior descending artery ligation using 7-0 silk suture. Isoflurane (3-5%) inhalation was used for general anaesthesia, and subcutaneous or intraperitoneal injection of Buprenorphine (0.5mg/kg, twice a day, three days including operation day) was used for analgesia. ECT implantation was performed one week after MI induction during the “subacute phase” of MI. Left ventricular ejection fraction (EF) was evaluated six days after coronary artery ligation by echocardiography. Rats whose hearts showed more than 60% EF or preserved more than 80% of initial EF were excluded from subsequent experiments.

[00101] A total of 10 rats were randomly divided into two groups: rats implanted with ECTs (Tx group; n=5) and sham-operated rats (sham group; n=5). The LV anterior wall was exposed through redo-left thoracotomy. Using 7-0 silk sutures, the anterior infarcted myocardium was covered with one whole ME-ECT, folded in half, along the LV circumferential direction. For the sham-operated group, a thoracotomy was performed one week after coronary ligation; however, no ECT implantation was performed (**FIG. 8A**). Hearts were harvested 4 weeks after treatment or sham operation and prepared for Masson’s trichrome and immunohistochemistry.

[00102] *Cardiac Functional Assessment*

[00103] Transthoracic echocardiography was performed by an investigator blinded to group assignment using a high resolution Vevo2100 system (VisualSonics, Toronto, Canada) and 21-MHz imaging transducer (MS250; VisualSonics). Evaluations were performed before MI, ECT implantation (six days after MI induction), and two and four weeks after implantation. Ejection fraction was calculated by single plane area-length method. Regional wall motion was traced, calculated and visualised using the VevoStrain application (VisualSonics).

[00104] *Histologic Measurement of Left Ventricular Remodeling*

[00105] To quantify the LV remodelling after MI with or without ECT implantation, five 8µm frozen sections with 50µm interval were stained with Masson's trichrome. Sections were imaged under Olympus DP72 optical microscope (Olympus, Tokyo, Japan) and analysed using NIH ImageJ. Just like Tang's report, a series of morphometric parameters were measured in each section including total LV area, scar area, risk region area, LV wall thickness in the risk and non-infarcted regions, and LV expansion index. Risk area was defined as the LV area between the two edges of the infarct scar. Wall thickness was the average of 5 measurements equally distributed within risk and non-risk area. The LV expansion index was calculated from LV circumference and wall thickness to evaluate both LV dilation and wall thinning simultaneously: Expansion Index = (endocardial circumference/epicardial circumference) x (noninfarcted region wall thickness/risk region wall thickness).

[00106] *Statistical Analysis*

[00107] The data were analysed using JMP software for Windows (version 10.0.2, SAS Institute Inc., Cary, NC). Results are presented as mean ± standard error of the mean (SEM). Comparisons between two groups were made with the unpaired t-test unless otherwise noted. Mann-Whitney U test was used for non-normal distributions. Comparisons between more than 2 groups were made with one-way or two-way repeated analysis of variance (ANOVA) followed by Tukey's test as post hoc. A p-value of less than 0.05 was considered significant.

[00108] Results

[00109] Multiple CV cell lineages were induced from hiPSCs to generate LF-ECTs using the lineage distribution shown to generate an optimal linear hiPSC-derived ECTs (**FIG. 5A**, **FIGS. 19A-19B**). Two distinct CV cell differentiation protocols were employed to generate either predominantly cardiac troponin-T (cTnT)⁺ CM and vascular endothelial (VE)-cadherin (CD144)⁺ endothelial cells (ECs) or to generate predominantly platelet-derived growth factor receptor beta (PDGFRβ; CD140b)⁺ vascular mural cells (MCs). Induced CV cells from these two protocols were then mixed to adjust final EC and MC concentrations to represent 10 to 20% of total cells to facilitate the *in vitro* expansion of vascular cells within ECTs and subsequent *in vivo* vascular coupling between ECTs and recipient myocardium (**FIG. 5A**). The calculated

composition of CMs, ECs, and MCs for ECT preparation was $44.2 \pm 0.6\%$, $15.9 \pm 0.5\%$, and $13.0 \pm 0.4\%$, respectively (n=107 constructs).

[00110] LF-ECTs composed of CM+EC+MC cells harvested on d15, collagen I, and Matrigel were generated. In order to investigate the relationship between LF-ECT geometry and LF-ECT structural and functional maturation, tissue molds with distinct patterns characterized by various post lengths and spacing were fabricated (**FIG. 5B**; **FIG. 20**). Three general categories of tissue molds were prepared: 1) molds with 7mm post lengths (PL), arrayed at a staggered position to facilitate formation of a mesh structure with bundles and junctions (PL7, ME-ECT); 2) molds with multiple 16mm long posts to support formation of parallel linear bundles without junctions (PL16, ML-ECT); and 3) molds with peripheral anchors but no internal posts to generate a plain sheet without central pores (PL0, PS-ECT) (**FIG. 5C**). During preliminary experiments, ECTs adhered to the PDMS posts during gel compaction. To prevent this cell adhesion, the protocol was modified to coat each mold with Pluronic F127 prior to use.

[00111] Similar to linear ECTs, each construct exhibited rapid gel compaction and acquired characteristic geometries dependent on the mold design (**FIGS. 5C-D**). Those structures were maintained even after release from tissue molds (**FIG. 5C**). All constructs started intrinsic spontaneous beating *in vitro* within 3 days and then continued beating throughout the duration of culture. ECT cross-sectional surface area was measured after ECT formation on day 0 and at intervals for two weeks of culture to calculate gel compaction during tissue maturation. Following gel compaction, d14 ME-ECTs and ML-ECTs tissue areas were comparable and significantly less than d14 PS-ECTs [$89.5 \pm 3.1 \text{ mm}^2$ (n=10) and $75.6 \pm 1.6 \text{ mm}^2$ (n=6), versus $165.9 \pm 11.0 \text{ mm}^2$ (n=8), respectively, ($P < 0.001$, **FIG. 5E**)]. The mean bundle widths in ME-ECTs and ML-ECTs were comparable [$0.49 \pm 0.08 \text{ mm}$ (n=10) and $0.49 \pm 0.04 \text{ mm}$ (n=6), **FIG. 5F**], however, ML-ECTs showed greater variation in bundle width likely due to substantially longer bundle lengths ($P < 0.001$, Mann-Whitney U test, **FIG. 5G**). Additionally, the impact of initial geometry on d14 cell viability was determined using Live/Dead assay (**FIG. 5H**), which showed that cell viability was greatest in ME-ECTs and lowest in PS-ECTs (**FIG. 5I**), indicating that both the presence of pores and ME geometry facilitated *in vitro* LF-ECT cell survival.

[00112] Next, the relationship between LF-ECT geometry and 3D CM alignment was determined using whole mount confocal imaging and a custom 3D cell orientation analysis program. Sequential cardiac troponin-T (cTnT) immunostained 2D confocal microscopic image stacks were reconstructed and then myofiber orientation was calculated within each geometric class of LF-ECT (**FIGS. 6A-C**). ME-ECTs showed higher values for the concentration parameter (κ) versus PS-ECTs ($P < 0.05$), representing greater myofiber alignment relative to the local bundle long axis (**FIG. 6D**). The impact of LF-ECT geometry on electromechanical properties was then explored using a custom intact muscle test system. Maximum paced capture rates and excitation threshold voltages were similar among three groups (**FIGS. 6E-F**), however, active stress at 2Hz/5V pacing in ME-ECTs and ML-ECTs was higher than in PS-ECTs ($P < 0.05$, **FIG. 6G**). This was consistent with greater cell viability and increased alignment versus PS-ECTs. Of note, maximum capture rates, excitation threshold voltages, and active stress for ME-ECTs and ML-ECTs were similar to comparable functional measures in hiPSC-derived (CM+EC+MC) linear ECTs in the instant inventors' previous study, consistent with comparable lineage compositions.

[00113] Once the structural and functional advantage of the ME-ECT geometry were determined, the impact of altered initial cell seeding number and seeding density on cell viability and *in vitro* LF-ECT structural and functional maturation were explored. ME-ECTs were generated with increasing initial cell numbers [6×10^6 (6M), 9×10^6 (9M), or 12×10^6 (12M) cells] by using a standard cell density of 15×10^6 cells/ml and increased initial cell/matrix volume from 400 μ l to 600 μ l or 800 μ l and a fourth group was generated with 2-fold higher cell density [12×10^6 cells at 30×10^6 cells/ml (12MH)] in 400 μ l (12MH, **FIG. 7A**). In the standard density groups, 9M and 12M ME-ECTs exhibited wider bundles than 6M ECTs and final bundle width positively correlated with initial seeding cell number and gel volume ($P < 0.001$, **FIG. 7B**). The 12MH ME-ECTs also formed wider bundles at day 14 compared to the standard 6M ME-ECTs despite the same initial gel volume, consistent with higher cell death and reduced gel compaction, though the difference was not statistically significant.

[00114] Based on 3D histology, 9M and 12M ME-ECTs showed more sparsely distributed CMs, mainly located along the ME-ECT surfaces, compared to 6M ECTs (**FIG. 7C**). The relationship between initial cell number and density on cell survival was also assessed for each group (**FIG. 7D**). The percentage of dead cells on d14 in 9M was more than three-fold greater

than in 6M ($P<0.01$) with even higher dead cell percentages for the 12M and 12MH groups, supporting the original 6M maximal seeding number and density ($P<0.001$) as optimal for the ME-ECT geometry. Consistent with the lower cell death of 6M ME-ECTs, the highest active stress was noted in 6M ME-ECTs versus 9M, 12M ($P<0.001$), and 12MH ($P<0.01$, **FIG. 7E**). Somewhat surprisingly, increasing cell density from 15×10^6 to 30×10^6 cells/ml produced lower active stress, suggesting that nutrient availability within the gel may impact cell survival and function.

[00115] The impact of prolonged 28-day *in vitro* culture on 6M ME-ECT maturation was then investigated. The 28-day ME-ECT constructs showed significantly more rapid contraction and relaxation and captured higher pacing frequency versus 14-day constructs (**FIGS. 8A-C**) and average maximum capture rate increased to 6.9 Hz (**FIG. 8D**). Active stress force-frequency relations (FFR) were determined up to 4.5 Hz (270 bpm) while 14-day constructs displayed a progressively negative FFR. On the contrary, 28-day constructs displayed a positive FFR analogous to Bowditch effect in native human myocardium (**FIG. 7E**). According to myofiber alignment analysis, 28-day constructs showed greater alignment than 14-day constructs (**FIG. 7F**).

[00116] To further understand the mechanism of functional maturation during prolonged *in vitro* LF-ECT culture, gene expression analysis (qPCR) was performed for selected CM genes relevant to CM structure and function. The expression level of cTnT in 28-day constructs was significantly lower than in 14-day ones (**FIG. 8G**). Additionally, the percentage of CMs among all cells in cTnT and DAPI stained images of ECTs was quantified, which showed a reduced volume fraction of CM in 28-day constructs compared to 14-day constructs [30.9 ± 6.2 (n=4) versus 56.8 ± 14 % (n=3), $p=0.11$ (**FIGS. 22A-22C**)]. Therefore, the lower expression level of cTnT transcripts is consistent with an attrition in the number of CMs with prolonged *in vitro* culture. To compare the characteristics of individual CMs in ECTs, other genes were examined and their values were normalized to cTnT expression level of each group. 28-day constructs showed a significantly greater ratio of MLC2v and MLC2a, indicating a predominance of maturing ventricular CM (**FIG. 8G**). The increase of multiple transcripts consistent with CM maturation was also noted, including KCNJ2 and KCND3, related to inward rectifier potassium current (I_{K1}) and transient outward potassium current (I_{to}), respectively. Along with those

advanced ion channel functions, increased expression of GJA1 (coding connexin43) is consistent with the rapid contraction and relaxation of the d28 LF-ECTs (**FIG. 8H**).

[00117] To develop the LF-ECT surgical implant method, one-half of an ME-ECT was initially implanted onto an uninjured immune tolerant rat heart similar to the inventors' approach for linear ECTs. Histology was performed four weeks after the implantation. ME-ECT engraftment and vascular coupling between the recipient myocardium and implanted ECT grafts was confirmed using host venous injection of fluorescent dye-conjugated lectin to identify perfused vessels within the engrafted tissue (**FIGS. 23A-23B**). The *in vivo* potential of LF-ECT implantation to recover cardiac structure and function following myocardial infarction was then determined in an immune tolerant rat MI model (**FIG. 9A**). A total of 10 rats were randomly divided into two groups: rats implanted with ECTs (n=5) and sham-operated controls (n=5). A whole ECT, folded in half, was implanted over the anterolateral left ventricular (LV) surface in the treated group (**FIG. 9B**). All implanted and sham-operated rats survived the 4-week post-implant observation period with no tumor formation. Although diastolic LV area increased in both groups (**FIG. 9C**), ECT implantation improved ejection fraction (**FIG. 9D**), cardiac output (**FIG. 9E**), and regional LV radial and longitudinal strain at 4 weeks (**FIGS. 9F-G**). Both increased radial and longitudinal strain were noted at the infarct region following LF-ECT implantation as well as a compensatory increased anterior mid- and base-longitudinal strain in the non-infarcted regions in sham-operated animals.

