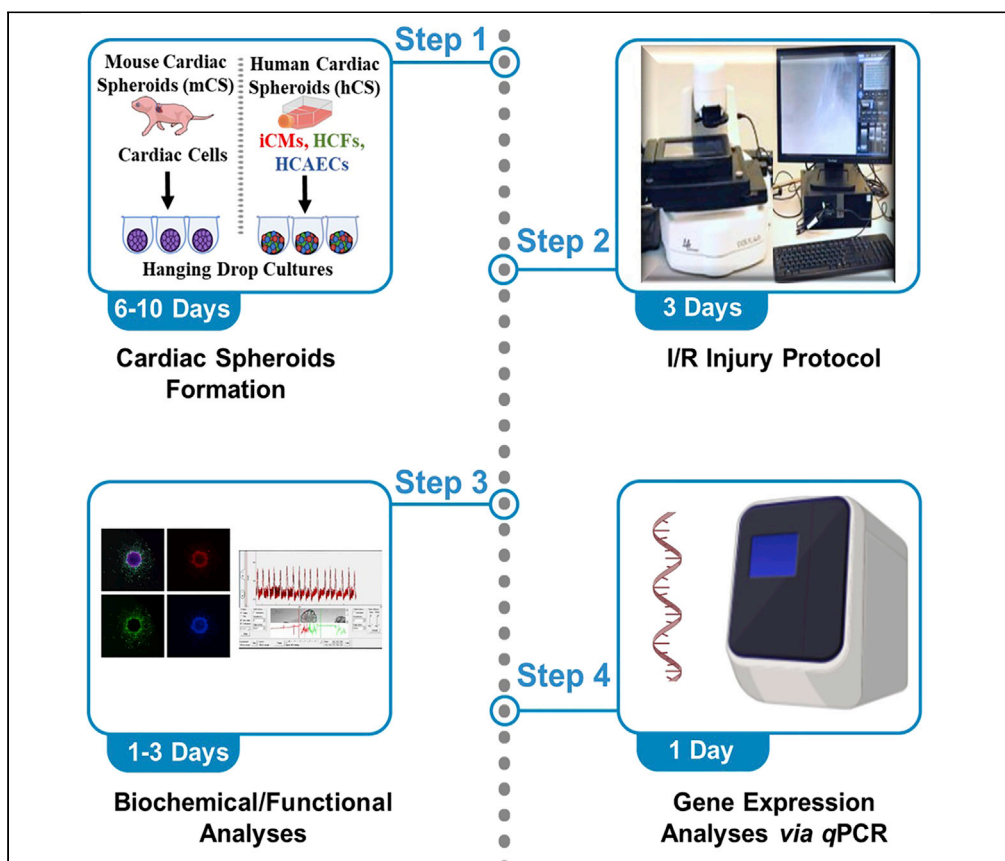


Protocol

In vitro modeling of myocardial ischemia/reperfusion injury with murine or human 3D cardiac spheroids



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Highlights

This protocol describes a clinically relevant *in vitro* myocardial infarction model

I/R injury is modeled by changing pathophysiological oxygen concentrations

I/R cardiac spheroids (CSs) can be generated by either murine or human cells

I/R CSs can be used to measure viability, contractility, and gene expression changes

Myocardial infarction (MI) is the primary cause of death worldwide, but there are no clinically relevant models to study MI. Here, we describe an ischemia/reperfusion (I/R) injury model typical of MI using mouse or human 3D *in vitro* cardiac spheroids (CSs). First, we demonstrated the culture and maintenance of CSs. Then, we detailed how to expose CSs to pathophysiological oxygen concentrations to induce I/R injury. The protocol can be used in combination with viability, contractility, and mRNA expression level measurements.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

In vitro modeling of myocardial ischemia/reperfusion injury with murine or human 3D cardiac spheroidsPoonam Sharma,^{1,2,3,4,5,*} Clara Liu Chung Ming,⁴ and Carmine Gentile^{2,3,4,6,*}¹The University of Newcastle, Newcastle, NSW 2308, Australia²University of Sydney, Sydney, NSW 2000, Australia³Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney, NSW 2065, Australia⁴University of Technology Sydney, Sydney, NSW 2007, Australia⁵Technical contact⁶Lead contact*Correspondence: p.sharma@uon.edu.au (P.S.), carmine.gentile@uts.edu.au (C.G.)
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SUMMARY

Myocardial infarction (MI) is the primary cause of death worldwide, but there are no clinically relevant models to study MI. Here, we describe an ischemia/reperfusion (I/R) injury model typical of MI using mouse or human 3D *in vitro* cardiac spheroids (CSs). First, we demonstrated the culture and maintenance of CSs. Then, we detailed how to expose CSs to pathophysiological oxygen concentrations to induce I/R injury. The protocol can be used in combination with viability, contractility, and mRNA expression level measurements. For complete details on the use and execution of this protocol, please refer to Sharma et al. (2022).

