Journal of Visualized Experiments
A model of ischemic heart disease and a video-based comparison of cardiomyocyte contraction using hiPSC-dervived cardiomyocytes
--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61104R2
Full Title:	A model of ischemic heart disease and a video-based comparison of cardiomyocyte contraction using hiPSC-dervived cardiomyocytes
Section/Category:	JoVE Medicine
Keywords:	Ischemic heart disease; hypoxia; Myocardial infarction; Human induced pluripotent stem cells; cellular differentiation; Cardiomyocytes
Corresponding Author:	Ken Takahashi, Ph.D. Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences Okayama, Okayama JAPAN
Corresponding Author's Institution:	Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences
Corresponding Author E-Mail:	takah-k2@okayama-u.ac.jp
Order of Authors:	Yun Liu
	Yin Liang
	Mengxue Wang
	Chen Wang
	Heng Wei
	Keiji Naruse
	Ken Takahashi, Ph.D.
Additional Information:	

Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Okayama, Japan

1   **TITLE:**
2   Model of Ischemic Heart Disease and Video-Based Comparison of Cardiomyocyte Contraction
3   Using hiPSC-Derived Cardiomyocytes
4
5   **AUTHORS AND AFFILIATIONS:**
6   Yun Liu[1*], Yin Liang[1*], Mengxue Wang[1*], Chen Wang[1], Heng Wei[2], Keiji Naruse[1], Ken Takahashi[1]
7
8   [1]Department of Cardiovascular Physiology, Graduate School of Medicine, Dentistry and
9   Pharmaceutical Sciences, Okayama University, Okayama, Japan
10   [2]Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan
11   *These authors are equally contributed
12
13   **Email addresses of co-authors:**
14   Yun Liu (pdx9527@163.com)
15   Yin Liang (m13130484071@163.com)
16   Mengxue Wang (maoqiu985@163.com)
17   Chen Wang (wangchen11228@gmail.com)
18   Heng Wei (wei.heng.28m@st.kyoto-u.ac.jp)
19   Keiji Naruse (knaruse@okayama-u.ac.jp)
20
21   **Corresponding author:**
22   Ken Takahashi (takah-k2@okayama-u.ac.jp)
23

24   **KEYWORDS:**
25   Ischemic heart disease; hypoxia; Myocardial infarction; Human induced pluripotent stem cells;
26   cellular differentiation; Cardiomyocytes
27

28   **SUMMARY:**
29   We present a model of ischemic heart disease using cardiomyocytes derived from human
30   induced pluripotent stem cells, together with a method for quantitative evaluation of tissue
31   damage caused by ischemia. This model can provide a useful platform for drug screening and
32   further research on ischemic heart disease.
33

34   **ABSTRACT:**
35   Ischemic heart disease is a significant cause of death worldwide. It has therefore been the subject
36   of a tremendous amount of research, often with small-animal models such as rodents. However,
37   the physiology of the human heart differs significantly from that of the rodent heart,
38   underscoring the need for clinically relevant models to study heart disease. Here, we present a
39   protocol to model ischemic heart disease using cardiomyocytes differentiated from human
40   induced pluripotent stem cells (hiPS-CMs) and to quantify the damage and functional impairment
41   of the ischemic cardiomyocytes. Exposure to 2% oxygen without glucose and serum increases the
42   percentage of injured cells, which is indicated by staining of the nucleus with propidium iodide,
43   and decreases cellular viability. These conditions also decrease the contractility of hiPS-CMs as
44   confirmed by displacement vector field analysis of microscopic video images. This protocol may

45　furthermore provide a convenient method for personalized drug screening by facilitating the use
46　of hiPS cells from individual patients. Therefore, this model of ischemic heart disease, based on
47　iPS-CMs of human origin, can provide a useful platform for drug screening and further research
48　on ischemic heart disease.
49
50　**INTRODUCTION:**
51　Ischemic heart disease (IHD) is recognized worldwide as the leading cause of death, and it was
52　estimated to be responsible for more than nine million fatalities in 2016[1]. The prevalence of
53　cardiovascular disease continues to rise, and globalization seems to have contributed to the
54　prevalence of heart disease risk factors in developing countries. Therefore, the study of IHD is
55　becoming increasingly more urgent[2].
56
57　Experimental models of cardiovascular disease are critical for studying the mechanisms of
58　disease, accuracy of diagnosis, and development of new therapies. Several experimental models
59　have been proposed by many laboratories. It is of utmost importance to choose a model with
60　significant advantages; feasibility, repeatability and similarity to human disease are key factors
61　in the selection of cardiovascular disease models[3]. Human induced pluripotent stem cells (hiPSCs)
62　carrying specific cardiomyopathy-associated mutations are a promising alternative to animal
63　models[4]. Although many strategies have been described for inducing cardiomyocytes using
64　iPSCs[5-9], here we provide a simple method to produce an IHD model using cardiomyocytes
65　differentiated from hiPSCs, in which laborious selection of cardiomyocyte using markers is not
66　applied. In this method, cardiomyocytes are selected functionally by analyzing spontaneous
67　contraction.
68
69　Induction of cardiomyocytes using hiPSCs avoids animal sacrifice and technically difficult surgery.
70　Establishing animal models of IHD requires challenging surgical techniques[10]. Perfectly simulating
71　the various pathophysiological aspects of human heart disease such as heart rate and action
72　potentials from the physiology of rodents is nearly impossible. Coupled with the morality and
73　ethics of using animal models, the development of new experimental models other than animal
74　models is imperative. Cardiomyocytes differentiated from human iPS cells better mimic the
75　physiological state of the human heart. In this protocol, we establish a model of IHD using
76　cardiomyocytes derived from hiPS cells (hiPS-CMs). In our model, the deprivation of oxygen and
77　glucose leads to a decrease in the contractile force and viability of hiPS-CMs. Our method
78　provides a new approach to model IHD and demonstrates a new platform for the study of this
79　disease.
80
81　**PROTOCOL:**
82
83　**1. hiPSC maintenance culture**
84
85　1.1. Coat a six-well culture plate with laminin (**Table of Materials**).
86
87　1.1.1. Dilute laminin to 0.5 μg/mL in phosphate buffered saline (PBS).
88

89   1.1.2. Add diluted laminin to a six-well plate at a volume of 2.0 mL/well.

90

91   1.1.3. Incubate the plate at 37 °C for 30 min.

92

93   1.2. Remove the laminin solution from the wells.

