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Sarcomere Shortening of Pluripotent Stem Cell-Derived Cardiomyocytes Using Fluorescent-tagged Sarcomere Proteins.

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Corresponding Author:	Hideki Uosaki, M.D., Ph.D. Jichi Medical University Shimotsuke, Tochigi JAPAN
Corresponding Author's Institution:	Jichi Medical University
Corresponding Author E-Mail:	uosaki.hideki@jichi.ac.jp
Order of Authors:	Razan Ahmed
	Nawin Chanthra
	Tatsuya Anzai
	Keiichiro Koiwai
	Tomoki Murakami
	Hiroaki Suzuki
	Yutaka Hanazono
	Hideki Uosaki
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TITLE:

Sarcomere Shortening of Pluripotent Stem Cell-Derived Cardiomyocytes Using Fluorescent-tagged Sarcomere Proteins.

AUTHORS AND AFFILIATIONS:

Razan E Ahmed[1,*], Nawin Chanthra[1,*], Tatsuya Anzai[1,2], Keiichiro Koiwai[3,4], Tomoki Murakami[4], Hiroaki Suzuki[4], Yutaka Hanazono[1], Hideki Uosaki[1]

[1]Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan

[2] Department of Pediatrics, Jichi Medical University, Tochigi, Japan

[3] Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, Tokyo, Japan

[4] Department of Precision Mechanics, Faculty of Science and Engineering, Chuo University, Tokyo, Japan

[*]These authors contributed equally

Corresponding Author:

Hideki Uosaki

uosaki.hideki@jichi.ac.jp

Email Addresses of Co-authors:

Razan E Ahmed (d2029@jichi.ac.jp)

Nawin Chanthra (nawin2020@jichi.ac.jp)

Tatsuya Anzai (r1204at@jichi.ac.jp)

Keiichiro Koiwai (koiwai@go.tuat.ac.jp)

Tomoki Murakami (murakami@nano.mech.chuo-u.ac.jp)

Hiroaki Suzuki (suzuki@mech.chuo-u.ac.jp)

Yutaka Hanazono (hanazono@jichi.ac.jp)

KEYWORDS:

Pluripotent stem cell, cardiomyocyte, sarcomere shortening, live imaging, fluorescent-tagged sarcomere proteins, microcontact printing

SUMMARY:

This method can be used to examine sarcomere shortening using pluripotent stem cell-derived cardiomyocytes with fluorescent-tagged sarcomere proteins.

ABSTRACT:

Pluripotent stem cell-derived cardiomyocytes (PSC-CMs) can be produced from both embryonic and induced pluripotent stem (ES/iPS) cells. These cells provide promising sources for cardiac disease modeling. For cardiomyopathies, sarcomere shortening is one of the standard physiological assessments that are used with adult cardiomyocytes to examine their disease phenotypes. However, the available methods are not appropriate to assess the contractility of PSC-CMs, as these cells have underdeveloped sarcomeres that are invisible under phase-contrast microscopy. To address this issue and to perform sarcomere shortening with PSC-CMs, fluorescent-tagged sarcomere proteins and fluorescent live-imaging were used. Thin Z-lines and an M-line reside at both ends and the center of a sarcomere, respectively. Z-line proteins - α-Actinin (ACTN2), Telethonin (TCAP), and actin-associated LIM protein (PDLIM3) – and one M-line protein - Myomesin-2 (Myom2) were tagged with fluorescent proteins. These tagged proteins can be expressed from endogenous alleles as knock-ins or from adeno-associated viruses (AAVs). Here, we introduce the methods to differentiate mouse and human pluripotent stem cells to cardiomyocytes, to produce AAVs and to perform and analyze live-imaging. We also describe the methods for producing polydimethylsiloxane (PDMS) stamps for a patterned culture of PSC-CMs, which facilitates the analysis of sarcomere shortening with fluorescent-tagged proteins. To assess sarcomere shortening, time-lapse images of the beating cells were recorded at a high framerate (50–100 frames per second) under electrical stimulation (0.5-1 Hz). To analyze sarcomere length over the course of cell contraction, the recorded time-lapse images were subjected to SarcOptiM, a plug-in for ImageJ/Fiji. Our strategy provides a simple platform for investigating cardiac disease phenotypes in PSC-CMs.

INTRODUCTION:

Cardiovascular diseases are the leading cause of mortality worldwide[1] and cardiomyopathy represents the third cause of cardiac-related deaths[2]. Cardiomyopathy is a collective group of diseases that affect cardiac muscles. The recent developments of induced pluripotent stem (iPS) cells and the directed-differentiation of iPS cells toward cardiomyocytes (PSC-CMs) have opened the door for studying cardiomyocytes with patient genome as an *in vitro* model of cardiomyopathy. These cells can be used to understand the pathophysiology of cardiac diseases, to elucidate their molecular mechanisms, and to test different therapeutic candidates[3]. There is a tremendous amount of interest, thus, patient-derived iPS cells have been generated (e.g., hypertrophic cardiomyopathy [HCM][4,5], arrhythmogenic right ventricular cardiomyopathy [ARVC][6], dilated cardiomyopathy [DCM][7], and mitochondrial-related cardiomyopathies[8,9]). Because one of the characteristics of cardiomyopathy is the dysfunction and disruption of sarcomeres, a valid tool that uniformly measures sarcomere function is needed.

Sarcomere shortening is the most widely used technique to assess sarcomere function and the contractility of adult cardiomyocytes derived from animal models and humans. To perform sarcomere shortening, well-developed sarcomeres that are visible under phase-contrast are required. However, PSC-CMs cultured in vitro display underdeveloped and disorganized sarcomeres and, therefore, are unable to be used to properly measure sarcomere shortening[10]. This difficulty to properly assess the contractility of PSC-CMs hinders their usage as a platform to assess cardiac functions in vitro. To assess PSC-CMs contractility indirectly, atomic force microscopy, micro-post arrays, traction force microscopy, and impedance measurements have been used to measure the effects of the motion exerted by these cells on their surroundings[11-13]. More sophisticated and less invasive video-microscopy recordings of actual cellular motion (e.g., SI8000 from SONY) can be used to alternatively assess their contractility, however, this method doesn't directly measure sarcomere motion or force generation kinetics[14].

To directly measure sarcomere motion in PSC-CMs, new approaches, such as fluorescent-tagging to sarcomere protein, are emerging. For example, Lifeact is used to label filamentous actin (F-actin) to measure sarcomere motion[15,16]. Genetically modified iPS cells are another option for tagging sarcomere proteins (e.g., α-actinin [ACTN2] and Myomesin-2 [MYOM2]) by fluorescent protein[17–19].

In this paper, we describe how to perform time-lapse imaging for measuring sarcomere shortening using Myom2-TagRFP (mouse embryonic stem [ES] cells) and ACTN2-mCherry (human iPS cells). We also show that a patterned culture facilitates sarcomere alignment. In addition, we describe an alternative method of sarcomere labeling, using adeno-associated viruses (AAVs), which can be widely applied to patient-derived iPS cells.

PROTOCOL:

1. Differentiation of mouse pluripotent stem cells

1.1. Maintenance of mouse ES cells

1.1.1. Maintenance medium: Mix 50 mL of fetal bovine serum (FBS), 5 mL of L-alanine-L-glutamine, 5mL of non-essential amino acid (NEAA), 5 mL of 100 mM Sodium Pyruvate, and 909 μl of 55 mM 2-Mercaptoethanol with 450 mL of Glasgow Minimum Essential Medium (GMEM). Supplement Leukemia inhibitory factor (LIF), CHIR-99021, and PD0325901 at a final concentration of 1000 U/mL, 1 μM, and 3 μM, respectively. Sterilize the medium through a 0.22 μm filter.

1.1.2. 10% FBS medium: Mix 55 mL of FBS, 5.5 mL of L-alanine-L-glutamine, 5.5 mL of Sodium Pyruvate, and 5.5 mL of NEAA to 500 mL of Dulbecco's Modified Eagle Medium (DMEM) high glucose. Filtrate the medium through a 0.22 μm filter to sterilize.

1.1.3. Culture SMM18 mouse ES cells in which TagRFP was knocked into the *Myom2* locus on a gelatinized 6 cm dish in maintenance medium as previously described[18]. Passage every 2-3 days.

1.2. Preparation of serum-free differentiation (SFD) medium

1.2.1. Basal SFD: Mix 250 mL of Ham's F-12, 750 mL of Iscove's Modified Dulbecco's Medium (IMDM), 10 mL of B27 supplement minus Vitamin A, 5 mL of N2 supplement, 10 mL of L-alanine-L-glutamine, 5 mL of 10% bovine serum albumin in phosphate-buffered saline (PBS), and 10 mL of penicillin and streptomycin (10,000 U/mL). Filter through 0.22 μm strainer to sterilize.

1.2.2. Dissolve ascorbic acid at 5 mg/mL in distilled water and filter through 0.22 μm strainer to sterilize.

1.2.3. Dilute 13 μL of 1-Thioglycerol to 1 mL of IMDM. Herein, refer to this diluted 1-Thioglycerol as MTG.

1.2.4. Add 10 μL of ascorbic acid (5 mg/mL) and 3 μL of MTG to 1 mL of basal SFD on the day of use. Herein, refer to this mixture as complete SFD.

1.3. Day 0, embryoid body (EB) formation for differentiation

1.3.1. Harvest SMM18 mouse ES cells with a recombinant trypsin-like protease (rTrypsin) and count the cells.

1.3.2. Centrifuge 5 x 10^5 cells at 300 x g for 3 min in 4 °C, resuspend in 10 mL of complete SFD, and seed into a 10-cm Petri dish. Culture the cells at 37 °C and 5% CO_2 for 50 h.

1.4. Differentiation day 2

1.4.1. Add Activin A, human vascular endothelial growth factor (hVEGF), and bone morphogenetic protein 4 (BMP4) to complete SFD at a final concentration of 5 ng/mL, 5 ng/mL, and 1.9 ng/mL, respectively.

NOTE: BMP4 concentrations may differ depending on the lot of BMP4. Test several concentrations in a small-scale trial prior to using a new lot and determine the best concentration for cardiac differentiation.

1.4.2. Transfer EBs from a Petri dish into a 15 mL tube and centrifuge at 50-100 x g for 3 min at 4 °C.

1.4.3. Meanwhile, add the medium prepared in step 1.4.1 to the Petri dish to protect the remaining EBs being dry.

1.4.4. Aspirate the supernatant from the 15 mL tube, resuspend the EBs with the medium in the Petri dish, and transfer the EB solution back to the dish. Then, cultivate the EBs at 37 °C and 5% CO_2 for 46 h.

1.5. Differentiation day 4

1.5.1. Gelatinize a 10 cm tissue culture-treated dish with 5 to 10 mL of 0.1% gelatin for at least 5 min. Aspirate gelatin right before seeding cells.

1.5.2. Prepare medium: Mix basic fibroblast growth factor (bFGF), FGF10, and hVEGF to complete SFD at 5 ng/mL, 25 ng/mL, and 5 ng/mL final concentrations, respectively. For a 10 cm dish, prepare 10 mL.

1.5.3. Transfer cells from the Petri dish to a 15-mL tube. Add 5 mL of PBS to the Petri dish, wash several times, and transfer to the 15-mL tube to collect the remaining cells. Centrifuge at 50-100 x g, 4 °C, 3 min.

1.5.4. Aspirate supernatant, add 1 mL of rTrypsin, and incubate at 37 °C for 3 min.

1.5.5. Vortex briefly to dissociate EBs, add 9 mL of 10% FBS medium, vortex again, and count the cells.

1.5.6. Centrifuge 1.5 x 10^7 cells at 300 x g, 4 °C for 3 min, resuspend with the media prepared in step 1.4.2, and seed into the gelatinized dish. Incubate at 37 °C and 5% CO_2 for 2 days.

NOTE: By day 7 or 8, extensive beating of PSC-CMs can be observed.

1.6. Drug selection at differentiation day 7 and 9: Refeed the media with puromycin (2 μg/mL at the final concentration) to eliminate non-cardiomyocytes at day 7 and 9 of differentiation.

NOTE: Parental line of SMM18 is syNP4 mouse ES cells, harboring NCX1 promoter-driven puromycin-resistant gene[20].

1.7. Day 10, replate for future experiments

1.7.1. Coat a glass-bottom culture plate or a 35 mm imaging dish containing a polymer coverslip with 0.1% Gelatin. To enhance maturation, coat the dishes with laminin-511 E8 fragment (LN511-E8) at 1 μg/cm^2 for 30-60 min at room temperature[18]. To culture PSC-CMs in specific patterns of interest, please refer to steps 4 and 5 for preparing polydimethylsiloxane (PDMS) stamps.

1.7.2. To harvest SMM18 PSC-CMs, wash the dish twice with PBS, apply 1 mL of rTrypsin, and incubate 3 min at 37 °C.

1.7.3. Collect cells in 9 mL of 10% FBS medium, suspend, and count the cells. Plate the cells at 50,000-100,000 cells in one well of a 24-well plate or 250,000-500,000 cells in a 35 mm imaging dish.

1.7.4. Centrifuge a sufficient number of cells (300 x g, 3 min) and resuspend the cells with complete SFD supplemented with FBS (final concentration at 10%).

1.7.5. Incubate overnight and change the culture medium to complete SFD with puromycin.

1.7.6. From day 14, change the culture medium two to three times a week with complete SFD until day 21-28, when Myom2-RFP becomes prominent. For AAV-based transduction of fluorescent-tagged sarcomere proteins, please refer to Step 3.

2. Differentiation of human pluripotent stem cells

2.1. Preparation of differentiation media

2.1.1. RPMI+B27-Ins: mix 500 mL of RPMI 1640 medium, 10 mL of B27 minus insulin, and 5.25 mL of L-alanine-L-glutamine.

2.1.2. RPMI+B27+Ins: mix 500 mL of RPMI, 10 mL of B27 supplement, and 5.25 mL of L-alanine-L-glutamine.

2.2. Maintenance of human iPS cells

2.2.1. Passage human iPS cells twice a week with AK02N on LN511-E8 following previously published method with some modifications[21].

2.2.2. Harvest cells with a 3 min treatment of rTrypsin and collect into 10% FBS medium. Count cells and centrifuge at 300 x g for 3 min at 4 °C. Seed 75,000-125,000 cells in one well of 6 well plate with 2 mL of AK02N supplemented with LN511-E8 and Y27632 at the final concentration of 0.5 μg/mL (0.1 μg/cm^2) and 10 μM, respectively.

2.2.3. Incubate at 37 °C and 5% CO_2 and replace the medium the following day with 2 mL of AK02N without any supplement. Change media every two to three days and passage every three to four days.

2.3. Day -4: replate prior to differentiation

2.3.1. Coat a 6 well plate with 0.5 μg/cm^2 of LN511-E8 diluted in PBS. Then, incubate for at least 30 min at 37 °C and 5% CO_2 or 1 h at room temperature. Aspirate coating solution right before seeding cells.

2.3.2. Harvest human iPS cells with rTrypsin and count cells as in Step 2.2.2.

2.3.3. Centrifuge 1.25 x 10^5 cells for a well of a 6-well plate at 300 x g for 3 min at 4 °C and resuspend in 2 mL of AK02N supplemented with LN511-E8 (final concentration 0.5 μg/mL or 0.1 μg/cm^2) and Y27632 (final concentration 10 μM) per well.

2.3.4. Aspirate coating solution, seed resuspended cells into the coated plate, and incubate at 37 °C and 5% CO_2.

2.4. Day -3 and -1: replace medium with 2 mL of AK02N.

2.5. Day 0: Replace medium with 2 mL of RPMI+B27-Ins supplemented with CHIR99021 (final concentration 6 μM) per well to start differentiation.

2.6. Day 2: Replace medium with 2 mL of RPMI+B27-Ins with WntC59 (final concentration 2 μM) per well.

2.7. Day 4: Replace medium with 2 mL of RPMI+B27-Ins per well.

2.8. Day 7 and day 9: replace medium with 2 mL of RPMI+B27+Ins with puromycin (final concentration 10 μg/mL) per well to selectively culture PSC-CMs.

NOTE: ACTN2-mCherry line, used in this study, has a cassette of internal ribosomal entry site (IRES), puromycin-resistant gene inserted to the 3'-untranslated region (UTR) of TNNT2 locus,

and mCherry replacing the stop codon of ACTN2. To purify cardiomyocyte without knock-in, please refer to Steps 3 and 4.

2.9. Day 10: replate for future experiments

2.9.1. Coat a 35 mm imaging dish with a polymer coverslip with 0.5-1 μg/cm^2 of LN511-E8 diluted in 0.1% Gelatin. Incubate 2-4 h at room temperature for long-term viability. To culture PSC-CMs in desired patterns, please refer to steps 4 and 5 for preparing PDMS stamps.

2.9.2. To harvest human PSC-CMs, wash the dish twice with PBS, apply 1 mL of rTrypsin per well, and incubate 3 min at 37 °C.

2.9.3. Collect cells in 4 mL of 10% FBS medium, suspend, and count the cells. Plate 250,000-500,000 cells per 35 mm imaging dish.

2.9.4. Centrifuge a sufficient number of cells at 300 x g for 3 min at 4 °C, resuspend with RPMI+B27+Ins with puromycin (10 μg/mL), and plate on the coated 35 mm imaging dish.

2.9.5. Incubate overnight. The next morning, replace the culture medium with RPMI+B27+Ins with puromycin (10 μg/mL).

2.9.6. From day 14, change culture medium 2-3 times a week with RPMI+B27+Ins until day 21-28 for imaging. For AAV-based transduction of fluorescent-tagged sarcomere proteins, please refer to Step 3.

3. Fluorescent labeling of sarcomeres using adeno-associated viruses

3.1. Preparation before AAV production

3.1.1. Maintain HEK293T cells in DMEM supplemented with FBS (final concentration 10%) on a 10 cm tissue culture plate. Passage cells three times a week.

3.1.2. Prepare polyethylenimine (PEI) at 1 mg/mL. Mix 50 mg of polyethylenimine MAX 40000 and 40 mL of ultrapure water. Adjust pH to 7.0 using 1N NaOH. Then, make bring the final volume to 50 mL with ultrapure water and filter through a 0.25 μm strainer.

3.1.3. Prepare a shuttle vector with a sarcomere labeling gene (e.g., TCAP or PDLIM3 fused with a green fluorescent protein [GFP]), driven by a cardiomyocyte-specific promoter, such as cardiac troponin T (cTNT)[22].

NOTE: For this instance, we used monomeric enhanced GFP with mutations of V163A, S202T, L221V[23].

3.2. Day 0, passage HEK cells

3.2.1. When cells reach confluency, passage 2.0 x 10^7 HEK293T cells to a 15 cm tissue culture plate with 20 mL of DMEM with 10% FBS.

3.3. Day 1, transfection

3.3.1. Mix 13.5 μg of the shuttle vector, 26 μg of pHelper (a vector coding E2A, E4, and VA of adenovirus), 16.5 μg of pRC6 (a vector coding AAV2 Rep and AAV6 Cap genes), and 1 mL of DMEM without sodium pyruvate (DMEM-Pyr).

3.3.2. Mix 224 μL of PEI (1mg/mL, prepared in step 3.1.2) and 776 μL of DMEM-Pyr.

3.3.3. Mix and incubate the plasmid solution and the PEI solution at room temperature for 30 min.

3.3.4. Add the plasmid/PEI solution to the HEK293T cells prepared in step 3.2.

3.4. Day 2, medium change

3.4.1. At 24 h after transfection, change medium to DMEM-Pyr. Culture cells until harvesting AAV on day 7. AAV will be released into the culture media.

3.5. Day 7, AAV collection, concentration, and buffer substitution using minimal purification method[24]

3.5.1. Incubate a centrifugal ultrafiltration unit (100k molecular weight cut-off [MWCO]) with 5 mL of 1% BSA in PBS at room temperature for 15 min. Then, centrifuge the ultrafiltration unit at 500 x g for 2 min and aspirate both filtered and remaining solutions.

3.5.2. Transfer medium from the 15 cm dish that produced AAV to a new 50mL conical tube and centrifuge (500 x g, 5 min). Filter the supernatant through a 0.45 μm syringe strainer to remove cell debris and apply suspension to the ultrafiltration unit.