[00118] Consistent with functional recovery, LF-ECT implantation reduced histologic measures of scar size (**FIGS. 10A-C**) with a trend to reduce risk area (**FIG. 10D**), and increased viable myocardium and mean LV wall thickness in the risk area (**FIGS. 10D-F**). The lower expansion index in implanted rats also indicated reduced post-MI LV remodeling (**FIG. 10G**). All implanted rats exhibited ECT engraftment (n=5), and the retention of grafted hiPSC-derived cTnT⁺ CMs with sarcomeric structures (**FIGS. 11A-F**) and NG2⁺ mural cells (**FIGS. 11G-H**) was confirmed. Interestingly a small number of hiPSC-derived cells that costained for cTnT and NG2 was noted, consistent with expression of NG2 by immature cardiomyocytes or the less likely expression of cTnT by human cardiac pericytes. In addition, the distribution of vWF⁺ endothelial cells (**FIGS. 11I-J**) inside the ECT was confirmed. Most vWF⁺ endothelial cells were HNA negative, indicating the invasion of host-derived vasculature into the graft. LF-ECT implantation also resulted in vascular perfusion of the implanted construct as evidenced by the

presence of lectin staining using an intravenous injection of fluorescent lectin that was subsequently visualized within implanted LF-ECTs using histology (**FIGS. 24A-24B**).

[00119] Finally, as a proof of feasibility for scaling up the LF-ECT method for large animal pre-clinical studies a 3 cm final width extra-large format ME-ECTs (XLF-ECT) was generated from a 4 cm square wide mold based on the PL7 design and containing 24 million cells (**FIGS. 11K-L**). Similar to the original ME-ECTs, the spontaneously beating XLF-ECTs were easily collected from the mold without damaging the ECT, indicating an excellent potential for scalability of the ME-ECT format.

[00120] Discussion

[00121] Multiple formations (cell species, lineage, matrix composition, geometry) of ECTs have been reported, and these microtissues are suitable for *in vitro* drug toxicity screening and disease modelling. Several have been used as implantable grafts in rodents, though they are too small for larger animal trials or clinical translation. The instant example describes the successful incorporation, survival, and functional maturation of hiPSC-derived cardiac and vascular cells in a scalable, porous LF-ECT suitable for *in vivo* translation. Several technical aspects of this approach are worth noting. Thin PDMS sheets were used to design the 3D molds used to generate LF-ECTs, and once coated with Pluronic F127, the LF-ECTs remained detached from the adjacent posts and the bottom during *in vitro* maturation, facilitating gel compaction and final removal from the mold. Comparison of 3 distinct geometries, all using the cell composition of CM+EC+MC shown to be optimal for linear ECTs, identified the PL7 ME-ECT as displaying preferred structural and functional properties. PL0 PS-ECTs had higher cell death *in vitro*. They also showed reduced cell alignment and lower active force compared to other structures, which indicates poorer mechanical loading and suggests a less optimal pre-implant construct.

[00122] The PL16 ML-ECT mold had the longest posts but produced ECTs with variable bundle widths, most likely due to uneven cell distribution during gel pouring or to variations in mechanical loading from the ends of these longer bundles versus the shorter PL7 ME-ECT bundles. Interestingly, ML-ECTs also showed a higher percentage of dead cells than ME-ECTs. Without wishing to be bound by theory, it is believed that this may be related to the irregular bundle shapes and loading. PL7 ME-ECTs had reproducibly uniform bundle widths and

increased cell alignment as well as structural durability during *in vitro* handling and *in vivo* implantation. Accordingly, ME-ECTs are believed to have preferred scalability and reproducibility compared to ML-ECTs.

[00123] In addition to active stress, capture rate and excitation threshold voltage during force measurement analysis were measured as an index of CM electrophysiological maturation. There was no difference among three geometries, and each value was comparable to CM+EC+MC type linear ECTs in the inventors' previous study. Thus, those parameters were not as affected by tissue geometry as by the cellular composition or duration of *in vitro* maturation.

[00124] Next, with the aim of delivering more cardiac cells and generating thicker constructs, the initial seeding cell number was increased at the same or doubled cell density per construct using the ME-ECT format. However, somewhat surprisingly, the original 6M cells showed the best cell survival and ECT function, indicating preferred range and/or limit to cell number and density with this formulation.

[00125] In one study, the instant inventors found that inclusion of ECs and MCs in linear h-iPSC ECTs resulted in more prominent sarcomeric structural maturation. Here the impact of prolonging *in vitro* 3D culture to 4 weeks on the electromechanical maturation of ME-ECTs was investigated. Although 28-day constructs showed a trend towards lower active stress at 1.5Hz pacing versus 14-day constructs, they display a positive force-frequency relationship, more rapid force generation and relaxation cycle, and captured higher pacing frequency. This is believed to be the first report to demonstrate a positive force-frequency relationship in hiPSC-derived ECTs. Despite increased CM maturation, CM content decreased in 28-day constructs compared to 14-day constructs (**FIGS. 22A-22C**), suggesting that a prolonged *in vitro* environment is likely less physiologic than *in vivo*. Furthermore, extended *in vitro* culture beyond 28 days was associated with migration of cells from the floating LF-ECTs to the construct mold, which resulted in some tethering of the construct. Therefore, the different constructs may be preferably used in different capacities. For example, it may be preferable to use the 14-day LF-ECT constructs as grafts for implantation and the 28-day culture LF-ECT constructs as platforms for drug screening and disease modelling.

[00126] With respect to translation of the LF-ECT paradigm to clinical therapies, it is noted that in an immune tolerant xenograft rat model, *in vivo* survival of LF-ECT grafted cells was confirmed on both non-MI and MI hearts. The invasion of host-derived vasculature into the graft and vascular integration between the host and the graft was confirmed histologically. LF-ECT implantation attenuated ventricular remodeling and recovered cardiac function after myocardial infarction. Importantly, LF-ECT contributed to the preservation of viable myocardium at the risk region leading to the maintenance of wall thickness and the prevention of scar formation. According to regional strain analysis, the regional wall motion at and around the risk region recovered significantly compared to the sham operated controls. Direct and local functional integration of ECTs and host myocardium was confirmed by the inventors' current analytic approach at the four-week follow-up period.

[00127] In conclusion, LF-ECTs were generated from hiPSC-derived cardiac cells using PDMS tissue molds coated with Pluronic F127. LF-ECT geometry alters cell survival, tissue maturation, and function, identifying PL7 ME-ECT as the preferred geometry. LF-ECTs cell composition also alters survival, maturation, and function, identifying 6M as optimal for cell survival and force production with continued maturation of CM performance with extended *in vitro* 3D culture to 28 days. LF-ECTs survived *in vivo* and improved cardiac function after MI. The scale of 15x15mm LF-ECTs can be expanded to larger formats such as 30x30mm XLF-ECT for large animal trials. Accordingly, scalable large-format ECTs are feasible for hiPSC-based preclinical and clinical cardiac regeneration paradigms.

[00128] EXAMPLE 3

[00129] As an alternative to electrical stimulation (ES), which increases cell maturity but can be invasive and toxic, optogenetic pacing (OP) was investigated as a stimulation method for excitable tissue. As illustrated in **FIG. 12**, hiPSCs were virally transfected with the light-sensitive channelrhodopsin (ChR) ion channels. In particular, as illustrated in **FIGS. 13A-B**, the hiPSCs were transfected with ChIEF light sensitive ion channel expressing tdTomato. Following transfection, the hiPSCs were optically stimulated (**FIGS. 14A-B**) and contractile stress was measured at an intrinsic beat rate and optical pacing (**FIG. 15**). As shown in **FIG. 16**, the mean active stress of the paced ECTs was decreased as compared to the control. Additionally, the chronic optical pacing improved ECT functional maturation (**FIGS. 17A-C**).

[00130] In view thereof, it was concluded that hiPSCs may be successfully transfected with AAV-ChIEF-tdTomato with a low rate of cell death (**FIGS. 18A-D**), and these transfected hiPSCs may form ECTs which undergo normal gel compaction and being beating similar to control. In addition, the transfected hiPSC ECTs may be OP above the intrinsic rate with a pulsed 470 nm LED. While chronic seven-day OP did not visibly damage the ECTs, the OP improved electrophysiology of the ECTs as defined by higher maximum capture rates, improved force-frequency relationship, and reduced beat-to-beat hysteresis which indicated better Ca^{2+} cycling. Furthermore, preliminary histology showed greater cardiomyocyte organization. Accordingly, it is believed that optogenetic pacing is a non-contact method for CM and ECT conditioning.

[00131] Throughout this document, various references are mentioned. All such references are incorporated herein by reference, including the references set forth in the following list:

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[00132] It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the subject matter disclosed herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. An engineered cardiac tissue, comprising a plurality of induced pluripotent stem cell-derived cardiac cells arranged in a configuration suitable for implantation onto the surface of a heart.
2. The engineered cardiac tissue of claim 1, wherein the plurality of induced pluripotent stem cell-derived cardiac cells are arranged in a square, mesh, or parallel configuration.
3. The engineered cardiac tissue of claim 1, wherein the plurality of induced pluripotent stem cell-derived cardiac cells are arranged in a mesh configuration.
4. The engineered cardiac tissue of claim 1, wherein the plurality of induced pluripotent stem cell-derived cardiac cells are arranged in a configuration having a length of about 15 mm to about 40 mm and a width of about 15 mm to about 40 mm.
5. The engineered cardiac tissue of claim 1, wherein the heart is a human heart.
6. The engineered cardiac tissue of claim 1, wherein plurality of induced pluripotent stem cell-derived cardiac cells are selected from the group consisting of cardiomyocytes, endothelial cells, mural cells, fibroblasts, and combinations thereof.
7. The engineered cardiac tissue of claim 6, wherein the tissue comprises between 10 and 20% endothelial cells, by weight.
8. The engineered cardiac tissue of claim 6, wherein the tissue comprises between 10 and 20% mural cells, by weight.
9. The engineered cardiac tissue of claim 6, wherein the tissue comprises, by weight, between 10 and 20% endothelial cells and between 10 and 20% mural cells.

10. The engineered cardiac tissue of any one of claims 7-9, wherein the concentration of cells facilitates *in vitro* expansion of vascular cells within the engineered cardiac tissue and subsequent *in vivo* vascular coupling between the engineered cardiac tissue and a recipient's myocardium.
11. The engineered cardiac tissue of claim 1, comprising an initial cell number of 6M or less.
12. The engineered cardiac tissue of claim 1, wherein at least one of the induced pluripotent stem cells-derived cardiac cells is transfected with a light-sensitive ion channel.
13. The engineered cardiac tissue of claim 12, wherein the light-sensitive ion channel is channelrhodopsin.
14. A mold for forming an engineered cardiac tissue, the mold comprising a polymeric base material having an outer surface and a shape configured to produce an engineered cardiac tissue in a configuration suitable for implantation onto the surface of the heart.
15. The mold of claim 14, wherein the polymeric base material of the mold comprises polydimethylsiloxane.
16. The mold of claim 15, wherein the polydimethylsiloxane forms the outer surface of the mold.
17. The mold of claim 14, further comprising at least one post arranged and disposed in the mold such that the mold is configured to form a pore in the engineered cardiac tissue.
18. The mold of claim 14, further comprising a plurality of posts arranged and disposed in the mold such that the mold is configured to form a mesh configuration in the engineered cardiac tissue.

19. A method of forming an engineered cardiac tissue, the method comprising:
providing a mold including an outer surface;
pouring a cell solution onto the outer surface of the mold; and
forming the engineered cardiac tissue from the cell solution, the engineered cardiac tissue having a geometry corresponding to a geometry of the mold.
20. The method of claim 19, wherein the cell solution comprises a mixture of cells and matrix.
21. The method of claim 19, wherein forming the engineered cardiac tissue includes culturing the cell solution on the mold.
22. The method of claim 21, wherein the cell solution is cultured for 14 days.
23. The method of claim 19, wherein the cell solution comprises a plurality of induced pluripotent stem cell-derived cardiac cells.
24. The method of claim 23, wherein at least one of the induced pluripotent stem cell-derived cardiac cells is transfected with a light-sensitive ion channel.
25. The method of claim 24, further comprising optogenetic pacing of the engineered cardiac tissue.
26. A method of treating a myocardial infarction, comprising implanting an engineered cardiac tissue onto a heart of a subject in need thereof, the engineered cardiac tissue comprising a plurality of induced pluripotent stem cell-derived cardiac cells arranged in a configuration suitable for implantation onto the surface of the heart.