94

95   1.3. Seed hiPSCs in 2 mL of iPSC maintenance medium (**Table of Materials**) onto the coated wells
96   at a density of 3×10^4 cells/well without drying the surface.

97

98   1.4. Subculture the hiPSCs.

99

100   1.4.1. Prepare 0.5× cell dissociation enzymes (**Table of Materials**) solution.

101

102   1.4.1.1. Mix 10 μL of 0.5 M Ethylenediaminetetraacetic (EDTA) and 10 mL of PBS to make 0.5 mM
103   EDTA/PBS.

104

105   1.4.1.2. Dilute 10 mL of 1× cell dissociation enzymes to 0.5× by adding 10 mL of 0.5 mM
106   EDTA/PBS.

107

108   1.4.2. Aspirate the spent medium from the wells.

109

110   1.4.3. Add 2 mL/well of PBS to wash the cells, then discard the PBS.

111

112   1.4.4. Add 800 μL of the 0.5× cell dissociation enzymes, and incubate the cells for 7 min at 37 °C
113   in a humidified incubator containing 5% CO_2.

114

115   NOTE: 0.05% trypsin-EDTA can be used to dissociate cells.

116

117   1.4.5. Gently remove the 0.5× cell dissociation enzymes solution.

118

119   1.4.6. Wash the cells with 2 mL PBS, then discard the PBS.

120

121   NOTE: Be gentle because the cells easily detach after adding cell dissociation enzymes solution.

122

123   1.4.7. Add 1 mL of iPS maintenance medium (**Table of Materials**) containing 10 μM Y-27632.

124

125   NOTE: Adding Y-27632 increases the survival rate of hiPSCs.

126

127   1.4.8. Dislodge cells using a cell scraper, and collect them in a 15 mL centrifuge tube.

128

129   1.4.9. Count the number of cells. Adjust the cell density to 1.5×10^4 cells/mL by adding iPS
130   maintenance medium containing 10 μM Y-27632.

131

132   NOTE: Do not use centrifugation in order to prevent cell damage.

133

134 1.4.10. Seed 2 mL of cell mixture on the laminin-coated six-well plate (final density:
135 3×10^4 cells/well).

136

137 1.4.11. Incubate the cells at 37 °C in a humidified incubator containing 5% CO_2.

138

139 1.4.12. Replace the culture medium with iPS maintenance medium without Y-27632 on days 1,
140 4, 5, and 6.

141

142 1.5. Subculture the cells on day 7.

143

144 NOTE: Subculture the cells by day 7 in order to inhibit random differentiation of hiPSCs.

145

146 **2. Induction of cardiac differentiation of hiPSCs**

147

148 2.1. Coat a 96-well culture plate with laminin (**Table of Materials**).

149

150 2.1.1. Dilute laminin to 1.675 μg/mL with PBS.

151

152 2.1.2. Add the diluted laminin solution to a 96-well plate at a volume of 0.1 mL/well.

153

154 2.1.3. Incubate the plate at 37 °C for 30 min.

155

156 2.2. Seed hiPSCs onto a 96-well plate at a density of 3×10^4 cells/well.

157

158 2.3. Proliferate hiPSCs at 37 °C in a humidified incubator containing 5% CO_2.

159

160 2.3.1. One day later, replace the medium with 200 μL/well iPS growth medium (**Table of**
161 **Materials**).

162

163 2.3.2. Change the medium every day for an additional 2–3 days until the cells reach 70%–80%
164 confluency.

165

166 2.4. Apply differentiation media.

167

168 2.4.1. Aspirate the spent medium and replace it slowly with 200 μL/well of pre-warmed
169 differentiation medium A (**Table of Materials**).

170

171 2.4.2. Place the plate at 37 °C in a humidified incubator containing 5% CO_2.

172

173 2.4.3. After 48 h, aspirate the medium and slowly replace it with 200 μL/well of pre-warmed
174 differentiation medium B (**Table of Materials**).

175

176 2.4.4. Place the plate at 37 °C in a humidified incubator containing 5% CO_2.

177

NOTE: It is **extremely important** that the differentiation media are kept fresh. They will gradually lose their differentiation effect, typically within two weeks. If necessary, aliquot fresh medium and store at −20 °C.

2.5. Apply cardiomyocyte maintenance medium.

2.5.1. After 48 h, aspirate the medium and slowly replace it with 200 µL/well of pre-warmed cardiomyocyte maintenance medium (**Table of Materials**).

2.5.2. Place the plate at 37 °C in a humidified incubator containing 5% CO_2.

2.5.3. Replace the cardiomyocyte differentiation medium every other day up to day 30.

NOTE: It is important that the duration of the application of differentiation media A and B is accurately set to 48 h to ensure the precise expression sequence of genes required for differentiation.

3. Exposure to ischemia

3.1. Deprive the culture medium of nutrients and oxygen.

3.1.1. Prepare Dulbecco's modified Eagle medium (DMEM) without glucose and serum.

3.1.2. Aspirate culture medium from the wells of the 96-well plate containing hiPS-CMs.

3.1.3. Add the nutrient-deprived medium to the wells at a volume of 200 µL/well.

3.2. Place the culture plate in a hypoxic incubator (**Table of Materials**).

3.3. Infuse nitrogen gas, and maintain the internal oxygen concentration at 2% and CO_2 concentration at 5% for 24 h.

3.4. Proceed to desired analyses: *e.g.*, viability assay, assessment of contractility, or assessment of cellular damage.

4. Assessment of cellular viability using MTT assay

4.1. Use the MTT assay kit (**Table of Materials**) to quantitatively assess the viability of cells.

NOTE: Exposure to 1 mM hydrogen peroxide in DMEM for 1 h can be used to damage cells (positive control). Exposure to 0 mM hydrogen peroxide in DMEM can be used for the negative control.

221   4.1.1. Add 10 µL of MTT reagent to the cells using a repeating pipettor.

222

223   4.1.2. Mix gently for one minute on an orbital shaker.

224

225   4.1.3. Incubate the cells for 3–4 h at 37 °C in a 5% CO_2 incubator. After incubation, the formazan
226   produced in the cells will appear as dark crystals in the bottom of the wells.

227

228   4.1.4. After removing the supernatant, dissolve the insoluble formazan crystals in 100 µL of
229   dimethyl sulfoxide solution (DMSO). This solution will dissolve the formazan crystals, producing
230   a purple solution.