3.5.3. Centrifuge at 2000 x g for 90 min or until concentrating the culture supernatant 0.5 to 1 mL.

3.5.4. Aspirate filtered medium and apply 15 mL of PBS to the ultrafiltration unit.

3.5.5. Repeat centrifugation until the concentrate becomes 0.5-1 mL.

3.5.6. Repeat 3.5.4 and 3.5.5.

3.5.7. Transfer concentrated AAV to a new 1.5 mL tube and store at 4 °C or -20 °C.

NOTE: AAV can be used in P1 facilities but follow local rules and regulations. AAV can be produced by conventional methods as well.

3.6. Calculation of AAV titer

3.6.1. Mix 5 μL of AAV, 195 μL of DMEM-Pyr, and 10 U of benzonase and incubate at 37 °C for 1 h.

3.6.2. Add 200 μL of proteinase K buffer (0.02 M Tris HCl and 1% SDS) and 5 μL of proteinase K (20 mg/mL) and incubate at 37 °C for 1 h.

3.6.3. Carefully prepare 400 μL of a 25:24:1 Phenol/chloroform/isoamyl alcohol solution, vortex for 1 min, and centrifuge at 20,000 x g for 1 min.

3.6.4. Transfer 200 μL of the aqueous phase to a new 1.5 mL tube, which will yield approximately half of the original AAV genomes.

3.6.5. Add 1 μL of Glycogen (20 mg/mL) to 20 μL of 3 M CH_3COONa (pH 5.2) and vortex. Add 250 μL of 2-Propanol to 100 μL of 100% ethanol and vortex again.

3.6.6. Incubate at -80 °C for 15 min. Then centrifuge at 20,000 x g for 30 min at 4 °C.

3.6.7. Aspirate supernatant and add 70% ethanol to the tube. Then, centrifuge at 20,000 x g, 4 °C for 5 min.

3.6.8. Aspirate supernatant and air dry until the pellet becomes clear.

3.6.9. Add 200 μL of Tris-Ethylenediaminetetraacetic acid (TE; pH 8.0) to resolve the AAV genomes. Then, dilute the sample 100-fold with TE.

3.6.10. Prepare a standard with pAAV-CMV-Vector at 6.5 ng/μL with TE to obtain 10^9 vector genomes (vg)/ μL. Then, make a series of 10-fold dilution from 10^4 to 10^8 with TE.

3.6.11. Mix 1 μL of sample DNA (or the standards), 0.4 μL of primers (5 μM), 3.6 μL of distilled water, and 5 μL of SYBR Green master mix. Primers, located on ITR, are 5'-GGAACCCCTAGTGATGGAGTT-3' and 5'-CGGCCTCAGTGAGCGA-3'.

3.6.12. Perform real-time PCR with the following condition: Initial denature at 95 °C for 60 s, 40 cycles of denaturing at 95 °C for 15 s, and annealing and extension at 60 °C for 30 s, followed by melting curve.

3.6.13. Based on the standards and Ct values, a real-time PCR machine provides the copy number of vector genome in 1 μL of a sample. Calculate original AAV titer using the following equation: a copy number provided by real-time PCR (vg/μL) x 8 x 10^3 x 2, wherein 8 x 10^3 as a dilution factor during AAV genome isolation, and 2 as the difference factor of AAV (single strand) and plasmid (double strand).

3.7. Transduction to PSC-CMs

3.7.1. Count PSC-CMs in an extra well or extra dish.

3.7.2. Dilute AAVs (1 x 10^4 to 1 x 10^6 vg/cell) to make up 50 μL with PBS. Apply AAVs at the multiplicity of infection (MOI) of 1 x 10^4 to 1 x 10^6 vg/cell to PSC-CMs and culture PSC-CMs for 3 days with AAV in the corresponding differentiation media for mouse and human PSC-CMs, then change media to culture medium without AAV.

3.7.3. Use PSC-CMs for live-cell imaging after 7 days or more post-transduction.

4. [Optional] AAV-based purification of PSC-CMs

4.1. Preparation of AAV

4.1.1. Prepare AAV as described in Step 3 using a shuttle vector expressing blasticidin-resistant gene under the control of cTNT promoter.

4.2. Transduction to differentiating iPS cells

4.2.1. Differentiate human iPS cells for 4 days following the protocol described in Step 2 and count the number of cells in an extra well.

4.2.2. After changing medium at day 4, apply AAVs at the MOI of 1 x 10^5 vg/cell to differentiating PSCs in RPMI+B27-Ins media.

4.2.3. At day 7, refresh medium with RPMI+B27+Ins and add 2.5-10 μg/mL of blasticidin.

4.2.4. At day 10, PSC-CMs are ready to replate.

5. Preparation of PDMS stamps

5.1. Design the device pattern of 200 μm strips along with 10-25 μm grooves in between the strips using a computer-aided design (CAD) drawing software.

5.2. Draw the pattern of devices onto a chromium photomask coated with AZP1350 using UV light of a maskless lithography tool.

5.3. Develop the pattern on the photomask in a series of positive photoresist developer (e.g., chromium etchant) and rinse with DI water.

5.4. Dehydrate a silicon wafer on a hot plate at 120 °C for 15 min.

5.5. Allow the wafer to cool to room temperature and spin-coat a negative photoresist SU-8 3010 to make a height of 10-20 μm with 1500 rpm for 30 s using a spin-coater.

5.6. Soft bake the wafer in two steps on a hot plate according to the manufacturer's protocol.

5.7. After the wafer cools to room temperature, load the wafer onto the mask aligner.

5.8. Use a mask aligner to align the mask on the wafer and expose the wafer to UV light.

5.9. Conduct the post-exposure bake to the wafer in two steps on a hot plate according to the manufacturer's protocol.

5.10. Develop the wafer in a series of SU-8 developer and 2-Propanol, then dry the wafer with a nitrogen stream.

5.11. Transfer the wafer into a Petri dish of a suitable size.

5.12. Mix PDMS elastomer and its curing agent in a ratio 10:1 w/w and pour the mixture into the Petri dish.

5.13. Degas the PDMS in a desiccator until all air bubbles disappear, then cure PDMS on hot plate at 80 °C for 2 h.

5.14. Peel the cured PDMS off from the master mold using a tweezer, then cut out the portion with the design to be a PDMS stamp.

NOTE: Shape can be square, however, an octagonal shape transfers the pattern better at the edge.

6. Patterned culture of pluripotent stem cell-derived cardiomyocytes

6.1. Remove dust from the surface of PDMS stamps using mending tape.

6.2. Submerge the stamps into 70% ethanol to sterilize. Then, blow ethanol off the surface of the stamps using an air duster.

6.3. Apply 5-10 μL of 0.5 wt% 2-methacryloyloxyethyl phosphorylcholine (MPC)

polymer/ethanol on the surface of PDMS stamps.

NOTE: Uneven distribution of MPC polymer may cause a disrupted pattern.

6.4. Incubate 10-30 min until MPC polymer is completely dried.

6.5. Place the stamps in contact with coverslips of a glass-bottom culture plate or a 35 mm imaging dish with a polymer coverslip and put a weight (e.g., a AAA battery) on the stamp for 10 min.

6.6. Remove the weight and stamps. Then, confirm that the pattern has been transferred under microscope.

NOTE: Stamped plates/dishes can be stored up to 1 week at room temperature.

6.7. Wash the stamped wells/dishes with PBS two times.

6.8. Dilute LN511-E8 with PBS at 2-4 μg/mL and coat the dish with LN511-E8 at 0.5-1 μg/cm^2. For human PSC-CMs, dilute LN511-E8 with 0.1% gelatin solution instead of PBS. Then, incubate for at least 1 h (optimally, more than 4 h).

6.9. Plate cells as described in previous sections.

7. Time-lapse imaging of sarcomeres under fluorescent microscope

7.1. Turn on and connect the microscope, associated computer, and also all of the required peripherals.

7.2. To perform time-lapse imaging, capture time-lapse images with the highest magnification (100X objective lens with oil emersion).

7.3. Select live-imaging conditions. To obtain good representative data, try to adjust to the highest framerate (minimum of 20 ms or 50 frames per second is recommended). Set the shutter open and apply a necessary binning (4 X 4) and a crop of the acquisition area to achieve the shortest intervals between images during the time-lapse imaging.

NOTE: The setting may vary depending on the configurations of microscopes. The camera needs to be high-sensitivity and is able to transfer the data to the connected PC fast enough. To this end, we used ORCA flash with Camera-link. We have tested a spinning confocal microscopy and have acquired images at 400 frames per second.

7.3.1. [**Optional**] If the beating rate of the cells is low, evoke the cells by electrical field stimulation.

7.4. Run the time-lapse record

7.4.1. Ensure that the imaged fields remain in focus during recording the time-lapse image.

7.4.2. Save the time-lapse images into an appropriate folder.

8. Analysis of time-lapse imaging using SarcOptiM, an ImageJ/Fiji plugin

8.1. Load a series of time-lapse images into ImageJ. For Olympus VSI format, open files through OlympusViewer Plugin.

8.2. Adjust brightness and contrast of the image to observe the sarcomere pattern clearly (**Image | Adjust | Brightness/Contrast**).

8.3. Open SarcOptiM by clicking **More tools** menu (>>) and selecting **SarcOptiM**.

8.4. Calibrate the program by pressing Ctrl + Shift + P and 1 μm button on the toolbar and following the instructions of the dialog boxes.

8.5. Draw a line across the region of the sarcomere that will be used to measure the sarcomere shortening.

8.6. Start sarcomere shortening analysis by pressing **SingleCell (AVI)** on the toolbar. Representative data is shown in **Figure 1** and **Figure 2**.

REPRESENTATIVE RESULTS:

Measuring sarcomere shortening using knock-in PSC-CMs reporter lines. Sarcomere-labeled PSC-CMs were used to measure sarcomere shortening. The lines express Myom2-RFP and ACTN2-mCherry from endogenous loci. TagRFP was inserted to *Myom2*, coding M-proteins that localize to the M-line, while mCherry was knocked-in to *ACTN2*, coding α-Actinin, which localizes to the Z-line[18, 25]. Time-lapse images were obtained and used to determine sarcomere shortening as presented in **Figures 1** and **2** and **Movie 1-3**.

To overcome the disorganized sarcomere of PSC-CMs, specific PDMS stamps were used to culture PSC-CMs in the stripe pattern. This patterned culture promoted an elongated cell shape and a more organized sarcomere pattern compared to cells cultured in the non-pattern area (**Figures 2B** and **2C**). With this advantage, the patterned culture promoted better contraction of the cells and provided a smooth sarcomere length profile as shown in **Movies 2** and **3** and **Figure 2D**.

Fluorescent tagging of Z-line protein using AAV vectors. To visualize the Z-line of PSC-CMs without generating knock-in iPS cells, fluorescent-tagged Z-line proteins were expressed using AAV transduction. Two of small Z-line proteins, Telethonin (TCAP) and Actin-associated LIM protein (PDLIM3) with GFP, were tagged and packaged using the AAV6 capsid (**Figure 3A**). Once PSC-CMs were differentiated and purified, AAVs were transduced to PSC-CMs (**Figure 3B**). The

transduced PSC-CMs expressed sarcomeric GFP signals along the PSC-CMs as early as three days post-transduction (**Figures 3C** and **3D**). Typically, the transduction of AAV at an MOI of 10^5 vg/cell is sufficient to visualize fluorescent-tagged sarcomere proteins and a higher titer may cause non-specific localization of GFP to cytoplasm though it increases overall GFP intensity.

Purification of PSC-CMs using AAV vectors. Current methods rely on the drug selection cassette that is already on the genome of PSC-CMs, either transgenic or knock-in line. However, it is labor-intensive to produce such a line from patient-derived iPS cells. As AAV vectors have been demonstrated to drive the expression of fluorescent-labeled Z-line proteins without the need for knock-in, we sought to establish the purification method without knock-ins as well (**Figure 4**). To this end, a new AAV vector, which encodes blasticidin-resistant gene under the control of cTNT promoter, was constructed (**Figure 4A**). The AAV (MOI of 10^5 vg/cell) was transduced to differentiating human iPS cells at day 4. Then cells were treated with 2.5-10 μg/mL of blasticidin (need to titrate for each cell line) between days 7 and 9 (**Figure 4B**). At day 14, the purity of PSC-CMs was more than 90% (**Figure 4C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sarcomere shortening of the mouse PSC-CMs derived from the Myom2-TagRFP cell line. A. The timeline for mouse PSC-CM differentiation. **B.** Representative images for sarcomere shortening in different time points with measuring regions as indicated by yellow bars. Scale bar = 10 μm. **C.** Sarcomere length profile during contraction of the cardiomyocytes that was stimulated with electricity at 1 Hz. The framerate was 50 frames per second. The pixel size was 0.26 μm.

Figure 2: Representative data showing sarcomere shortening of the human PSC-CMs derived from the ACTN2-mCherry cell line in non-patterned and patterned culture. A. The timeline for human PSC-CM differentiation. **B.** and **C.** Cardiomyocytes cultured in non-patterned cultures show disorganized sarcomere patterns (B) while patterned cultures promote a good alignment of the sarcomere (C). Measuring regions presented by yellow bars. **D.** Corresponding sarcomere length profiles obtained during cell contraction induced by electrical stimulation at 0.5 Hz and a 100 frames per second frame rate. Pixel size = 0.26 μm, scale bar = 10 μm.

Figure 3: Mouse PSC-CMs after AAV transduction for 3 days. A. Schematic vector map of AAV for sarcomere labeling. A sarcomere protein (gene of interest, GOI) is linked to GFP with a Gly-Gly-Gly-Ser linker (L) and expressed under the control of cardiac troponin T (cTNT) promoter. **B.** The timeline for mouse PSC-CM differentiation and AAV transduction. **C.** and **D.** Representative images showing clear sarcomere localization and the corresponding sarcomere length profile of TCAP-GFP (C) and PDLIM3-GFP (D) after 3 days of transduction into PSC-CMs generated from the Myom2-TagRFP cell line. Scale bar = 10 μm.

Figure 4: Blasticidin Purification of human PSC-CMs without knock-in.
A. Schematic vector map of AAV, in which a blasticidin-resistance gene cassette (BSR) is inserted downstream to cTNT promoter. **B.** The timeline of human PSC-CMs differentiation,

transduction, and blasticidin selection. **C.** Representative data showing percentage of cTNT + cells in human PSC-CMs (transduced 10^5 vg/ cell AAV6 on day 4 then treated with 2.5 μg/mL blasticidin on days 7 and 9).

Movie 1: Fluorescent time-lapse video of mouse PSC-CMs generated from the Myom2-TagRFP cell line. RFP signals showed a sarcomere pattern after PSC-CM culture for 28 days. The cells showed beating synchronously when stimulated with electricity at 1 Hz. The time-lapse images were acquired every 20 ms with a 100X lens. Scale bar = 5 μm.

Movie 2: Fluorescent time-lapse video of the human PSC-CMs with ACTN2-mCherry cultured on a non-patterned culture dish. The PSC-CMs expressing ACTN2-mCherry on a non-patterned culture dish not only showed disorganization of sarcomere but also presented a waving contraction, for which it is difficult to determine sarcomere shortening. The cells were stimulated with electricity at 0.5 Hz and images acquired every 10 ms with a 100X lens. Scale bar = 10 μm.

Movie 3: Fluorescent time-lapse video of the human PSC-CMs with ACTN2-mCherry cultured on a patterned culture dish. The patterned culture promoted alignment of the sarcomere and forced the cells to a rod shape. This method allowed the sarcomere shortening in PSC-CMs to be determined more easily. The video was obtained by stimulating the cells with electricity at 0.5 Hz. The framerate was 100 frames per second. Scale bar = 10 μm.

Supplemental CAD files. CAD files for creating stamps with strips of 200 μm width and grooves of 10 μm (Stamp_200x10.dxf), 25 μm (Stamp_200x25.dxf), and 50 μm (Stamp_200x50.dxf).

DISCUSSION:

PSC-CMs have great potential to be utilized as an in vitro platform to model heart disease and to test the effects of drugs. Nevertheless, an accurate, unified method to assess PSC-CMs functions must first be established. Most of functional tests work with PSC-CMs, e.g., electrophysiology, calcium transient, and metabolisms[26], and one of the first patient-derived PSC-CM studies was about long-QT syndrome[27]. However, contractility, one of the most important functions of a cardiomyocyte, is still difficult to assess. With adult cardiomyocytes, sarcomere shortening is widely used. In contrast, due to the underdeveloped and disorganized sarcomere of PSC-CMs, the standard method for sarcomere shortening does not work with these cells. Therefore, we have presented an alternative method for examining sarcomere shortening in PSC-CMs using fluorescent-tagged sarcomere proteins. As demonstrated, proteins localized to the M-line (MYOM2) or Z-line (ACTN2, TCAP, and PDLIM3) fused with fluorescent proteins can be used for this approach. We have also shown that fluorescent-tagged proteins can be expressed from endogenous loci or by AAVs. AAVs provide more flexibility for expressing fluorescent-tagged proteins than do endogenous loci, as AAVs can be applied to any type of patient-derived PSC-CMs. In contrast, expressing proteins from endogenous loci may have a lesser effect on sarcomere function, as the expression level of the genes is tightly regulated and can also be used for monitoring the maturation of PSC-CMs[18].

Even though Myom2-RFP, ACTN2-RFP, and Lifeact were all used to examine the sarcomere shortening[16, 18, 19], it is still unclear if these proteins interfere with sarcomere function. Recently, Lifeact was reported to disturb actin organization and cellular morphology[28]. It is also important to note that fusion patterns (i.e., the GFP fusion site at N-term or C-term of target protein) also affect the sarcomere function[29]. Therefore, before being used widely, it is important to thoroughly assess whether fluorescent-tagged sarcomere proteins interfere with sarcomere function and whether protein-protein interactions occur in sarcomeres in vitro, in vivo, and/or in adult cardiomyocytes. This repertoire of fluorescent-tagged sarcomere proteins provides a good starting point for future protein-engineering options (i.e., shortening the sarcomere proteins to only localization signals). Selecting proteins to tag is another key to success. We have tagged another Z-line protein with GFP, however, this protein displayed only a cytoplasmic distribution rather than localizing to the sarcomere. For live-imaging, protein stability may also play a role, as, for example, if a tagged protein is unstable, the signal level will be lower. The photostability of the fluorescent protein is also important, as unstable protein signals will be easily quenched during imaging.

To examine the contractility of PSC-CMs using methods other than described, indirect measurements of force generated by PSC-CMs (e.g., micro-post arrays, traction force microscopy) or motion (e.g., high-resolution motion detection using SI8000)[11–14] can be used. Our method can be combined with these methods or with dye-based action potential/calcium transient measurements. The combinatorial approach may provide further information on how a disease causes dysfunction in patient-derived PSC-CMs.

One of the challenges in sarcomere shortening in PSC-CMs is to find a good sarcomere that moves linearly, otherwise, the cells may easily come off the line for sarcomere detection of SarcOptiM and cause unstable sarcomere shortening results. Here, we demonstrate that using a patterned culture generated with PDMS stamps may provide a more stable and linear sarcomere movement. A patterned culture is also known to support the maturation of PSC-CMs[16], which is important for sarcomere function.

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DISCLOSURES:

H.U. has filed a patent related to this manuscript.

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TITLE:

Sarcomere Shortening of Pluripotent Stem Cell-Derived Cardiomyocytes Using Fluorescent-tagged Sarcomere Proteins.

AUTHORS AND AFFILIATIONS:

Razan E Ahmed[1,*], Nawin Chanthra[1,*], Tatsuya Anzai[1,2], Keiichiro Koiwai[3,4], Tomoki Murakami[4], Hiroaki Suzuki[4], Yutaka Hanazono[1], Hideki Uosaki[1]

[1]Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan

[2] Department of Pediatrics, Jichi Medical University, Tochigi, Japan

[3] Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, Tokyo, Japan

[4] Department of Precision Mechanics, Faculty of Science and Engineering, Chuo University, Tokyo, Japan

[*] The authors equally contributed

Corresponding Author:
Hideki Uosaki
uosaki.hideki@jichi.ac.jp
Tel: +81-285-58-7450

Email Addresses of Co-authors:
Razan E Ahmed (d2029@jichi.ac.jp)
Nawin Chanthra (nawin2020@jichi.ac.jp)
Tatsuya Anzai (r1204at@jichi.ac.jp)
Keiichiro Koiwai (koiwai@go.tuat.ac.jp)
Tomoki Murakami (murakami@nano.mech.chuo-u.ac.jp)
Hiroaki Suzuki (suzuki@mech.chuo-u.ac.jp)
Yutaka Hanazono (hanazono@jichi.ac.jp)

KEYWORDS:

Pluripotent stem cell, cardiomyocyte, sarcomere shortening, live imaging, fluorescent-tagged sarcomere proteins, microcontact printing

SUMMARY:

We summarized the method to examine sarcomere shortening using pluripotent stem cell-derived cardiomyocytes with fluorescent-tagged sarcomere proteins.