BEFORE YOU BEGIN

- All kit reagents must be kept on ice during the procedures, thawed, and used in dark. Refer to [key resources table](#) for a complete list of materials and equipment.
- Prepare and prewarm culture media at 37°C for a minimum of 30 min prior to the beginning of each respective section of this protocol.
 - Human Cardiac Spheroid Medium (HCS) - A mixture of iCells (Human induced pluripotent stem cells derived cardiomyocyte) Maintenance Medium, Human Cardiac Fibroblasts Growth (HCFG) Medium, and Human MesoEndo Cell Growth (HMECG) Medium at 2:1:1 proportion, respectively.
 - Complete Dulbecco's Modified Eagle Medium (DMEM) – DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (P/S), and 1% L-Glutamine.
 - iCells Plating Media.
 - iCells Maintenance Media (Thaw a day before use at 4°C).
 - Human Cardiac Fibroblasts Growth Medium.
 - Human MesoEndo Cell Growth Medium.
 - Phosphate Buffered Saline (10×, pH-7.4, Ca/Mg Free).
- For plating iCMs, pre-coat two T25 culture flasks with 2 mL of PBS containing 40 µg/mL of fibronectin and incubate at 37°C, 5% CO₂ for at least 4 h.
- Stock Solutions
 - LIVE/DEAD Viability/Cytotoxicity Kit: Ready to use kit including calcein-AM and ethidium homodimer.
 - Image-iT Hypoxia Reagent: Prepare 1 mM stock solution of Image-iT using DMSO and make a final concentration of 10 µM.