231

232   CAUTION: DMSO can irritate eyes, respiratory system, and skin. Wear suitable gloves and
233   eye/face protection.

234

235   4.1.5. Measure the absorbance of each sample with a microplate reader at a wavelength of
236   570 nm.

237

238   **5. Assessment of contractility of iPS-CMs**

239

240   5.1. If not installed, obtain Particle Image Velocimetry ImageJ plugin[11] at
241   https://sites.google.com/site/qingzongtseng/piv and install.

242

243   5.2. Using a phase contrast microscope, record the video images of hiPS-CMs using the 4×
244   objective lens at ~20 frames per second for ~10 s, and save as "analyze.avi". For a comparison
245   of contractility between before and after ischemia, confirm that the location of interest is
246   recorded.

247

248   NOTE: A microscope with an automated stage is useful to target the location of interest. Movie
249   file should be in .avi format for later analysis. If not, convert the movie to .avi.

250

251   5.3. Create folder structure as indicated in **Figure 1**. An example of "joblist.txt" is indicated in
252   supplementary file.

253

254   5.4. Analyze discrete two-dimensional vector fields of cellular displacement.

255

256   5.4.1. Launch Fiji (ImageJ) software[12], go to **Plugins > Macros > Edit** and open
257   "vector_analysis.ijm" (Supplementary Coding File).

258

259   5.4.2. Click **Run**. Analysis will be performed automatically.

260

261   NOTE: Displacement vectors D(x,y) are calculated for every 16 × 16 pixels between the reference
262   frame (the first frame) and all subsequent frames (frames 1 vs. 2, 1 vs. 3, 1 vs. 4, etc.). The result

263 is calculated for every frame and saved as "vec_x.txt" (x is a frame number). A vector of maximum
264 displacement, M(x, y), is defined for every (x, y) pair as follows:

265

266 $$M(x, y) = D_k(x, y)$$

267

268 where k represents the frame number at which $|D_k(x,y)|$ = max $[|D_2(x,y)|, |D_3(x,y)|, ..., |D_n$
269 $(x,y)|]$ and n denotes the last frame. The result is saved as "Max_vector.txt". $|M(x, y)|$ represents
270 the maximum displacement caused by cardiomyocyte contraction at the analysis point (x, y).
271 Contractility value C in arbitrary units is calculated as follows:

272

273 $$C = \sum_{xy} |M(x,y)|$$

274

275 This value is saved at column 7, row 1 in the "Max_vector.txt". C represents the sum of
276 displacement for all (x, y). The larger the portion where the displacement due to myocardial
277 contraction is large, the larger the value of C. The vector field of maximum displacement, M(x, y),
278 is overlaid with phase contrast image of the first frame ("Phase_contrast.png") and saved as
279 "Overlaid.png". As the magnitude of the displacement vector is calculated based on the first
280 frame of the video, it is preferable for the hiPS-CMs to be at resting diastolic period for the first
281 frame.

282

283 **6. Assessment of cellular damage using flow cytometry**

284

285 6.1. Dilute a 1 mg/mL solution of propidium iodide to 1:1,000 in PBS.

286

287 6.2. Stain the detached cells with propidium iodide.

288

289 6.2.1. Aspirate the medium and place it in an appropriately sized centrifuge tube.

290

291 6.2.2. Centrifuge the tube at 1,000 × g for 5 min, and carefully remove the supernatant so as not
292 to lose sedimented cells.

293

294 6.2.3. Incubate the cells with the diluted propidium iodide solution at room temperature for 15
295 min in the dark.

296

297 6.2.4. Centrifuge the tube at 1,000 × g for 5 min, and carefully remove the supernatant so as not
298 to lose sedimented cells.

299

300 6.2.5. Reconstitute the cells in ~1 mL of PBS.

301

302 NOTE: It is important to collect detached floating cells from the medium to accurately quantify
303 cellular damage.

304

305   6.3. Stain attached cells with propidium iodide.

307   6.3.1. Wash attached cells twice with PBS gently, then discard PBS.

309   6.3.2. Incubate the cells with the diluted propidium iodide solution for 15 min in the dark.

311   6.3.3. Aspirate the propidium iodide solution.

313   6.3.4. Detach the cells using 0.25% trypsin. Move the cell solution into a centrifuge tube.

315   6.3.5. Centrifuge the tube at 1,000 × g for 5 min, and carefully remove the supernatant so as not
316   to lose sedimented cells.

318   6.3.6. Reconstitute the cells in ~1 mL of PBS.

320   6.4. Mix the floating and attached cells for fluorescence activated cell sorting (FACS) analysis.

322   6.5. Pass the cell solution through a 30 μm filter.

324   NOTE: Passing the cells to a filter is very important for an accurate FACS measurement.

326   6.4. Analyze the samples using a FACS system.

328   **7. Immunostaining**

330   7.1. Fix the cell sample.

332   7.1.1. Aspirate the culture medium.

334   7.1.2. Add 4% paraformaldehyde in PBS for 10 min at room temperature.

336   7.1.3. Wash the cells three times with PBS.

338   NOTE: Fresh paraformaldehyde solution is recommended for optimal fixation.

340   7.2. Permeabilize the cells with 0.2% Triton X-100 for 15 min, then discard the reagent.

342   7.3. Add 3% bovine serum albumin to block the cells for 30 min.

344   7.4. Apply primary antibody.

346   7.4.1. Discard bovine serum albumin solution from the culture plate.

348   7.4.2. Incubate the cells with primary antibodies overnight at 4 °C.

349

350   7.4.3. Remove the antibody solution.

351

352   7.4.4. Wash the cells three times with PBS.

353

354   7.5. Apply secondary antibody.

355

356   7.5.1. Discard PBS solution from the culture plate.

357

358   7.5.2. Incubate the cells with secondary antibodies for 30 min at room temperature in the dark.

359

360   NOTE: Primary anti-cardiac troponin T (TNNT2) mouse monoclonal antibody is used at a dilution
361   of 1:750 in 3% BSA. The Alexa Fluor 488-conjugated secondary goat anti-mouse antibody is
362   diluted to 1:1,000 in 3% BSA.

363

364   7.6. Additional staining of nucleus and actin fibers.

365

366   7.6.1. Remove the antibody solution.

367

368   7.6.2. Incubate the cells in nucleus staining reagent (**Table of Materials**) and actin staining
369   reagent (**Table of Materials**) in PBS for 30 min at room temperature in the dark.

