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Supplemental Information

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Supplementary Materials for

Human Pluripotent Stem Cell-Derived Cardiac Tissue-Like Constructs for Repairing of the Infarcted Myocardium

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Supplemental experimental procedures

Nanofiber Fabrication

Poly(D,L-lactic-co-glycolic acid) (PLGA, 75/25, Sigma, USA) was mixed with tetrahydrofuran (THF, Wako, Japan) at different concentrations: 20%, 23%, and 25% (w/v); then, ionic surfactant sodium dodecyl sulphate (SDS, Wako, Japan) dissolved in de-ionized water was added to a final concentration of 0.92 g L^{-1}. For fluorescent labeling, PLGA solution was loaded with fluorescein isothiocyanate (FITC) or Alexa Fluor® 594 (Life Technologies, USA). PLGA nanofibers were fabricated by electrospinning at the voltage of 10 kV provided by a DC high-voltage generator (Tech Dempaz, Japan). The solution was loaded into a 1-mL syringe to which a needle with a 0.6-mm inner diameter was attached; the positive electrode of the high-voltage power supply was connected to the needle. A grounded rotating drum was used at the speed of 11.4 m s^{-1} to generate aligned nanofibers (ANFs); random nanofibers (RNFs) were generated without rotation. The thickness of nanofibers was controlled by varying the spin time: 10 min for high-density ANFs (H-ANFs, 11.3 ± 1.2 μm), 40 s for low-density ANFs (L-ANFs, 1.5 ± 0.1 μm) and 20 s for RNFs (1.5 ± 0.1 μm). The distance between the tip and collector was maintained at 8 cm. Before spinning, a layer of aluminium foil was attached to the drum for the fiber transfer procedure. Nanofibers were collected in the aluminium foil which was then peeled off and pressed onto the substrate by a thermal press machine (AS ONE, Japan) or transferred to a poly-dimethylsiloxane (PDMS) frame (1 × 1 cm^2); then, the foil was removed and nanofibers remained on the substrate or PDMS frame.

Electrophysiological Characterization

Extracellular recording of field potentials (FPs) was performed using the multielectrode array (MEA) data acquisition system (USB-ME64-System, Multi Channel Systems, Germany).

Signals were recorded from day 2 after CM seeding. The data were collected and processed using MC_Rack (Multi Channel Systems) or LabChart (ADInstruments, New Zealand).

Electrical activation was started by applying bipolar stimuli (±1500 mV, 40 μs) in the electrodes at the MEA centre. The local activation time (LAT) for a single electrode was determined by calculating the minimum of the first derivative plot of the original data. The isochronal map was constructed based on linear interpolation between the electrodes (Meiry et al., 2001), calculated using the Matlab function (Matlab, MathWorks, America). The amplitude, QT interval, and beating rate were determined by analyzing the wave form, and the corrected cQT interval was calculated by normalization to the CM beating rate using the Fridericia correction formula: cQT interval = QT interval/$\sqrt[3]{\text{RR interval}}$. To assess the effects of different drugs, E-4031, isoproterenol, propranolol, Verapamil and Quinidine were added to 1 mL of medium respectively between 6-14 day after cell seeding.

Electron Microscopy

Top view high-resolution images were obtained using a scanning electron microscope (SEM JCM-5000; JEOL Ltd., Japan) operating at 10 kV. CM samples were fixed with 4% paraformaldehyde (PFA; Wako) for 2 min at room temperature, washed twice with PBS, immersed in 30% ethanol for 30 min, and dehydrated in a series of ethanol concentrations (50%, 70%, 80%, 90%, and 100%) for 10 min per each step, followed by nitrogen drying. A 5-nm-thick platinum layer was deposited on the samples by sputtering (MSP 30T; Shinku Device, Japan).

For transmission electron microscopy (TEM), the samples were fixed with 2% glutaraldehyde (Distilled EM Grade, Electron Microscopy Sciences, USA) in NaHCa buffer (100 mM NaCl, 30 mM HEPES, 2 mM $CaCl_2$, adjusted to pH 7.4 with NaOH) and successively post-fixed

with 0.25% OsO4/0.25% $K_4Fe(CN)_6$, then with 1% tannic acid, and finally with 50 mM uranyl acetate. The samples were washed, dehydrated in a series of ethanol, and embedded in TABA EPON 812 resin (TAAB Laboratories Equipment Ltd, UK). After polymerization at 65°C, ultrathin sections (60–100 nm) were cut perpendicular to PLGA fibers using an ultramicrotome (Leica FC6, Austria), mounted on EM grids, stained with lead citrate, and analyzed by TEM (JEOL JEM1400, Japan).

Histology

Tissues were washed three times with PBS, fixed in 4% PFA in PBS, and embedded in paraffin. Thin sections were cut, stained with hematoxylin and eosin (Muto chemical corporation, Japan). Capillary density and inflammatory reactions were assessed by immunohistolabeling for CD31 (mouse monoclonal IgG, 1:50; Dako: M0823) or CD68 (mouse monoclonal IgG, 1:100; Abcam: 955) respectively. The sections were observed under a CKX41 microscope (Olympus) or a BIOREVO fluorescence microscope (KEYENCE Corporation).