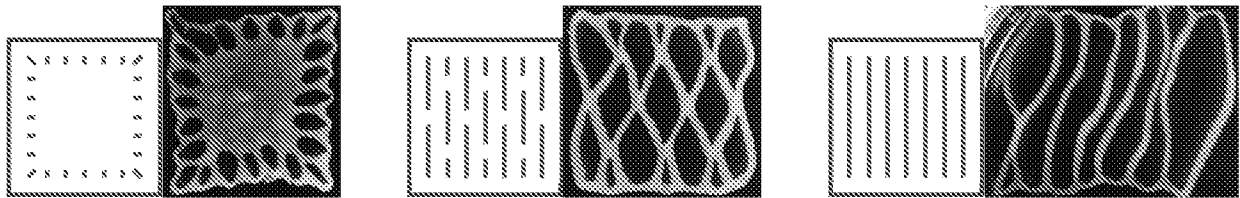


FIG. 1

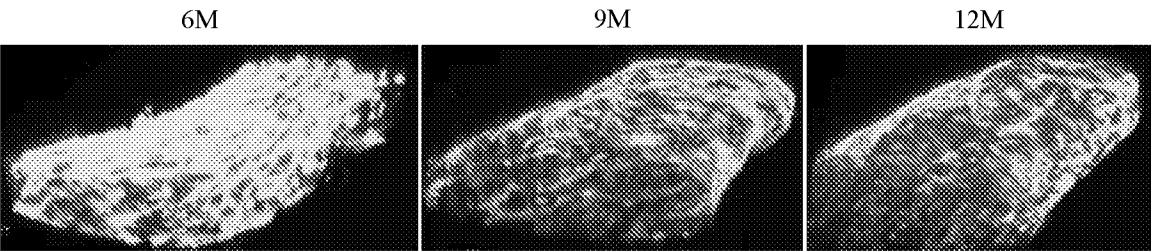


FIG. 2

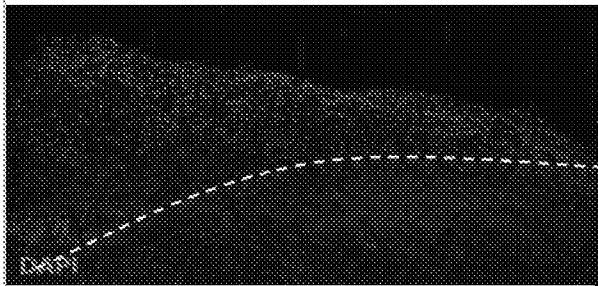


FIG. 3A

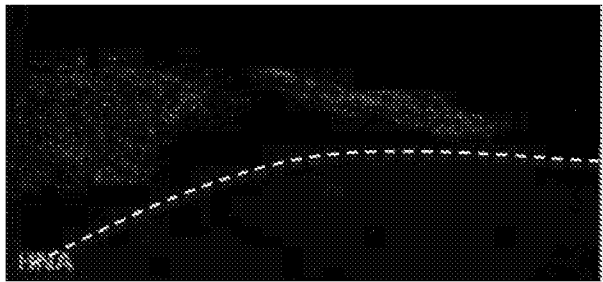


FIG. 3B

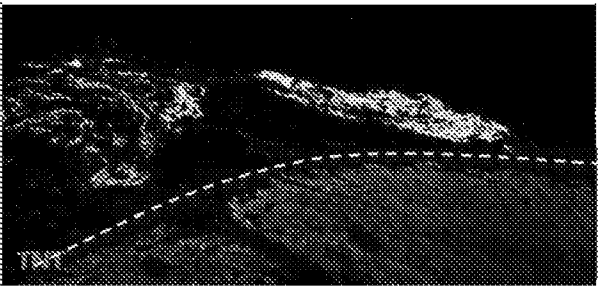


FIG. 3C

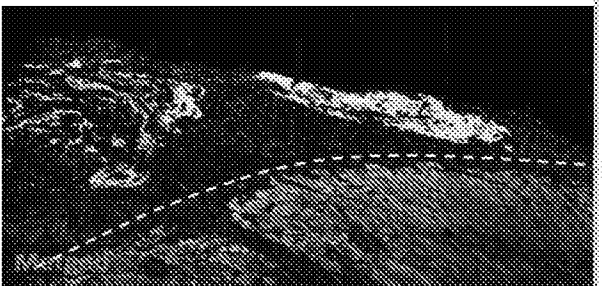


FIG. 3D

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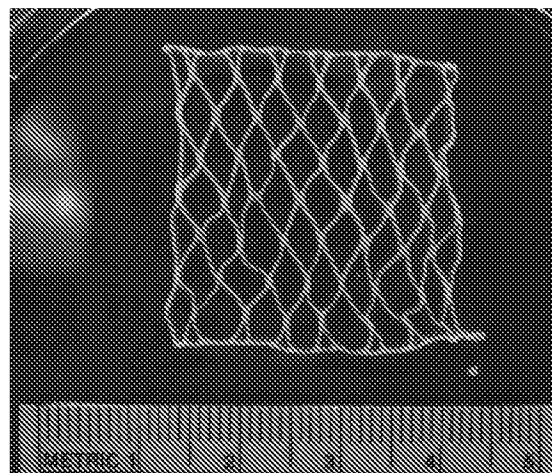
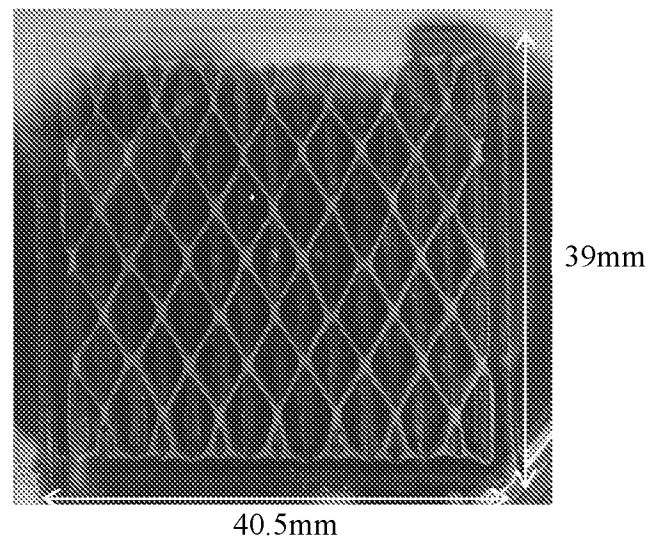