370

371   NOTE: 4',6-diamidino-2-phenylindole (DAPI) or Hoechst 33342 can be used for nuclear staining.

372

373   7.6.3. Wash the cells three times with PBS.

374

375   7.7. Capture fluorescence images using a fluorescence microscope.

376

377   **REPRESENTATIVE RESULTS:**

378   Successfully differentiated cells show spontaneous contraction under the microscope (**Video 1**).
379   Typically, 50% of wells show spontaneous contraction in <20 days (**Supplementary Figure 1**).
380   Cardiac marker protein (e.g., cTnT) can be used to confirm successful differentiation (**Figure 2**).

381

382   Typically, cells from the ischemic group show lower viability in MTT assays (**Figure 3A**) and
383   contractility (**Figures 3E,F**, **Supplementary Figure 2**) than those from normoxic control group.
384   Also, the ratio of propidium iodide-positive cells is higher in the ischemic group than in the control
385   group (**Figures 3B–D**), which indicates higher cellular damage.

386

387   **FIGURE LEGENDS:**

388

389   **Figure 1: Folder structure for displacement vector analysis using imageJ.** "joblist.txt" describes
390   path to movie files each line. If there are three files to analyze, there will be three folders (movie1,
391   movie2, and movie3), in which "analyze.avi" (the movie of interest) should be placed. After the

392 analysis is performed by "vector_analysis.ijm" code, files will be generated (indicated in blue) in
393 each movie folder. Information stored in each file is explained in detail in the main text.

394

395 **Figure 2: Representative image of cardiac marker staining.** Expression of the cardiac marker
396 protein cardiac troponin T (cTnT). Blue: 4',6-diamidino-2-phenylindole (DAPI), red: actin, green:
397 cTnT. Inset: striated expression of cTnT, which corresponds to the sarcomere structure. Scale bar,
398 50 μm. This figure has been modified from Wei et al.[13]

399

400 **Figure 3: Representative images of cellular viability assay, assessment of cellular damage, and**
401 **contractility.** (**A**) Comparison of cellular viability (MTT assay). Higher absorbance indicates higher
402 viability. $n = 5$ for each condition. (**B**, **C**, and **D**) Flow cytometry analysis of propidium iodide (PI)-
403 stained cells. Ischemic cells exhibit a reduced forward scatter intensity and increased PI
404 fluorescence, compared with the control. (**D**) Percentage of PI-stained cells in the flow cytometry
405 analysis. $n = 3$ for each condition. (**E**) Analysis of iPS-CMs contractility using ImageJ software. The
406 red and blue vectors indicate the largest and smallest contractions, respectively. Scale bar, 100
407 μm. (**F**) Quantitative analysis of the iPS-CM contractility using code. $n = 3$ for control and $n = 8$ for
408 ischemic condition. Error bars represent standard error of the mean. For the statistical analysis,
409 unpaired two-tailed Student's t-test was performed. *: $p < 0.05$, **: $p < 0.01$. ***: $p < 0.0001$.
410 This figure is cited from Wei et al.[13]

411

412 **Video 1: Differentiated cells show spontaneous contraction under the microscope.**

413

414 **Supplementary Figure 1: Kaplan-Meier analysis of percentage of samples without spontaneous**
415 **contraction**. 50% of iPS-CM samples showed contraction on day 20. On day 30, 64.4% of the
416 samples showed contraction. $n = 83$.

417

418 **Supplementary Figure 2: Assessment of cardiac contractility**. Scaled contractility obtained by
419 dividing contractility C by the number of analysis points (x, y) was plotted for control and ischemic
420 groups before and after the exposure to 24 h hypoxia. $n = 3$ for control and $n = 8$ for ischemic
421 condition.

422

423 **Supplementary Figure 3: Assessment of the content of cardiomyocytes**. Immunostaining of
424 cardiac marker protein on a 96 well plate. Green: cTnT, Blue: DAPI. Rate of the differentiated cells
425 was calculated to be 20.7 ± 9.6% by dividing the area of cTnT-stained region by that of DAPI-
426 stained region. Scale bar = 1 mm. $n = 4$ for control condition.

427

428 **DISCUSSION:**
429 Researchers often use laboratory small-animal models to conduct IHD experiments. Here, we
430 developed a cell culture model of human IHD to conduct such experiments.

431

432 The main problem a user of this protocol may face is low rate of differentiated cardiomyocytes.
433 Several steps should be taken with great care to improve the rate of successful differentiation:
434 (a) be gentle when pipetting because the cells easily detach after addition of the cell dissociation
435 enzymes reagent, (b) adding Y-27632 increases the survival rate of iPS cells when detaching, and

436 (c) the timing of medium changes at the beginning of differentiation should be precisely 48 h
437 apart to ensure controlled expression of genes involved in differentiation.

439 Regarding the extracellular matrix proteins used for coating the surface of culture plate, other
440 materials than laminin we used in this protocol can be used. For example, Matrigel[14,15], and
441 gelatin[16,17] are used for the feeder-free maintenance culture of hiPSCs. According to Haraguchi
442 et al., hiPSCs seeded on Matrigel were successfully differentiated into cardiac cell sheet[18].

444 There are a number of preceding studies regarding culture models for disease modeling using
445 cardiomyocytes derived from hiPSCs. Regarding the modeling of ischemia-reperfusion injury,
446 manipulations of the cellular environment, such as hyperkalemia, acidosis, and lactate
447 accumulation, have been introduced[19]. Other methods include cell pelleting to hinder oxygen
448 diffusion[20] and metabolic inhibition using cyanide[21]. In the current protocol, cell injury was
449 achieved by relatively simple method, namely 24 h deprivation of oxygen and nutrients. However,
450 care should be taken to consider accurate pathophysiological processes of the ischemic heart
451 disease, as there are indeed differences between the disease in vivo and the disease model of
452 the current protocol, such as the presence or absence of three-dimensional cellular environment
453 and blood.

455 Regarding the technological aspect of the evaluation of cardiomyocyte function, Toepfer et al.
456 reported a MatLab-based algorithm to determine sarcomere contraction and relaxation in hiPS-
457 CMs[22]. Smith et al. reported an advanced method of high-throughput electrophysiological
458 analysis of excitable cells derived from hiPS-CMs, using sophisticated nanotopographically
459 patterned multielectrode arrays[23,24]. The advantage of our protocol is that it only requires
460 conventional software and consumables, such as imageJ software and 96-well plates.