Immunostaining and Imaging

CMs were fixed in 4% PFA at room temperature for 30 min, permeabilized with 0.5% v/v Triton X-100 in Dulbecco's (D)-PBS at room temperature for 1 h, and incubated in blocking solution (5% v/v normal goat serum, 5% v/v normal donkey serum, 3% v/v bovine serum albumin, and 0.1% v/v Tween 20 in D-PBS) at 4°C for 16 h. CMs were then incubated with primary antibodies: anti-β-MHC (mouse monoclonal IgM, 1:100; Santa Cruz Biotechnology: SC-53089), anti-α-actinin (mouse monoclonal IgG, 1:1000; Sigma: A7811), and anti-cTnT (mouse monoclonal IgG, 1:200; Santa Cruz Biotechnology: SC-20025) at 4°C for 16 h. Cells were washed and incubated with appropriate secondary antibodies diluted 1:300 in blocking

buffer: DyLight-594 anti-mouse IgM (Jackson ImmnoResearch: 715-516-020), Alexa Fluor 594 anti-rabbit IgG (Jackson ImmnoResearch: 711-586-152), Alexa Fluor 594 anti-mouse IgG (Jackson ImmnoResearch: 715-586-150), and Alexa Fluor 488 anti-rabbit IgG (Jackson ImmnoResearch: 711-546-152) at room temperature for 1 h. Cell were counterstained with 300 nM 4'-6-diamidino-2-phenylindole (DAPI, Wako) at room temperature for 30 min to visualize the nuclei. Images were captured using a fluorescent or confocal microscopes (Olympus), and the orientation of CMs and nanofibers was evaluated by the Fourier component analysis using the ImageJ Directionality plugin (Woolley et al., 2011) which assessed the orientation distribution for each color channel. Tomography images were acquired and combined to form 3D images using the Optical Coherence Microscopy system and the white-light Linnik interferometer (OCM system, Panasonic).

For immunostaining after transplantation, tissues were rinsed with PBS, cut, immersed in 30% sucrose in PBS, and embedded in O.C.T. compound (Sakura Finetek USA, Inc.). Frozen sections were cut into 7-μm-thick slices using a cryostat (Leica CM 1950) and mounted on MAS-coated glass slides (Matsunami Glass Ind. Ltd.). After treatment with PBS or Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20, the sections were incubated with a mouse anti-cardiac troponin T antibody (2–10 μg/mL; Abcam Plc: ab8295), a rabbit anti-cardiac troponin I (rabbit monoclonal IgG, 1:100; Abcam Plc: ab52862) or a mouse anti-human nuclear antibody (HNA) (mouse monoclonal IgG, 1:200; MED Millipore: MAB1281) for 16 h at 4°C, followed by incubation with secondary anti-mouse Alexa 555-conjugated IgG (1:200; Life Technologies: A21422), anti-rabbit Alexa 555-conjugated IgG (1:200; Life Technologies: A21428), anti-mouse Alexa 488-conjugated IgG (1:200; Life Technologies: A11001) and anti-rabbit Alexa 488-conjugated IgG (1:200; Life Technologies: A11008). F-actin was stained using Alexa Fluor 647-labelled phalloidin (1:100; Life Technologies: A22287). The sections were mounted with the

ProLong Gold antifade reagent with DAPI (Life Technologies) and examined under a confocal laser scanning microscope (FV1200; Olympus Co.) at the excitation wavelengths of 405, 488, 543, and 635 nm.

Flow Cytometry

HiPSCs-CMs cultured on different substrates were harvested using TrypLE Express solution (Life Technologies), fixed in 4% PFA at room temperature for 30 min, permeabilized with 0.5% v/v Triton X-100 in Dulbecco′s (D)-PBS at room temperature for 30 min, incubated with anti-cTnT antibodies (mouse monoclonal IgG, 1:200; Santa Cruz Biotechnology: SC-20025) or isotype-matched antibodies (BD Phosphoflow: 557782) at 37 ◦C for 30 min, washed with D-PBS, and incubated with Alexa Fluor 488 anti-mouse IgG (1:500; Jackson ImmnoResearch: 715-546-150). Cells were then washed twice with D-PBS and analyzed using a FACS Canto II flow cytometer (BD Biosciences, USA) and the FlowJo software (Treestar Inc., USA). Data shown are representative of at least three independent experiments.

qPCR

Total RNA was harvested using Trizol (Life Technologies), and RNA concentration was measured using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized and analyzed by qPCR using the SYBR Green PCR MasterMix (Life Technologies) and the qBiomarker Validation PCR Array (IPHS-102A; Qiagen, USA) in a 96-well format following the manufacturer's instructions. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min; the reactions were performed in a StepOnePlus Real-Time PCR system (Life Technologies). The gene expressions were measured by ddCt method relative to house keep

gene (GAPDH). Heatmaps were generated by the R package open-source software for bioinformatics. The clustering order was produced with Ward.D clustering algorithm.