ABSTRACT:

Pluripotent stem cell-derived cardiomyocytes (PSC-CMs) can be produced from both embryonic and induced pluripotent stem (ES/iPS) cells. These cells provide promising sources for cardiac disease modeling. For cardiomyopathies, sarcomere shortening is one of the standard physiological assessments that are used with adult cardiomyocytes to examine their disease phenotypes. However, the available methods are not appropriate to assess the contractility of PSC-CMs as they have underdeveloped sarcomere that is invisible under phase-contrast microscopy. To address the issue and perform sarcomere shortening with PSC-CMs, we describe fluorescent-tagged sarcomere proteins and fluorescent live-imaging. Thin Z-lines and M-line reside at both ends and the center of a sarcomere, respectively. We tagged Z-line proteins—α-Actinin (ACTN2), Telethonin (TCAP), and actin-associated LIM protein (PDLIM3), and M-line protein—Myomesin-2 (Myom2) with fluorescent proteins. These tagged proteins can be expressed from endogenous alleles as knock-ins or from adeno-associated viruses (AAVs). Here, we introduce the methods to differentiate mouse and human pluripotent stem cells to cardiomyocytes, produce AAVs, and perform and analyze live-imaging. We also describe the methods of producing polydimethylsiloxane (PDMS) stamps for a patterned culture of PSC-CMs, which facilitates the analysis of sarcomere shortening with fluorescent-tagged proteins. To assess sarcomere shortening, time-lapse images of the beating cells at a high framerate (50–100 frames per second) were recorded under the electrical stimulation (0.5–1 Hz). To analyze sarcomere length over the course of cell contraction, the recorded time-lapse images were subjected to SarcOptiM, a plug-in for ImageJ/Fiji. Our strategy would provide a simple platform to investigate the cardiac disease phenotypes in PSC-CMs.

INTRODUCTION:

Cardiovascular diseases are the leading cause of mortality worldwide[1], and cardiomyopathy represents the third cause of cardiac-related deaths[2]. Cardiomyopathy is a collective group of diseases, affecting cardiac muscles. The recent developments of induced pluripotent stem (iPS) cells and directed-differentiation of iPS cells toward cardiomyocytes (PSC-CMs) have opened the door for studying cardiomyocytes with patient genome as *in vitro* model of cardiomyopathy. These cells can be used to understand the pathophysiology of cardiac diseases, elucidate their molecular mechanisms, and to test different therapeutic candidates[3]. There is a tremendous amount of interests, thus, patient-derived iPS cells have been derived—*e.g.* hypertrophic cardiomyopathy (HCM)[4, 5], arrhythmogenic right ventricular cardiomyopathy (ARVC)[6], dilated cardiomyopathy (DCM)[7], and mitochondrial-related cardiomyopathies[8, 9]. Because one of the characteristics of cardiomyopathy is dysfunction and disruption of sarcomeres, a valid tool that uniformly measures sarcomere function is in need.

Sarcomere shortening is the most widely used technique to assess the sarcomere function and contractility of adult cardiomyocytes derived from animal models and humans. To perform sarcomere shortening, well-developed sarcomere that can be visible under phase-contrast is required. However, PSC-CMs cultured *in vitro* display underdeveloped and disorganized sarcomeres, and it is unable to properly measure sarcomere shortening[10]. This difficulty to properly assess the contractility of PSC-CMs hinders their usage as a platform to assess cardiac functions *in vitro*. To assess PSC-CMs contractility indirectly, atomic force microscopy, micro-post arrays, traction force microscopy, and impedance measurements are used to measure the effects of the motion exerted by those cells on their surroundings[11–13]. More sophisticated and less invasive video-microscopy recording actual cellular motion (e.g. SI8000 from SONY) can be used to alternatively assess the contractility, however, it doesn't directly measure sarcomere motion nor force generation kinetics[14].

To directly measure sarcomere motion in PSC-CMs, new approaches such as fluorescent-tagging to sarcomere protein are emerging. For example, Lifeact is used to label filamentous actin (F-actin) to measure sarcomere motion[15, 16]. Genetically modified iPS cells are another option to tag sarcomere protein—e.g. α-actinin (ACTN2) and Myomesin-2 (MYOM2)—by fluorescent protein[17–19].

In this paper, we describe the details of how to perform time-lapse imaging for measuring sarcomere shortening using Myom2-TagRFP (mouse embryonic stem [ES] cells) and ACTN2-mCherry (human iPS cells). We also show that a patterned culture facilitates the sarcomere alignment. In addition, we describe an alternative method of sarcomere labeling, using adeno-associated viruses (AAVs), which can be widely applied to patient-derived iPS cells.

PROTOCOL:

1. Differentiation of Mouse Pluripotent Stem Cells

1.1. Maintenance of mouse ES cells.

1.1.1. Maintenance medium: Mix 50 mL of fetal bovine serum (FBS), 5 mL of L-alanine-L-glutamine, 5mL of non-essential amino acid (NEAA), 5 mL of 100 mM Sodium Pyruvate, 909 µl of 55 mM 2-Mercaptoethanol to 450 mL of Glasgow Minimum Essential Medium (GMEM). Supplement Leukemia inhibitory factor (LIF), CHIR-99021, and PD0325901 at the final concentration of 1000 U/mL, 1 µM, and 3 µM, respectively. Sterilize the medium through a 0.22-µm filter.

1.1.2. 10% FBS medium: Mix 55 mL of FBS, 5.5 mL of L-alanine-L-glutamine , 5.5 mL of Sodium Pyruvate, and 5.5 mL of NEAA to 500 mL of Dulbecco's Modified Eagle Medium (DMEM) high glucose. Filtrate the medium through a 0.22-µm filter to sterilize.

1.1.3. Culture SMM18 mouse ES cells, in which TagRFP was knocked into *Myom2* locus, on a gelatinized 6-cm dish in the maintenance medium as previously described[18]. Passage every 2–3 days.

1.2. Preparation of serum-free differentiation (SFD) medium:

1.2.1. Basal SFD: Mix 250 mL of Ham's F-12, 750 mL of Iscove's Modified Dulbecco's Medium (IMDM), 10 mL of B27 supplement minus Vitamin A, 5 mL of N2 supplement, 10 mL of L-alanine-L-glutamine, 5 mL of 10% bovine serum albumin in phosphate-buffered saline (PBS), 10 mL of penicillin and streptomycin (10,000 U/mL). Filtrate through 0.22 µm filter to sterilize.

1.2.2. Dissolve ascorbic acid at 5 mg/mL in distilled water and filtrate through 0.22 µm to sterilize.

1.2.3. Dilute 13 µL of 1-Thioglycerol to 1 mL of IMDM. Herein, refer to this diluted 1-Thioglycerol as MTG.

1.2.4. Add 10 µl of ascorbic acid (5 mg/mL) and 3 µl of MTG to 1 mL of basal SFD on the day of use. Herein, refer to this mixture as complete SFD.

1.3. Day 0, embryoid body (EB) formation for differentiation:

1.3.1. Harvest SMM18 mouse ES cells with a recombinant trypsin-like protease (rTrypsin) and count cell number.

1.3.2. Centrifuge 5 x 10^5 cells at 300 x g for 3 min in 4 °C, resuspend in 10 mL of complete SFD, and seed into a 10-cm petri dish. Culture the cells at 37 °C and 5% CO_2 for 50 h.

1.4. Differentiation day 2:

1.4.1. Add Activin A, human vascular endothelial growth factor (hVEGF), and bone morphogenetic protein 4 (BMP4) to complete SFD at the final concentration of 5 ng/mL, 5 ng/mL, and 1.9 ng/mL, respectively.

Note: BMP4 concentration might differ depending on the lots of BMP4. Test several concentrations in a small-scale trial prior to use a new lot and define the best concentration for cardiac differentiation.

1.4.2. Transfer EBs from a petri dish into a 15-ml tube and centrifuge at 50-100 x g for 3 min at 4 °C.

1.4.3. Meanwhile, add the medium prepared in step 1.4.1 to the petri dish to protect the remaining EBs being dry.

1.4.4. Aspirate the supernatant from the 15-mL tube, resuspend the EBs with the medium in the petri dish, and transfer back to the dish. Then, cultivate the EBs at 37 °C and 5% CO_2 for 46 h.

1.5. Differentiation day 4:

1.5.1. Gelatinize a 10-cm tissue culture-treated dish with 5 to 10 mL of 0.1% gelatin for at least 5 min. Aspirate gelatin right before seeding the cells.

1.5.2. Prepare medium: Mix basic fibroblast growth factor (bFGF), FGF10, and hVEGF to complete SFD at the final concentration 5 ng/ml, 25 ng/ml, and 5 ng/ml respectively. For a 10-cm dish, prepare 10 mL.

1.5.3. Transfer cells in the petri dish to a 15-ml tube. Add 5 ml of PBS to the petri dish, wash several times, and transfer to the 15-mL tube to collect the remaining cells. Centrifuge at 50–100 x g, 4 °C, 3 min.

1.5.4. Aspirate supernatant, add 1 ml of rTrypsin, and incubate at 37 °C for 3 min.

1.5.5. Vortex briefly to dissociate EBs, add 9 ml of 10% FBS medium, vortex again, and count cell number.

1.5.6. Centrifuge 1.5 x 10^7 cells at 300 x g, 4 °C for 3 min, resuspend with the media prepared in step 1.4.2, and seed into the gelatinized dish. Incubate at 37 °C and 5% CO_2 for 2 days.

Note: By day 7 or 8, extensive beatings of PSC-CMs can be observed.

1.6. Drug selection at differentiation day 7 and 9: Refed the media with puromycin (2 μg/mL at the final concentration) to eliminate non-cardiomyocytes at day 7 and 9 of differentiation.

Note: Parental line of SMM18 is syNP4 mouse ES cells, harboring NCX1 promoter-driven puromycin-resistant gene[20].

1.7. Day 10, replate for future experiments:

1.7.1. Coat a glass-bottom culture plate or a 35-mm imaging dish with a polymer coverslip with 0.1% Gelatin. To enhance maturation, coat the dishes with laminin-511 E8 fragment (LN511-E8) at 1 μg/cm^2 for 30 min to 1 h at the room temperature[18]. To culture PSC-CMs in desired patterns, please refer to steps 4 and 5 for preparing polydimethylsiloxane (PDMS) stamps.

1.7.2. To harvest SMM18 PSC-CMs, wash the dish twice with PBS, then apply 1 mL of rTrypsin, and incubate 3 min at 37 °C.

1.7.3. Collect cells to 9 mL of 10% FBS medium, suspend, and count the cell number. Plating cell number is 50,000–100,000 cells in a well of a 24-well plate, and 250,000–500,000 cells in a 35-mm imaging dish.

1.7.4. Centrifuge a sufficient number of cells (300 x g, 3 min), resuspend them with complete SFD supplemented with FBS (final concentration at 10%).

1.7.5. Incubate overnight, and change culture medium to complete SFD with puromycin.

1.7.6. From day 14, change culture medium two to three times a week with complete SFD until day 21–28 when Myom2-RFP becomes prominent. For AAV-based transduction of fluorescent-tagged sarcomere proteins, please refer to Step 3.

2. Differentiation of Human Pluripotent Stem Cells

2.1. Preparation of differentiation media:

2.1.1. RPMI+B27-Ins: mix 500 ml of RPMI 1640 medium, 10 ml of B27 minus insulin, and 5.25 mL of L-alanine-L-glutamine.

2.1.2. RPMI+B27+Ins: mix 500 ml of RPMI, 10 ml of B27 supplement, and 5.25 mL of L-alanine-L-glutamine.

2.2. Maintenance of human iPS cells:

2.2.1. Passage human iPS cells twice a week with AK02N on LN511-E8 following previously published method with some modifications[21].

2.2.2. Harvest cells with a 3-min treatment of rTrypsin and collect into 10% FBS medium. Count cells and centrifuge down at 300 x g for 3 min at 4 °C. Seed 75,000–125,000 cells in a well of 6-well plate with 2 mL of AK02N supplemented with LN511-E8 and Y27632 at the final concentration of 0.5 µg/ml (0.1 µg/cm^2) and 10 µM, respectively.

2.2.3. Incubate at 37 °C and 5% CO_2 and change the medium on the following day with 2 ml of AK02N without any supplement. Change media every two to three days and passage every three to four days.

2.3. Day –4, replate prior to differentiation:

2.3.1. Coat a 6-well plate with 0.5 µg/cm^2 of LN511-E8 diluted in PBS. Then, incubate for at least 30 min at 37 °C and 5% CO_2 or 1h at room temperature. Aspirate coating solution right before seeding cells.

2.3.2. Harvest human iPS cells with rTrypsin and count cell number as Step 2.2.2.

2.3.3. Centrifuge 1.25 x 10^5 cells for a well of a 6-well plate at 300 x g for 3 min at 4 °C and resuspend in 2 ml of AK02N supplemented with LN511-E8 (final concentration 0.5 µg/ml or 0.1 µg/cm^2) and Y27632 (final concentration 10 µM) per well.

2.3.4. Aspirate coating solution and seed resuspended cells into the coated plate, incubate at 37 °C and 5% CO_2.

2.4. Day –3 and –1, refed the media with 2 mL of AK02N.

2.5. Day 0: Change the medium to 2 ml of RPMI+B27-Ins supplemented with CHIR99021 (final concentration 6 µM) per well to start differentiation.

2.6. Day 2: Change the medium to 2 ml of RPMI+B27-Ins with WntC59 (final concentration 2 µM) per well.

2.7. Day 4: Change the medium to 2 ml of RPMI+B27-Ins per well.

2.8. Day 7 and day 9, medium change to 2 ml of RPMI+B27+Ins with puromycin (final concentration 10 µg/ml) per well to selectively culture PSC-CMs.

Note: ACTN2-mCherry line, we used in this study, has a cassette of internal ribosomal entry site (IRES) and puromycin-resistant gene inserted to 3'-untranslated region (UTR) of TNNT2 locus, and

mCherry replacing the stop codon of ACTN2. To purify cardiomyocyte without knock-in, please refer to Step 3 and 4.

2.9. Day 10, replate for future experiments:

2.9.1. Coat a 35-mm imaging dish with a polymer coverslip with 0.5–1 μg/cm^2 of LN511-E8 diluted in 0.1% Gelatin. Incubate 2–4 h at room temperature for the long-term viability. To culture PSC-CMs in desired patterns, please refer to steps 4 and 5 for preparing PDMS stamps.

2.9.2. To harvest human PSC-CMs, wash the dish twice with PBS, then apply 1 mL of rTrypsin to a well, and incubate 3 min at 37 °C.

2.9.3. Collect cells to 4 mL of 10% FBS medium, suspend, and count the cell number. Plating cell number is 250,000–500,000 cells in a 35-mm imaging dish.

2.9.4. Centrifuge a sufficient number of cells at 300 x g for 3 min at 4 °C, resuspend them with RPMI+B27+Ins with puromycin (10 μg/ml), and plate them on the coated 35-mm imaging dish.

2.9.5. Incubate overnight, and change culture medium to RPMI+B27+Ins with puromycin (10 μg/ml).

2.9.6. From day 14, change culture medium two to three times a week with RPMI+B27+Ins until day 21–28 for imaging. For AAV-based transduction of fluorescent-tagged sarcomere proteins, please refer to Step 3.

3. Fluorescent Labeling of Sarcomere Using Adeno-associated Viruses

3.1. Preparation before AAV production:

3.1.1. Maintain HEK293T cells in DMEM supplemented with FBS (final concentration 10%) on a 10-cm tissue culture plate. Passage cells three-time a week.

3.1.2. Prepare polyethylenimine (PEI) at 1 mg/mL. Mix 50mg of polyethylenimine MAX 40000 and 40 mL of ultrapure water. Adjust pH to 7.0 using 1N NaOH. Then, make it up to 50 mL with ultrapure water, and filtrate through a 0.25-μm filter.

3.1.3. Prepare a shuttle vector with a sarcomere labeling gene, e.g. TCAP or PDLIM3 fused with a green fluorescent protein (GFP), driven by a cardiomyocyte-specific promoter, such as cardiac troponin T (cTNT) promoter[22].

Note: For this instance, we used monomeric enhanced GFP with mutations of V163A, S202T, L221V[23].

3.2. Day 0, passage HEK cells: When cells reach to confluent, passage $2.0x10^7$ HEK293T cells to a 15-cm tissue culture plate with 20 mL of DMEM with 10% FBS.

3.3. Day 1, transfection:

3.3.1. Mix 13.5 μg of the shuttle vector, 26 μg of pHelper (a vector coding E2A, E4, and VA of adenovirus), 16.5 μg of pRC6 (a vector coding AAV2 Rep and AAV6 Cap genes), and 1 mL of DMEM without sodium pyruvate (DMEM-Pyr).

3.3.2. Mix 224 μL of PEI (1mg/mL, prepared in step 3.1.2) and 776 μL of DMEM-Pyr.

3.3.3. Mix and incubate the plasmid solution and the PEI solution at room temperature for 30 min.

3.3.4. Add the plasmid/PEI solution to the HEK293T cells prepared in step 3.2.

3.4. Day 2, medium change: At 24 h after transfection, change medium to DMEM-Pyr. Culture cells until harvesting AAV on day 7. AAV will be released into the culture media.

3.5. Day 7, AAV collection, concentration, and buffer substitution using minimal purification method[24]:

3.5.1. Incubate a centrifugal ultrafiltration unit (100k molecular weight cut-off [MWCO]) with 5 mL of 1% BSA in PBS at room temperature for 15 min. Then, centrifuge the ultrafiltration unit at 500 x g for 2 min, and aspirate both filtered solution and remaining solution.

3.5.2. Transfer medium from the 15-cm dish that produced AAV to a new 50-mL conical tube and centrifuge (500 x g, 5 min). Filtrate the supernatant through a 0.45-μm syringe filter for removing cell debris and apply it to the ultrafiltration unit.

3.5.3. Centrifuge at 2,000 x g for 90 min or until concentrating the culture supernatant 0.5 to 1 mL.

3.5.4. Aspirate filtered medium and apply 15 mL of PBS to the ultrafiltration unit.

3.5.5. Repeat centrifugation until the concentrate becomes 0.5–1 mL.

3.5.6. Repeat 3.5.4 and 3.5.5 again.

3.5.7. Transfer concentrated AAV to a new 1.5 mL tube and store at 4 °C or -20 °C.

Note: AAV can be used in P1 facilities but follow local rules and regulations.
Note: AAV can be produced by conventional methods as well.

3.6. Calculation of AAV titer:

3.6.1. Mix 5 µL of AAV, 195 µL of DMEM-Pyr, and 10 U of benzonase, and incubate at 37 °C for 1 h.

3.6.2. Add 200 µL of proteinase K buffer (0.02 M Tris HCl and 1% SDS) and 5 µL of proteinase K (20 mg/mL) and incubate at 37 °C for 1 h.

3.6.3. CAUTION: Add 400 µL of 25:24:1 Phenol/chloroform/isoamyl alcohol, vortex for 1 min, and centrifuge at 20,000 x g for 1 min.

3.6.4. Transfer 200 µL of the aqueous phase to a new 1.5-mL tube, which yields approximately half of the original AAV genomes.

3.6.5. Add 1 µL of Glycogen (20 mg/mL) and 20 µL of 3 M CH_3COONa (pH 5.2), and vortex. Add 250 µL of 2-Propanol and 100 µL of 100% ethanol, and vortex again.

3.6.6. Incubate at -80 °C for 15 min, then centrifuge at 20,000 x g for 30 min at 4 °C.

3.6.7. Aspirate supernatant and replace it with 70% ethanol. Then, centrifuge at 20,000 x g, 4 °C for 5 min.

3.6.8. Aspirate supernatant and air dry until the pellet becomes clear.

3.6.9. Add 200 µL of Tris-Ethylenediaminetetraacetic acid (TE; pH 8.0) to resolve the AAV genomes. Then, dilute the sample 100-fold with TE.

3.6.10. Prepare a standard with pAAV-CMV-Vector at 6.5 ng/µL with TE, which equals to 10^9 vector genomes (vg)/µL. Then, make a series of 10-fold dilution for 10^4 to 10^8 with TE.