462 With respect to the oxygen level in the heart, the oxygen pressure in veins that reach the heart
463 is thought to be 40 mmHg[25]. According to McDougal et al., the extracellular oxygen pressure
464 under hypoxia is estimated to be <12.8 mmHg[26]. By applying the method of Rounds et al.[27], the
465 oxygen pressure in the culture medium (salinity: 35‰) under the hypoxic condition (2% oxygen)
466 at 37 °C treated with the current protocol is calculated to be 14.9 mmHg, which is higher than
467 the estimation above. Interestingly, Al-Ani et al. reported that there is a gradient of oxygen
468 pressure in the culture medium, and the oxygen pressure is affected by cell type, seeding density,
469 and medium volume[28]. Typically, the oxygen concentration at the bottom of the culture plate
470 where cells reside is the lowest. Therefore, the effect of depth in the culture medium would
471 further reduce the effective oxygen pressure near hiPS-CMs. In order to induce sufficient damage
472 to hiPS-CMs using hypoxic condition, careful attention must be paid to the depth of medium and
473 the density of cells.

475 Our hiPS-CM model, which is very close to the physiological conditions of human cardiac
476 myocytes, advantageously mimics human IHD. Animal model-based approaches include ethical,
477 technical, and academic issues. Particularly, in vivo models require an advanced technique of
478 microsurgery to achieve reproducible data: e.g., occlusion of the anterior descending branch of
479 the left coronary artery in rodents[3]. The hiPS-CM model described herein overcomes these

480 critical barriers and provides a useful, relevant, and repeatable platform for cardiovascular
481 disease.
482
483 However, some limitations should be noted. An obvious difference between iPS-induced
484 cardiomyocytes and normal cardiomyocytes is the absence of T-tubules[29], and we did not include
485 humoral factors such as tissue damage induced by leukocytes and activation of a complement
486 system in our study. Moreover, the rate of differentiated cardiomyocytes in this model (20.7 ±
487 9.6%, **Supplementary Figure 3**) should be improved. The recent publication by Halloin et al.
488 describes a scalable, chemically defined method to induce hiPS-CM purity of >95% by chemical
489 WNT pathway modulators without requirement of any selection by markers[30]. Nevertheless, our
490 model of human IHD is relatively simple and clinically applicable (e.g., drug screening using
491 patient-derived iPS cells). Our model is also a unique platform to further elucidate mechanisms
492 underlying IHDs.
493

501

502 **REFERENCES:**
503 1   Naghavi, M., Global Burden of Disease Self-Harm, C. Global, regional, and national burden
504   of suicide mortality 1990 to 2016: systematic analysis for the Global Burden of Disease
505   Study 2016. *BMJ.* **364** l94, (2019).
506 2   Nowbar, A. N., Gitto, M., Howard, J. P., Francis, D. P., Al-Lamee, R. Mortality From
507   Ischemic Heart Disease. *Circulation: Cardiovascular Quality and Outcomes.* **12** (6),
508   e005375, (2019).
509 3   Oh, J. G., Ishikawa, K. Experimental Models of Cardiovascular Diseases: Overview.
510   *Methods in Molecular Biology.* **1816** 3-14, (2018).
511 4   Brodehl, A. et al. Human Induced Pluripotent Stem-Cell-Derived Cardiomyocytes as
512   Models for Genetic Cardiomyopathies. *International Journal of Molecular Sciences.* **20**
513   (18), (2019).
514 5   Garbern, J. C. et al. Inhibition of mTOR Signaling Enhances Maturation of Cardiomyocytes
515   Derived from Human Induced Pluripotent Stem Cells via p53-Induced Quiescence.
516   *Circulation.* 10.1161/CIRCULATIONAHA.119.044205, (2019).
517 6   Horikoshi, Y. et al. Fatty Acid-Treated Induced Pluripotent Stem Cell-Derived Human
518   Cardiomyocytes Exhibit Adult Cardiomyocyte-Like Energy Metabolism Phenotypes. *Cells.*
519   **8** (9), (2019).
520 7   Hu, D. et al. Metabolic Maturation of Human Pluripotent Stem Cell-Derived
521   Cardiomyocytes by Inhibition of HIF1alpha and LDHA. *Circulation Research.* **123** (9), 1066-
522   1079, (2018).
523 8   Correia, C. et al. Distinct carbon sources affect structural and functional maturation of

cardiomyocytes derived from human pluripotent stem cells. *Scientific Reports.* **7** (1), 8590, (2017).

9   Yang, X. et al. Tri-iodo-l-thyronine promotes the maturation of human cardiomyocytes-derived from induced pluripotent stem cells. *Journal of Molecular and Cellular Cardiology.* **72** 296-304, (2014).

10   Tamargo, J. et al. Genetically engineered mice as a model for studying cardiac arrhythmias. *Frontiers in Bioscience.* **12** 22-38, (2007).

11   Tseng, Q. et al. Spatial organization of the extracellular matrix regulates cell-cell junction positioning. *Proceedings of the National Academy of Sciences of the United States of America.* **109** (5), 1506-1511, (2012).

12   Schneider, C. A., Rasband, W. S., Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods.* **9** (7), 671-675, (2012).

13   Wei, H., Wang, C., Guo, R., Takahashi, K., Naruse, K. Development of a model of ischemic heart disease using cardiomyocytes differentiated from human induced pluripotent stem cells. *Biochemical and Biophysical Research Communications.* **520** (3), 600-605, (2019).

14   Ghaedi, M., Niklason, L. E. Human Pluripotent Stem Cells (iPSC) Generation, Culture, and Differentiation to Lung Progenitor Cells. *Methods in Molecular Biology.* **1576** 55-92, (2019).

15   Hou, J. et al. Retaining mTeSR1 Medium during Hepatic Differentiation Facilitates Hepatocyte-Like Cell Survival by Decreasing Apoptosis. *Cellular Physiology and Biochemistry.* **51** (4), 1533-1543, (2018).

16   Yoshida, K. et al. Differentiation of mouse iPS cells into ameloblast-like cells in cultures using medium conditioned by epithelial cell rests of Malassez and gelatin-coated dishes. *Medical Molecular Morphology.* **48** (3), 138-145, (2015).

17   Guzzo, R. M., Drissi, H. Differentiation of Human Induced Pluripotent Stem Cells to Chondrocytes. *Methods in Molecular Biology.* **1340** 79-95, (2015).