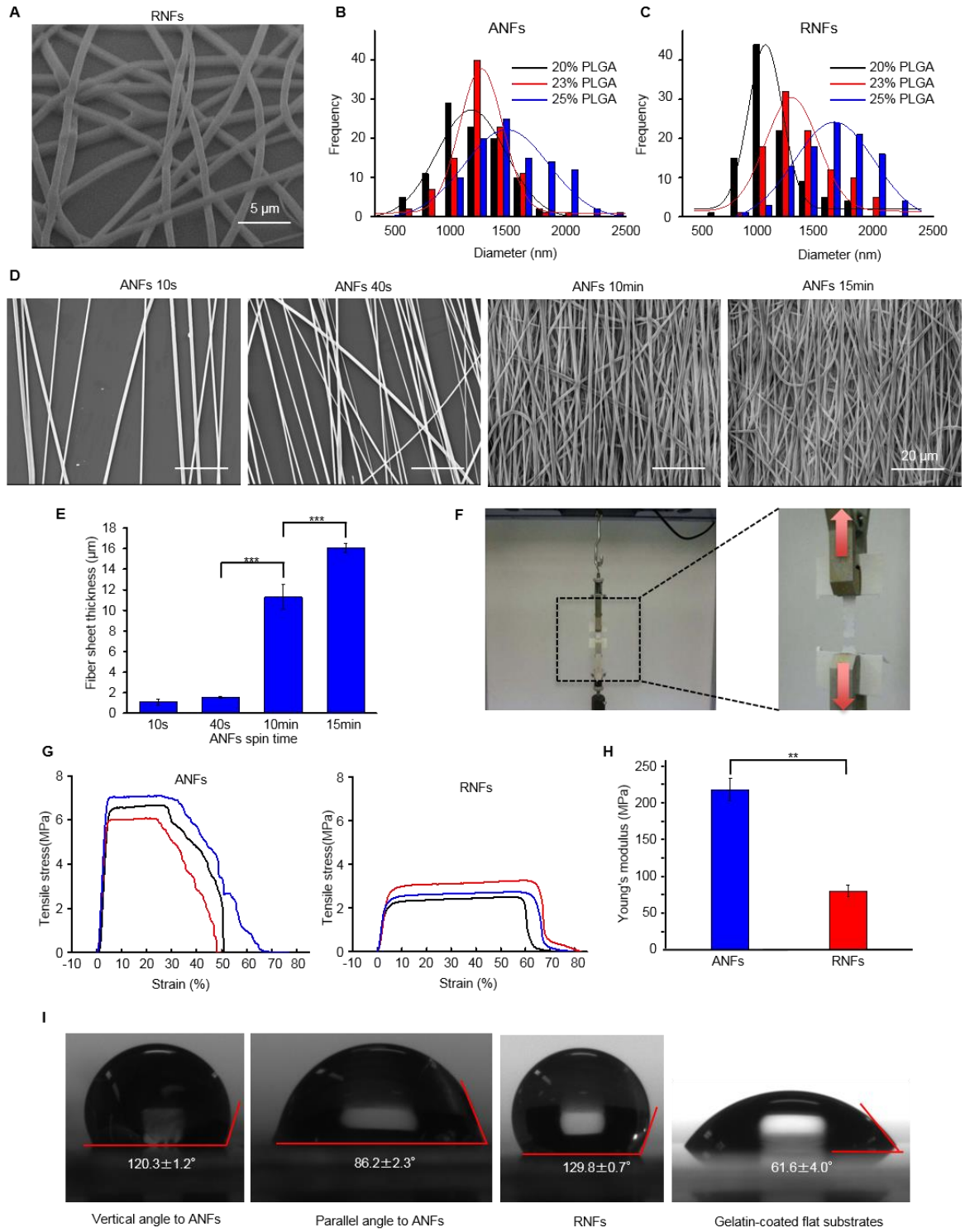

Figure S1. Characteristics of nanofibers. Related to Figure 1. (A) A representative electron microscopy image of randomly arranged nanofibers (RNFs). **(B, C)** Diameter distribution of aligned nanofibers (ANFs, **B**) and RNFs **(C)** fabricated with different concentrations of poly(D,L-lactic-co-glycolic acid) (PLGA). **(D)** Electron microscopy images

of ANFs manufactured using different spin times (10 s, 40 s, 10 min, and 15 min). **(E)** ANFs thickness depending on the spin time. Data are represented as means ± SD, n = 4 independent experiments. ***p < 0.001 by One-way ANOVA followed by Tukey's post hoc test. **(F)** Photographs of the experimental setup. Specimen gauge length and width were determined using a Shimadzu Autograph AGS-X micro-tensile tester (Shimadzu Corp.) with a 1N load cell and digital video extensometer, setting the cross-head speed at 10 mm min^{-1}. The rigidity was calculated using Trapezium X with an initial linear region of the stress-strain curve. **(G)** Stress-strain curves of aligned nanofibers (ANFs) and random nanofibers (RNFs). **(H)** Young's modulus of ANFs and RNFs. Data are represented as means ± SD, n = 3 independent experiments. **p < 0.01 by Student's t test. **(I)** Contact angle measurement of ANF/RNF and gelatin-coated flat substrates. The sessile drop method was used to measure the contact angle of a water droplet on the substrate using a microscope with a CCD camera. A 2-μL water droplet was deposited onto the substrate and the water/substrate interface was photographed. The edge of the droplet was then analyzed using a sessile drop-fitting model. Data are represented as means ± SD, n = 3 independent experiments.