FIG. 4

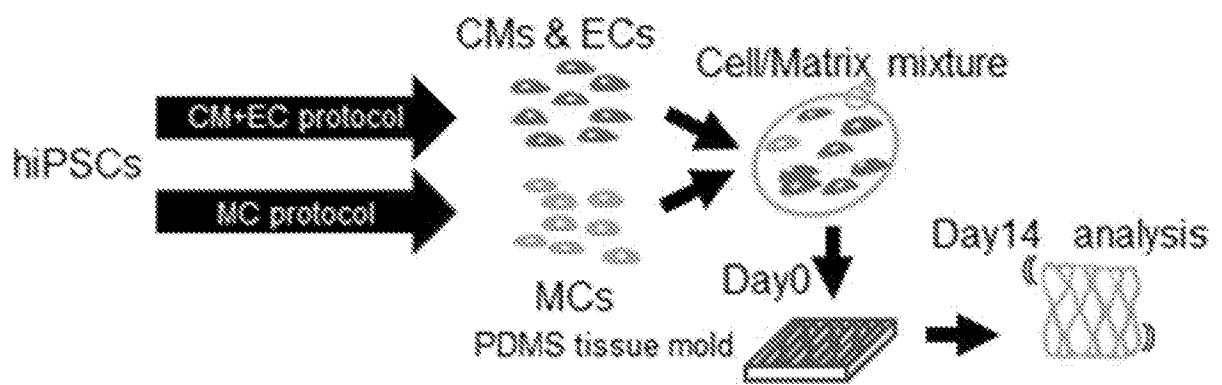


FIG. 5A

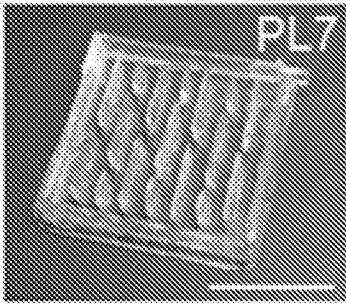


FIG. 5B

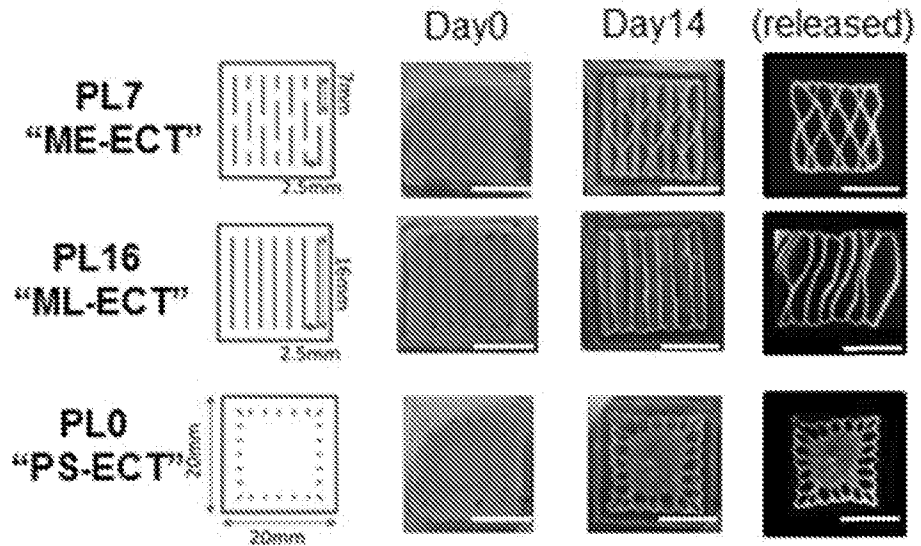


FIG. 5C

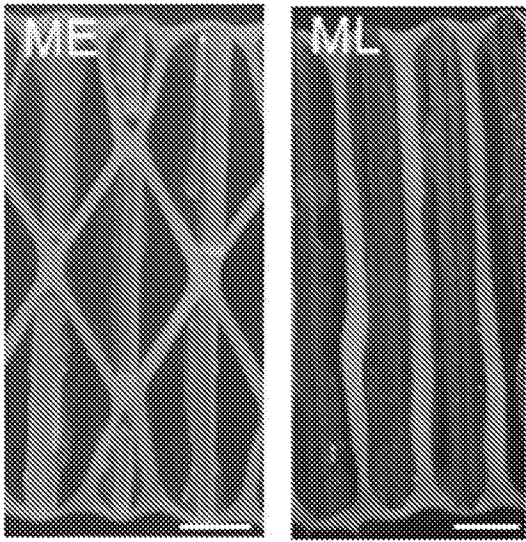


FIG. 5D

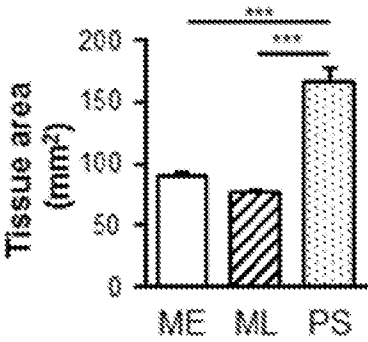


FIG. 5E

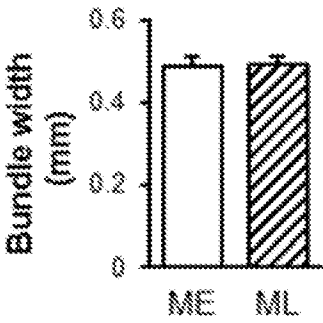


FIG. 5F

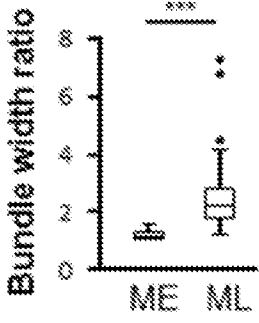


FIG. 5G

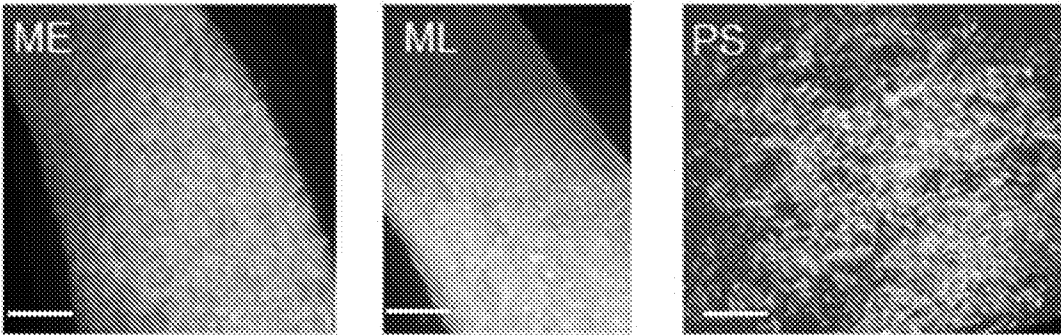


FIG. 5H

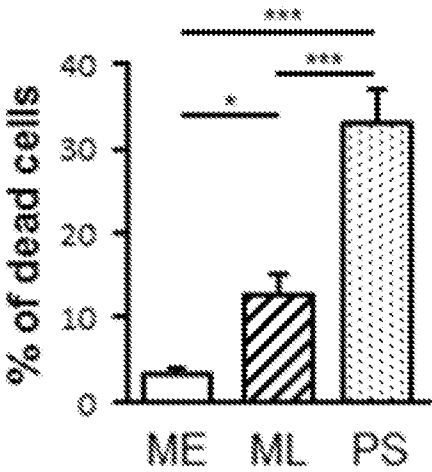


FIG. 5I

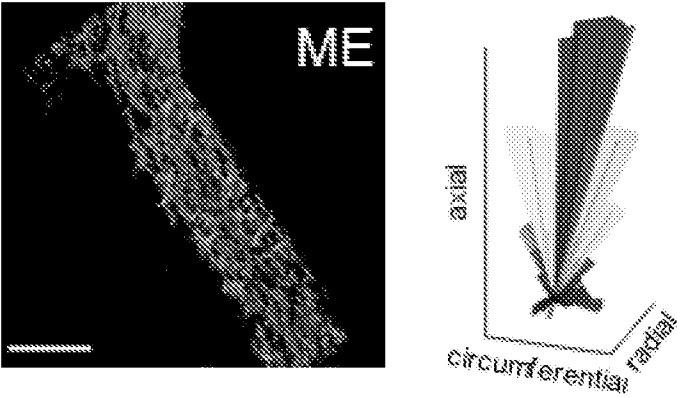


FIG. 6A

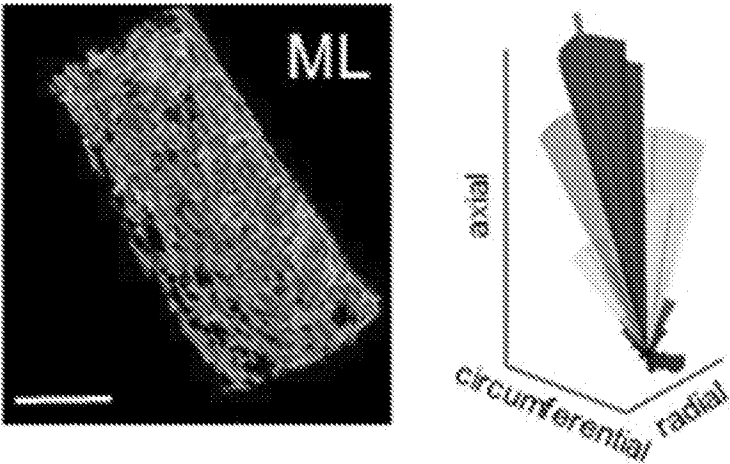


FIG. 6B

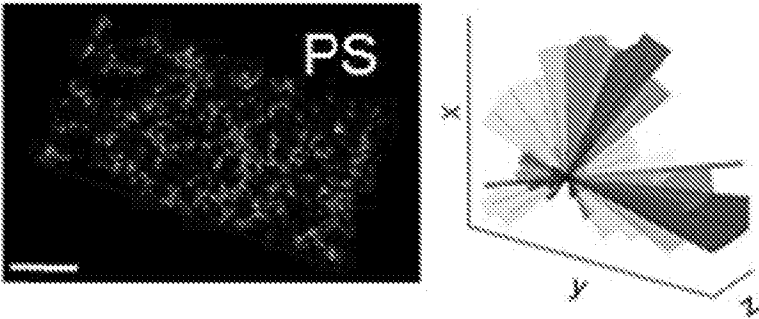


FIG. 6C

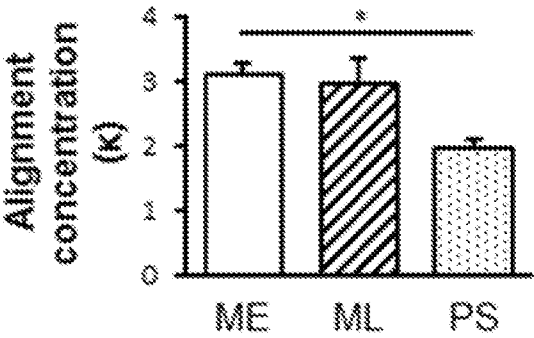


FIG. 6D

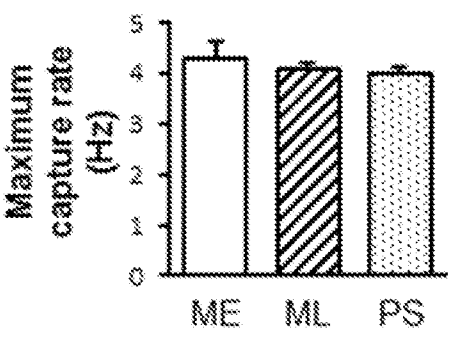


FIG. 6E

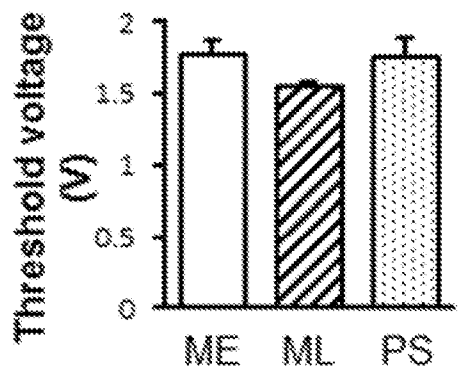


FIG. 6F

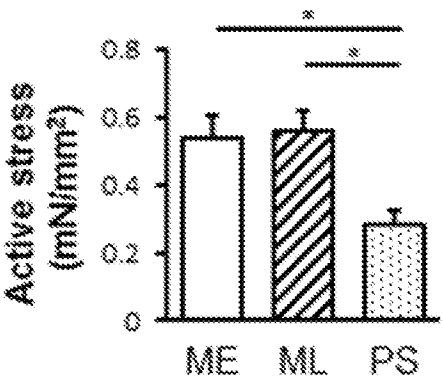


FIG. 6G

Group	Cell number	Matrix volume	Cell density
6M	6 million	400μL	1x
9M	9 million	600μL	1x
12M	12 million	800μL	1x
12MH	12 million	400μL	2x

FIG. 7A

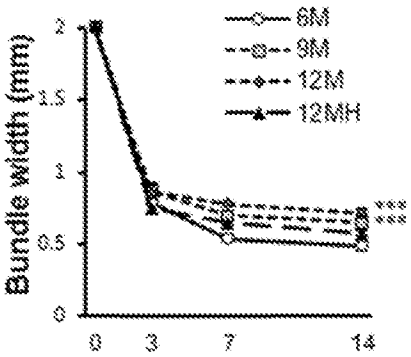


FIG. 7B

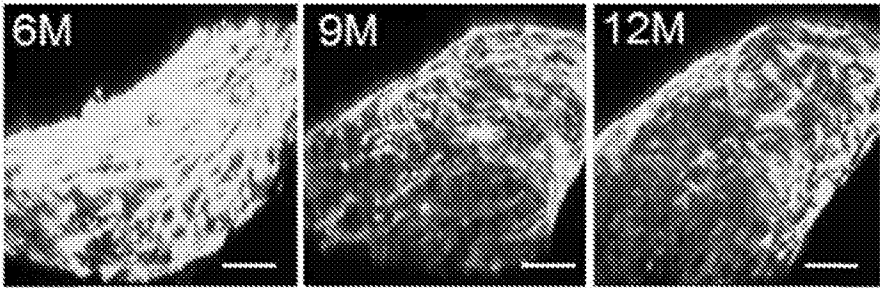


FIG. 7C

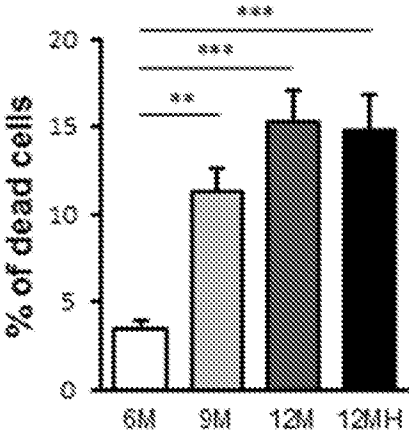


FIG. 7D

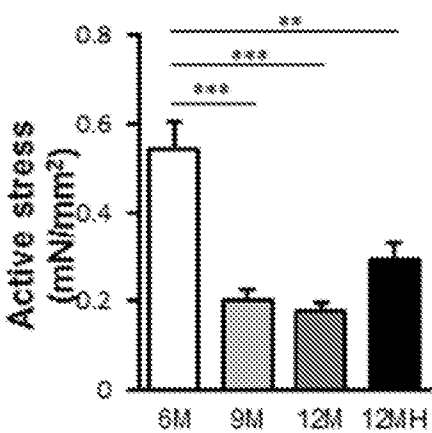


FIG. 7E

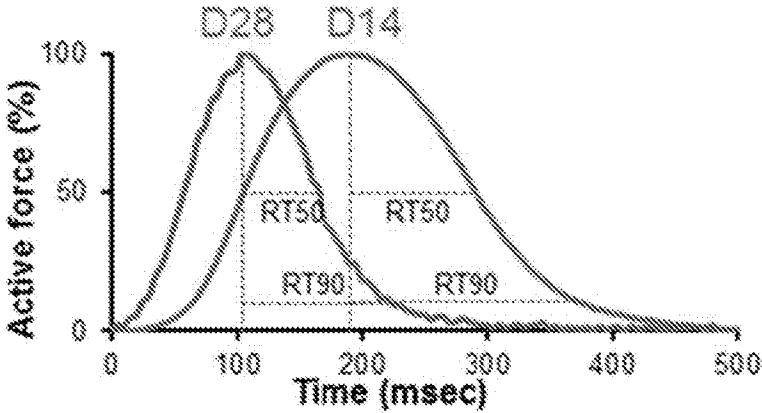


FIG. 8A

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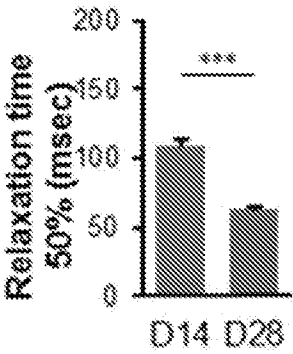


FIG. 8B

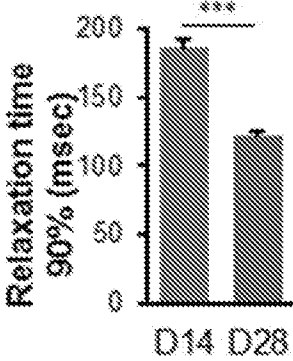


FIG. 8C

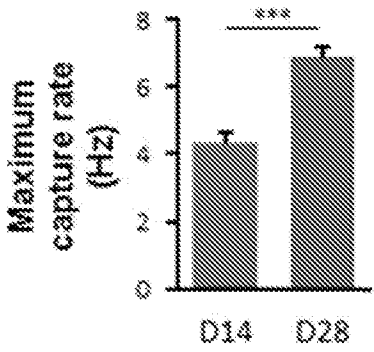


FIG. 8D

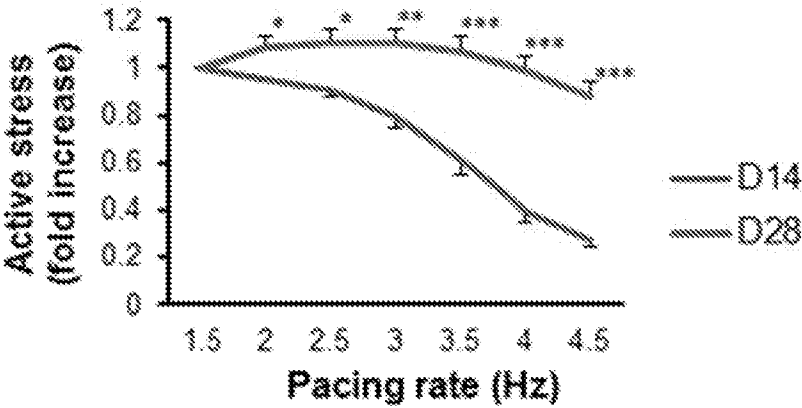


FIG. 8E

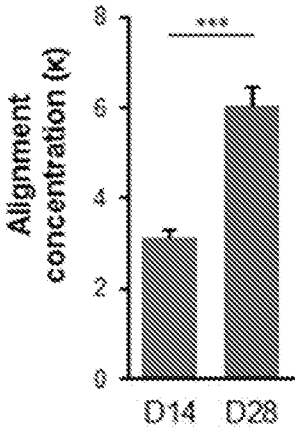


FIG. 8F

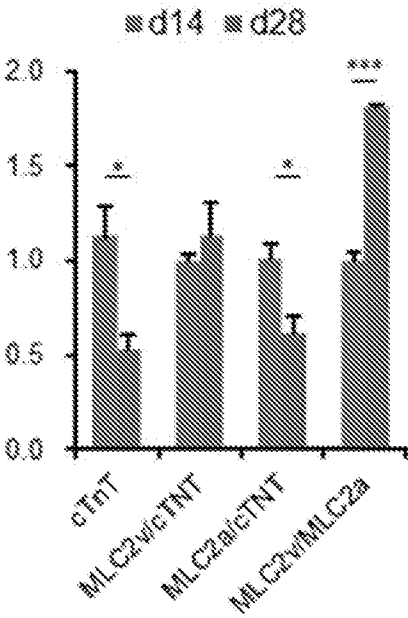


FIG. 8G

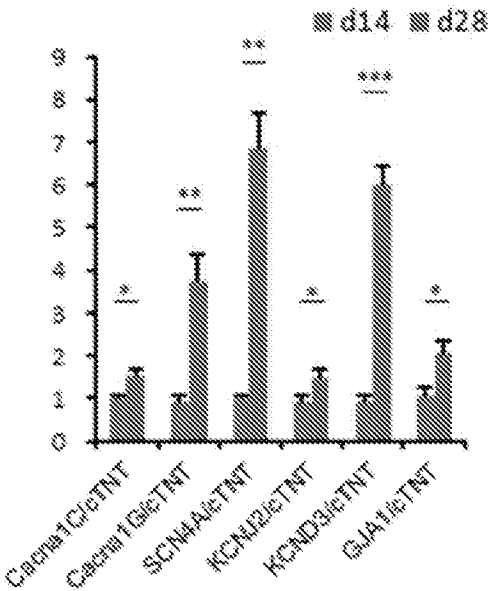


FIG. 8H

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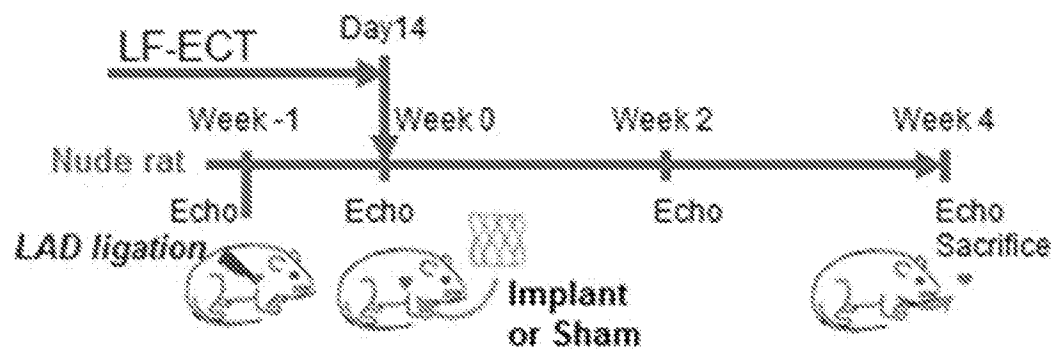