3.6.11. Mix 1 µL of sample DNA (or the standards), 0.4 µL of primers (5 µM), 3.6 µL of distilled water, and 5 µL of SYBR Green master mix. Primers, located on ITR, are 5'-GGAACCCCTAGTGATGGAGTT-3' and 5'-CGGCCTCAGTGAGCGA-3'.

3.6.12. Perform real-time PCR with the following condition: Initial denature at 95 °C for 60 s, and then 40 cycles of denaturing at 95 °C for 15 s and annealing and extension at 60 °C for 30 s, followed by melting curve.

3.6.13. Based on the standards and Ct values, a real-time PCR machine provides the copy number of vector genome in 1 µL of a sample. Calculate original AAV titer using the following equation: a copy number provided by real-time PCR (vg/µL) x 8 x 10^3 x 2, wherein 8 x 10^3 as a dilution factor during AAV genome isolation, and 2 as the difference factor of AAV (single strand) and plasmid (double strand).

3.7. Transduction to PSC-CMs:

3.7.1. Count cell number of PSC-CMs in an extra well or an extra dish.

3.7.2. Dilute AAVs ($1x10^4$ to $1x10^6$ vg/cell) to make up 50 µL with PBS. Apply AAVs at the multiplicity of infection (MOI) of $1x10^4$ to $1x10^6$ vg/cell to PSC-CMs and culture PSC-CMs for 3 days with AAV in the corresponding differentiation media for mouse and human PSC-CMs, then change media to normal ones without AAV.

3.7.3. Use PSC-CMs for live-cell imaging after 7 days or more post-transduction.

4. [Optional] AAV-based purification of PSC-CMs

4.1. Preparation of AAV:

4.1.1. Prepare AAV as following the steps described in Step 3 using a shuttle vector expressing blasticidin-resistant gene under the control of cTNT promoter.

4.2. Transduction to differentiating iPS cells:

4.2.1. Differentiate human iPS cells for 4 days following the protocol described in Step 2 and count the number of cells in an extra well.

4.2.2. After changing media at day 4, apply AAVs at the MOI of $1x10^5$ vg/cell to differentiating PSCs in RPMI+B27-Ins media.

4.2.3. At day 7, refresh media with RPMI+B27+Ins and add 2.5-10 µg/mL of blasticidin.

4.2.4. At day 10, PSC-CMs are ready to replate.

5. Preparation of PDMS stamps

5.1. Design the device pattern of 200 µm strips along with 10–25 µm grooves in between the strips using a computer-aided design (CAD) drawing software.

5.2. Draw the pattern of devices onto a chromium photomask coated with AZP1350 by using UV light of a maskless lithography tool.

5.3. Develop the pattern on the photomask in a series of positive photoresist developer, Chromium etchant, and rinse with DI water.

5.4. Dehydrate a silicon wafer by baking it on a hot plate at 120 °C for 15 min.

5.5. Allow the wafer to cool to room temperature, and then spin-coat a negative photoresist SU-8 3010 to make a height of 10–20 µm with 1,500 rpm for 30 sec using a spin-coater.

5.6. Soft bake the wafer in two steps on a hot plate according to the manufacturer's protocol.

5.7. After the wafer cooled to room temperature, load the wafer onto the mask aligner.

5.8. Using a mask aligner, align the mask on the wafer and expose the wafer to UV light.

5.9. Conduct the post-exposure bake to the wafer in two steps on a hot plate according to the manufacturer's protocol.

5.10. Develop the wafer in a series of SU-8 developer and 2-Propanol, then dry the wafer with a nitrogen stream.

5.11. Transfer the wafer into a petri dish of a suitable size.

5.12. Mix PDMS elastomer and its curing agent in a ratio 10:1 w/w, and pour it into the Petri dish.

5.13. Degas the PDMS in a desiccator until all air bubbles disappear, then cure the PDMS on a hot plate at 80 °C for 2 h.

5.14. Peel the cured PDMS off from the master mold using a tweezer, then cut out the portion with the design to be a PDMS stamp.

Note: Shape can be square, however, an octagonal shape transfers the pattern better at the edge.

6. Patterned Culture of Pluripotent Stem Cell-derived Cardiomyocytes

6.1. Remove dust from the surface of PDMS stamps using mending tape.

6.2. Submerge the stamps into 70% ethanol to sterilize. Then, blow ethanol off the surface of the stamps using an air duster.

6.3. Apply 5–10 µL of 0.5wt% 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer/ethanol on the surface of PDMS stamps.

Note: Uneven distribution of MPC polymer may cause a disrupted pattern.

6.4. Incubate 10–30 min until MPC polymer is completely dried.

6.5. Place the stamps in contact with coverslips of a glass-bottom culture plate or a 35-mm imaging dish with a polymer coverslip, and put a weight (*e.g.* a AAA battery) on a stamp for 10

min.

6.6. Remove the weight and stamps. Then, confirm the pattern is transferred under microscope.

Note: Stamped plates/dishes can be stored up to 1 week at room temperature.

6.7. Wash the stamped wells/dishes with PBS for 2-times.

6.8. Dilute LN511-E8 with PBS at 2–4 μg/mL and coat the dish with LN511-E8 at 0.5–1 μg/cm^2. For human PSC-CMs, dilute LN511-E8 with 0.1% gelatin solution instead of PBS. Then, incubate for at least 1 h (optimally, more than 4 h).

6.9. Plate cells as described in the previous sections.

7. Time-lapse Imaging of Sarcomere Under Fluorescent Microscope

7.1. Turn on and connect the microscope, associated computer, and also all of the required peripherals.

7.2. Perform time-lapse imaging: Capture time-lapse images with the highest magnification (100X objective lens with oil emersion)..

7.3. Select live-imaging conditions. To obtain good representative data, try to adjust to the highest framerate (minimum of 20 ms or 50 frames per second is recommended). Set the shutter open and apply a necessary binning (4 X 4) and a crop of the acquisition area to achieve the shortest intervals between images during the time-lapse imaging.

Note: The setting may vary depending on the configurations of microscopes. The camera needs to be high-sensitivity and is able to transfer the data to the connected PC fast enough. To this end, we used ORCA flash with Camera-link. We have tested a spinning confocal microscopy, and acquired images at 400 frames per second.

7.3.1. [**Optional**] If the beating rate of the cells is low, evoke the cells by electrical field stimulation.

7.4. Run the time-lapse record:

7.4.1. Ensure that the imaged fields remain in focus during recording the time-lapse image.

7.4.2. Save the time-lapse images into an appropriate folder.

8. Analysis of Time-lapse Imaging Using SarcOptiM, an ImageJ/Fiji Plugin

8.1. Load a series of time-lapse images into ImageJ. For Olympus VSI format, open files through

OlympusViewer Plugin.

8.2. Adjust brightness and contrast of the image to observe the sarcomere pattern clearly (Image -> Adjust -> Brightness/Contrast).

8.3. Open SarcOptiM by clicking “More tools” menu (>>), and then selecting “SarcOptiM”.

8.4. Calibrate the program by pressing CTRL+SHIFT+P and “1 µm” button on the toolbar, and following the instructions of the dialog boxes.

8.5. Draw a line across the region of the sarcomere that will be measured the sarcomere shortening.

8.6. Start sarcomere shortening analysis by pressing “SingleCell (AVI)” button on the toolbar. Representative data is shown in **Figure 1 and Figure 2**.

REPRESENTATIVE RESULTS:

Measuring sarcomere shortening using knock-in PSC-CMs reporter lines.

We used sarcomere-labeled PSC-CMs to measure sarcomere shortening. The lines express Myom2-RFP and ACTN2-mCherry from endogenous loci. TagRFP was inserted to *Myom2*, coding M-protein that localize to M-line, while mCherry was knocked-in to *ACTN2*, coding α-Actinin that localize to Z-line[18, 25]. Time-lapse images were obtained and used to determine sarcomere shortening as presented in **Figures 1, 2, and Movie 1–3**.

To overcome the disorganized sarcomere of PSC-CMs, we used specific PDMS stamps to culture PSC-CMs in the stripe pattern. This patterned culture promoted elongated cell shape and a more organized sarcomere pattern compared to the cells that culture in the non-pattern area (**Figure 2B and C**). With this advantage, the patterned culture promoted better contraction of the cells and provided a smooth sarcomere length profile as shown in **Movie 2, 3, and Figure 2D**.

Fluorescent tagging of Z-line protein using AAV vectors.

To visualize the Z-line of PSC-CMs without generating knock-in iPS cells, we expressed fluorescent-tagged Z-line proteins using AAV transduction. We tagged two of small Z-line proteins, Telethonin (TCAP) and Actin-associated LIM protein (PDLIM3) with GFP, and packaged them using the AAV6 capsid (**Figure 3A**). Once PSC-CMs were differentiated and purified, we transduced AAVs to PSC-CMs (**Figure 3B**). The transduced PSC-CMs expressed sarcomeric GFP signals along the PSC-CMs as early as three days post-transduction (**Figure 3C-D**). Typically, the transduction of AAV at the MOI of 10^5 vg/cell is sufficient to visualize fluorescent-tagged sarcomere proteins, and a higher titer may cause non-specific localization of GFP to cytoplasm though it increases overall GFP intensity.

Purification of PSC-CMs using AAV vectors.

Current method relies on the drug selection cassette that is already on the genome of PSC-CMs, either transgenic or knock-in line. However, it is labor-intensive to produce such a line from each of patient-derived iPS cells. As we demonstrated that AAV vectors drive the expression of fluorescent-labeled Z-line proteins without the need for knock-in, we sought to establish the purification method without knock-in as well (**Figure 4**). To this end, we constructed a new AAV vector, which encode blasticidin-resistant gene under the control of cTNT promoter (**Figure 4A**). The AAV (MOI of 10^5 vg/cell) was transduced to differentiating human iPS cells at day 4, then cells were treated with 2.5-10 μg/mL of blasticidin (need to titrate for each cell line) between days 7 and 9 (**Figure 4B**). At day 14, the purity of PSC-CMs was more than 90% (**Figure 4C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sarcomere shortening of the mouse PSC-CMs derived from the Myom2-TagRFP cell line.

A. The timeline for mouse PSC-CM differentiation. **B.** Representative images for sarcomere shortening in different time points with measuring regions as indicated by yellow bars. **C.** Sarcomere length profile during contraction of the cardiomyocytes that was stimulated with electricity at 1 Hz. The framerate was 50 frames per second. The pixel size was 0.26 μm. Scale bar

= 10 µm.

Figure 2: Representative data showing sarcomere shortening of the human PSC-CMs derived from the ACTN2-mCherry cell line in non-patterned and patterned culture.
A. The timeline for human PSC-CM differentiation. **B.** The cardiomyocytes cultured in non-patterned culture showed disorganized sarcomere pattern whereas **B.** patterned culture promoted well alignment of the sarcomere. **B.** Representative images for sarcomere shortening with measuring regions as presented by yellow bars, and **C.** corresponding sarcomere length profile during cell contraction which electrical stimulation at 0.5 Hz. The framerate was 100 frames per second. The pixel size was 0.26 µm. Scale bar = 10 µm.

Figure 3: Mouse PSC-CMs after AAV transduction for 3 days.
A. Schematic vector map of AAV for sarcomere labeling. A sarcomere protein (gene of interest, GOI) is linked to GFP with a Gly-Gly-Gly-Ser linker (L) and expressed under the control of cardiac troponin T (cTNT) promoter. B**.** The timeline for mouse PSC-CM differentiation and AAV transduction. **C-D.** Representative images showing clear sarcomere localization and the corresponding sarcomere length profile of TCAP-GFP (C) and PDLIM3-GFP (D) after 3 days of transduction into PSC-CMs generated from the Myom2-TagRFP cell line. Scale bar = 10 µm.

Figure 4: Blasticidin Purification of human PSC-CMs without knock-in.
A. Schematic vector map of AAV, in which a blasticidin-resistance gene cassette (BSR) is inserted downstream to cTNT promoter. **B.** The timeline of human PSC-CMs differentiation, transduction, and blasticidin selection. **C.** Representative data showing percentage of cTNT + cells in human PSC-CMs (transduced 10^5 vg/ cell AAV6 on day 4 then treated with 2.5 µg/ml blasticidin on days 7 and 9).

Movie 1: Fluorescent time-lapse video of mouse PSC-CMs generated from the Myom2-TagRFP cell line.
RFP signals showed a sarcomere pattern after culturing the PSC-CMs for 28 days. The cells showed beating synchronously when stimulated with electricity at 1 Hz. The time-lapse images were acquired every 20 ms with a 100X lens. Scale bar = 5 µm.

Movie 2: Fluorescent time-lapse video of the human PSC-CMs with ACTN2-mCherry cultured on a non-patterned culture dish.
The PSC-CMs expressing ACTN2-mCherry on a non-patterned culture dish not only showed disorganization of sarcomere, but also presented a waving contraction which is difficult to determine sarcomere shortening. The cells were stimulated with electricity at 0.5 Hz and acquired the images every 10 ms with a 100X lens. Scale bar = 10 µm.

Movie 3: Fluorescent time-lapse video of the human PSC-CMs with ACTN2-mCherry cultured on a patterned culture dish.
The patterned culture promoted the alignment of the sarcomere and forced cells to rod-shape. This method allowed us to determine the sarcomere shortening of the PSC-CMs easier. This video was obtained by stimulating the cells with electricity at 0.5 Hz. The framerate was 100

frames per second. Scale bar = 10 µm.

Supplemental CAD files: CAD files for creating stamps with strips of 200 µm width and grooves of 10 µm (Stamp_200x10.dxf), 25 µm (Stamp_200x25.dxf), and 50 µm (Stamp_200x50.dxf).

DISCUSSION:

PSC-CMs is a material with great potential that can be utilized as an *in vitro* platform to model heart diseases and test the effects of drugs. Nevertheless, before being able to efficiently use them, we must establish an accurate, unified method to assess PSC-CMs functions. Most of the functional tests work with PSC-CMs, *e.g.* electrophysiology, calcium transient, and metabolisms[26]. Therefore, one of the first patient-derived PSC-CM studies was about long-QT syndrome[27]. However, contractility, one of the most important functions as a cardiomyocyte, is still difficult to assess. With adult cardiomyocytes, sarcomere shortening is widely used. In contrast, due to the underdeveloped and disorganized sarcomere of PSC-CMs, the standard method for sarcomere shortening does not work with PSC-CMs. Therefore, we presented here an alternative method to examine sarcomere shortening of PSC-CMs using fluorescent-tagged sarcomere proteins. We demonstrated that the proteins localized to M-line (MYOM2) or Z-line (ACTN2, TCAP, and PDLIM3) fused with fluorescent proteins can be used for this approach. We also showed that fluorescent-tagged proteins can be expressed from endogenous loci or by AAVs. AAVs provide more flexibility to express fluorescent-tagged proteins than endogenous loci, as they can be applied to any type of patient-derived PSC-CMs. In contrast, expressing the proteins from endogenous loci may have lessor alteration to the sarcomere function as the expression level of the genes is tightly regulated, and it can also be used for monitoring the maturation of PSC-CMs[18].

Even though Myom2-RFP, ACTN2-RFP, and Lifeact were all used to examine the sarcomere shortening[16, 18, 19], it is still unclear if they interfere with the sarcomere function. Recently, Lifeact was reported to disturb actin organization and cellular morphology[28]. It is also important to note that fusion patterns (*i.e.* the GFP fusion site at N-term or C-term of target protein) also affect the sarcomere function[29]. Therefore, before being used widely, it is important to extensively assess if these fluorescent-tagged sarcomere proteins do not interfere with sarcomere function or protein-protein interaction in sarcomere *in vitro* and, if possible, *in vivo* or in adult cardiomyocytes. As we provided a repertoire of fluorescent-tagged sarcomere proteins to start with, we may have better options in near future by protein-engineering (*i.e.* shortening the sarcomere proteins to only localization signals). Selecting protein to tag is another key to success. We have tagged another Z-line protein with GFP, however, it only displayed cytoplasmic distribution rather than localization to the sarcomere. For live-imaging, protein stability might also play the role. For example, if a tagged protein is unstable, the signal level will be lower. The photostability of the fluorescent protein is also important as unstable ones will be easily quenched during the imaging.

To examine the contractility of PSC-CMs other than the method described here, indirect measurements of force generated by PSC-CMs (micro-post arrays, traction force microscopy) or motion (high-resolution motion detection using SI8000)[11–14] are used. The current method can be combined with such methods or dye-based action potential/calcium transient measurement. The combinatorial approach may provide us further implication on how a disease cause

dysfunction in patient-derived PSC-CMs.

One of the challenges in sarcomere shortening in PSC-CMs is to find a good sarcomere that moves linearly, otherwise, it may easily come off from the line for sarcomere detection of SarcOptiM and cause unstable sarcomere shortening results. Here, we demonstrated a patterned culture using PDMS stamps may provide a more stable and linear movement of a sarcomere. A patterned culture is also known to support the maturation of PSC-CMs[16], which is important for sarcomere function.

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DISCLOSURES:

H.U. has filed a patent related to this manuscript.