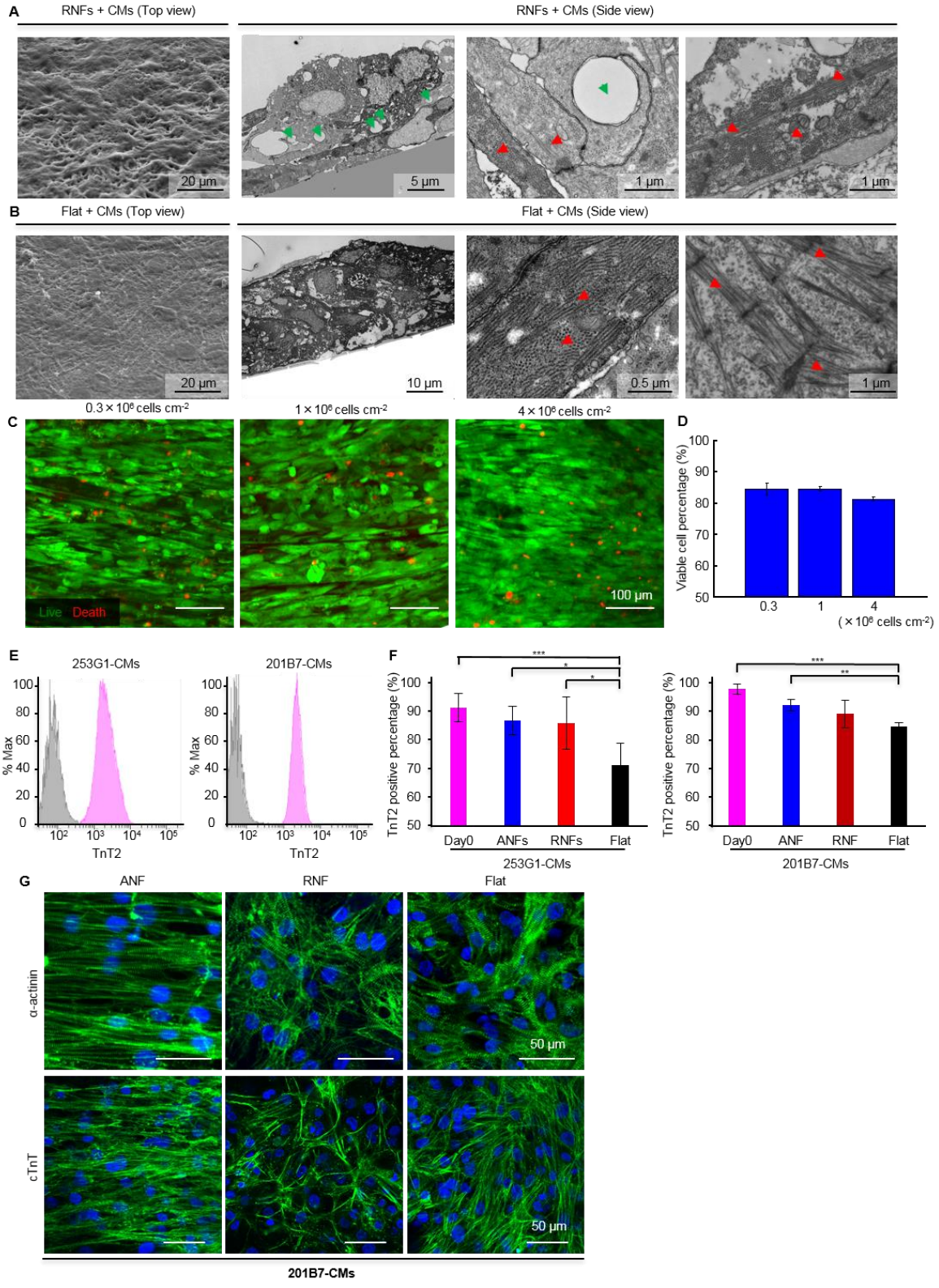

Figure S2. Tissue formed on different substrates. Related to Figure 1 and Figure 2. (A) Scanning electron microscopy **(**SEM, top view) and transmission electron microscopy (TEM, side view) images of cardiomyocytes (CMs) cultured on random nanofibers (RNFs) for 14 days. **(B)** SEM (top view) and TEM (side view) images of CMs cultured on Flat for 14 days. The green and red arrows indicate nanofibers and sarcomeric bundles in the actin-myosin system, respectively. **(C, D)** Viability of CTLCs with different cell densities on day 6. Data

are represented as means ± SD, n = 3 independent experiments. **(E)** Flow cytometry data of cTnT positive cell (hiPS cell line: 253G1; 201B7) on day 0. **(F)** Flow cytometry analysis of CMs on different substrates: aligned nanofibers (ANFs), random nanofibers (RNFs), and gelatin-coated flat substrate (Flat) for 14 days. Data are represented as means ± SD. For 253G1**,** Day 0: n = 32; ANFs: n = 3; RNFs: n = 3; Flat: n = 3; For 201B7, n = 3 (n represents independent experiments for all the groups). *$p<0.05$, **$p<0.01$ and ***$p < 0.001$ by One-way ANOVA followed by Tukey's post hoc test. **(G)** Immunostaining images of α-actinin and cTnT (green). Cardiomyocytes (CMs, 201B7) were cultured on different substrates for 14 days.