FIG. 9A

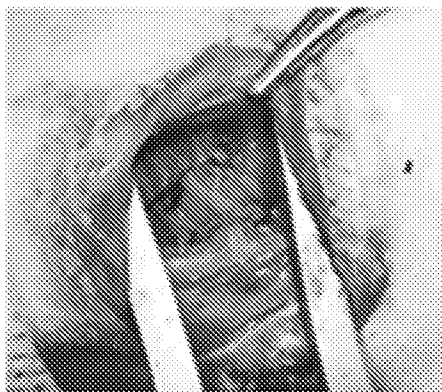


FIG. 9B

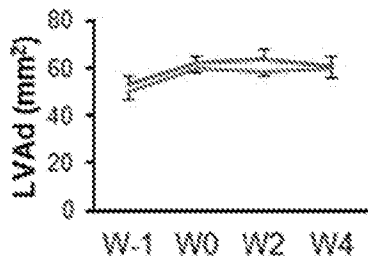


FIG. 9C

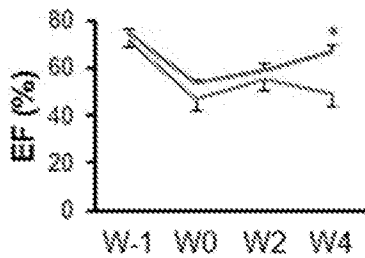


FIG. 9D

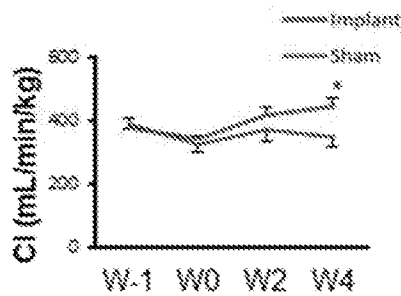


FIG. 9E

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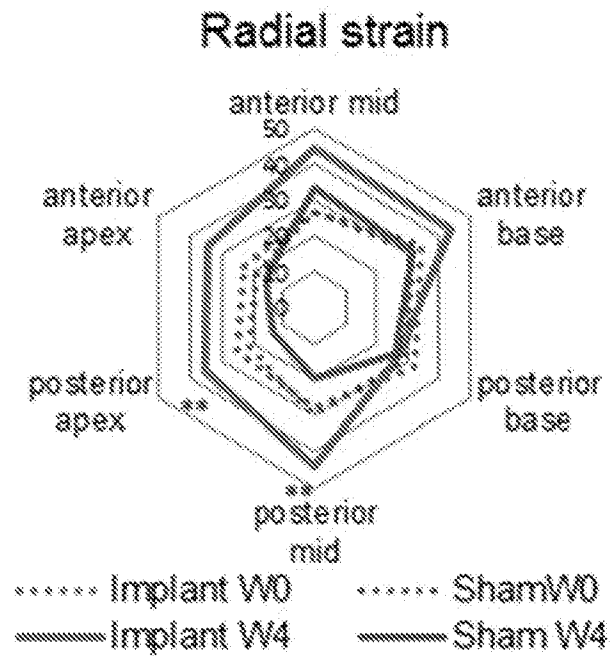


FIG. 9F

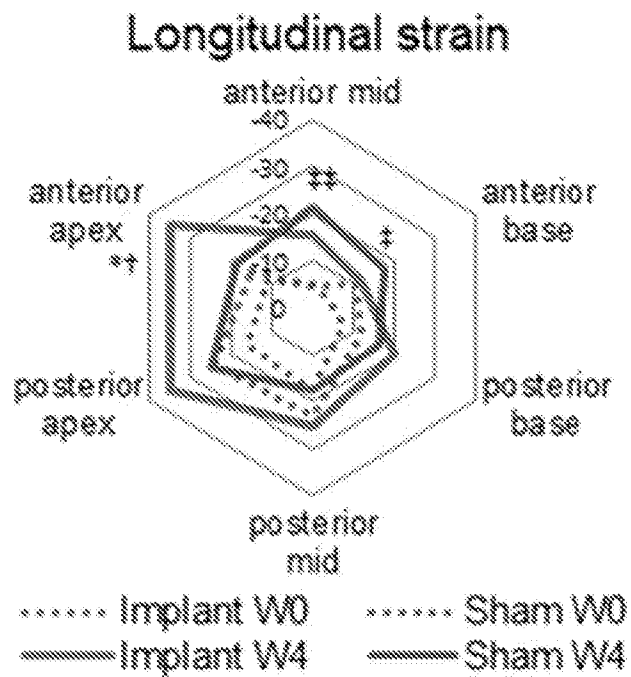


FIG. 9G

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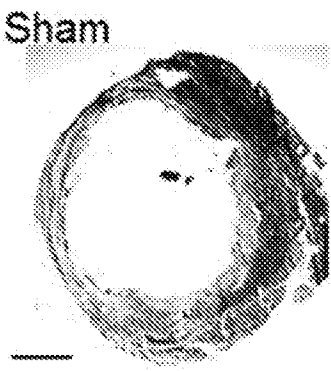


FIG. 10A

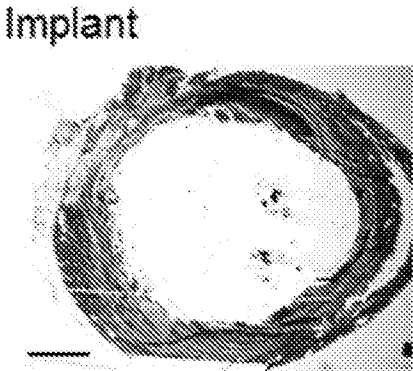


FIG. 10B

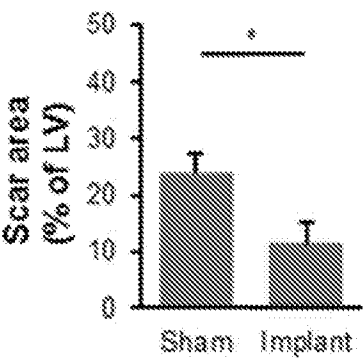


FIG. 10C

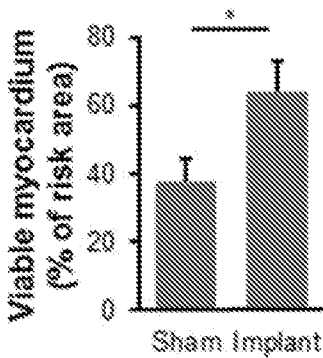


FIG. 10D

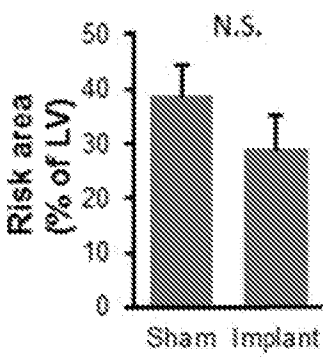


FIG. 10E

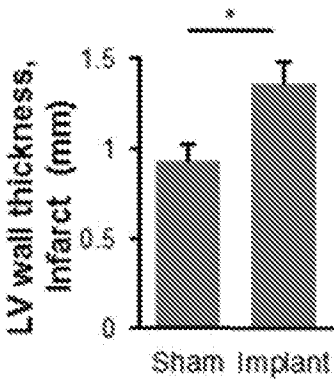


FIG. 10F

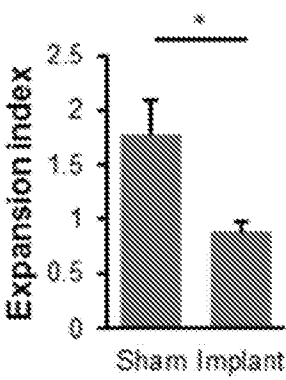


FIG. 10G

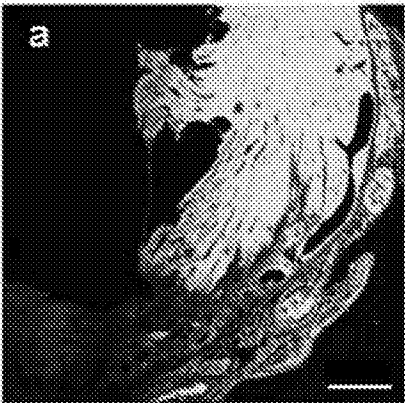


FIG. 11A

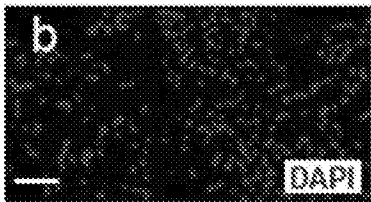


FIG. 11B



FIG. 11C

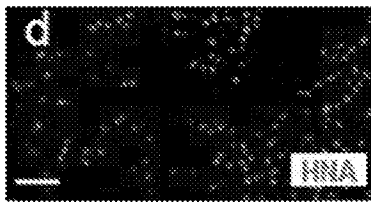


FIG. 11D



FIG. 11E

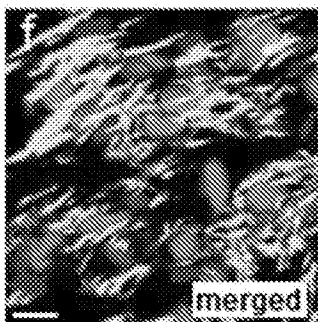


FIG. 11F

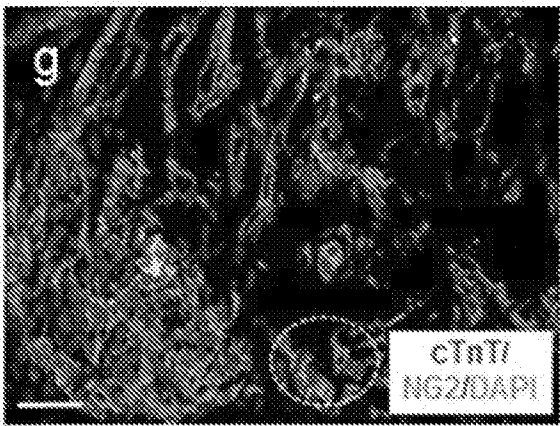
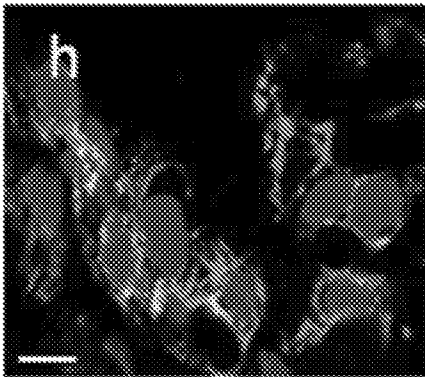


FIG. 11G



cTnT/NG2/DAPI

FIG. 11H

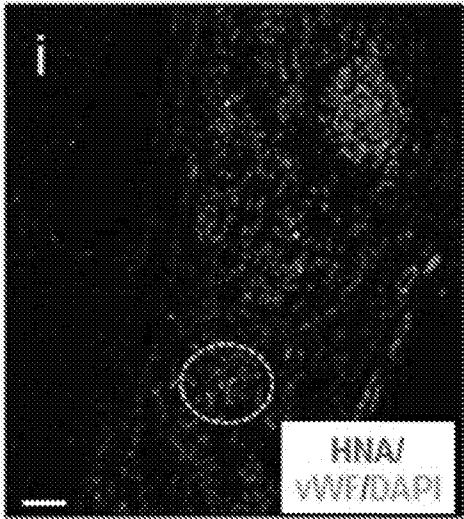


FIG. 11I

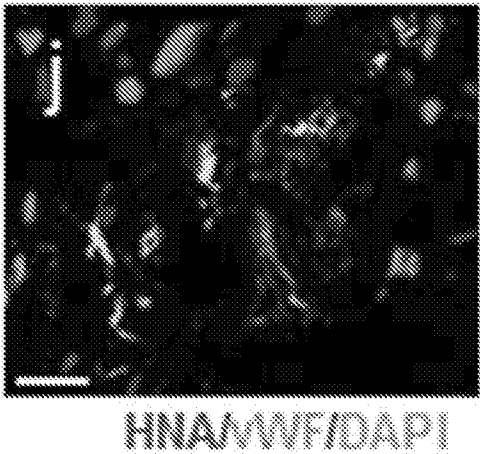


FIG. 11J

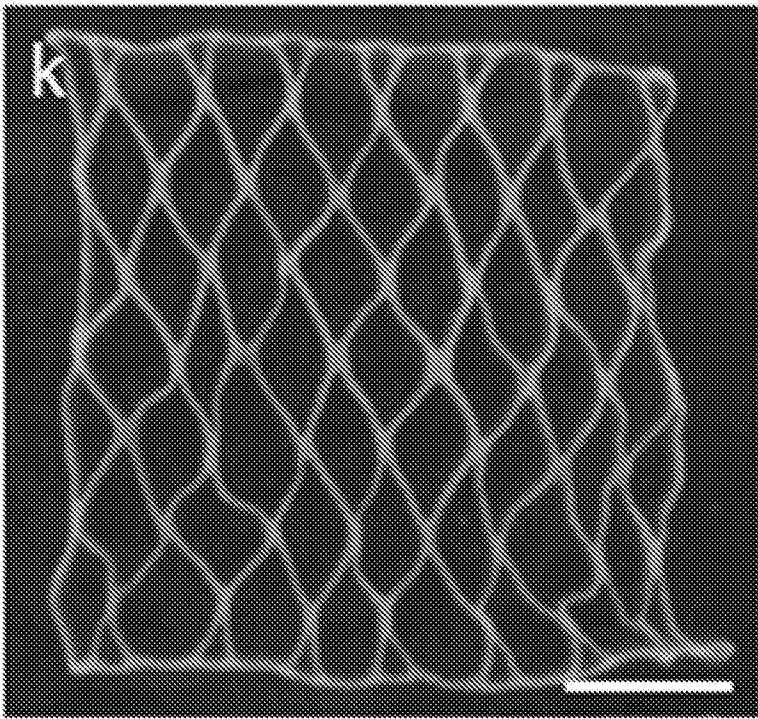


FIG. 11K

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Formation of Light Sensitive ECTs and ECT Analysis

