Supporting information

Label-free Raman spectroscopy for assessing purity and maturation of hiPSC-derived cardiac tissue.

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1. Methods

The preparation of iCell/fibroblast spheroids

Cryopreserved hiPSC-CMs (iCell^2, Lot:105451, Cellular Dynamics International (CDI), USA) and human ventricular cardiac fibroblasts (NHCF-V, CC-2904, Lonza, Switzerland) were thawed in a prepared medium (Plating Media, CDI). The iCell and fibroblasts were mixed at different ratios (0%, 25%, 50%, 75%, 100%) in the plating medium and 1,000 cells were seeded into a v-bottom 96-well plates (Wako, Pure Chemical Industries) to obtain spheroids according to a previous report [1]. The medium was changed to maintenance medium (CDI) after 2 d.

Preparation of hiPSCs and differentiation into CMs

HiPSCs (253G1; Riken, Saitama, Japan) were maintained in primate embryonic stem cell medium (ReproCELL, Kanagawa, Japan) with the addition of basic fibroblast growth factor (bFGF; ReproCELL). The mouse embryonic fibroblast cells (ReproCELL) were treated with Mitomycin C (0.5 mg/mL; Wako, Pure Chemical Industries, Tokyo, Japan) for 2 h and used as the feeder layer. CM differentiation was performed as previously reported [2] in StemPro 34 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 2 mM L-glutamine (Thermo Fisher Scientific), 50 μg/mL ascorbic acid (Wako, Pure Chemical Industries), and 400 mM 1-thioglycerol (Sigma-Aldrich, St. Louis, MO, USA). hiPSCs were dissociated by using Accmax™ (Nacalai Tesque, Kyoto, Japan) for 5 min, and the induction was initiated in a bioreactor (ABLE Corporation & Biott Co., Tokyo, Japan). The following factors (BMP4, activin A, bFGF, and VEGF (R&D Systems, Minneapolis, MN, USA), and IWR-1 and IWP-2 (Sigma-Aldrich) were added on the corresponding days: BMP4 (day 0–1); activin A, BMP4, and bFGF (day 1–4); IWR-1 and IWP-2 (day 4–6); and VEGF and bFGF (after day 6).

The clinical grade hiPSC cell line (QHJI14s04) was established using peripheral blood mononuclear cells collected from a healthy HLA homozygous donor[3-4]. The cells were maintained using a feeder-free and

xeno-free culture system. The cardiomyogenic differentiation was induced as previously described[3-4]. Briefly, the embryoid bodies were generated in EZ plates (Iwaki, Shizuoka, Japan) in Stem Fit Ak03N without bFGF and with 10 μM Y-27632 (Wako Pure Chemical Industries, Osaka, Japan) and BMP-4. After embryonic body (EB) formation, the culture medium was replaced with differentiation medium containing Stem Fit Ak03N without bFGF and supplemented with several human recombinant proteins, including BMP-4 and activin A, bFGF, and VEGF as well as the small molecule inhibitor of Wnt production 3 (Stemgent, Lexington, MA, USA), SB431542 (Sigma-Aldrich), and dorsomorphin (Sigma-Aldrich). After cardiac differentiation, the EB was dissociated with Accumax for 10 min and used for flow cytometry analysis performed on day 14.

Flow Cytometry

HiPSC-CMs were fixed in 4% paraformaldehyde (PFA) at 25 degree 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 (1:200; sc-20025, Santa Cruz Biotechnology, Dallas, TX, USA) or isotype-matched antibody (BD Phosphoflow: 557782) at 4 ˚C for 8 h. The cells were then washed with D-PBS, and then incubated with Alexa Fluor 488 anti-mouse IgG (1:1,000; A11029; Thermo Fisher Scientific). Cells were then washed twice with D-PBS and analyzed using a FACS Canto II flow cytometer (BD Biosciences, CA, USA) and the FlowJo software (Treestar Inc., OR, USA).