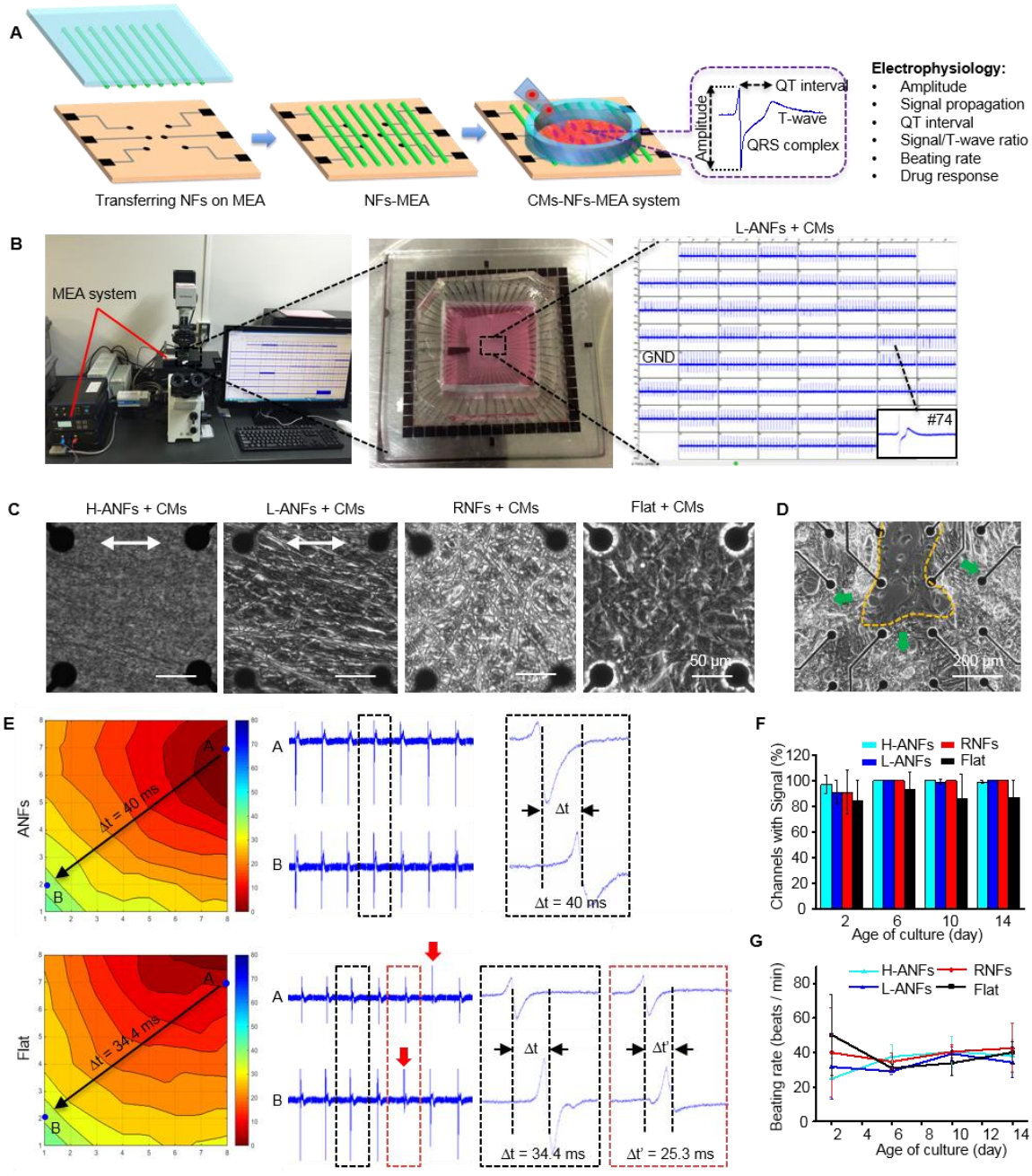

Figure S3. Extracellular recording of cardiomyocytes (CMs) using the microelectrode array (MEA). Related to Figure 3. (A) Schematic representation of cardiac tissue-like construct (CTLC) integration into the MEA system. The encircled image is a representative electrogram of the field potential (FP) recorded from CMs, illustrating the parameters to be analyzed. **(B)** Images of the MEA system and MEA chip with CTLC. The enlarged images indicate the homogeneous electrical signals recorded by electrodes. **(C)** Phase contrast images of CMs on different substrates: high-density and low-density aligned nanofibers (H-ANFs and L-ANFs, respectively), random nanofibers (RNFs), and gelatin-coated flat substrates (Flat). The white arrows mark ANFs orientation. **(D)** Images of the Flat sample on day 6 with CMs clusters marked by green arrows. The dashed line marks the area with few remaining CMs. **(E)** Homogeneity and regularity of CM beating on ANFs and Flat. Activation maps (left) illustrate homogeneous propagation of spontaneous contractions; contraction regularity is shown by a series of beatings (right) recorded from point A to B, with a delay of Δt. The red arrows mark irregular beating which resulted in different Δt = 25.3

ms. **(F)** Channels recording field potential (n = 6–8 independent biological replicates). **(G)** CM beating rate at different culture times (n = 3–5 independent biological replicates). Data are represented as means ± SD.