Institutional permissions

Mouse CSs were generated under our approved protocol number RESP_17/55 from the Animal Ethics Committee at the Northern Sydney Local Health District St Leonards, NSW, Australia. Ethics approval for the use of neonatal mice to generate mouse CSs is essential. Human CSs are prepared from commercially available cells and do not need ethics approval.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Donkey Anti-mouse Secondary Antibody Alexa Fluor 594	Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA	RRID: AB_2340854
Donkey Anti-mouse Secondary Antibody Alexa Fluor 488	Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA	RRID: AB_2340846
Donkey Anti-mouse Secondary Antibody Cy3	Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA	RRID: AB_2340813
Mouse Monoclonal [1C11] Anti-human Cardiac Troponin T- Texas Red	Abcam, Cambridge, Massachusetts, USA	RRID: AB_306445
Mouse Monoclonal Anti-human CD31/PECAM	BD Pharmingen, San Diego, California, USA	RRID: AB_2739574
Mouse Monoclonal [V9] to vimentin- Alexa Fluor 488	Abcam, Cambridge, Massachusetts, USA	RRID: AB_2916318
Biological samples		
Mouse hearts for the isolation of cardiac cells	Please refer to (Sharma et al., 2022)	N/A
Chemicals, peptides, and recombinant proteins		
Bovine Serum Albumin (BSA)	Sigma-Aldrich, AU	Cat# A1933
DMEM	Sigma-Aldrich, AU	Cat# D5671
Fetal Bovine Serum (FBS)	Gibco, Thermo Fisher Scientific, AU	Cat# 10099141
Fibronectin (bovine)	Sigma-Aldrich, AU	Cat# F1141-1MG
HCF Growth Medium	Cell Applications, Sigma-Aldrich, AU	Cat# 316-500
MesoEndo Cell Growth Medium	Cell Applications, Sigma-Aldrich, AU	Cat# 212-500
Image-iT Hypoxia Reagent	Thermo Fisher Scientific, AU	Cat# H10498
iCell Cardiomyocyte Maintenance Medium	FUJIFILM Cellular Dynamics, Inc., Madison, Wisconsin, USA	Cat# R1057
L-glutamine	Gibco, Thermo Fisher Scientific, AU	Cat# 25030081
NucBlue Live Ready Probe Reagent (Hoechst 33342)	Invitrogen, Thermo Fisher Scientific, AU	Cat# R37605
Paraformaldehyde	Sigma-Aldrich	Cat# P6148
Penicillin/Streptomycin	Gibco, Thermo Fisher Scientific	Cat# 15140122
Phosphate-Buffered Saline	Sigma-Aldrich, AU	Cat# D8537
iCell Cardiomyocyte Plating Medium	FUJIFILM Cellular Dynamics, Inc., Madison, Wisconsin, USA	Cat# R1057
Sodium Azide	Sigma-Aldrich, AU	Cat# S2002
Trypsin-EDTA, 0.25%	Gibco, Thermo Fisher Scientific, AU	Cat# 25200072
Trypan Blue Solution, 0.4%	Gibco, Thermo Fisher Scientific, AU	Cat# 15250061
Triton	Sigma-Aldrich, AU	Cat# X100
Vectashield Mounting Media	Vector Laboratories, Abacus dx, AU	Cat# H-1000-10
70 µm Strainer	Corning, Sigma-Aldrich, AU	Cat# CLS431751
Critical commercial assays		
LIVE/DEAD Viability/Cytotoxicity Kit	Invitrogen, Thermo Fisher Scientific, AU	Cat# L3224
Human Cardiovascular Disease RT2 qPCR Kit	QIAGEN, AU	Cat# 330231
RT2 First Strand Kit	QIAGEN, AU	Cat# 330404
Mouse Cardiovascular Disease RT2 qPCR Kit	QIAGEN, AU	Cat# 330231
miRNeasy Mini Kit	QIAGEN, AU	Cat# 217004
Syber Green Rox qPCR Mastermix	QIAGEN, AU	Cat# 330523
Neonatal Heart Dissociation Kit Mouse/Rat	Miltenyi Biotec, AU	Cat# 130-098-373
Experimental models: Cell lines		
Human Cardiac Fibroblasts (HCFs)	Cell Applications, Sigma-Aldrich, AU	Cat# 306AK-05a
Human Coronary Artery Endothelial Cells (HCAECs)	Cell Applications, Sigma-Aldrich, AU	Cat# 300K-05a

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human iPSC-derived Cardiomyocytes (iCMs)	FUJIFILM Cellular Dynamics, Inc., Madison, Wisconsin, USA	Cat# R1057
Experimental models: Organisms/strains		
Organism - Mouse Strain - C57-Bl/6 Age - 0–5 days Gender – Female/male	Sharma et al. (2022)	N/A
Software and algorithms		
Prism	GraphPad	N/A
Qiagen Online Software at Geneglobe	QIAGEN, AU	N/A
EVOS™ Microscope System Suitable for Live Imaging	Invitrogen, Thermo Fisher Scientific, AU	N/A
ImageJ Software	National Institutes of Health, University of Wisconsin, USA	N/A
Other		
Fine Scissors	Kent Scientific Corporation, Torrington, Connecticut, USA	Cat# INS600124
Tissue Culture Flasks (T25)	Thermo Fisher Scientific, AU	Cat# 156367
3D 384 Well Hanging Drop Culture (HDC) Plate	3D Biomatrix, Bioquote LTD, York, UK	Cat# HDP-1384
C Tubes	Miltenyi Biotec, AU	Cat# 130-093-237
GentleMACS™ Dissociator	Miltenyi Biotec, AU	Cat# 130-093-235

STEP-BY-STEP METHOD DETAILS

Isolation of neonatal mouse cardiac cells and generation of mouse CSs

⌚ Timing: 6–10 days

Mouse cardiac cells were isolated from 0–5-day-old mice hearts using Neonatal Mouse Heart Dissociation Kit. All the experiments including animals were conducted under the approved protocol.

1. Take the neonatal heart dissociation kit and prepare the enzyme mixture and enzyme mixture 2 as shown below and place both mixtures immediately on ice.

Enzyme mix 1		Enzyme mix 2		
Enzyme P	Buffer X	Buffer Y	Enzyme A	Enzyme D
62.5 µL	2,300 µL	25 µL	12.5 µL	100 µL

Note: After preparing the solutions of lyophilized powders supplied, prepare aliquots of 20 mL to avoid repeated freeze-thaw-cycles and then store aliquots at –20°C. This will let solutions stable for 6 months after reconstitution.