18   Haraguchi, Y., Matsuura, K., Shimizu, T., Yamato, M., Okano, T. Simple suspension culture system of human iPS cells maintaining their pluripotency for cardiac cell sheet engineering. *Journal of Tissue Engineering and Regenerative Medicine.* **9** (12), 1363-1375, (2015).

19   Chen, T., Vunjak-Novakovic, G. In vitro Models of Ischemia-Reperfusion Injury. *Regenerative Engineering and Translational Medicine.* **4** (3), 142-153, (2018).

20   Strijdom, H., Genade, S., Lochner, A. Nitric Oxide synthase (NOS) does not contribute to simulated ischaemic preconditioning in an isolated rat cardiomyocyte model. *Cardiovascular Drugs and Therapy.* **18** (2), 99-112, (2004).

21   Cavalheiro, R. A. et al. Potent cardioprotective effect of the 4-anilinoquinazoline derivative PD153035: involvement of mitochondrial K(ATP) channel activation. *PLoS One.* **5** (5), e10666, (2010).

22   Toepfer, C. N. et al. SarcTrack. *Circulation Research.* **124** (8), 1172-1183, (2019).

23   Smith, A. S. T. et al. NanoMEA: A Tool for High-Throughput, Electrophysiological Phenotyping of Patterned Excitable Cells. *Nano Letters.* 10.1021/acs.nanolett.9b04152, (2019).

24   Smith, A. S., Macadangdang, J., Leung, W., Laflamme, M. A., Kim, D. H. Human iPSC-derived cardiomyocytes and tissue engineering strategies for disease modeling and drug

568   screening. *Biotechnology Advances.* **35** (1), 77-94, (2017).

569   25   Sitkovsky, M., Lukashev, D. Regulation of immune cells by local-tissue oxygen tension:
570   HIF1 alpha and adenosine receptors. *Nature Reviews Immunology.* **5** (9), 712-721, (2005).

571   26   McDougal, A. D., Dewey, C. F., Jr. Modeling oxygen requirements in ischemic
572   cardiomyocytes. *Journal of Biological Chemistry.* **292** (28), 11760-11776, (2017).

573   27   Rounds, S. A., Wilde, F. D., Ritz, G. F. Chapter A6. Section 6.2. Dissolved oxygen. Report
574   No. 09-A6.2, (Reston, VA, 2006).

575   28   Al-Ani, A. et al. Oxygenation in cell culture: Critical parameters for reproducibility are
576   routinely not reported. *PLoS One.* **13** (10), e0204269, (2018).

577   29   Cadet, J. S., Kamp, T. J. A Recipe for T-Tubules in Human iPS Cell-Derived Cardiomyocytes.
578   *Circulation Research.* **121** (12), 1294-1295, (2017).

579   30   Halloin, C. et al. Continuous WNT Control Enables Advanced hPSC Cardiac Processing and
580   Prognostic Surface Marker Identification in Chemically Defined Suspension Culture. *Stem*
581   *Cell Reports.* **13** (2), 366-379, (2019).