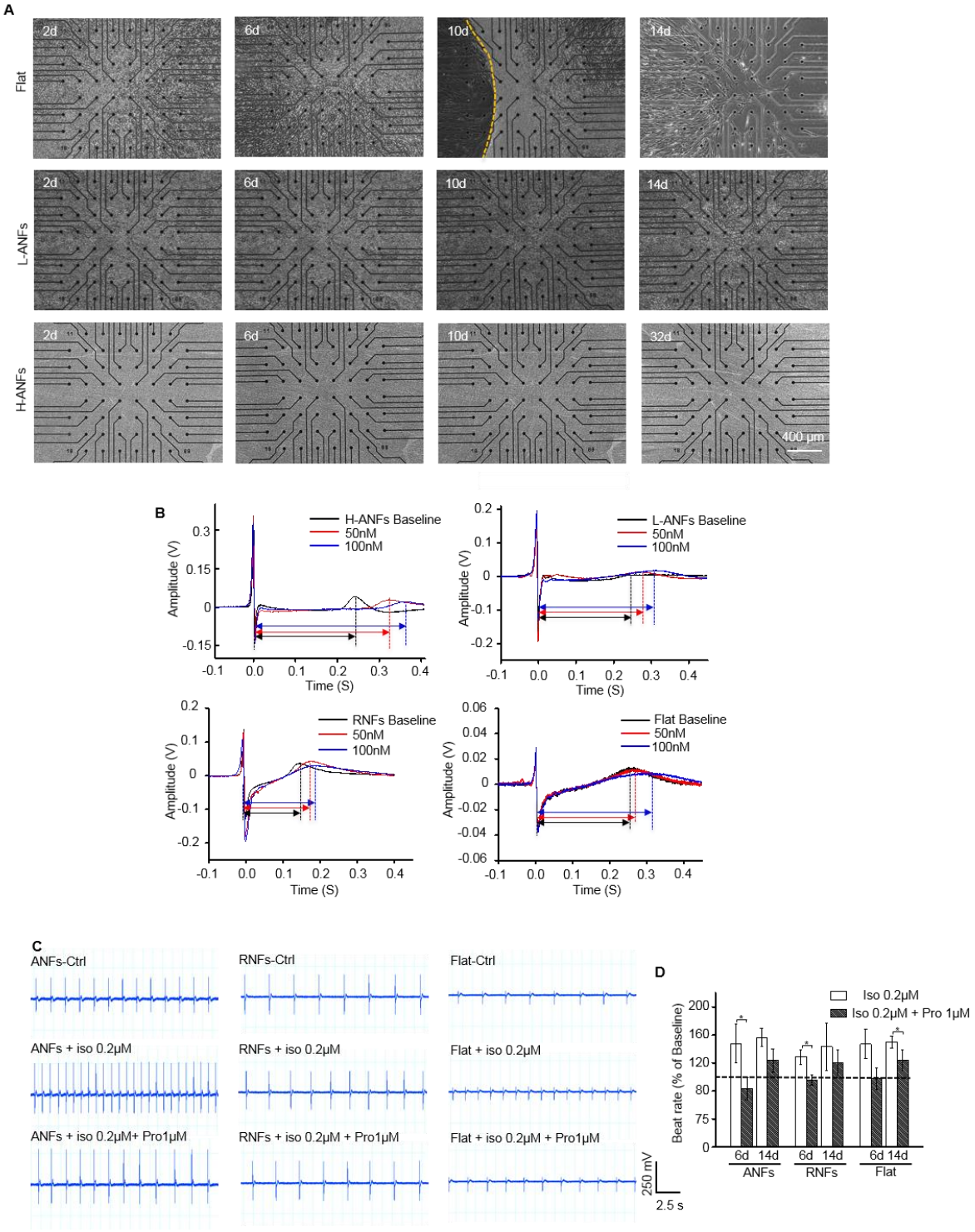

Figure S4. Long term culture and drug effects on cardiomyocytes (CMs). Related to Figure 3. (A) Long-term culture of cardiomyocytes (CMs) on gelatin-coated flat substrates (Flat) and low-density and high-density aligned nanofibers (L-ANFs and H-ANFs, respectively). The dashed line marks the area where the CM sheet peeled off from the

substrates; the CM sheet totally peeled off from Flat on day 14. Cardiac tissue-like constructs (CTLCs) created on H-ANFs were sustained for over 32 days. **(B)** Prolongation of the repolarization phase after E4031 application. **(C)** Representative beating of CMs treated with isoproterenol (Iso) and propranolol (Pro) and cultured on different substrates. **(D)** Effects of Iso and Pro on CM beating rate. Data are represented as means ± SD, n = 3–4 independent biological replicates. *p < 0.05 by Student's t test.