Promoting maturation of hiPSC-CM tissue by using traveling wave

The devices were prepared using a protocol modified from a previous report[5-6]. A polydimethylsiloxane (PDMS) well (SYLGARD 184; Dow Corning, Midland, MI, USA) with an

inner diameter of 8 mm was prepared using a tissue puncher. The well and a 3-mm PDMS pillar were aligned and attached to the bottom of quartz plates to facilitate the following Raman recording. The device was precoated with laminin511-E8 fragments (MATRIXOME, Osaka, Japan) at 37 °C for 30 min before cell plating. Single 253G1 CMs were filtered using a 40-μm cell strainer (BD Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) and resuspended at a density of 0.5×10^6 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) containing 20% fetal bovine serum (Gibco, USA) and 1% laminin511-E8 fragments, 3μM Y-27632. The medium was changed with prewarmed DMEM containing 5% fetal bovine serum after day 2 and the medium change was performed every 3–4 days. The traveling wave will spontaneously originate in the close-loop tissue from day 2. As for the control group (without training of traveling wave), the traveling wave was terminated by changing the medium at room temperature. All samples were maintained for 14 d before being used for further analysis. For the hypoxia culture, cardiac tissues were cultured in a hypoxia incubator (Waken tech, Japan) with oxygen set at 5% for 5 d.

Immunostaining and imaging

The spheroids or cardiac tissue 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 [0.1% (v/v) Tween-20, 5% (v/v) normal donkey serum, 3% (v/v) bovine serum albumin, and 5% (v/v) normal goat serum in D-PBS] at 4°C for 16 h. The samples were then incubated with the primary antibodies anti-α-actinin (1:1,000; A7811; Sigma-Aldrich), anti-MYL2 (1:200; 10906-1-AP; Proteintech), anti-troponin T2 (TnT2; 1:200; SC-20025; Santa Cruz Biotechnology), anti-connexin 43 (Cx43; 1:200; C6219; Sigma-Aldrich),

anti-cytochrome *c* (Cyt c; 1:100; sc-13561, Santa Cruz Biotechnology), anti-myoglobin (1:200; ab77232; Abcam) or anti-β-MHC (1:100; SC-53089; Santa Cruz Biotechnology) at 4°C overnight. The samples were then rinsed with PBS and incubated with secondary antibodies diluted at 1:300 in a blocking buffer. Thereafter, the tissues were rinsed with PBS and incubated with the secondary antibodies Alexa Fluor 594 anti-mouse IgG (715-586-150; Jackson Immuno Research, West Grove, PA, USA), DyLight-594 anti-mouse IgM (715-516-020; Jackson Immuno Research), Alexa Fluor 647 anti-rabbit IgG (A21245; Thermo Fisher Scientific), and Alexa Fluor 488 anti-rabbit IgG (A21206; Thermo Fisher Scientific) at a dilution of 1:300 in blocking buffer at room temperature for 1 h. 4′-6-Diamidino-2-phenylindole (DAPI; 300 nM; Wako Pure Chemical Industries, Ltd.) was used to counterstain nuclei at room temperature for 30 min, after which images were captured using a confocal microscope (FV1200; Olympus or NIKON A1; Nikon).

Live/dead assay

The cardiac tissue was stained by using a LIVE/DEAD kit (Thermo Fisher Scientific) according to the manual. Briefly, the tissue was washed with PBS once. The 1 μL A and 5 μL B were mixed with 2 mL PBS and added into the wells with tissue for 30 min. Fluorescent images were captured using a confocal microscope (NIKON A1; Nikon). Images were processed and analyzed using the ImageJ software (NIH, Bethesda, MD, USA).

Transmission electron microscopy (TEM)

Cardiac tissues were fixed with 2.5% glutaraldehyde for 2 h. The samples were then post-fixed with 1% osmium tetraoxide for 1.5 h and dehydrated through a graded series of ethanol (50–100%) and propylene oxide. The tissues were then embedded in epoxy resin, sliced using an ultramicrotome (Ultracut E; Reichert-Jung, Vienna, Austria), and stained with uranyl acetate and lead citrate. The cardiac tissues were observed using a transmission electron microscope (H-7650; Hitachi Co., Tokyo, Japan).