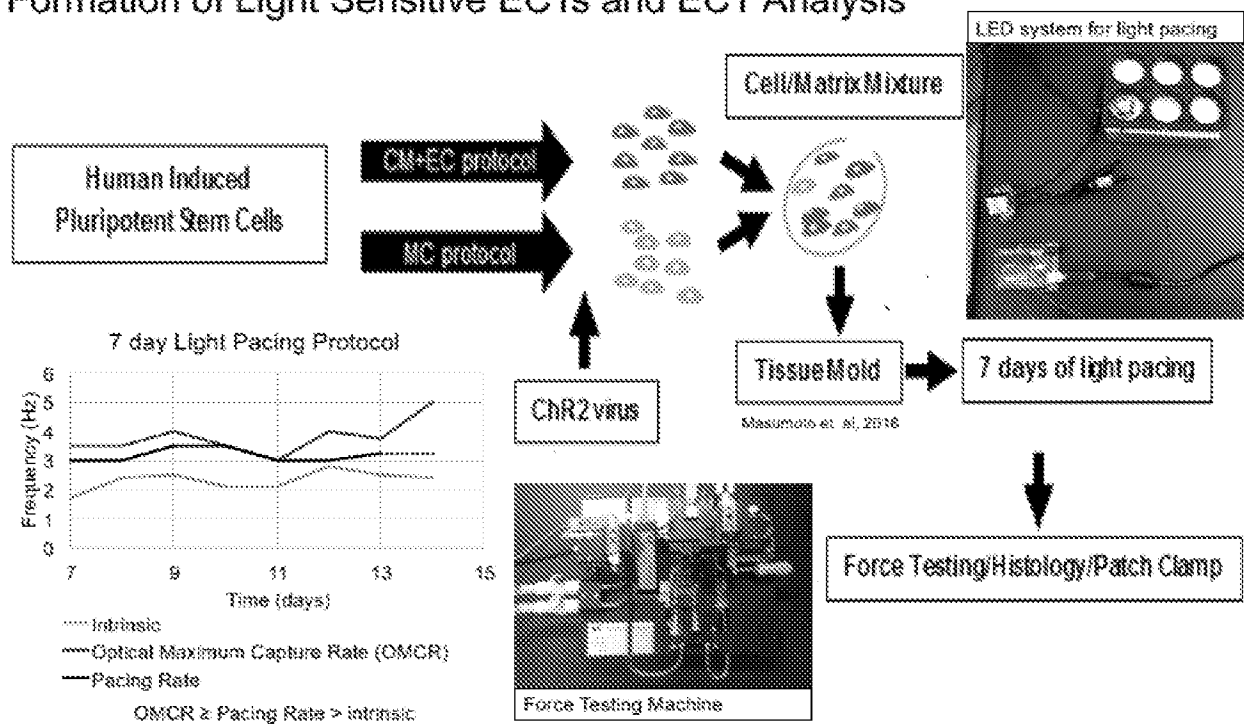
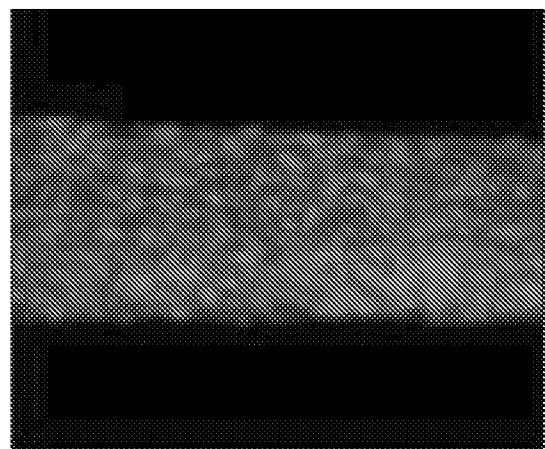
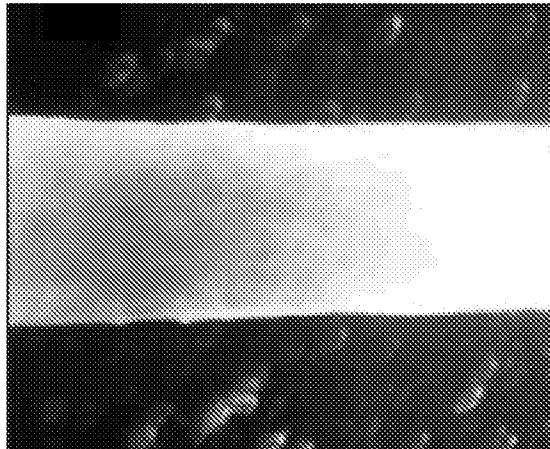


FIG. 12



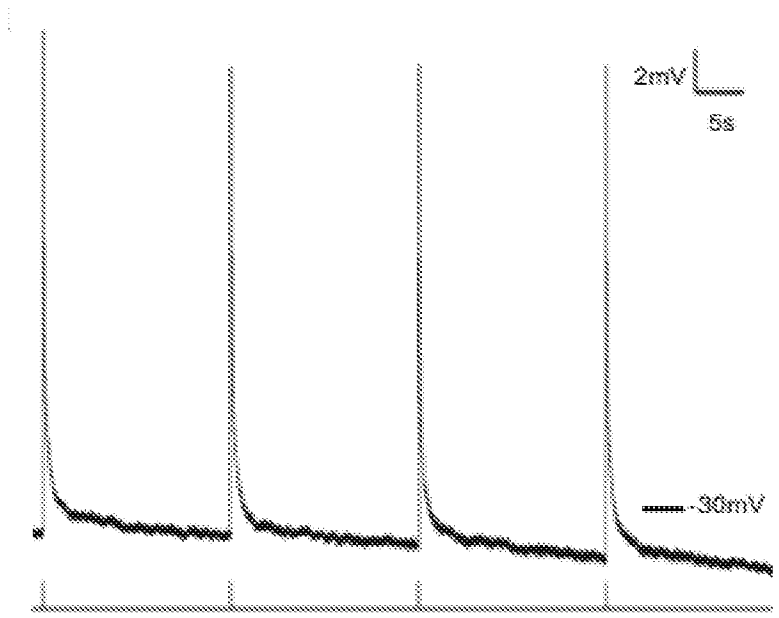


FIG. 14A

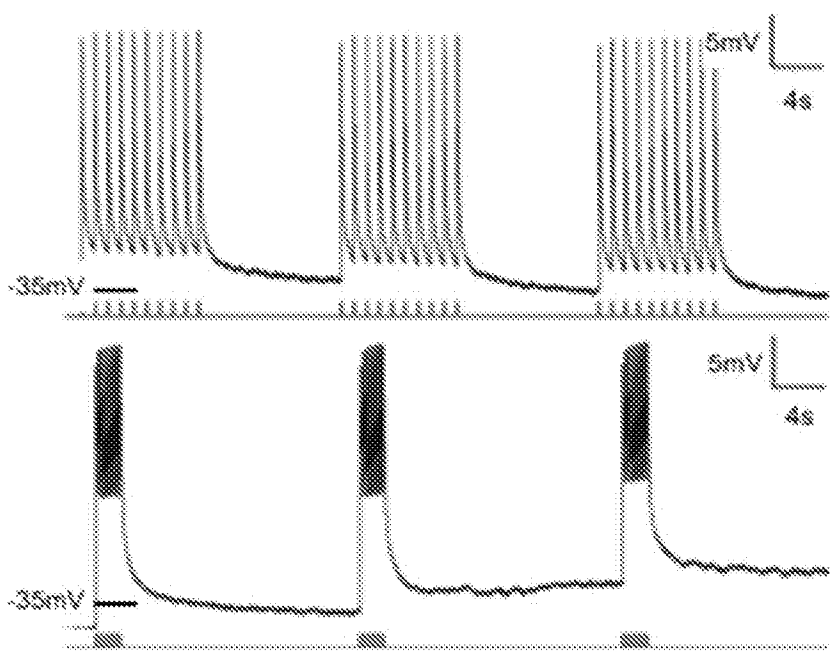


FIG. 14B

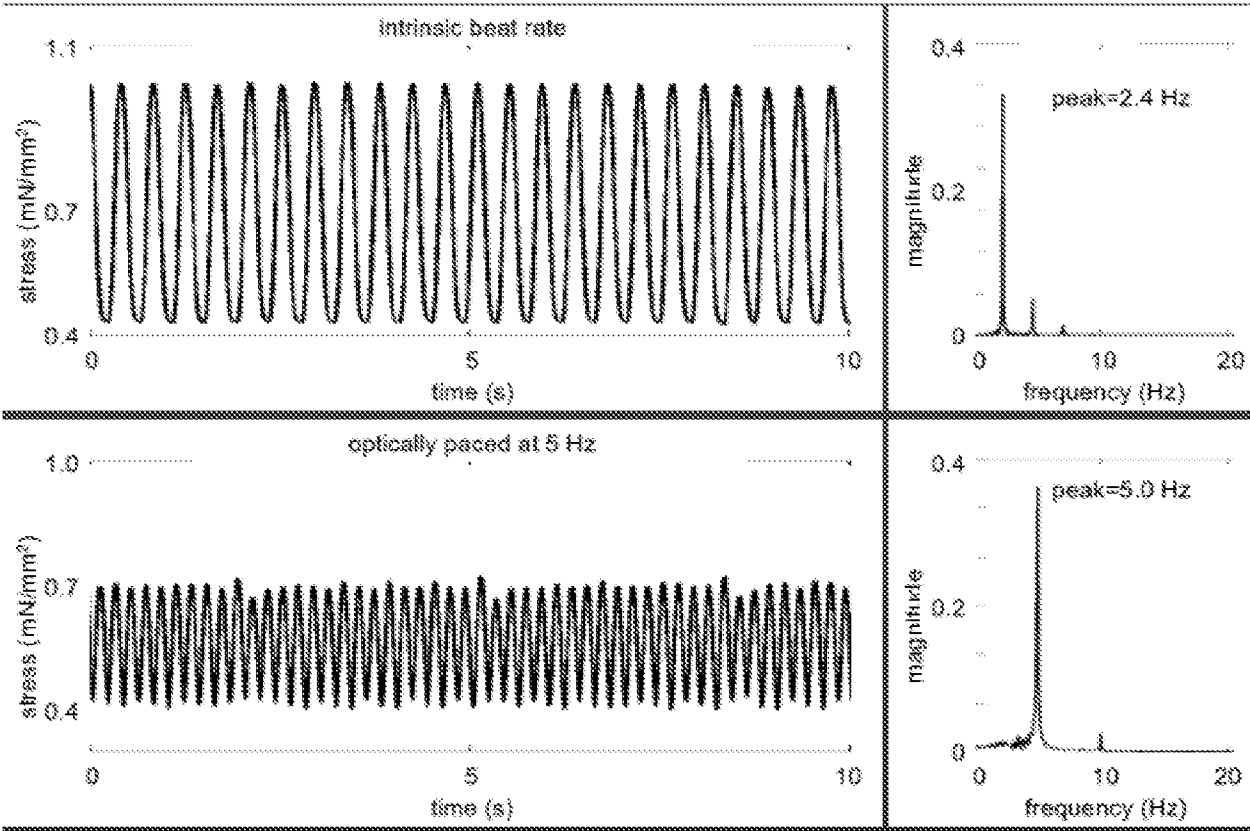


FIG. 15

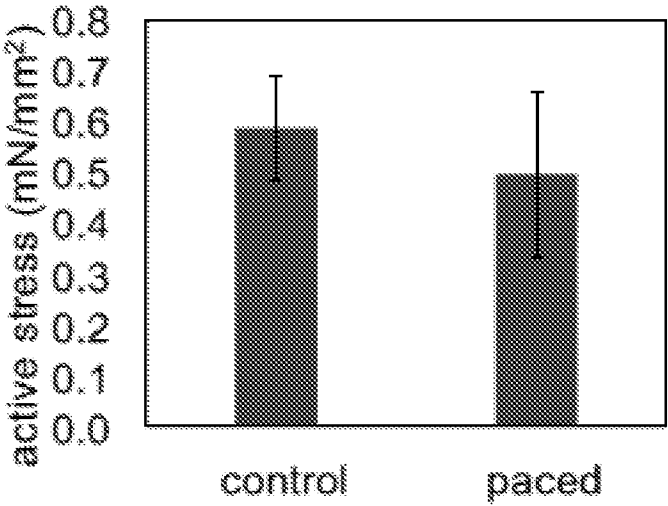


FIG. 16

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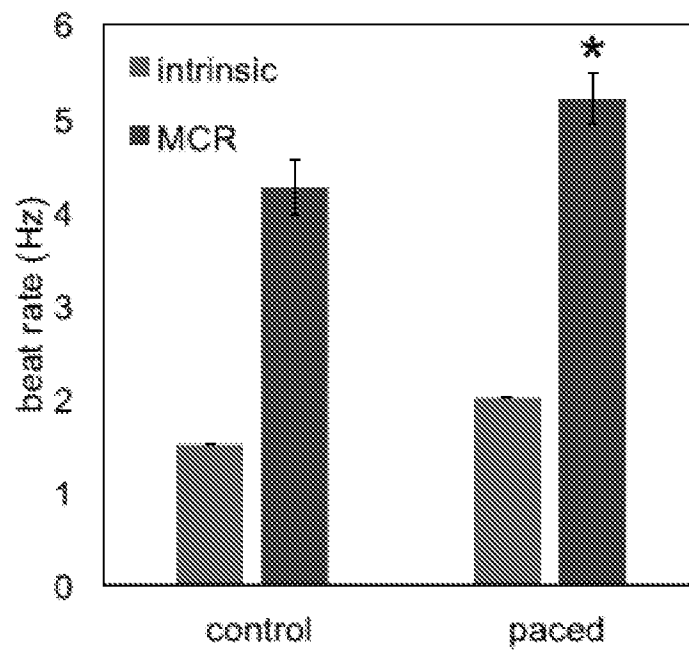