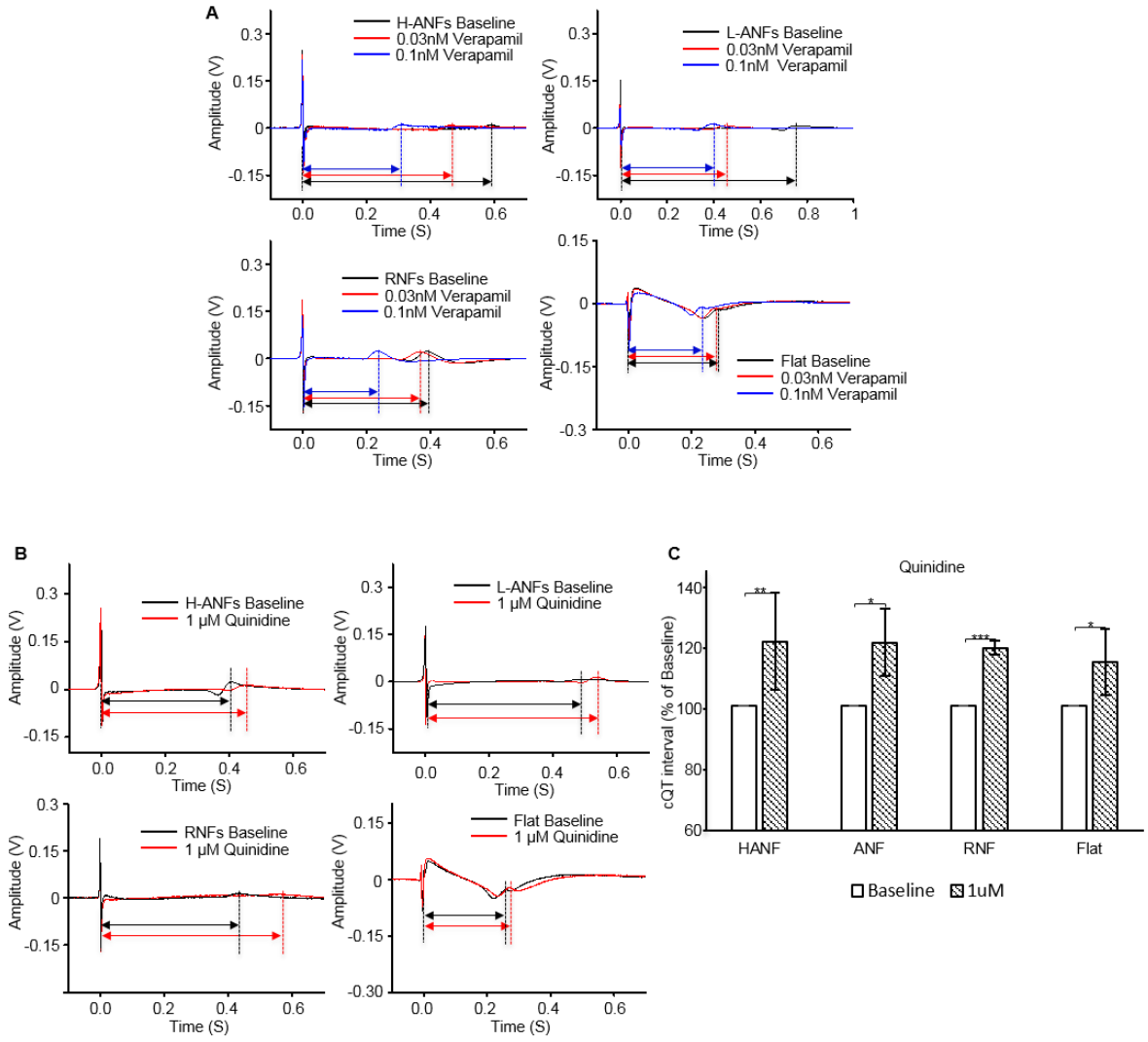

Figure S5. Drug effects on cardiomyocytes (CMs). Related to Figure 3. (A) Shortening of the repolarization phase after verapamil application. **(B)** Prolongation of the repolarization phase after quinidine application. **(C)** Effects of quinidine on corrected QT interval (cQT interval) of CM beating. Data are represented as means ± SD, n = 3–7 independent biological replicates. *p < 0.05, **p < 0.01, and ***p < 0.001 by Student's t test.

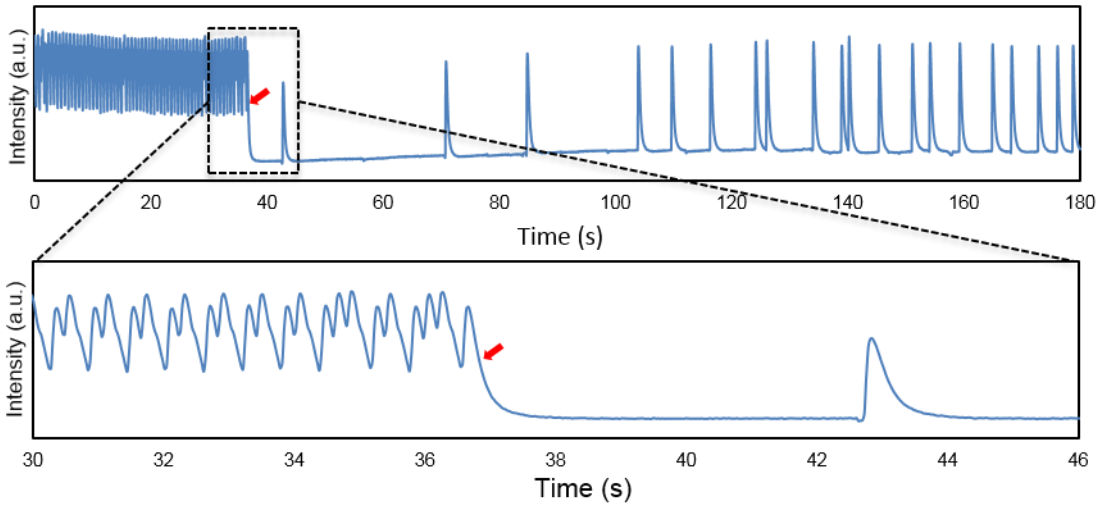

Figure S6 Ca^{2+} transients of the GCaMP3-positive CTLC on a host CM sheet with spiral waves. Related to Figure 4. The recording lasted for 3 min and the red arrow marked the moment when the spiral wave was terminated by the coupling of CTLC with the host CM sheet.