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Figure 1

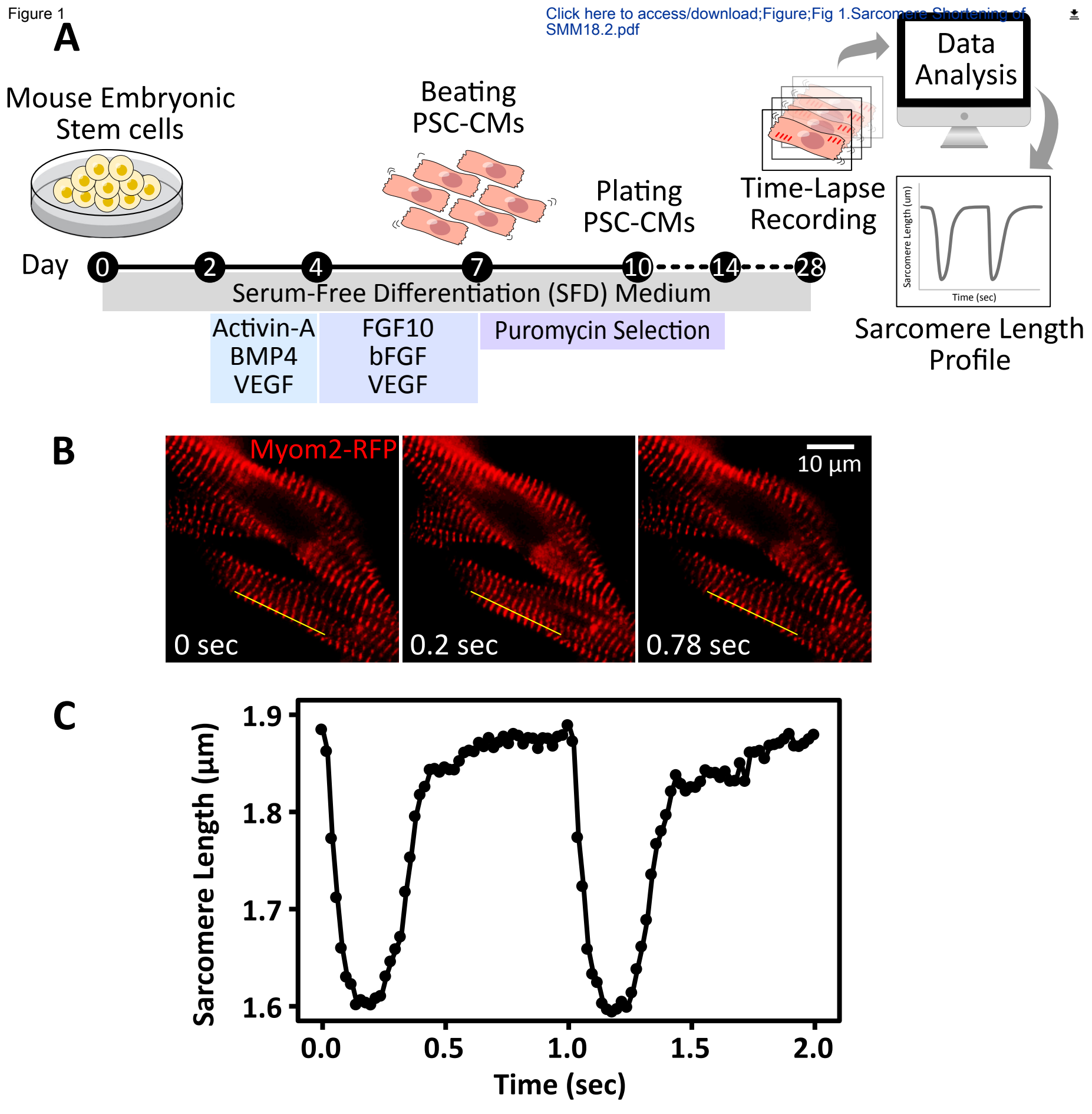
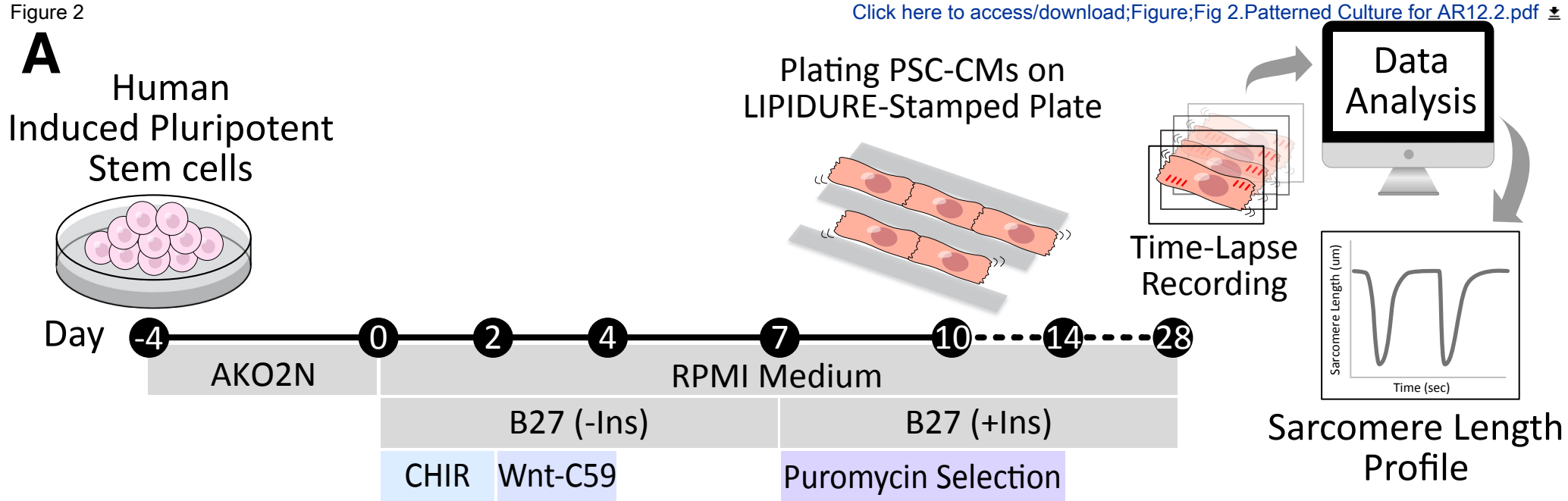
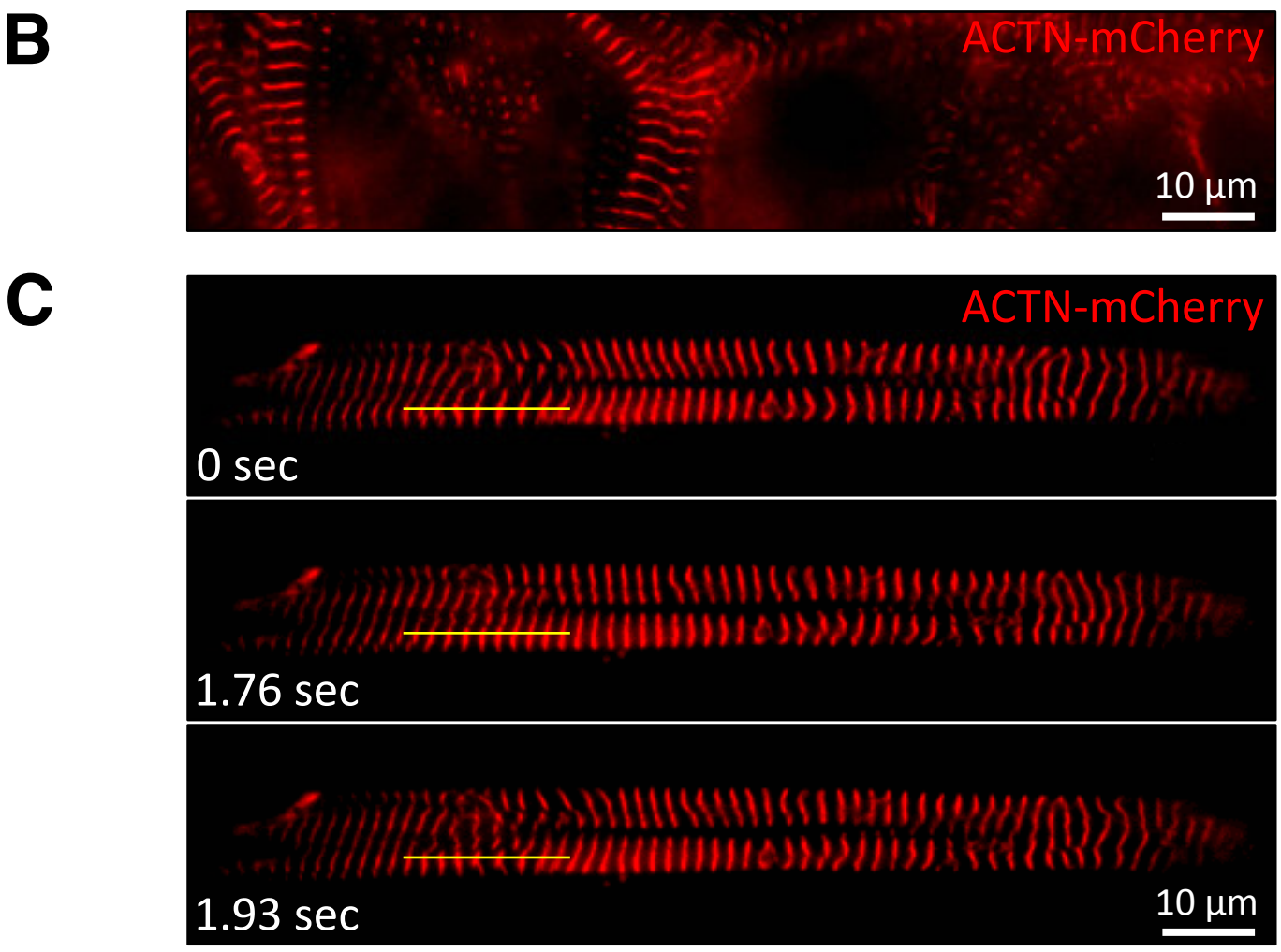
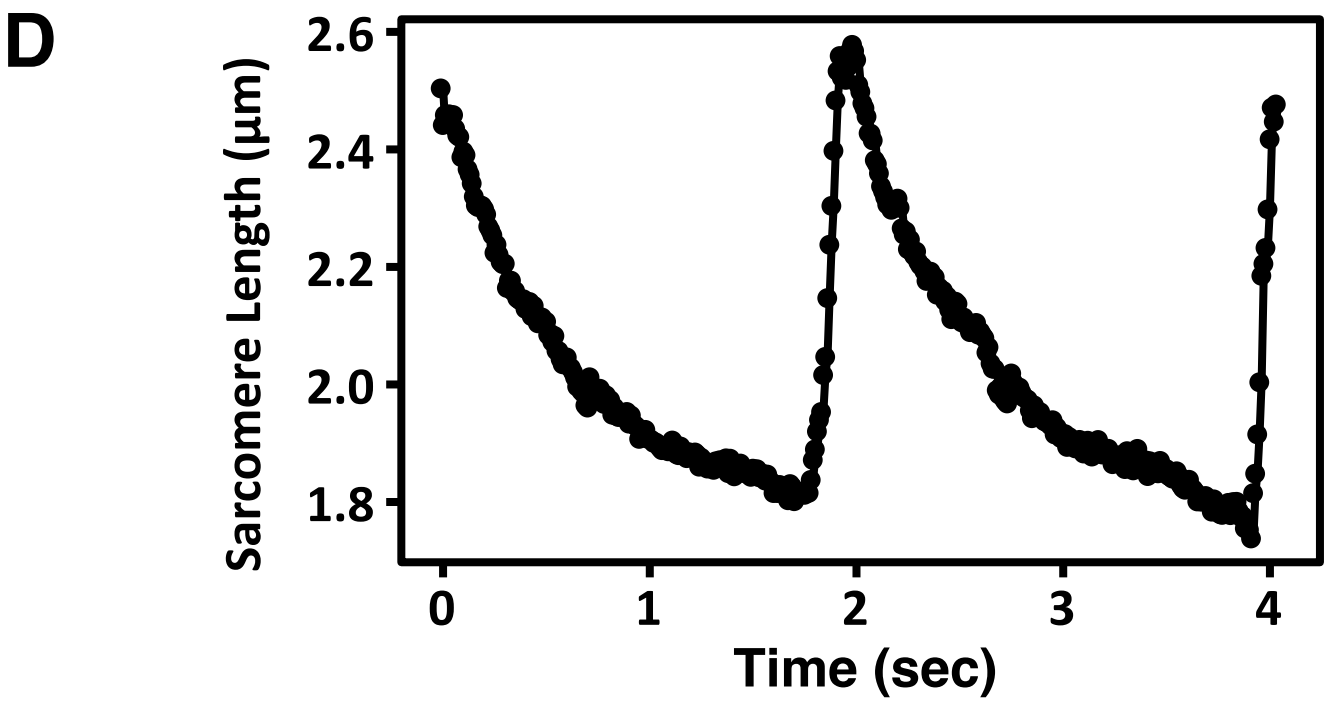


Figure 2







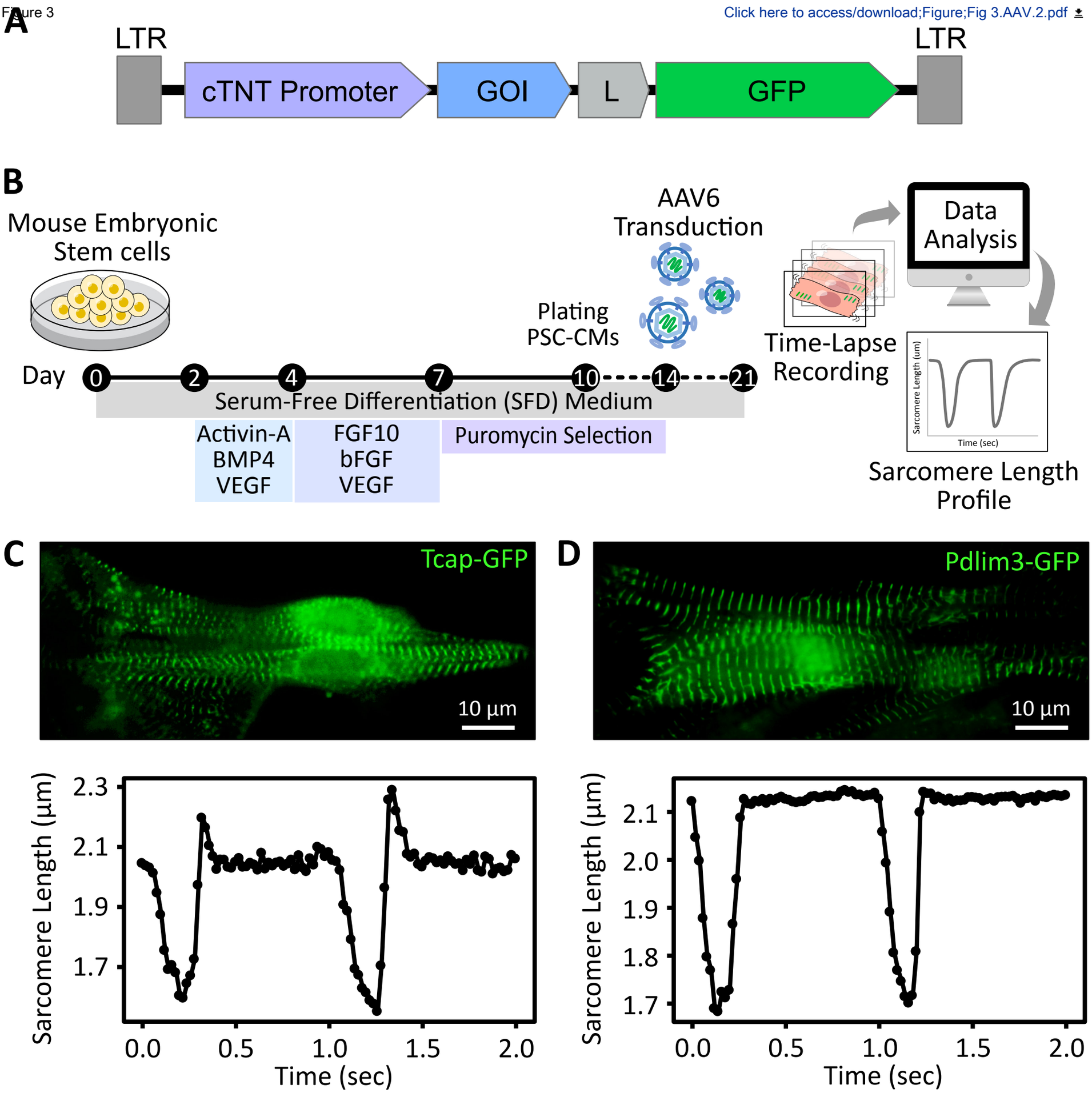
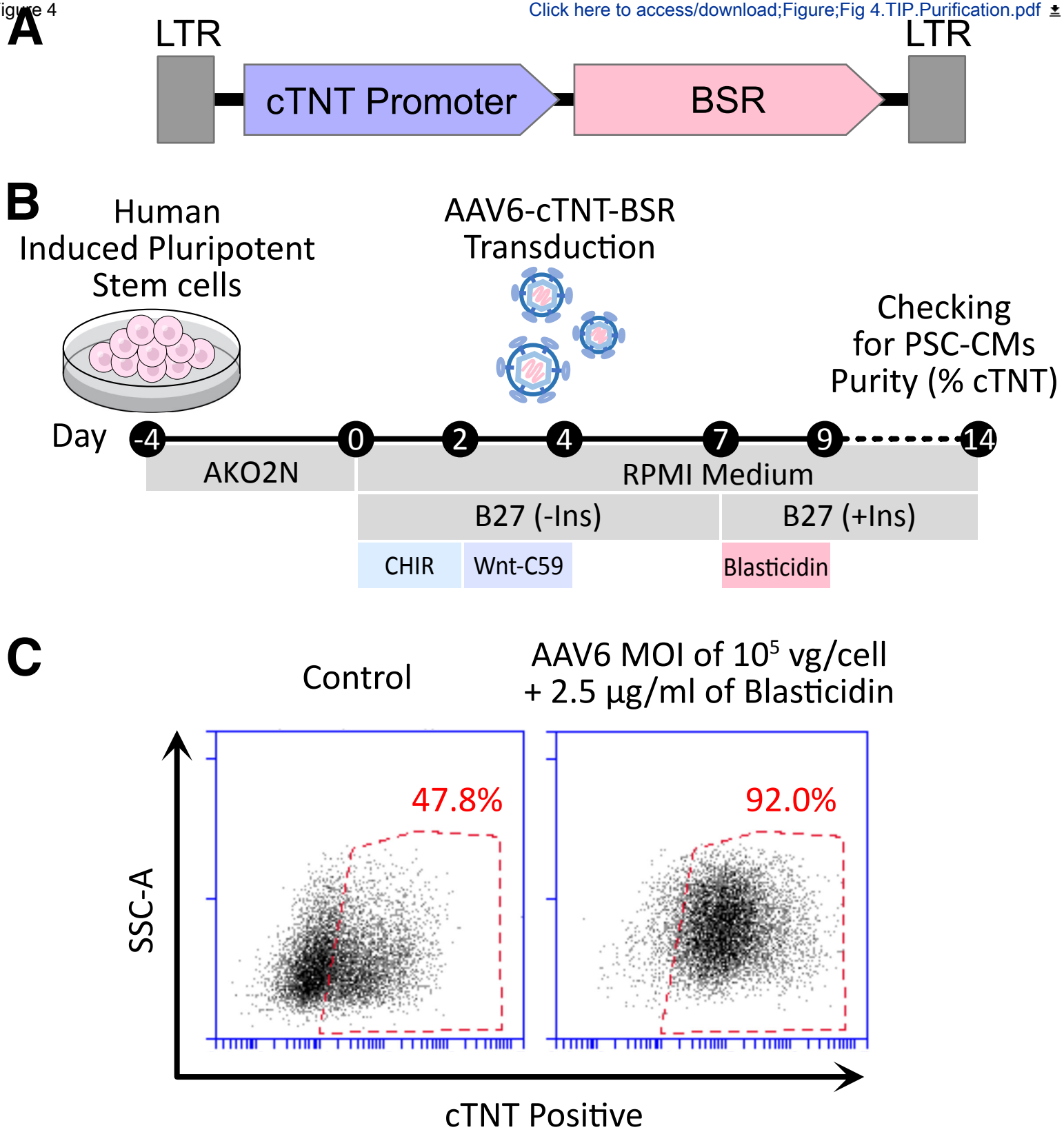
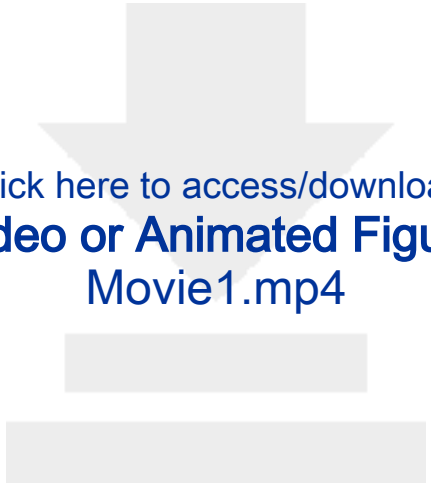


Figure 4



Movie 1



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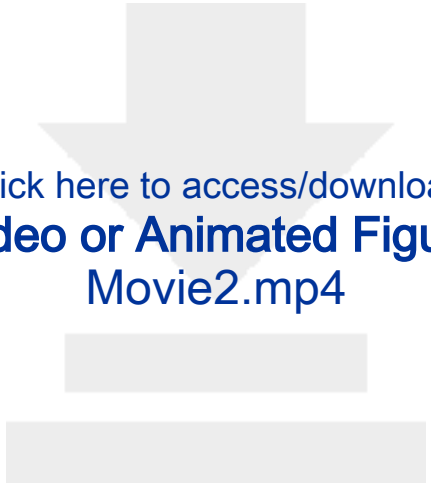
Video or Animated Figure

Movie1.mp4

Movie 2



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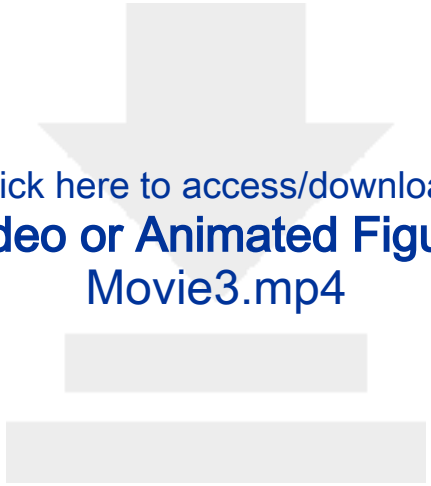
Video or Animated Figure