FIG. 17A

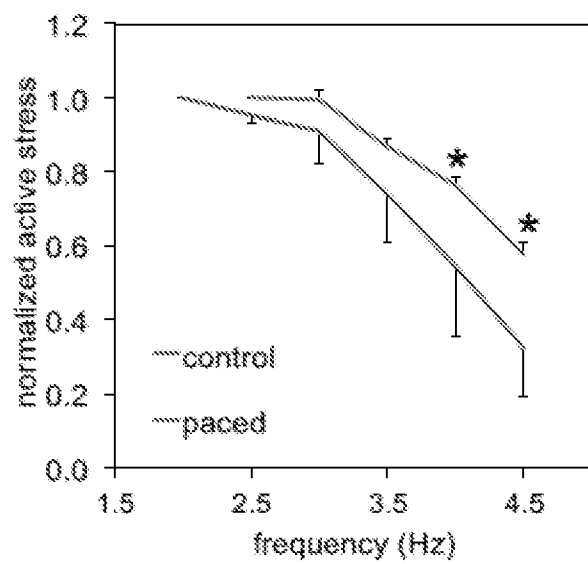


FIG. 17B

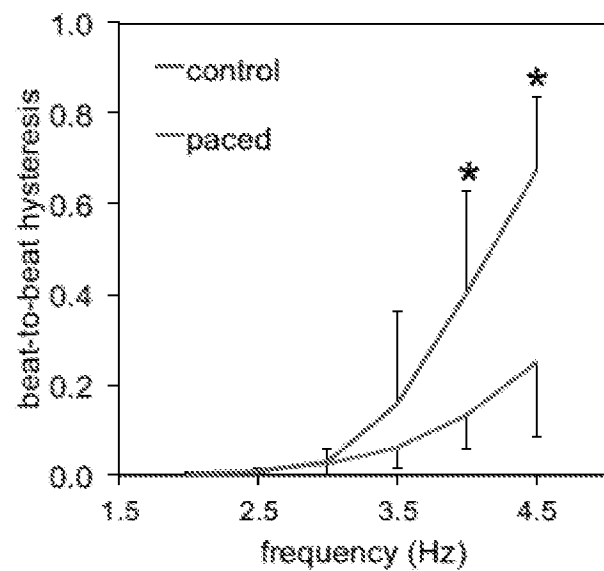


FIG. 17C

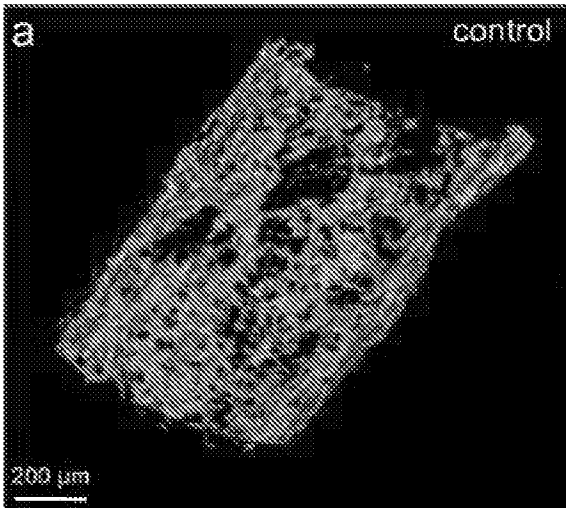


FIG. 18A

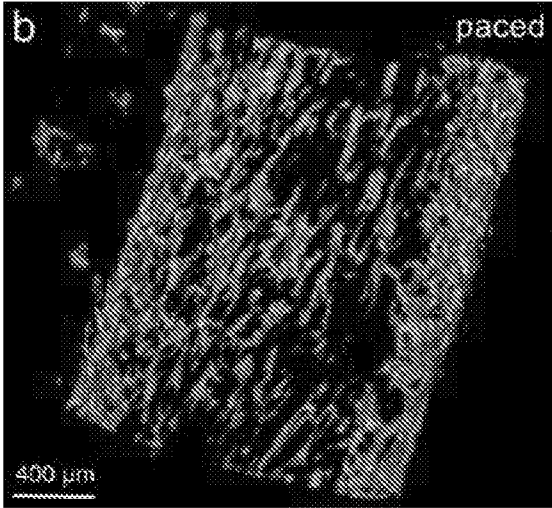


FIG. 18B

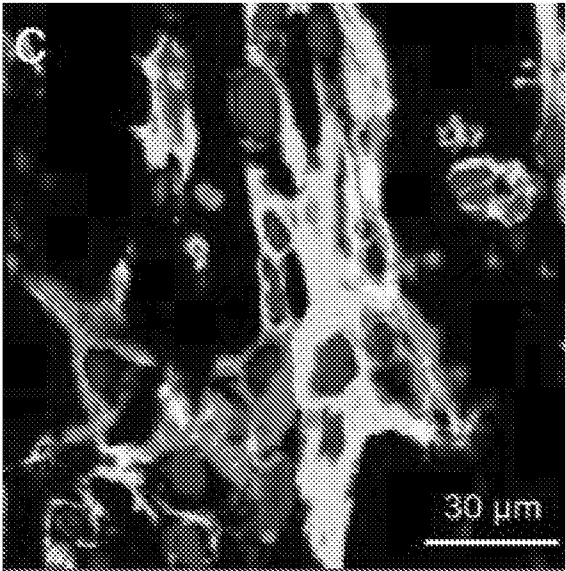


FIG. 18C

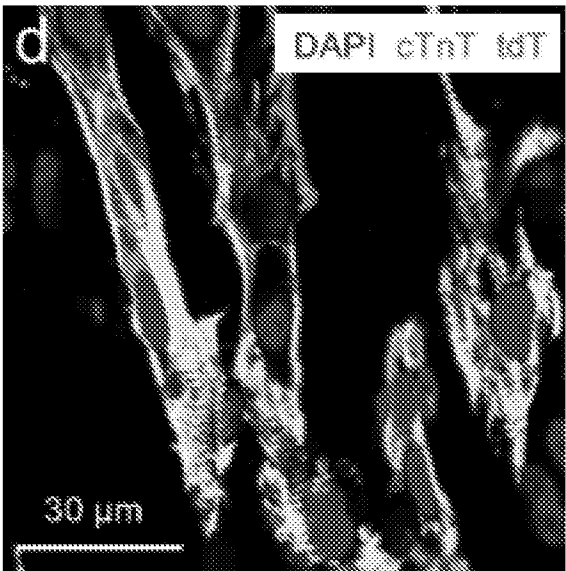


FIG. 18D

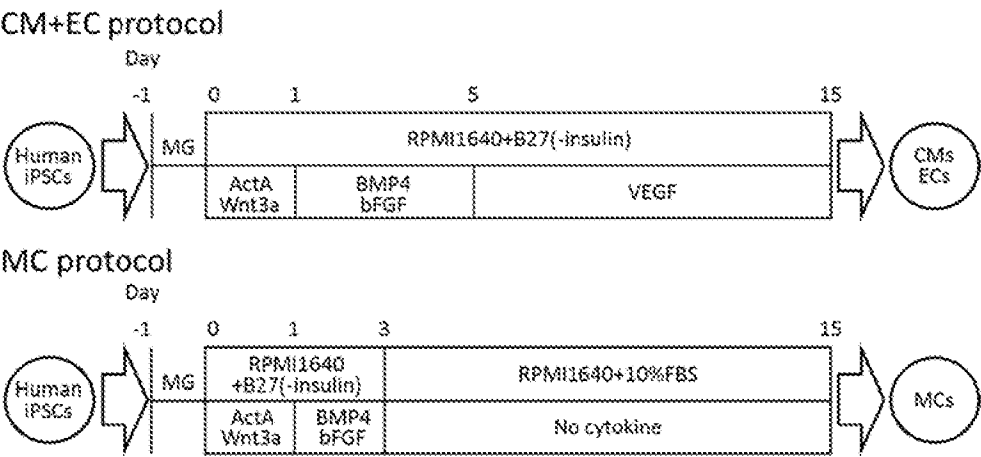


FIG. 19A

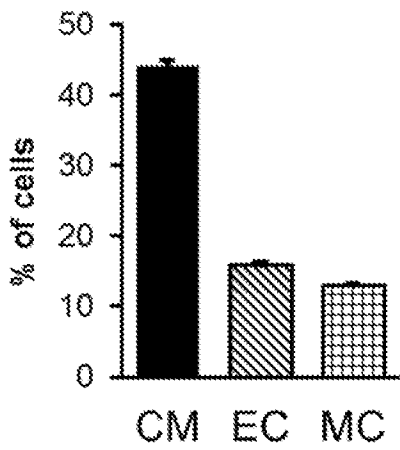


FIG. 19B

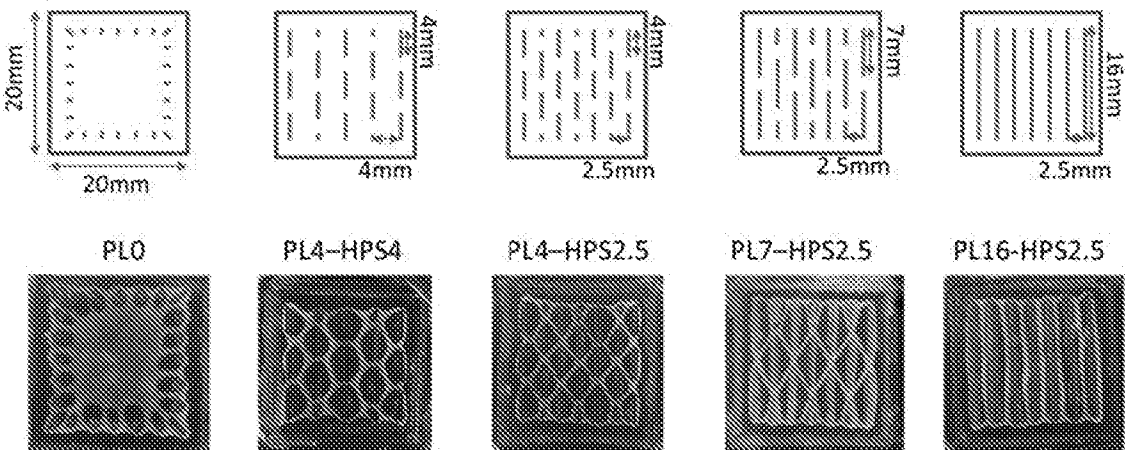


FIG. 20

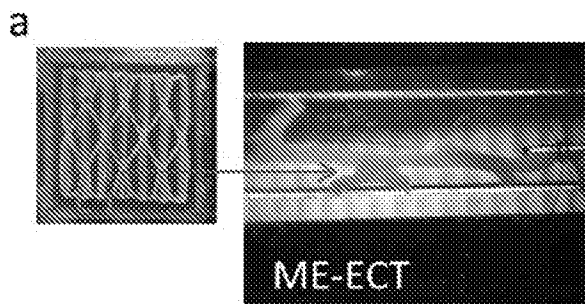


FIG. 21A

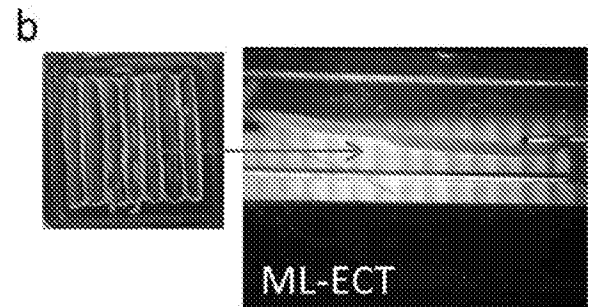


FIG. 21B

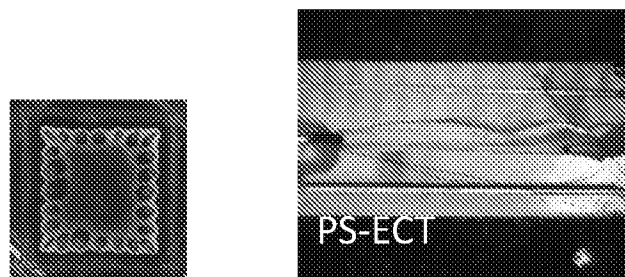


FIG. 21C

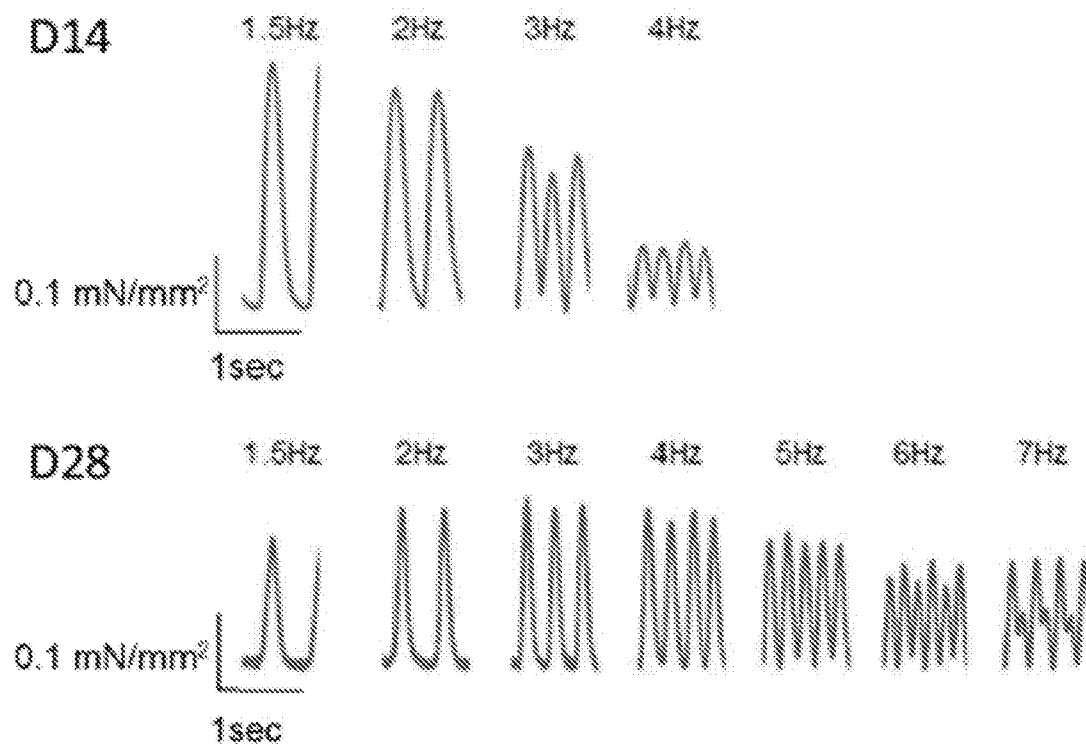


FIG. 22A

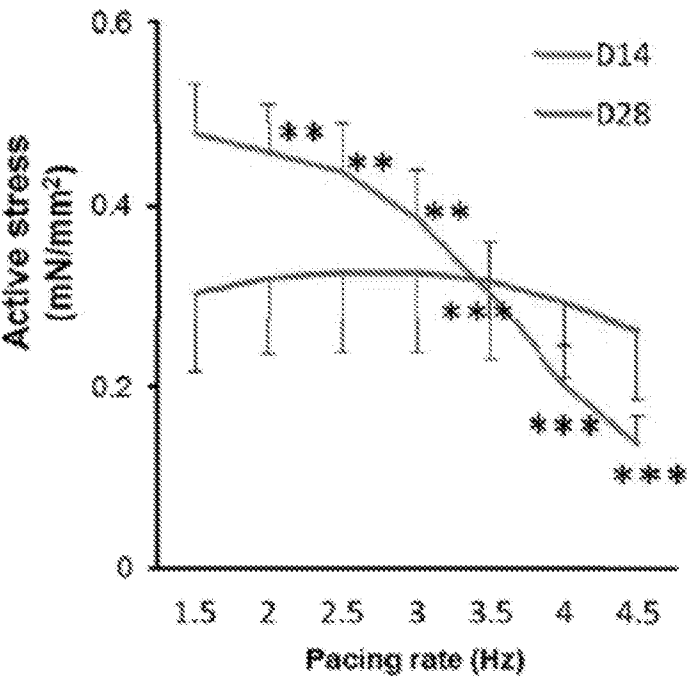


FIG. 22B

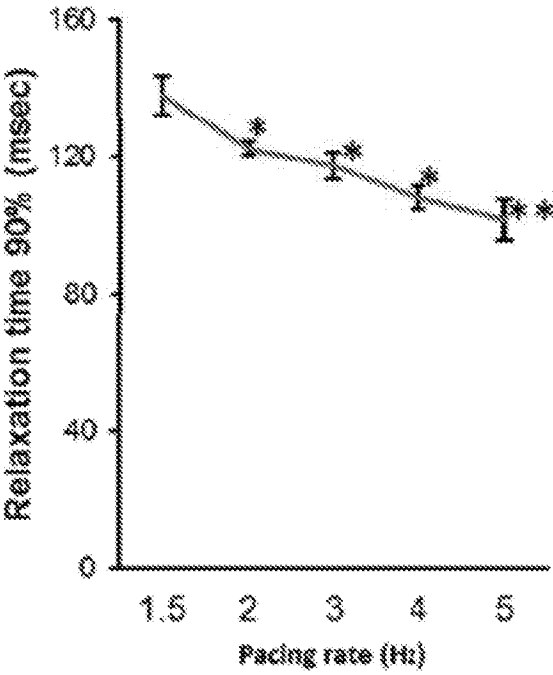


FIG. 22C

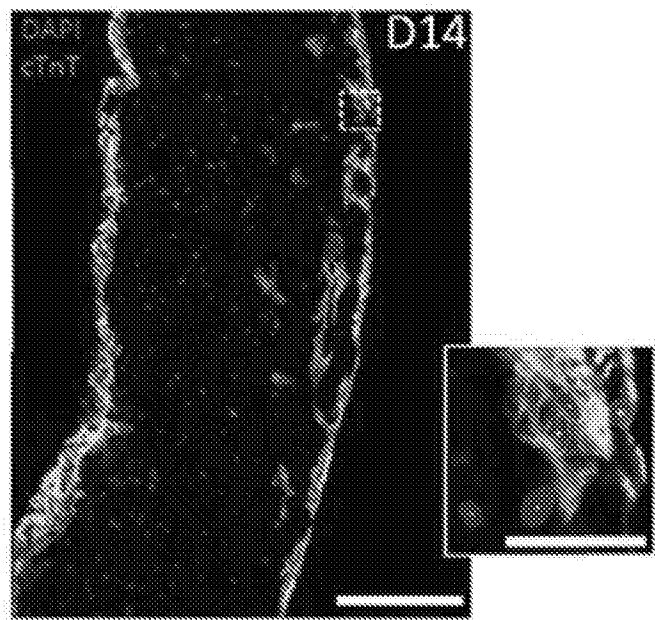


FIG. 23A

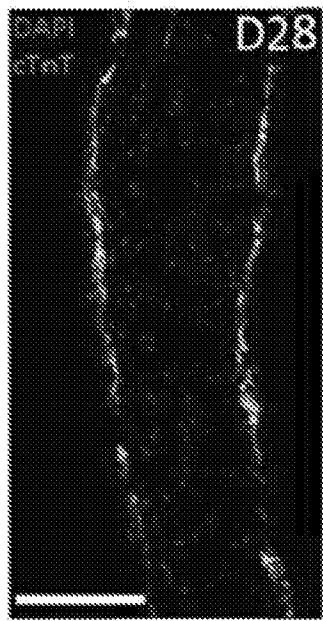


FIG. 23B

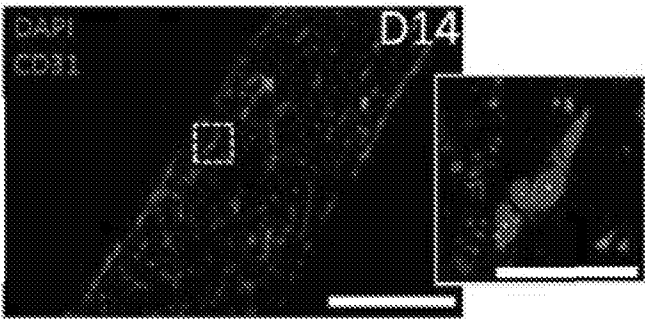


FIG. 23C

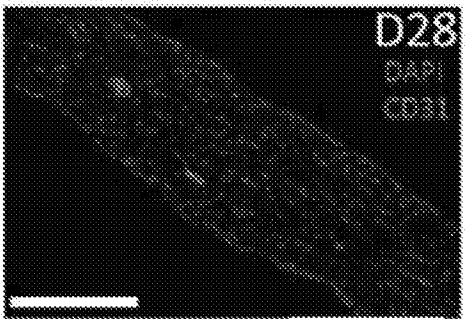


FIG. 23D

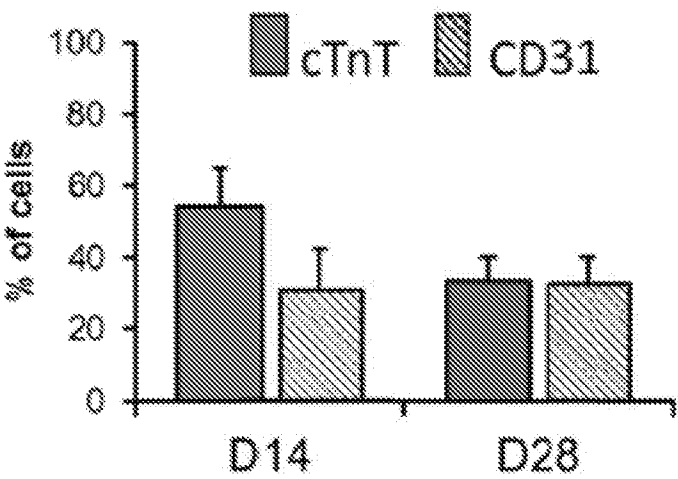


FIG. 23E

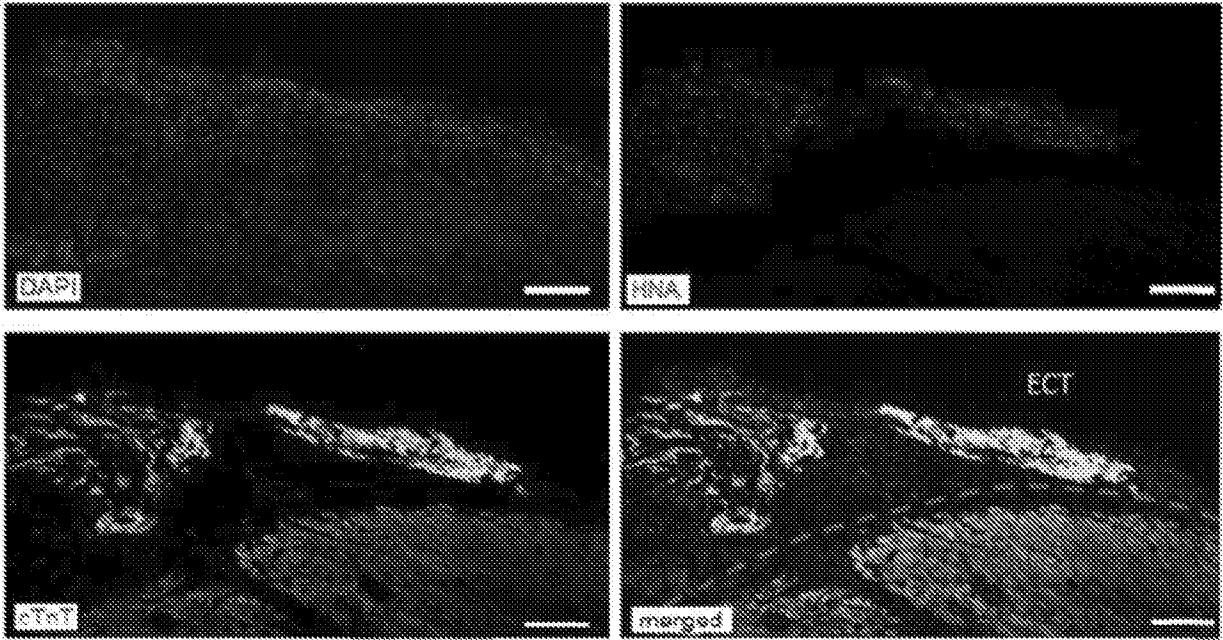


FIG. 24A

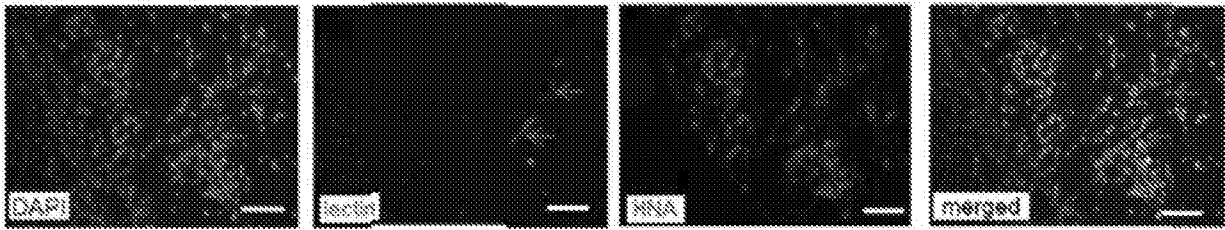


FIG. 24B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/13001

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 5/071, 5/074, 5/0789, 5/0797, 5/095; A61K 35/34, 35/44; A61L 27/54 (2017.01)