A

Parameters	Condition
Cardiomyocytes differentiation time	3 weeks, 7 weeks
CTLC coating treatment protein type	Laminin, Collagen, No-coated
Protein concentration	Laminin (1 μg / cm² ; 10 μg / cm²), Collagen (5 μg / cm² ; 50 μg / cm²)
CTLC coating treatment temperature and time	Room temperature 5 min, 37°C 5 min, 37°C overnight
Protein solution type	PBS, Medium
Cell culture time on ANFs	1 day, 2 day, 7 day
Fibrin sealant	Beriplast P, No glue

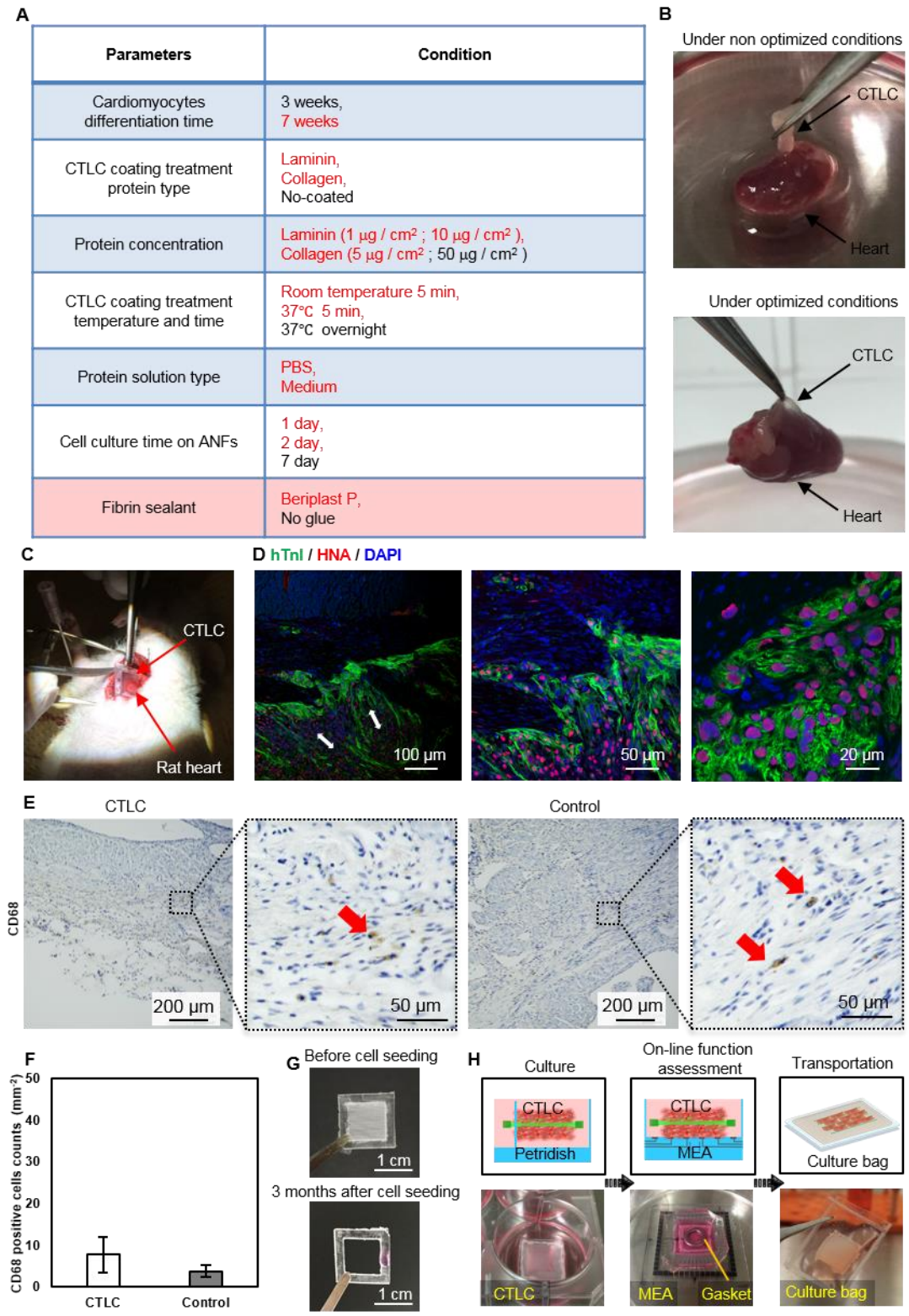

Figure S7. Preparation of a cardiac tissue-like construct (CTLC) for transplantation). Related to Figure 5 and Figure 6. (A) Condition screening by the *in vitro* attachment to mouse hearts. To improve CTLC attachment to the heart, a number of experimental conditions were screened. The conditions marked in red were used for transplantation. **(B)** CTLC attachment was assessed *in vitro* by testing whether the mouse heart-bound CTLC could sustain the weight of the heart. **(C)** Transplantation of CTLC on a rat heart**. (D)** Double immunostaining of consecutive sections from the *in vivo* transplanted CTLC for human

cardiac troponin I (hTnI) and human nuclear antigen (HNA); nuclei were stained with DAPI. The white arrow indicated the alignment of CMs. **(E)** Long-term degradation of nanofibers. Aligned nanofibers (ANFs) were mounted onto a PDMS frame (top) and immersed in medium after seeding of cardiomyocytes (CMs); ANFs would degrade within 3 month (bottom). **(E)** Immunohistochemical analysis on peri-ischemic zone in MI heart 4 week after transplantation of CTLC (left) and acellular control (right). The sections are immunostained with CD68 antibodies. The red arrow marked the CD68-positive cells. **(F)** The CD68 positive cells density in CTLC and control group. Data are represented as means ± SD, n = 3 rats. **(G)** Preparation and transport of the CTLC. After cell seeding, the CTLC can be functionally evaluated before transportation and used for other applications. A gasket is used to fix the CTLC on the MEA for signal recording.

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