Movie2.mp4

Movie 3



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Video or Animated Figure

Movie3.mp4

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_R1.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-Thioglycerol	Sigma-Aldrich	M6145-25	
2-Mercaptoethanol (55mM)	Thermo Fisher Scientific	21985-023	
2-methacryloyloxyethyl phosphorylcholine (MPC) polymer	NOF Corp.	LIPIDURE-CM5206	
2-Propanol	Fujifilm wako	166-04836	
35-mm imaging dish with a polymer coverslip (µ-Dish 35 mm, high)	ibidi	81156	
AAVproR Helper Free System (AAV6) (vectors; pHelper, pRC6, pAAV-CMV-Vector)	Takara	6651	
ACTN2-mCherry (AR12, AR21) hiPSCs	N.A.		We inserted IRES-puromycin resistant casette to 3' UTR of TNNT2 locus and mCherry around the stop codon of ACTN2 in 610B1 hiPSC line, following a method describe elsewhere (Anzai, Methods Mol Biol, in press)
B-27 Supplement (50X), serum free	Thermo Fisher Scientific	17504-044	
B-27 Supplement, minus insulin	Thermo Fisher Scientific	A18956-01	
B27 supplement (50X), minus Vitamin A	Thermo Fisher Scientific	12587-010	
Benzonase (25 U/µL)	Merck Millipore	70746	
Blasticidin S Hydrochloride	Fujifilm wako	029-18701	
BMP-4, Human, Recombinant	R&D Systems, Inc.	314-BP-010	
Bovine Serum Albumin	Sigma-Aldrich	A4503-100g	
C59, Wnt Antagonist (WntC59)	abcam	ab142216	
CAD drawing software	Robert McNeel and Associates, WA, USA	Rhinoceros 6.0	
Centrifugal ultrafiltration unit (100k MWCO), Vivaspin-20	Sartorius	VS2042	
CHIR99021	Cayman	13122	
Chromium etchant	Nihon Kagaku Sangyo Co., Ltd., Japan	N14B	
Chromium mask coated with AZP1350	Clean Surface Technology Co., Japan	CBL2506Bu-AZP	
Dr. GenTLE Precipitation Carrier (20mg/mL Glycogen, 3 M Sodium Acetate (pH 5.2))	Takara	9094	
Dulbecco's Modified Eagle's Medium (DMEM) - high glucose	Sigma-Aldrich	D6429-500	
Dulbecco's Modified Eagle's Medium (DMEM) - high glucose, without sodium pyruvate	Sigma-Aldrich	D5796	
Ethanol (99.5)	Fujifilm wako	057-00456	
Fetal Bovine Serum	Moregate	59301104	
FGF-10, Human, Recombinant	R&D Systems, Inc.	345-FG-025	
Fibroblast Growth Factor(basic), human, recombinant	Fujifilm wako	060-04543	
Gelatin from porcine skin powder	Sigma-Aldrich	G1890-100g	
Glasgow Minimum Essential Medium (GMEM)	Sigma-Aldrich	G5154-500	
GLASS BOTTOM culture plates	MatTek	P24G-1.5-13-F/H	
Ham's F-12	Thermo Fisher Scientific	11765-062	
Iscove's Modified Dulbecco's Medium (IMDM)	Thermo Fisher Scientific	12440-061	
L-alanine-L-glutamine (GlutaMAX Supplement, 200mM)	Thermo Fisher Scientific	35050-061	
L(+)-Ascorbic Acid Sodium Salt	Fujifilm wako	196-01252	
Laminin-511 E8 fragment (LN511-E8, iMatrix-511)	Nippi	892012	
Mask aligner	Union Optical Co., Ltd., Japan	PEM-800	
Maskless lithography tool	NanoSystem Solutions, Inc., Japan	D-Light DL-1000	
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific	11140-050	
Millex-HV Syringe Filter Unit, 0.45 µm, PVDF (0.45-µm filter)	Merck Millipore	SLHVR33RS	
Myom2-RFP (SMM18)	N.A.		Developed in our previous paper (Chanthra, Sci Rep, 2020)
N-2 Supplement (100X)	Thermo Fisher Scientific	17502-048	
ORCA-Flash4.0 V3 digital CMOS camera	Hamamatsu	C13440-20CU	
PD0325901	Stemgent	04-0006-10	
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140-122	
Petri dish	Sansei medical co. Ltd	01-004	
Phenol/Chloroform/Isoamyl alcohol（25:24:1）	Nippon Gene	311-90151	
Polydimethylsiloxane (PDMS) elastomer	Dow Corning Corp., MI, USA	SILPOT 184	
polyethylenimine MAX (MW. 40,000)	Polyscience	24765-1	
Positive photoresist developer	Tokyo Ohka Kogyo Co., Ltd., Japan	NMD-3	
PowerUp SYBR Green Master Mix	Thermo Fisher Scientific	A25742	
Proteinase K	Takara	9034	
Puromycin Dihydrochloride	Fujifilm wako	166-23153	
Recombinant Human/Mouse/Rat Activin A Protein	R&D Systems, Inc.	338-AC-050	
Recombinant trypsin-like protease (rTrypsin; TrypLE express)	Thermo Fisher Scientific	12604-039	
RPMI1640 Medium	Thermo Fisher Scientific	11875-119	
Silicon wafer	Matsuzaki Seisakusyo Co., Ltd., Japan	N.A.	
Sodium Pyruvate (100 mM)	Thermo Fisher Scientific	11360-070	
Spin-coater	Mikasa Co., Ltd., Japan	MS-A100	
Spininng confocal microscopy	Oxford Instruments	Andor Dragonfly Spinning Disk System	
StemSure LIF, Mouse, recombinant, Solution (10^6U)	Fujifilm wako	195-16053	
SU-8 3010	Kayaku Advanced Materials, Inc., MA, USA	SU-8 3010	
SU-8 developer	Kayaku Advanced Materials, Inc., MA, USA	SU-8 developer	
Tris-EDTA	Nippon Gene	314-90021	
Vascular Endothelial Growth Factor-A165(VEGF), Human, recombinant	Fujifilm wako	226-01781	

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

: We have proofread and corrected some minor errors.

2. Please revise the following lines to avoid overlap with previously published work: 417-423

: We revised accordingly.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: GlutaMAX, TrypLE, iMatrix-511, Dr. GenTLE Precipitation Carrier, PowerUp SYBR Green Master Mix, Rhinoceros 6.0, LIPIDURE-CM5206, ORCA flash, Andor Dragonfly Spinning Disk, Olympus etc.

: We have removed commercial languages as much as possible, but we left some as we need to specify media and supplements, for which no generic terms are available.

4. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

b) Please specify the euthanasia method, but do not highlight it.

: In this study, we do not have anything related to animals.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

: We have revised accordingly to describe each steps as much as possible.

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

: We removed the last paragraph, in which we described future perspectives.

6. 3.6.6, 3.6.7: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

7. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

9. Please sort the Materials Table alphabetically by the name of the material.

: We revised accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Overall, this is a nice description of a method to evaluate the contractility of pluripotent stem cell-derived cardiomyocytes.

Major Concerns:

1) The authors should remove any "human" adjective from their text since they only described experiments in mouse PSC- derived cardiomyocytes.

: As you can see, we had experiments in human PSC-CMs in Section 2, and Figure 2. To make which PSC-CMs were used in each experiment, we added the adjectives either “mouse” or “human” in the respective figure legend.

2) In the introduction, they authors described ARVM, DCM and mitochondrial cardiomyopathy. Several works have been done in the field of hypertrophic cardiomyopathy that should be also cited, since in this case, such as in DCM, contractility is the most important (for ARVM,much less).

: We added HCM in the introduction and some references as listed below.

4. Carvajal-Vergara, X. *et al.* Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. *Nature*. **465** (7299), 808–812, doi: 10.1038/nature09005 (2010).

5. Lan, F. *et al.* Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. *Cell Stem Cell*. **12** (1), 101–113, doi: 10/f4k8b6

(2013).

Minor Concerns:

1) The AAV concentration should be given in MOI. It looks very high to me. In addition, please describe the medium used for the transduction. Do you do it in the absence or presence of serum and how long?

: We changed the AAV concentration to "MOI of $1x10^4$ to $1x10^6$ vg/cell" from "$1x10^4$ to $1x10^6$ vg/cell" following the reviewer's suggestion.

In terms of AAV concentration, we performed preliminary tests using pAAV-Troponin T promoter-EGFP. Then, we found that MOI of 10^4 vg/cell transduced ~50% of PSC-CMs, and MOI of more than or equal to 10^5 vg/cell transduced almost 100% of PSC-CMs. At MOI of 10^6 vg/cell, we observed non-specific expression in non-CMs. In the case that some fusion protein provided dimmer signals, we needed to increase MOI to 10^6 vg/cell, though.

The range of MOI of 10^4 to 10^6 vg/cell is not too high. For instance, Rapi et al (Mol Ther Methods Clin Dev, 2015) compared the dosage and serotypes of AAVs on PSC-CMs. Using scAAV2 and scAAV6, they demonstrated the efficient transduction at MOI of 10^4 to 10^6. Although we used ssAAV6 not scAAV6, the transduction efficiency is in a comparable range.

3) Lines 379- 380: please describe where are located the primers to titer the AAV (ITR, polyA, promoter…).

: Primers are located on the ITR. We added the description to the manuscript as follows.

> Primers, located on ITR, are 5'-GGAACCCCTAGTGATGGAGTT-3' and 5'-CGGCCTCAGTGAGCGA-3'.

2) Line 152: add a "a" between "petri dish into" and 15- mL tube.

4) Line 524: please correct 10^5 vg/cell (without "s" at the end of cell).

: Thank you for comments. We revised accordingly.

Reviewer #2:

Manuscript Summary:

The authors described methods for live imaging pluripotent stem cell (PSC)-derived cardiomyocytes using fluorescent-tagged sarcomere proteins. The fluorescent proteins were inserted into specific loci of the PSC lines for endogenous expression or introduced using adeno-associated viruses for exogeneous expression.

The manuscript is easy to read and well-constructed. My comments are as below.

Major Concerns:

#1 Since the image acquisition is one of the most important parts of the manuscript, section 6.1 and 6.1.1 should be described in more detail.

: We have updated the sections as follows:

7. Time-lapse Imaging of Sarcomere Under Fluorescent Microscope

7.1. Turn on and connect the microscope, associated computer, and also all of the required peripherals.

7.2. Perform time-lapse imaging: Capture time-lapse images with the highest magnification (100X objective lens with oil emersion)..

7.3. Select live-imaging conditions. To obtain good representative data, try to adjust to the highest framerate (minimum of 20 ms or 50 frames per second is recommended). Set the shutter open and apply a necessary binning (4 X 4) and a crop of the acquisition area to achieve the shortest intervals between images during the time-lapse imaging.

Note: The setting may vary depending on the configurations of microscopes. The camera needs to be high-sensitivity and is able to transfer the data to the connected PC fast enough. To this end, we used ORCA flash with Camera-link. We have tested a spinning confocal microscopy, and acquired images at 400 frames per second.

7.3.1. [Optional] If the beating rate of the cells is low, evoke the cells by electrical field stimulation.

7.4. Run the time-lapse record:

7.4.1. Ensure that the imaged fields remain in focus during recording the time-lapse image.

7.4.2. Save the time-lapse images into an appropriate folder.

#2 It is recommended that the authors include a timeline from the initiation of cardiomyocyte differentiation to the time-lapse imaging.

: We added the timeline for each experimental setting in the first panels of figures as the reviewer recommended.

Minor Concerns:

#3 Section 3.3.1 only describes the production of AAV6, but the authors also mention AAV2. Therefore, a related description should be included in the Protocol and Table of Materials.

: It may be confusing; however, AAV serotype is defined by a cap gene, while AAV2 rep gene is used for any serotypes. In our case, we used pRC6 to provide AAV2 rep and AAV6 cap genes. We modified the sentence to clarify the point as follows:

pRC6 (a vector coding AAV2 Rep and AAV6 Cap genes)

#4 In section 1.1.3, "which RFP was knocked in" should be corrected to "in which RFP was knocked in".

#5 In section 5.6, "the patter" should be replaced with "the pattern".

: Thank you for comment. We revised accordingly.

Reviewer #3:

Manuscript Summary:

The manuscript by Ahmed et. al. provides a detailed method for using fluorescently labeled sarcomeric proteins, either genome-encoded or delivered by AAV vectors, combined with micropatterning to measure sarcomere contractile dynamics in induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). The method for sarcomere measurement in the two genetically modified iPSC lines used here is a thorough step-by-step method for sarcomere measurement in patterned culture, which will be useful for any other lab with such genetically engineered cells. To make this method more broadly applicable, the authors describe the generation of AAVs to fluorescently label Z disks via overexpression of Z disk proteins, which would allow their measurement method to be extended to other iPSC lines. This overexpression approach, in particular, would be of interest to many labs, and has potential to be a valuable contribution as a method. However, the usefulness of this latter part of the method is not fully fleshed out in this manuscript and requires additional information in order to be fully useful. My detailed concerns are below.

Major Concerns:

1. To use viral transduction as a tool to measure sarcomere shortening, it is assumed that AAV overexpression of Z-disk proteins does not alter contractility. The authors cite previous publications that use ACTN2 and Myom2 fusion proteins as knock-ins, but none that use AAVs to overexpress these proteins. Do the authors have data (or citations) that AAV transduction with these Z disk tags does not alter contractility?

: We added the sarcomere length profile of Tcap-GFP and Pdlim3-GFP in Figure 3. Compared to the control (that is not shown in the figure), we did not observe any obvious difference. You might think Tcap-GFP result looks shaggy, which is due to the longer exposure time for live imaging as Tcap-GFP is dimmer than Pdlim3-GFP. We are trying to make new AAVs with brighter GFP mutants such as mGreenLantern, but the new ones have not been available due to the short time of revision.

2. Related to concern 1 - for AAV design, the precise fluorescent fusion proteins are an integral part of this process, as fusions that inactivate the proteins could be detrimental to sarcomere function (for instance in Sparrow et al 2019, C terminal GFP fusion to thin filament proteins disrupted sarcomere function, while N terminal fusion was tolerated). The fusion proteins (specifically TCAP-GFP and PDLIM3-GFP) should be

described in more detail, or references with the fusion proteins should be provided. If possible, providing plasmid sequences and cloning details would allow easier use of this system.

: In Figure 3, we added the vector scheme of AAV. Both Tcap and Pdlim3-GFP are C-term fusion with GGGS linker. We agree that it is very important which side the fusion is. We have experienced that one of fusion protein we have made showed different trace of sarcomere shortening depending on the fusion pattern (data not shown). We have added the discussion of the importance of this point and included the reference (Sparrow et al).

It is also important to note that fusion pattern (i.e. the GFP fusion site at N-term or C-term of target protein) also affect the sarcomere function[29].

Minor Concerns:

1. The differentiation protocol provided relies on antibiotic selection for purification of CMs, which is only applicable to the knockin lines used in the protocol and not more generally. Specifically, one could not use this protocol to differentiate alternate cell lines in order to use them for AAV induction.

: It is very true. To generalize the protocol, we also added AAV-based PSC-CMs selection method (Section 4 & Figure 4). With the method, we transduce AAV expressing a blasticidin-resistant gene under the control of cardiac troponin T promoter to the differentiating PSCs, and successfully purified PSC-CMs with blasticidin selection.

2. Step 4.4 - where is the silicon wafer sourced? I do not see it in the materials.

3. Though the photolithography pattern is described, it is somewhat hard to visualize. A figure showing the geometry used, and potentially the CAD file used to create the stamp, would add to the reproducibility of the method.

: Thank you for the comments. We added the source of the silicon wafer in the material table, and the CAD file as the supplement.

REFERENCES:

Sparrow Alexander J., Sievert Kolja, Patel Suketu, Chang Yu-Fen, Broyles Connor N., Brook Frances A., Watkins Hugh, Geeves Michael A., Redwood Charles S., Robinson Paul, et al. (2019). Measurement of Myofilament-Localized Calcium Dynamics in Adult Cardiomyocytes and the Effect of Hypertrophic Cardiomyopathy Mutations. Circulation Research 124, 1228-1239.

Division of Regenerative Medicine
Center for Molecular Medicine, Jichi Medical University
3311-1Yakushiji, Shimotsuke,
Tochigi 329-0498, Japan.
TEL : +81-285-58-7450
FAX : +81-285-44-5205

Dec 1, 2020

Prof. Sara Pahlavan
Guest Editor, ***Journal of Visualized Experiments***:

Dear Prof. Pahlavan,

Thank for the opportunity to make our manuscript better through the fair review. We are submitting our revised manuscript entitled "Sarcomere Shortening of Pluripotent Stem Cell-Derived Cardiomyocytes Using Fluorescent-tagged Sarcomere Proteins". We believe that the revised manuscript is now suitable to the Method Collections of "Methods toward maturation of human pluripotent stem cell-derived cardiomyocytes for in vitro and in vivo biomedical applications" in ***Journal of Visualized Experiments***.

We have revised our manuscript according to the editorial and reviewers' comments. Most importantly, we added section 4 and figure 4 to address the comment (Minor #1) from the Reviewer #3. In figure 3, we added panels in the response to the comment #1 and #2 from the reviewer #3. We also added discussion related to the comment #2. We revised section #6.1 and 6.1.1 (now #7.1 to #7.4.2) to detail the image acquisition to address the editorial comment #5 and the comment #1 from the reviewer #2. We also added timeline panels to each figure to address the comment #2 from the Reviewer #2. Please find attached file for the detail responses to the editorial and reviewers' comments.

We strongly believe that the method detailed in this manuscript—sarcomere shortening with fluorescent-tagged proteins in pluripotent stem cell-derived cardiomyocytes—will appeal to the readers and viewers of the method collections. We look forward to hearing a positive reply from you. Thank you for your kind effort on handling our manuscript in advance and giving us the opportunity to contribute to the exciting method collection.

Yours sincerely,

Hideki Uosaki, M.D., Ph.D.
Associate Professor,
Division of Regenerative Medicine, Center for Molecular Medicine
Jichi Medical University
3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, JAPAN
TEL: +81-285-58-7450
E-mail: uosaki.hideki@jichi.ac.jp

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TITLE:

Sarcomere Shortening of Pluripotent Stem Cell-Derived Cardiomyocytes Using Fluorescent-tagged Sarcomere Proteins.

AUTHORS AND AFFILIATIONS:

Razan E Ahmed[1,*], Nawin Chanthra[1,*], Tatsuya Anzai[1,2], Keiichiro Koiwai[3,4], Tomoki Murakami[4], Hiroaki Suzuki[4], Yutaka Hanazono[1], Hideki Uosaki[1]

[1]Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan

[2] Department of Pediatrics, Jichi Medical University, Tochigi, Japan

[3] Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, Tokyo, Japan

[4] Department of Precision Mechanics, Faculty of Science and Engineering, Chuo University, Tokyo, Japan

[*] The authors equally contributed

Corresponding Author:
Hideki Uosaki
uosaki.hideki@jichi.ac.jp
Tel: +81-285-58-7450

Email Addresses of Co-authors:
Razan E Ahmed (d2029@jichi.ac.jp)
Nawin Chanthra (nawin2020@jichi.ac.jp)
Tatsuya Anzai (r1204at@jichi.ac.jp)
Keiichiro Koiwai (koiwai@go.tuat.ac.jp)
Tomoki Murakami (murakami@nano.mech.chuo-u.ac.jp)
Hiroaki Suzuki (suzuki@mech.chuo-u.ac.jp)
Yutaka Hanazono (hanazono@jichi.ac.jp)

KEYWORDS:

Pluripotent stem cell, cardiomyocyte, sarcomere shortening, live imaging, fluorescent-tagged sarcomere proteins, microcontact printing

SUMMARY:

We summarized the method to examine sarcomere shortening using pluripotent stem cell-derived cardiomyocytes with fluorescent-tagged sarcomere proteins.

ABSTRACT:

Pluripotent stem cell-derived cardiomyocytes (PSC-CMs) can be produced from both embryonic ~~stem cells~~ and induced pluripotent stem (ES/iPS) cells. These cells provide promising sources for cardiac disease modeling. For cardiomyopathies, sarcomere shortening is one of the standard physiological assessments that are used with adult cardiomyocytes to examine their disease phenotypes. However, the available methods are not appropriate to assess the contractility of PSC-CMs as they have underdeveloped sarcomere that is invisible under phase-contrast microscopy. To address the issue and perform sarcomere shortening with PSC-CMs, we describe fluorescent-tagged sarcomere proteins and fluorescent live-imaging. Thin Z-lines and M-line reside at both ends and the center of a sarcomere, respectively. We tagged Z-line proteins—α-Actinin (ACTN2), Telethonin (TCAP), and actin-associated LIM protein (PDLIM3), and M-line protein—Myomesin-2 (Myom2) with fluorescent proteins. These tagged proteins can be expressed from endogenous alleles as knock-ins or from adeno-associated viruses (AAVs). Here, we introduce the methods to differentiate mouse and human pluripotent stem cells to cardiomyocytes, produce AAVs, and perform and analyze live-imaging. We also describe the methods of producing polydimethylsiloxane (PDMS) stamps for a patterned culture of PSC-CMs, which facilitates the analysis of sarcomere shortening with fluorescent-tagged proteins. To assess sarcomere shortening, time-lapse images of the beating cells at a high ~~frame-rate~~framerate (50–100 frames per second) were recorded under the electrical stimulation (0.5–1 Hz). To analyze sarcomere length over the course of cell contraction, the recorded time-lapse images were subjected to SarcOptiM, a plug-in for ImageJ/Fiji. Our strategy would provide a simple platform to investigate the cardiac disease phenotypes in PSC-CMs.