Quantitative polymerase chain reaction (qPCR)

Total RNA in hiPSC-CM tissue was harvested by using Trizol reagent (Life Technologies, CA, USA) according to manufacturer instructions, and RNA concentration was determined by using a spectrophotometer (NanoDrop1000, Thermo Fisher Scientific). cDNA was synthesized with a synthesis kit (TaKaRa, Shiga, Japan) and analyzed by qPCR using SYBR Green PCR master mix (Life Technologies) and a qBiomarker validation PCR array (IPHS-102A; Qiagen, Hilden, Germany) according to manufacturer instructions. The cycling conditions are the following: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 70 s. Reactions were performed in a StepOnePlus real-time PCR system (Life Technologies). Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method and relative to glyceraldehyde 3-phosphate dehydrogenase expression.

Line-illumination Raman microscope

Before imaging, the cardiac tissue was washed with PBS. To stop the spontaneous beating during recording, 4-degree Live cell imaging solution (Thermo Fisher) was used as an imaging medium. The cardiac tissues were then imaged using our homemade line-illumination Raman microscope.

The Raman hyperspectral datasets were recorded by a home-built slit-scanning confocal Raman microscope[7] equipped with a 532 nm continuous-wave laser (millennia eV; Spectra-Physics) with a power density of 5 mW/μm^2. Briefly, the line-shape laser was produced using cylindrical lenses and focused on the sample using a ×25 water immersion objective lens (NA 1.15 CFI75 Apochromat; Nikon). The Raman scattered light went through the same objective lens and was collected by a spectrograph (MK-300; Bunkoukeiki) through a long-pass edge filter (LP03-532RU-25; Semrock). The light dispersed by the grating (600 L/mm) was then recorded by using a cooled CCD camera (PIXIS 400 B; Teledyne Princeton Instruments) with an exposure time of 5 s. The spectral resolution was 3 cm^{-1}. 400 spectra on the line were collected with one exposure. Images were acquired by scanning with a single-axis galvanometer mirror. Each high-resolution Raman image contains 400 pixels × 126 pixels for a total of 100,800 spectra per image for Fig. 1 and 400 pixels × 189 pixels for Fig. 4.

Data processing

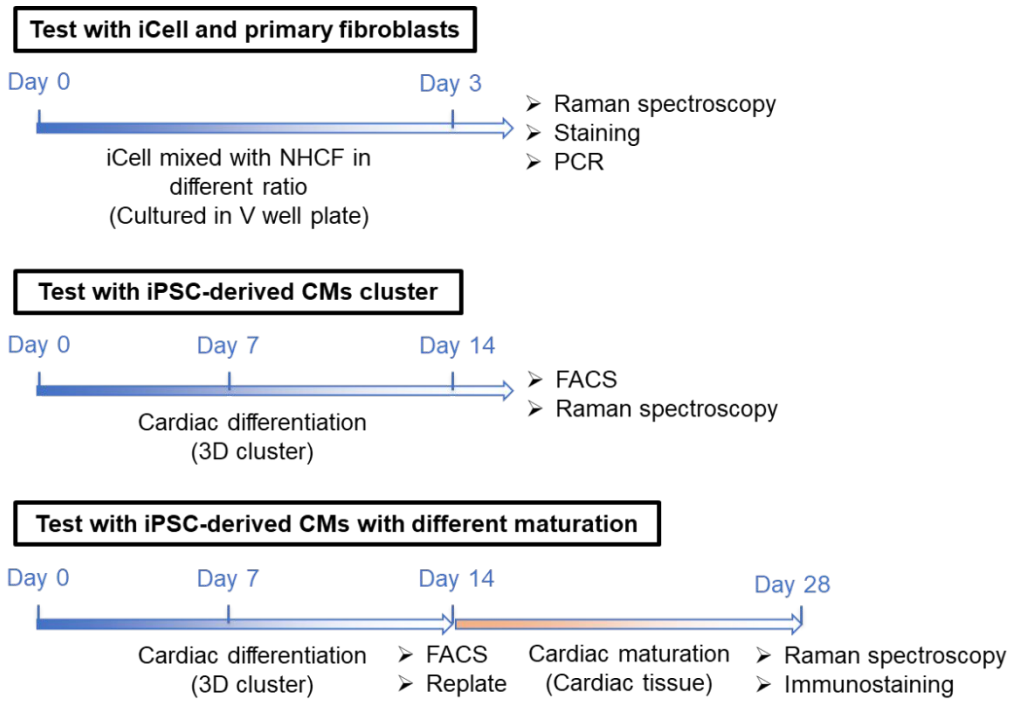
Cosmic rays were removed by the median filter and singular value decomposition (SVD) was used for noise reduction and loading vectors contributing to the image contrast were selected. The peak area of Raman intensity was used to quantify the intensity of each Raman peak. The intensity distribution of the designated Raman shift was mapped and shown as Raman images.