582

Figure 1

```
[input]
 vector analysis – movie_temp ┬ joblist.txt
                              ├ movie1 – analyze.avi
                              ├ movie2 – analyze.avi
                              ├ movie3 – analyze.avi
                                       …


[output]
 vector analysis – movie_temp ┬ joblist.txt
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                              │        ├ vec_1.txt
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                              │           …
                              │        ├ Max_vector.txt
                              │        └ Overlaid.png
                              ├ movie2 ┬ analyze.avi
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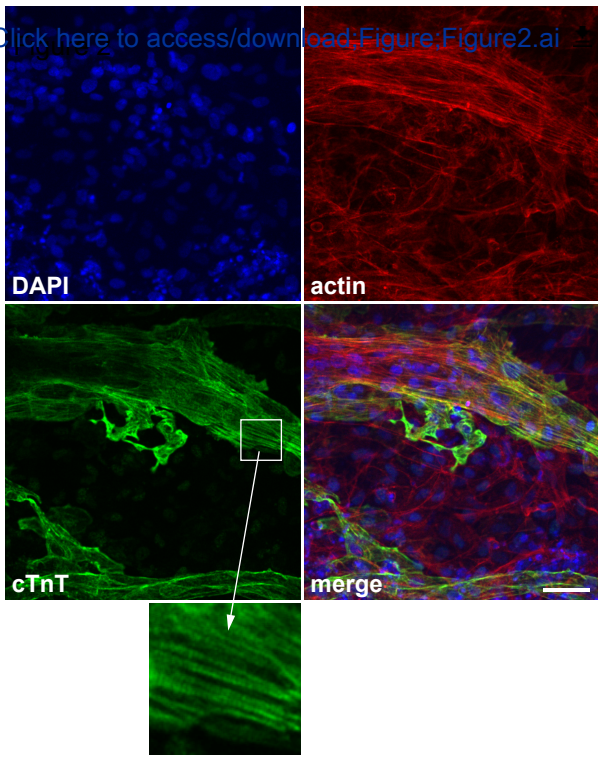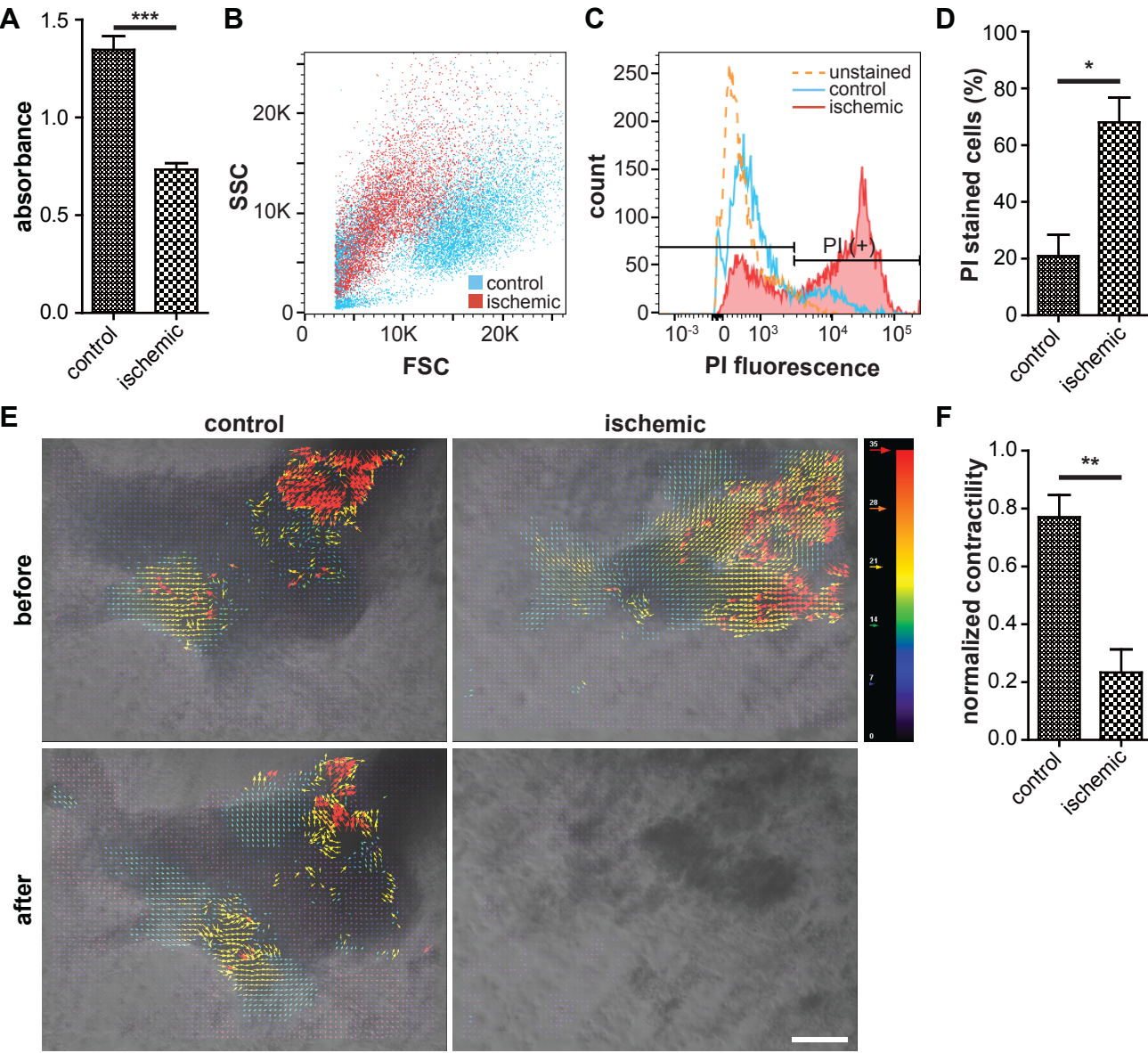
Figure 3 (revised)

Video

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12.5 2-2 before.wmv

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Actin staining reagent (ActinRed 555 ReadyProbes Reagent)	Thermo Fisher Scientific, Waltham, MA, USA	R37112
Anti cardiac troponin T antibody	Thermo Fisher Scientific, Waltham, MA, USA	MA5-12960
Cardiomyocyte maintenance medium (PSC Cardiomyocyte Differentiation Kit)	Thermo Fisher Scientific, Waltham, MA, USA	A2921201
Cell dissociation enzymes (TrypLE Select)	Thermo Fisher Scientific, Waltham, MA, USA	12563011
Differentiation medium A (PSC Cardiomyocyte Differentiation Kit)	Thermo Fisher Scientific, Waltham, MA, USA	A2921201
Differentiation medium B (PSC Cardiomyocyte Differentiation Kit)	Thermo Fisher Scientific, Waltham, MA, USA	A2921201
FACS Aria III system	BD Biosciences, San Jose, CA, USA	
Fluorescenct microscope	KEYENCE, Osaka, Japan	BZ-X710
Goat anti-mouse Alexa Fluor 488 secondary antibody	Thermo Fisher Scientific, Waltham, MA, USA	A28175
Human iPS cells	RIKEN, Tsukuba, Japan	201B7
Hypoxic incubator	SANYO, Osaka, Japan	MCO-175M
iPSC growth medium (Essential 8 Medium)	Thermo Fisher Scientific, Waltham, MA, USA	A1517001
iPSC maintenance medium (StemFit AK02N)	Ajinomoto, Tokyo, Japan	RCAK02N
Laminin (iMatrix-511)	Nippi, Tokyo, Japan	iMatrix-511
MTT assay kit (MTT Cell Proliferation Assay Kit)	Cayman Chemical Company, Ann Arbor, MI, USA	10009365
Nucleus staining reagent (NucBlue Fixed Cell ReadyProbes Reagent)	Thermo Fisher Scientific, Waltham, MA, USA	R37606
Triton X-100	Nacalai Tesque, Kyoto, Japan	35501-02

Comments/Description

March 26, 2020
Dr. Xiaoyan Cao
Review Editor
Journal of Visualized Experiments

Dear Editor,

Please find enclosed our revised manuscript titled "A model of ischemic heart disease and a video-based comparison of cardiomyocyte contraction using hiPSC-dervived cardiomyocytes," which we request you to consider for publication in *Journal of Visualized Experiments*.