INTRODUCTION:

Cardiovascular diseases are the leading cause of mortality worldwide[1], and cardiomyopathy represents the third cause of cardiac-related deaths[2]. Cardiomyopathy is a collective group of diseases, affecting cardiac muscles. The recent developments of induced pluripotent stem (iPS) cells ~~(iPSCs)~~ and directed-differentiation of iPS cell~~C~~s toward cardiomyocytes (PSC-CMs) have opened the door for studying cardiomyocytes with patient genome as *in vitro* model of cardiomyopathy. These cells can be used to understand the pathophysiology of cardiac diseases, elucidate their molecular mechanisms, and to test different therapeutic candidates[3]. There is a tremendous amount of interests, thus, patient-derived iPS ~~Cs~~ cells have been derived—*e.g.* hypertrophic cardiomyopathy (HCM)[4, 5], arrhythmogenic right ventricular cardiomyopathy (ARVC)[6], dilated cardiomyopathy (DCM)[7], and mitochondrial-related cardiomyopathies[8, 9]. Because one of the characteristics of cardiomyopathy is dysfunction and disruption of sarcomeres, a valid tool that uniformly measures sarcomere function is in need.

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Sarcomere shortening is the most widely used technique to assess the sarcomere function and contractility of adult cardiomyocytes derived from animal models and humans. To perform sarcomere shortening, well-developed sarcomere that can be visible under phase-contrast is required. However, PSC-CMs cultured *in vitro* display underdeveloped and disorganized sarcomeres, and it is unable to properly measure sarcomere shortening[10]. This difficulty to properly assess the contractility of PSC-CMs hinders their usage as a platform to assess cardiac functions *in vitro*. To assess PSC-CMs contractility indirectly, atomic force microscopy, micro-post arrays, traction force microscopy, and impedance measurements are used to measure the effects of the motion exerted by those cells on their surroundings[11–13]. More sophisticated and less invasive video-microscopy recording actual cellular motion (e.g. SI8000 from SONY) can be used to alternatively assess the contractility, however, it doesn't directly measure sarcomere motion nor force generation kinetics[14].

To directly measure sarcomere motion in PSC-CMs, new approaches such as fluorescent-tagging to sarcomere protein are emerging. For example, Lifeact is used to label filamentous actin (F-actin) to measure sarcomere motion[15, 16]. Genetically modified iPS ~~Cs~~ cells are another option to tag sarcomere protein—e.g. α-actinin (ACTN2) and Myomesin-2 (MYOM2)—by fluorescent protein[17–19].

In this paper, we describe the details of how to perform time-lapse imaging for measuring sarcomere shortening using Myom2-TagRFP (mouse embryonic stem [ES] cells) and ACTN2-mCherry (human iPS ~~Cs~~cells). We also show that a patterned culture ~~facilitate~~facilitates the sarcomere alignment. In addition, we describe an alternative method of sarcomere labeling, using adeno-associated viruses (AAVs), which can be widely applied to patient-derived iPS ~~Cs~~cells.

PROTOCOL:

1. Differentiation of Mouse Pluripotent Stem Cells

1.1. Maintenance of mouse ES cells.

1.1.1. Maintenance medium: Mix 50 mL of fetal bovine serum (FBS), 5 mL of L-alanine-L-glutamine~~GlutaMAX~~, 5mL of non-essential amino acid (~~MEM~~ NEAA), 5 mL of 100 mM Sodium Pyruvate, 909 µl of 55 mM 2-Mercaptoethanol to 450 mL of Glasgow Minimum Essential Medium (GMEM). Supplement Leukemia inhibitory factor (LIF), CHIR-99021, and PD0325901 at the final concentration of 1000 U/mL, 1 µM, and 3 µM, respectively. ~~Filtrate~~ Sterilize the medium through a 0.~~22~~ 22-µm filter ~~to sterilize~~.

1.1.2. 10% FBS medium: Mix 55 mL of ~~fetal bovine serum~~FBS, 5.5 mL of L-alanine-L-glutamine ~~GlutaMax~~, 5.5 mL of Sodium Pyruvate, and 5.5 mL of NEAA to 500 mL of Dulbecco's Modified Eagle Medium (DMEM) high glucose. Filtrate the medium through a 0.~~22~~ 22-µm filter to sterilize.

1.1.3. Culture SMM18 mouse ~~embryonic stem (ES) cells~~ES cells, in which TagRFP was knocked into *Myom2* locus, on a gelatinized 6-cm dish in the maintenance medium as previously described[18] ~~(Chanthra, Sci Rep, 2020)~~. Passage every 2–3 days.

1.2. Preparation of serum-free differentiation (SFD) medium:

1.2.1. Basal SFD: Mix 250 mL of Ham's F-12, 750 mL of Iscove's Modified Dulbecco's Medium (IMDM), 10 mL of B27 supplement minus Vitamin A, 5 mL of N2 supplement, 10 mL of L-alanine-L-glutamine~~GlutaMax~~, 5 mL of 10% ~~Bovine~~ bovine ~~s~~Serum ~~Albumin~~ albumin in phosphate-buffered saline (PBS), 10 mL of penicillin and streptomycin (10,000 U/mL)~~Pen Strep~~. Filtrate through 0.22 µm filter to sterilize.

1.2.2. Dissolve ascorbic acid at 5 mg/mL in distilled water and filtrate through 0.22 µm to sterilize.

1.2.3. Dilute 13 µL of 1-Thioglycerol to 1 mL of IMDM. Herein, refer to this diluted 1-Thioglycerol as MTG.

1.2.4. Add 10 µl of ascorbic acid (5 mg/mL) and 3 µl of MTG to 1 mL of basal SFD on the day of use. Herein, refer to this mixture as complete SFD.

1.3. Day 0, embryoid body (EB) formation for differentiation:

1.3.1. Harvest SMM18 mouse ES cells with a recombinant trypsin-like protease (rTrypsin)~~TrypLE~~ and count cell number.

1.3.2. Centrifuge 5 x 10^5 cells at 300 ~~G~~ x g for 3 min in 4 °C, resuspend in 10 mL of complete SFD,

and seed into a 10-cm petri dish. Culture the cells at 37 °C and 5% CO_2 for 50 h.

1.4. Differentiation day 2:

1.4.1. Add Activin A, human vascular endothelial growth factor (hVEGF), and bone morphogenetic protein 4 (BMP4) to complete SFD at the final concentration of 5 ng/mL, 5 ng/mL, and 1.9 ng/mL, respectively.

Note: BMP4 concentration might differ depending on the lots of BMP4. Test several concentrations in a small-scale trial prior to use a new lot and define the best concentration for cardiac differentiation.

1.4.2. Transfer EBs from a petri dish into a 15-ml tube and centrifuge at 50-100 x g for 3 min at 4 °C.

1.4.3. Meanwhile, add the medium prepared in step 1.4.1 to the petri dish to protect the remaining EBs being dry.

1.4.4. Aspirate the supernatant from the 15-mL tube, resuspend the EBs with the medium in the petri dish, and transfer back to the dish. Then, cultivate the EBs at 37 °C and 5% CO_2 for 46 h.

1.5. Differentiation day 4:

1.5.1. Gelatinize a 10-cm tissue culture-treated dish with 5 to 10 mL of 0.1% gelatin for at least 5 min. Aspirate gelatin right before seeding the cells.

1.5.2. Prepare medium: Mix basic fibroblast growth factor (bFGF), FGF10, and hVEGF to complete SFD at the final concentration 5 ng/ml, 25 ng/ml, and 5 ng/ml respectively. For a 10-cm dish, prepare 10 mL.

1.5.3. Transfer cells in the petri dish to a 15-ml tube. Add 5 ml of PBS to the petri dish, wash several times, and transfer to the 15-mL tube to collect the remaining cells. Centrifuge at 50–100 x g, 4 °C, 3 min.

1.5.4. Aspirate supernatant, add 1 ml of TrypLErTrypsin, and incubate at 37 °C for 3 min.

1.5.5. Vortex briefly to dissociate EBs, add 9 ml of 10% FBS medium, vortex again, and count cell number.

1.5.6. Centrifuge 1.5 x 10^7 cells at 300 x g, 4 °C for 3 min, resuspend with the media prepared in step 1.4.2, and seed into the gelatinized dish. Incubate at 37 °C and 5% CO_2 for 2 days.

Note: By day 7 or 8, extensive beatings of PSC-CMs can be observed.

1.6. Drug selection at differentiation day 7 and 9: Refed the media with puromycin (2 μg/mL at the final concentration) to eliminate non-cardiomyocytes at day 7 and 9 of differentiation.

Note: Parental line of SMM18 is syNP4 mouse ES cells, harboring NCX1 promoter-driven puromycin-resistant gene[20].

1.7. Day 10, replate for future experiments:

1.7.1. Coat a glass-bottom culture plate or a 35-mm imaging dish with a polymer coverslip~~a μ-Dish 35-mm dish~~ with 0.1% Gelatin. To enhance maturation, coat the dishes with laminin-511 E8 fragment (LN511-E8)~~iMatrix-511~~ at 1 μg/cm^2 for 30 min to 1 h at the room temperature[18]. To culture PSC-CMs in desired patterns, please refer to steps 4 and 5 for preparing polydimethylsiloxane (PDMS) stamps.

1.7.2. To harvest SMM18 PSC-CMs, wash the dish twice with PBS, then apply 1 mL of ~~TrypLE~~rTrypsin, and incubate 3 min at 37 °C.

1.7.3. Collect cells to 9 mL of 10% FBS medium, suspend, and count the cell number. Plating cell number is 50,000–100,000 cells in a well of a 24-well plate, and 250,000–500,000 cells in a 35-mm imaging dish~~a 35-mm glass-bottom dish~~.

1.7.4. Centrifuge a sufficient number of cells (300 x g, 3 min), resuspend them with complete SFD supplemented with FBS (final concentration at 10%).

1.7.5. Incubate overnight, and change culture medium to complete SFD with puromycin.

1.7.6. From day 14, change culture medium two to three times a week with complete SFD until day 21–28 when Myom2-RFP becomes prominent. For AAV-based transduction of fluorescent-tagged sarcomere proteins, please refer to Step 3.

2. Differentiation of Human Pluripotent Stem Cells

2.1. Preparation of differentiation media:

2.1.1. RPMI+B27-Ins: mix 500 ml of RPMI 1640 medium, 10 ml of B27 minus insulin, and 5.25 mL of L-alanine-L-glutamine~~GlutaMAX~~.

2.1.2. RPMI+B27+Ins: mix 500 ml of RPMI, 10 ml of B27 supplement, and 5.25 mL of L-alanine-L-

glutamine~~GlutaMAX~~.

2.2. Maintenance of human iPS cells:

2.2.1. Passage human iPS cells twice a week with AK02N on ~~iMatrix-511~~LN511-E8 following previously published method with some modifications[21].

2.2.2. Harvest cells with a 3-min treatment of ~~TrypLE~~rTrypsin and collect into 10% FBS medium. Count cells and centrifuge down at 300 ~~G~~ x g for 3 min at 4 °C. Seed 75,000–125,000 cells in a well of 6-well plate with 2 mL of AK02N supplemented with ~~iMatrix-511~~LN511-E8 and Y27632 at the final concentration of 0.5 µg/ml (0.1 µg/cm^2) and 10 µM, respectively.

2.2.3. Incubate at 37 °C and 5% CO_2~~,~~ and change the medium on the following day with 2 ml of AK02N without any supplement. Change media every two to three ~~days, and~~days and passage every three to four days.

2.3. Day –4, replate prior to differentiation:

2.3.1. Coat a 6-well plate with 0.5 µg/cm^2 of ~~iMatrix-511~~LN511-E8 diluted in PBS. Then, incubate for at least 30 min at 37 °C and 5% CO_2 or 1h at room temperature. Aspirate coating solution right before seeding cells.

2.3.2. Harvest human iPS cells with ~~TrypLE~~rTrypsin and count cell number as Step 2.2.2.

2.3.3. Centrifuge 1.25 x 10^5 cells for a well of a 6-well plate at 300 ~~G~~ x g for 3 min at 4 °~~C, and~~C and resuspend in 2 ml of AK02N supplemented with ~~iMatrix-511~~LN511-E8 (final ~~conc.~~concentration 0.5 µg/ml or 0.1 µg/cm^2) and Y27632 (final ~~conc.~~concentration 10 µM) per well.

2.3.4. Aspirate coating solution and seed resuspended cells into the coated plate, incubate at 37 °C and 5% CO_2.

2.4. Day –3 and –1, refed the media with 2 mL of AK02N.

2.5. Day 0: Change the medium to 2 ml of RPMI+B27-~~INS~~ Ins supplemented with CHIR99021 (final ~~conc.~~concentration 6 µM) per well to start differentiation.

2.6. Day 2: Change the medium to 2 ml of RPMI+B27-~~INS~~ Ins with WntC59 (final ~~conc.~~concentration 2 µM) per well.

2.7. Day 4: Change the medium to 2 ml of RPMI+B27-~~INS~~ Ins per well.

2.8. Day 7 and day 9, medium change to 2 ml of RPMI+B27+~~INS~~ Ins with puromycin (final

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concentration 10 µg/ml) per well to selectively culture PSC-CMs.

Note: ACTN2-mCherry line, we used in this study, has a cassette of internal ribosomal entry site (IRES~~-~~) and ~~Puromycin-~~ puromycin-resistant gene ~~cassette~~ inserted to 3'' ~~-~~untranslated region (UTR) of TNNT2 locus, and mCherry replacing the stop codon of ACTN2. To purify cardiomyocyte without knock-in, please refer to Step 3 and 4.

2.9. Day 10, replate for future experiments:

2.9.1. Coat a 35-mm imaging dish with a polymer coverslip ~~µ-Dish 35-mm dish~~ with 0.5–1 µg/cm^2 of ~~iMatrix-511~~LN511-E8 diluted in 0.1% Gelatin. Incubate 2–4 h at room temperature for the long-term viability. To culture PSC-CMs in desired patterns, please refer to steps 4 and 5 for preparing PDMS stamps.

2.9.2. To harvest human PSC-CMs, wash the dish twice with PBS, then apply 1 mL of ~~TrypLE~~rTrypsin to a well, and incubate 3 min at 37 °C.

2.9.3. Collect cells to 4 mL of 10% FBS medium, suspend, and count the cell number. Plating cell number is 250,000–500,000 cells in a ~~µ-Dish~~ 35-mm imaging dish.

2.9.4. Centrifuge a sufficient number of cells at 300 ~~G~~ x g for 3 min at 4 °C, resuspend them with RPMI+B27+Ins with puromycin (10 µg/ml), and plate them on the coated 35-mm imaging dish ~~µ-Dish 35-mm dish~~.

2.9.5. Incubate overnight, and change culture medium to RPMI+B27+Ins with puromycin (10 µg/ml).

2.9.6. From day 14, change culture medium two to three times a week with RPMI+B27+Ins until day 21–28 for imaging. For AAV-based transduction of fluorescent-tagged sarcomere proteins, please refer to Step 3.

3. Fluorescent Labeling of Sarcomere Using Adeno-associated Viruses

3.1. Preparation before AAV production:

3.1.1. Maintain HEK293T cells in DMEM supplemented with FBS (final ~~conc.~~concentration 10%) on a 10-cm tissue culture plate. Passage cells three-time a week.

3.1.2. Prepare polyethylenimine (PEI) at 1 mg/mL. Mix 50mg of polyethylenimine MAX 40000 ~~(Polyscience)~~ and 40 mL of ultrapure water ~~(*e.g.* Milli-Q water)~~. Adjust pH to 7.0 using 1N NaOH. Then, make it up to 50 mL with ultrapure water, and filtrate through a 0.25-µm filter.

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3.1.3. Prepare a shuttle vector with a sarcomere labeling gene, e.g. TCAP or PDLIM3 fused with a green fluorescent protein (GFP), driven by a cardiomyocyte-specific promoter, such as cardiac troponin T (cTNT) promoter[22].

Note: For this instance, we used monomeric enhanced GFP with mutations of V163A, S202T, L221V~~novo2 mutation~~[23].

3.2. Day 0, passage HEK cells: When cells reach to confluent, passage $2.0x10^7$ HEK293T cells to a 15-cm tissue culture plate with 20 mL of DMEM with 10% FBS.

3.3. Day 1, transfection:

3.3.1. Mix 13.5 µg of the shuttle vector, 26 µg of pHelper (a vector coding E2A, E4, and VA of adenovirus), 16.5 µg of pRC6 (a vector coding AAV2 Rep and AAV6 ~~cap~~ Cap genes ~~from AAV2 and AAV6, respectively~~), and 1 mL of DMEM without sodium pyruvate (DMEM-Pyr).

3.3.2. Mix 224 µL of PEI (1mg/mL, prepared in step 3.1.2) and 776 µL of DMEM-Pyr.

3.3.3. Mix and incubate the plasmid solution and the PEI solution at room temperature for 30 min.

3.3.4. Add the plasmid/PEI solution to the HEK293T cells prepared in step 3.2.

3.4. Day 2, medium change: At 24 h after transfection, change medium to DMEM-Pyr. Culture cells until harvesting AAV on day 7. AAV will be released into the culture media.

3.5. Day 7, AAV collection, concentration, and buffer substitution using minimal purification method[24]:

3.5.1. Incubate a centrifugal ultrafiltration unit (100k molecular weight cut-off [MWCO] ~~MWCO, e.g. Vivaspin-20~~) with 5 mL of 1% BSA in PBS at room temperature for 15 min. Then, centrifuge the ultrafiltration unit at 500 ~~G~~ x g for 2 min, and aspirate both filtered solution and remaining solution.

3.5.2. Transfer medium from the 15-cm dish that produced AAV to a new 50-mL conical tube and centrifuge (500 ~~G~~x g, 5 min). Filtrate the supernatant through a 0.45-µm syringe filter for removing cell debris and apply it to the ultrafiltration unit.

3.5.3. Centrifuge at 2,000 ~~G~~ x g for 90 min or until concentrating the culture supernatant 0.5 to 1 mL.

3.5.4. Aspirate filtered medium and apply 15 mL of PBS to the ultrafiltration unit.

3.5.5. Repeat centrifugation until the concentrate becomes 0.5–1 mL.

3.5.6. Repeat 3.5.4 and 3.5.5 again.

3.5.7. Transfer concentrated AAV to a new 1.5 mL tube and store at 4 °C or -20 °C.

Note: AAV can be used in P1 facilities but follow local rules and regulations.
Note: AAV can be produced by conventional methods as well.

3.6. Calculation of AAV titer:

3.6.1. Mix 5 µL of AAV, 195 µL of DMEM-Pyr, and 10 U of benzonase, and incubate at 37 °C for 1 h.

3.6.2. Add 200 µL of proteinase K buffer (0.02 M Tris HCl and 1% SDS) and 5 µL of proteinase K (20 mg/mL) and incubate at 37 °C for 1 h.

3.6.3. CAUTION: Add 400 µL of 25:24:1 Phenol/chloroform/isoamyl alcohol ~~(Nippon Gene)~~, vortex for 1 min, and centrifuge at 20,000 x g~~15,000 rpm~~ for 1 min.

3.6.4. Transfer 200 µL of the aqueous phase to a new 1.5-mL tube, which yields approximately half of the original AAV genomes.

3.6.5. Add 1 µL of Glycogen (20 mg/mL ~~or Dr. GenTLE Precipitation Carrier~~) and 20 µL of 3 M CH_3COONa (pH 5.2), and vortex. Add 250 µL of 2-Propanol and 100 µL of 100% ethanol, and vortex again.

3.6.6. Incubate at -80 °C for 15 min, then centrifuge at 20~~15~~,000 ~~G~~x g~~rpm~~ for 30 min at 4 °C.

3.6.7. Aspirate supernatant and replace it with 70% ethanol. Then, centrifuge at 20~~15~~,000 ~~G~~x g~~rpm~~, 4 °C for 5 min.