Reconstructed Raman images at 2,930 cm^{-1} were used to present clear spheroid boundaries. Individual spheroid masks were then created and applied to the spectrum data without SVD processing to acquire the average spectrum of each spheroid used for the following quantitative

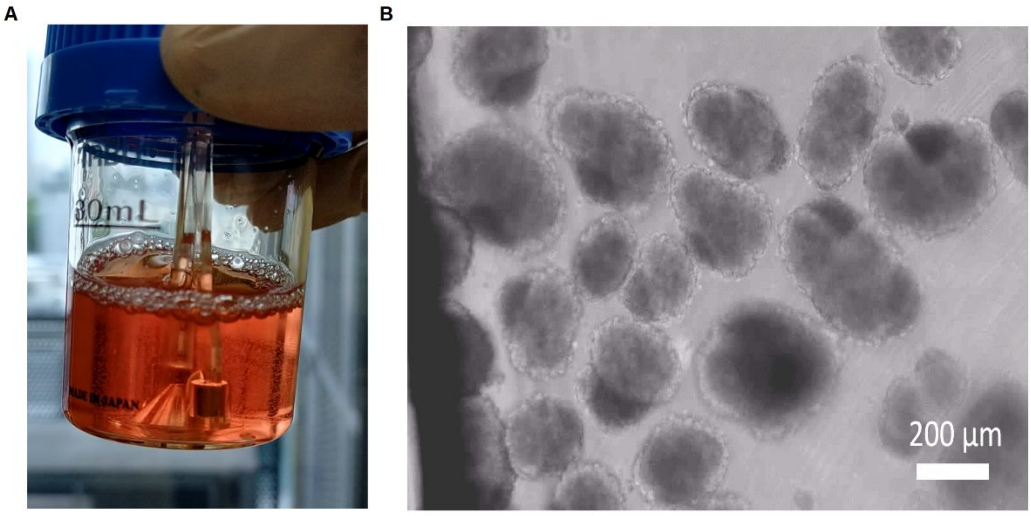
calculations. The Raman intensity of the target shifts was calculated after baseline correction to reduce any interference from adjacent Raman shifts. To compare the Raman intensities of the samples under various conditions, a Raman intensity of 2,930 cm^{-1} was used, which was assigned for the symmetric stretching mode of CH_3 and used as an internal reference for the normalization of the designated Raman shifts.

Statistics and Reproducibility

The quantitative data are presented as the mean ± standard deviation. The differences among different groups were analyzed using a one-tailed unpaired Student's *t*-test (between two groups) or one-way analysis of variance, followed by Tukey's post hoc test (among three or more groups). $P < 0.05$ was considered statistically significant.



Supplementary Figure 1. Schematic timing illustration of sample preparation and characterization. CM, cardiomyocytes\; FACS, fluorescence-activated cell sorting; iPSC, induced pluripotent stem cell



Supplementary Figure 2. Differentiation system of 3D cardiac spheroids. (A) Bioreactor for hiPSC differentiation into cardiomyocytes. (B) Cardiac spheroids at day 14 after initiation of differentiation. hiPSC, human induced pluripotent stem cell

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