We wish to thank reviewers for their relentless effort and attention to details to improve our manuscript. We believe most of the demands regarding the manuscript were met. We attach the point-by-point response to the reviewers' comments below.

We believe that the protocol described here is relevant to the scope of your journal and will be of interest to its readership. We look forward to hearing from you at your earliest convenience.

Sincerely,
Ken Takahashi, Ph.D.

Department of Cardiovascular Physiology, Graduate School of Medicine Dentistry and Pharmaceutical Sciences, Okayama University
2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
Email Address: takah-k2@okayama-u.ac.jp

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thank you for your suggestion. We proofread the manuscript.

2. 1.3: Please specify the culture media.

We specified the culture medium.

3. 1.4.13: The section title of 1.4 is "Subculture the hiPSCs". It is more logical if you change to "1.5. Subculture the cells on day 7".

The manuscript was revised as suggested.

4. 6.2.3: Please specify the incubation temperature.

Thank you for your suggestion. Temperature was described.

5. In the Figure and Table Legends section, please provide a title for the video (12.5 2-2 before.wmv).

We provided a title for the video as suggested.

Reviewers' comments:
Reviewer #4:
Major Concerns:
− The recent publication by Halloin et al. (Stem Cell Reports 2019; doi: 10.1016/j.stemcr.2019.09.001.) describes a scalable, chemically defined method to induce hPSC-CM purity of >95% by chemical WNT pathway modulators without requirement of any selection by markers. ROR1 was not used for CMs selection in that paper but to monitor efficiency of early cardiomyocyte induction. Since the paper describes one of the most recent and most efficient technologies to derive hPSC-CMs for experimental use, it must be cited in the current manuscript before publication is considered.

Thank you for your suggestion. We cited the publication by Halloin et al.

− Moreover, even if authors have not selected or enriched CMs in their method, they must still provide clear data and information on the CMs content achieved in their differentiation and subsequently used in their experiments. Again, in the paper by Halloin et al., authors will find methods how to quantitatively assess the CMs content in their culture e.g. by flow cytometry. Pls perform respective experiments and show the data; thank you.

We added an image-based analysis on the CM content in Supplementary Figure 3. The ratio of cardiomyocytes to total cells was calculated by dividing the area of cTnT-stained region by that of DAPI-stained region. The result was described in the limitation section.

Supplementary Figure 1
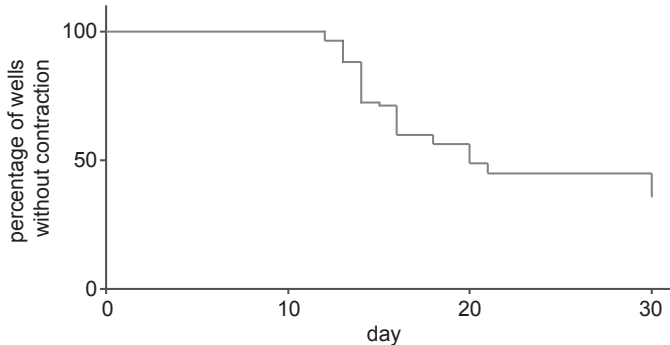
Supplementary Figure 2
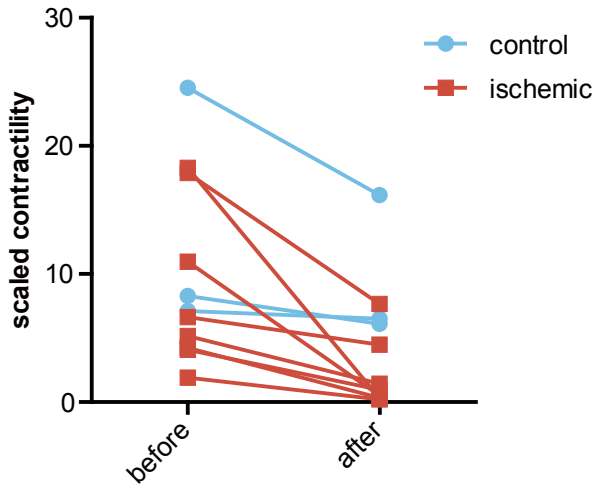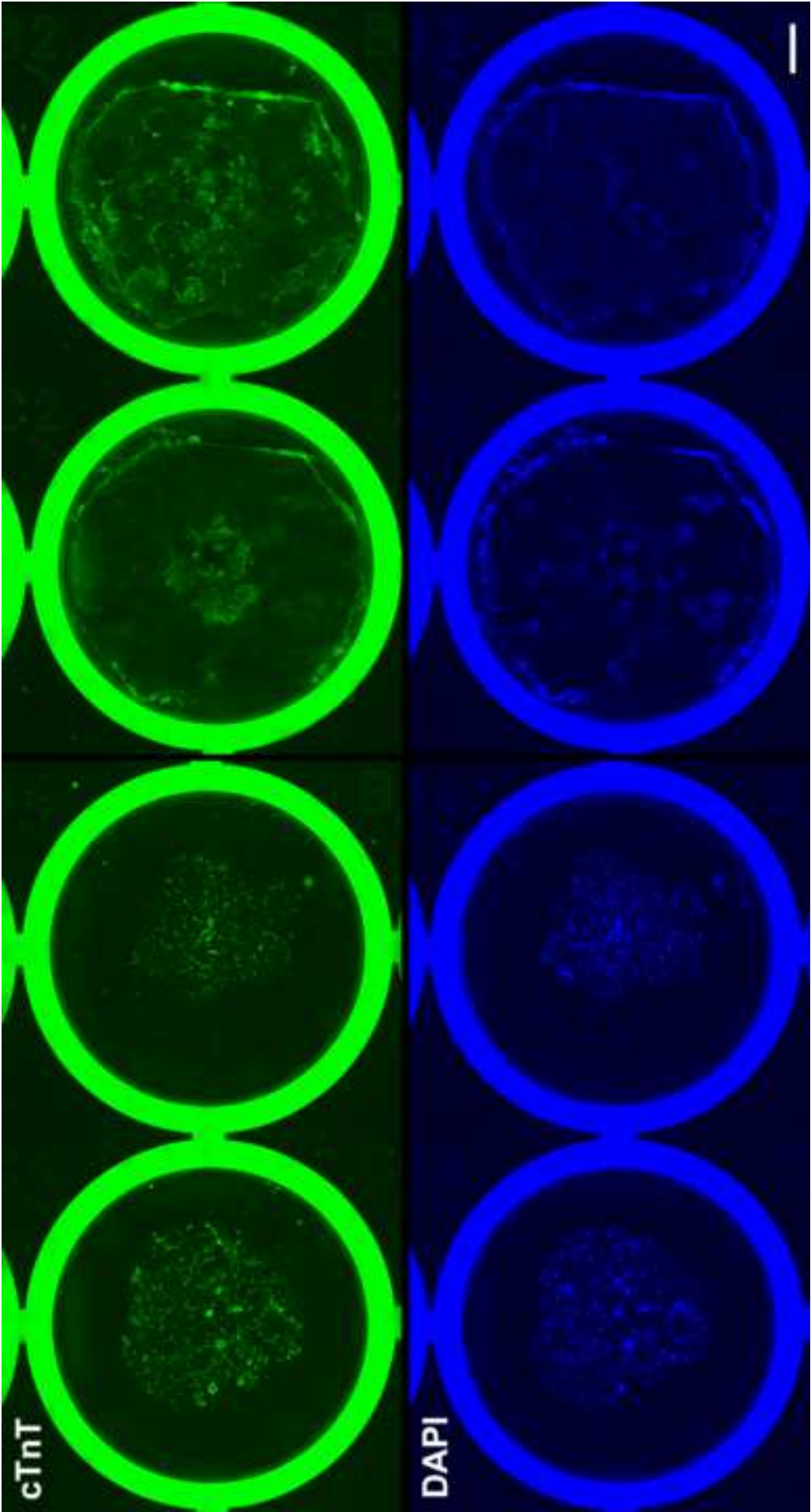
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