3.6.8. Aspirate supernatant and air dry until the pellet becomes clear.

3.6.9. Add 200 µL of Tris-Ethylenediaminetetraacetic acid~~EDTA~~ (TE; pH 8.0) to resolve the AAV genomes. Then, dilute the sample 100-fold with TE.

3.6.10. Prepare a standard with pAAV-CMV-Vector at 6.5 ng/µL with TE, which equals to 10^9 vector genomes (vg)/µL. Then, make a series of 10-fold dilution for 10^4 to 10^8 with TE.

3.6.11. Mix 1 µL of sample DNA (or the standards), 0.4 µL of primers (5 µM), 3.6 µL of distilled water, and 5 µL of ~~PowerUp~~ SYBR Green ~~Master~~ master ~~Mix~~mix. Primers, located on ITR, are 5′-GGAACCCCTAGTGATGGAGTT-3′ and 5′-CGGCCTCAGTGAGCGA-3′.

3.6.12. Perform real-time PCR with the following condition: Initial denature at 95 °C for 60 s, and

then 40 cycles of denaturing at 95 °C for 15 s and annealing and extension at 60 °C for 30 s, followed by melting curve.

3.6.13. Based on the standards and Ct values, a real-time PCR machine provides the copy number of vector genome in 1 µL of a sample. Calculate original AAV titer using the following equation: a copy number provided by real-time PCR (vg/µL) x 8 x 10^3 x 2, wherein 8 x 10^3 as a dilution factor during AAV genome isolation, and 2 as the difference factor of AAV (single strand) and plasmid (double strand).

3.7. Transduction to PSC-CMs:

3.7.1. Count cell number of PSC-CMs in an extra well or an extra dish.

3.7.2. Dilute AAVs (1x10^4 to 1x10^6 vg/cell) to make up 50 µL with PBS. Apply AAVs at the multiplicity of infection (MOI) of 1x10^4 to 1x10^6 vg/cell ~~1x10^4 to 1x10^6 vg/cells~~ to PSC-CMs and culture PSC-CMs for 3 days with AAV in the corresponding differentiation media for mouse and human PSC-CMs, then change media to normal ones without AAV.

~~3.7.2.~~

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3.7.3. Use PSC-CMs for live-cell imaging after 7 days or more post-transduction.

4. [Optional] AAV-based purification of PSC-CMs

4.1. Preparation of AAV:

4.1.1. Prepare AAV as following the steps described in Step 3 using a shuttle vector expressing blasticidin-resistant gene under the control of cTNT promoter.

4.2. Transduction to differentiating iPS cells:

4.2.1. Differentiate human iPS cells for 4 days following the protocol described in Step 2 and count the number of cells in an extra well.

4.2.2. After changing media at day 4, apply AAVs at the MOI of 1x10^5 vg/cell to differentiating PSCs in RPMI+B27-Ins media.

4.2.3. At day 7, refresh media with RPMI+B27+Ins and add 2.5-10 µg/mL of blasticidin.

4.2.4. At day 10, PSC-CMs are ready to replate.

~~4.~~5. Preparation of PDMS stamps

~~4.1.~~5.1. Design the device pattern of 200 µm strips along with 10–25 µm grooves in between the

strips using a computer-aided design (CAD) drawing software~~, Rhinoceros 6.0~~.

~~4.2.~~5.2. Draw the pattern of devices onto a chromium photomask coated with AZP1350 by using UV light of a maskless lithography tool.

~~4.3.~~5.3. Develop the pattern on the photomask in a series of positive photoresist developer, Chromium etchant, and rinse with DI water.

~~4.4.~~5.4. Dehydrate a silicon wafer by baking it on a hot plate at 120 °C for 15 min.

~~4.5.~~5.5. Allow the wafer to cool to room temperature, and then spin-coat a negative photoresist SU-8 3010 to make a height of 10–20 µm with 1,500 rpm for 30 sec using a spin-coater.

~~4.6.~~5.6. Soft bake the wafer in two steps on a hot plate according to the manufacturer's protocol.

~~4.7.~~5.7. After the wafer ~~Allow the wafer to cool~~cooled to room temperature ~~and then~~, load the wafer onto the mask aligner.

~~4.8.~~5.8. Using a mask aligner, ~~Align~~ align the mask on the wafer ~~using a mask aligner~~ and expose the wafer to UV light.

~~4.9.~~5.9. Conduct the post-exposure bake to the wafer in two steps on a hot plate according to the manufacturer's protocol.

~~4.10.~~5.10. Develop the wafer in a series of SU-8 developer and 2-Propanol, then dry the wafer with a nitrogen stream.

~~4.11.~~5.11. Transfer the wafer into a petri dish of a suitable size.

~~4.12.~~5.12. Mix PDMS elastomer and its curing agent in a ratio 10:1 w/w, and pour it into the Petri dish.

~~4.13.~~5.13. Degas the PDMS in a desiccator until all air bubbles disappear, then cure the PDMS on a hot plate at 80 °C for 2 h.

~~4.14.~~5.14. Peel the cured PDMS off from the master mold using a tweezer, then cut out the portion with the design to be a PDMS stamp.

Note: Shape can be square, however, an octagonal shape transfers the pattern better at the edge.

~~5.~~6. Patterned Culture of Pluripotent Stem Cell-derived Cardiomyocytes

~~5.1.~~6.1. Remove dust from the surface of PDMS stamps using mending tape.

5.2.6.2. Submerge the stamps into 70% ~~EtOH~~ ethanol to sterilize. Then, blow ethanol ~~EtOH~~ off the surface of the stamps using an air duster.

5.3.6.3. Apply 5–10 µL of 0.5wt% 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer~~LIPIDURE-CM5206~~/~~EtOH~~ ethanol on the surface of PDMS stamps.

Note: Uneven distribution of ~~LIPIDURE~~ MPC polymer may cause a disrupted pattern.

5.4.6.4. Incubate 10–30 min until MPC polymer ~~LIPIDURE~~ is completely dried.

5.5.6.5. Place the stamps in contact with coverslips of a glass-bottom culture plate ~~(MatTek)~~ or a 35-mm imaging dish with a polymer coverslip~~µ-Dish 35-mm dish (ibidi)~~, and put a weight (*e.g.* a AAA battery) on a stamp for 10 min.

5.6.6.6. Remove the weight and stamps. Then, confirm the pattern is transferred under microscope.

Note: Stamped plates/dishes can be stored up to 1 week at room temperature.

5.7.6.7. Wash the stamped wells/dishes with PBS for 2-times.

5.8.6.8. Dilute ~~iMatrix-511~~LN511-E8 with PBS at 2–4 µg/mL and coat the dish with ~~iMatrix-511~~LN511-E8 at 0.5–1– µg/cm^2. For human PSC-CMs, dilute ~~iMatrix-511~~LN511-E8 with 0.1% gelatin solution instead of PBS. Then, incubate for at least 1 h (optimally, more than 4 h).

5.9.6.9. Plate cells as described in the previous sections.

~~6.~~7. Time-lapse Imaging of Sarcomere Under Fluorescent Microscope

7.1. Turn on and connect the microscope, associated computer, and also all of the required peripherals.

7.2. Perform time-lapse imaging: Capture time-lapse images with the highest magnification (100X objective lens with oil emersion)~~, and the shortest intervals (20 ms, or 50 frames per second) as possible~~.

7.3. Select live-imaging conditions. To obtain good representative data, try to adjust to the highest framerate (minimum of 20 ms or 50 frames per second is recommended). Set the shutter open and apply a necessary binning (4 X 4) and a crop of the acquisition area to achieve the shortest intervals between images during the time-lapse imaging. ~~that will be provided the best representative data such as limiting exposure time to avoid phototoxicity and also reducing interval time in order to obtain the highest frame rate (20 ms, or 50 frames per second) as much as possible.~~

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Note: The setting may vary depending on the configurations of microscopes. The camera needs to be high-sensitivity and is able to transfer the data to the connected PC fast enough. To this end, we used ORCA flash with Camera-link. We have tested a spinning confocal microscopy, and acquired images at 400 frames per second.

7.3.1. [**Optional**] If the beating rate of the cells is ~~very~~ low, ~~it may be necessary to~~ evoke the cells by electrical field stimulation.

7.4. Run the time-lapse record:

7.4.1. Ensure that the imaged fields remain in focus during recording the time-lapse image.

7.4.2. Save the time-lapse images into an appropriate folder.

~~6.1.1.~~

~~**Note:** The setting may vary depending on the configurations of microscopes. To achieve the shortest intervals of time-lapse images, set the shutter open during the time-lapse imaging, and apply a binning to 4x4. The camera needs to be high-sensitivity and able to transfer the data to the connected PC fast enough. To this end, we used ORCA flash with Camera-link. Spinning disk confocal microscopy can be used as well. We have tested Andor Dragonfly Spinning Disk, and achieved as fast as 400 frames per second.~~

~~6.1.2. [**Optional**] If the beating rate of the cells is very low, it may be necessary to evoke the cells by electrical field stimulation.~~

~~7.~~8. Analysis of Time-lapse Imaging Using SarcOptiM, an ImageJ/Fiji Plugin

~~7.1.~~8.1. Load a series of time-lapse images into ImageJ. For Olympus ~~vsi~~ VSI format, open files through OlympusViewer Plugin.

~~7.2.~~8.2. Adjust brightness and contrast of the image to observe the sarcomere pattern clearly (Image -> Adjust -> Brightness/Contrast).

~~7.3.~~8.3. Open SarcOptiM by clicking “More tools” menu (>>), and then selecting “SarcOptiM”.

~~7.4.~~8.4. Calibrate the program by pressing CTRL+SHIFT+P and “1 µm” button on the toolbar, and following the instructions of the dialog boxes.

~~7.5.~~8.5. Draw a line across the region of the sarcomere that will be measured the sarcomere shortening.

~~7.6.~~8.6. Start sarcomere shortening analysis by pressing “SingleCell (AVI)” button on the toolbar. Representative data is shown in **Figure 1 and Figure 2**.

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REPRESENTATIVE RESULTS:

Measuring sarcomere shortening using knock-in PSC-CMs reporter lines.

We used sarcomere-labeled PSC-CMs to measure sarcomere shortening. The lines express Myom2-RFP and ACTN2-mCherry from endogenous loci. TagRFP was inserted to *Myom2*, coding M-protein that localize to M-line, while mCherry was knocked-in to *ACTN2*, coding α-Actinin that localize to Z-line[18, 25]. Time-lapse images were obtained and used to determine sarcomere shortening as presented in **Figures 1, 2, and Movie 1–3**.

To overcome the disorganized sarcomere of PSC-CMs, we used specific PDMS stamps to culture PSC-CMs in the stripe pattern. This patterned culture promoted elongated cell shape and a more organized sarcomere pattern compared to the cells that culture in the non-pattern area (**Figure ~~2A~~ 2B and ~~B~~C**). With this advantage, the patterned culture promoted better contraction of the cells and provided a smooth sarcomere length profile as shown in **Movie 2, 3, and Figure ~~2C~~2D**.

Fluorescent tagging of Z-line protein using AAV vectors.

To visualize the Z-line of PSC-CMs without generating knock-in iPS cell~~C~~s, we expressed fluorescent-tagged Z-line proteins using AAV transduction. We tagged two of small Z-line proteins, Telethonin (TCAP) and Actin-associated LIM protein (PDLIM3) with GFP, and packaged them using the AAV6 capsid (**Figure 3A**). Once PSC-CMs were differentiated and purified, we transduced AAVs to PSC-CMs (**Figure 3B**). ~~-~~The transduced PSC-CMs expressed sarcomeric GFP signals along the PSC-CMs as early as three days post-transduction (**Figure 3C-D**). Typically, the transduction of AAV at the MOI~~titer~~ of 10^5 vg/cell~~s~~ is sufficient to visualize fluorescent-tagged sarcomere proteins, and a higher titer may cause non-specific localization of GFP to cytoplasm though it increases overall GFP intensity.

Purification of PSC-CMs using AAV vectors.

Current method relies on the drug selection cassette that is already on the genome of PSC-CMs, either transgenic or knock-in line. However, it is labor-intensive to produce such a line from each of patient-derived iPS cells. As we demonstrated that AAV vectors drive the expression of fluorescent-labeled Z-line proteins without the need for knock-in, we sought to establish the purification method without knock-in as well (**Figure 4**). To this end, we constructed a new AAV vector, which encode blasticidin-resistant gene under the control of cTNT promoter (**Figure 4A**). The AAV (MOI of 10^5 vg/cell) was transduced to differentiating human iPS cells at day 4, then cells were treated with 2.5-10 μg/mL of blasticidin (need to titrate for each cell line) between days 7 and 9 (**Figure 4B**). At day 14, the purity of PSC-CMs was more than 90% (**Figure 4C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sarcomere shortening of the mouse PSC-CMs derived from the Myom2-TagRFP cell line.

A. The timeline for mouse PSC-CM differentiation. **B.** ~~Time line for mouse PSC-CM differentiation. A~~**B.** Representative images for sarcomere shortening in different time points with measuring regions as indicated by yellow bars. ~~B~~**C.** Sarcomere length profile during contraction of the

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cardiomyocytes that was stimulated with electricity at 1 Hz. The frame-rate was 50 frames per second. The pixel size was 0.26 µm. Scale bar = 10 µm.

Figure 2: Representative data showing sarcomere shortening of the human PSC-CMs~~cardiomyocytes~~ derived from the ACTN2-mCherry cell line in non-patterned and patterned culture.
A. The timeline for human PSC-CM differentiation. **B.** The cardiomyocytes cultured in non-patterned culture showed disorganized sarcomere pattern whereas **B.** patterned culture promoted well alignment of the sarcomere. **B.** Representative images for sarcomere shortening with measuring regions as presented by yellow bars, and **C.** corresponding sarcomere length profile during cell contraction which electrical stimulation at 0.5 Hz. The frame-rate was 100 frames per second. The pixel size was 0.26 µm. Scale bar = 10 µm.

Figure 3: Mouse PSC-CMs after AAV transduction for 3 days.
A. Schematic vector map of AAV for sarcomere labeling. A sarcomere protein (gene of interest, GOI) is linked to GFP with a Gly-Gly-Gly-Ser linker (L) and expressed under the control of cardiac troponin T (cTNT) promoter. B**.** The timeline for mouse PSC-CM differentiation and AAV transduction. **C-D.** Representative images showing clear sarcomere localization and the corresponding sarcomere length profile of TCAP-GFP (C) and PDLIM3-GFP (D) after 3 days of transduction into PSC-CMs generated from the Myom2-TagRFP cell line. Scale bar = 10 µm.

Figure 4: Blasticidin Purification of human PSC-CMs without knock-in.
A. Schematic vector map of AAV, in which a blasticidin-resistance gene cassette (BSR) is inserted downstream to cTNT promoter. **B.** The timeline of human PSC-CMs differentiation, transduction, and blasticidin selection. **C.** Representative data showing percentage of cTNT + cells in human PSC-CMs (transduced 10^5 vg/ cell AAV6 on day 4 then treated with 2.5 µg/ml blasticidin on days 7 and 9).

Movie 1: Fluorescent time-lapse video of mouse PSC-CMs generated from the Myom2-TagRFP cell line.
RFP signals showed a sarcomere pattern after culturing the PSC-CMs for 28 days. The cells showed beating synchronously when stimulated with electricity at 1 Hz. The time-lapse images were acquired every 20 ms with a 100X lens. Scale bar = 5 µm.

Movie 2: Fluorescent time-lapse video of the human PSC-CMs with ACTN2-mCherry cultured on a non-patterned culture dish.
The PSC-CMs expressing ACTN2-mCherry on a non-patterned culture dish not only showed disorganization of sarcomere, but also presented a waving contraction which is difficult to determine sarcomere shortening. The cells were stimulated with electricity at 0.5 Hz and acquired the images every 10 ms with a 100X lens. Scale bar = 10 µm.

Movie 3: Fluorescent time-lapse video of the human PSC-CMs with ACTN2-mCherry cultured on a patterned culture dish.
The patterned culture promoted the alignment of the sarcomere and forced cells to rod-shape.

This method allowed us to determine the sarcomere shortening of the PSC-CMs easier. This video was obtained by stimulating the cells with electricity at 0.5 Hz. The frame-rate was 100 frames per second. Scale bar = 10 µm.

Supplemental CAD files: CAD files for creating stamps with strips of 200 µm width and grooves of 10 µm (Stamp_200x10.dxf), 25 µm (Stamp_200x25.dxf), and 50 µm (Stamp_200x50.dxf).

DISCUSSION:

PSC-CMs is a material with great potential that can be utilized as an *in vitro* platform to model heart diseases and test the effects of drugs. Nevertheless, before being able to efficiently use them, we must establish an accurate, unified method to assess PSC-CMs functions. Most of the functional tests work with PSC-CMs, *e.g.* electrophysiology, calcium transient, and metabolisms[26]. Therefore, one of the first patient-derived PSC-CM studies was about long-QT syndrome[27]. However, contractility, one of the most important functions as a cardiomyocyte, is still difficult to assess. With adult cardiomyocytes, sarcomere shortening is widely used. In contrast, due to the underdeveloped and disorganized sarcomere of PSC-CMs, the standard method for sarcomere shortening does not work with PSC-CMs. Therefore, we presented here an alternative method to examine sarcomere shortening of PSC-CMs using fluorescent-tagged sarcomere proteins. We demonstrated that the proteins localized to M-line (MYOM2) or Z-line (ACTN2, TCAP, and PDLIM3) fused with fluorescent proteins can be used for this approach. We also showed that fluorescent-tagged proteins can be expressed from endogenous loci or by AAVs. AAVs provide more flexibility to express fluorescent-tagged proteins than endogenous loci, as they can be applied to any type of patient-derived PSC-CMs. In contrast, expressing the proteins from endogenous loci may have lessor alteration to the sarcomere function as the expression level of the genes is tightly regulated, and it can also be used for monitoring the maturation of PSC-CMs[18].

Even though Myom2-RFP, ACTN2-RFP, and Lifeact were all used to examine the sarcomere shortening[16, 18, 19], it is still unclear if they interfere with the sarcomere function. Recently, Lifeact was reported to disturb actin organization and cellular morphology[28]. It is also important to note that fusion patterns (*i.e.* the GFP fusion site at N-term or C-term of target protein) also affect the sarcomere function[29]. Therefore, before being used widely, it is important to extensively assess if these fluorescent-tagged sarcomere proteins do not interfere with sarcomere function or protein-protein interaction in sarcomere *in vitro* and, if possible, *in vivo* or in adult cardiomyocytes. As we provided a repertoire of fluorescent-tagged sarcomere proteins to start with, we may have better options in near future by protein-engineering (*i.e.* shortening the sarcomere proteins to only localization signals). Selecting protein to tag is another key to success. We have tagged another Z-line protein with GFP, however, it only displayed cytoplasmic distribution rather than localization to the sarcomere. For live-imaging, protein stability might also play the role. For example, if a tagged protein is unstable, the signal level will be lower. The photostability of the fluorescent protein is also important as unstable ones will be easily quenched during the imaging.

To examine the contractility of PSC-CMs other than the method described here, indirect measurements of force generated by PSC-CMs (micro-post arrays, traction force microscopy) or motion (high-resolution motion detection using SI8000)[11–14] are used. The current method can

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be combined with such methods or dye-based action potential/calcium transient measurement. The combinatorial approach may provide us further implication on how a disease cause dysfunction in patient-derived PSC-CMs.

One of the challenges in sarcomere shortening in PSC-CMs is to find a good sarcomere that moves linearly, otherwise, it may easily come off from the line for sarcomere detection of SarcOptiM and cause unstable sarcomere shortening results. Here, we demonstrated a patterned culture using PDMS stamps may provide a more stable and linear movement of a sarcomere. A patterned culture is also known to support the maturation of PSC-CMs[16], which is important for sarcomere function.

~~Tools provided by this study can be further enhanced and merged with other maturity assessment tools to meticulously measure PSC-CMs maturity. Moreover, those tools can be applied to patient-derived induced PSC-CMs. Knowing the exact level of maturity generated by currently available PSC-CMs will enable us to upgrade them to produce cells that can be clinically used on a wide scale. Furthermore, laboratory pharmacological assessment of the effects of cardiac drugs such as chronotropic drugs (*e.g.* beta-agonists) will be accurately done if we have tools that can measure even the minute differences in sarcomere shortening.~~

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DISCLOSURES:

H.U. has filed a patent related to this manuscript.

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