 CPC - A61K 35/12, 35/545, 35/34; C12N 5/0657, 5/0606, 5/0696; C08G 77/38; A61L 27/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	(MASUMOTO, H et al.) Human iPS Cell-Engineered Cardiac Tissue Sheets with Cardiomyocytes and Vascular Cells for Cardiac Regeneration. Scientific Reports. 22 October 2014; Vol. 4, No. 6716; pages 1-7; abstract; page 2, column 1, paragraph 2 – column 2, paragraph 1; page 3, column 1, paragraph 2 – column 2, paragraph 1; figure 2; DOI: 10.1038/srep06716.	1, 5-9, 10/7-9, 26 ----- 2-4, 11-13
X --- Y	(CHRISTOFOROU, N et al.) Induced Pluripotent Stem Cell-Derived Cardiac Progenitors Differentiate to Cardiomyocytes and Form Biosynthetic Tissues. PLoS One. 13 June 2013; Vol. 8, No. 6; pages 1-17; page 5, column 1, paragraph 5 – column 2, paragraph 1; page 15, column 1, paragraph 4; figures 7A-7G; DOI: 10.1371/journal.pone.0065963.	14-23 ----- 2-4, 24-25
Y	US 2014/0328806 A1 (BIRLA, RK) 06 November 2014; figure 3; paragraph [0012]	11
Y	US 2014/0295413 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 02 October 2014; paragraphs [0010]-[0011], [0220]; figure 25	12-13, 24-25

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 February 2017 (27.02.2017)

Date of mailing of the international search report

24 MAR 2017

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