2. Add sterile PBS in a 50 mL Falcon tube and place it on ice in the eskie.
3. Add sterile complete DMEM in a 50 mL Falcon tube and place it on ice.
4. Harvest the hearts from neonatal mice (up to 10).
 - a. Take a mouse and cut its head with sterile sharp scissor.
 - b. Make an incision from neck to upside down.
 - c. Cut the heart and put in ice-cold PBS Falcon tube. Do this for all mice.
 - d. Close the Falcon tube and gently vortex the tube to remove blood.

Note: To avoid the contamination of hearts use sterilized scissors and spray well the surrounding benchtop with 70% ethanol.

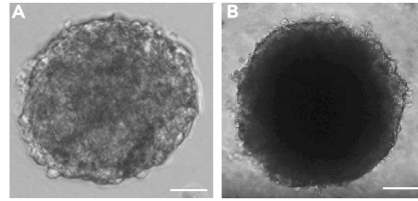


Figure 1. Cardiac spheroid formation from mouse and human cells

(A and B) Formation of CSs (A) Brightfield image of mCS on Day 6 and (B) Brightfield Image of hCS on Day 10. Scale Bar: 100 μ M.

5. At this point, take the enzyme mixture 1 and incubate it at 37°C for 5 min in a 5% CO₂ tissue culture incubator.
6. Then, spray well eskie with ethanol and place it under Biosafety cabinet (BSC).
7. Now, remove hearts from PBS with a plastic pipette and place them in a petri dish.
8. Then, quickly remove fat tissues from the hearts and chop them with a sharp blade into 1–2 mm³ pieces.
9. Then transfer chopped hearts into the gentleMACS C Tube.

Note: C tubes can be leaky if not sealed properly and can lead to contamination around the cap, therefore, the tube must be perfectly clean and tightly closed for proper functionality of the seal. In order to avoid sticking of the chopped heart pieces in C tube, invert the C tube and then tap it down a few times to bring all the content below.

10. Remove enzyme mixture 1 from the incubator and mix 2362.5 μ L of the solution with 137.5 μ L of enzyme mixture 2 to make 2.5 mL of total enzyme solution.
11. Add 2.5 mL of enzyme solution to the C Tube and close it tightly.
12. Invert the C tube and incubate it for 15 min in the 5% CO₂ cell culture incubator at 37°C.
13. After 15 min, attach C Tube onto the sleeve of the gentleMACS dissociator and run the gentleMACS program mr_neoheart_01.

Note: mr_neoheart_01 is a standard program of 37 s set up by the manufacturer for neonatal murine hearts which is critical for enzymatic digestion.

14. Then, repeat steps 12 and 13 three more times to complete 4 cycles each of 15 min incubation and 37 s gentleMACS dissociation.
15. After the program finishes, detach C Tube from the gentleMACS dissociator and add 7.5 mL of complete DMEM to resuspend the chopped hearts.
16. Strain the cell suspension with a 70 μ m strainer by placing it on a 50 mL Falcon tube.
17. Wash the strainer in the same tube with 3 mL of complete DMEM.
18. Then, centrifuge cell suspension at 300 \times g for 5 min at 4°C.
19. Aspirate supernatant completely and resuspend the pellet in 1 mL complete DMEM.
20. Count the total number of cells including cardiomyocytes, fibroblasts, and endothelial cells by mixing trypan blue and cell suspension (in 1:1 ratio) under the Olympus microscope using a hemocytometer.
21. Then adjust the volume of cell suspension with complete DMEM to plate 15 μ L of 30,000 mice cardiac cells per well in a 384 well HDC plate (Sharma et al., 2022).
22. Pipette 15 μ L of cell suspension into each well of the 384 well HDC plate either manually or by using a robotic multichannel pipette for automated liquid handling (Sharma and Gentile, 2021).
23. Pipette 1.5 mL of sterile PBS in each side of the channel around the Hanging Drop. (See [troubleshooting 1](#)).
24. Replace the media every 2–3 days until mouse CSs are formed (Figure 1).

Note: The volume of enzyme mixtures 1 and 2 remains the same for the number of mice up to 10.

△ **CRITICAL:** The number of mice is directly proportional to the number of cardiac cells. Therefore, to have a high yield of cardiac cells it is critical to use the number of mice closer to the upper range. Ideally, 5 mice of 2–3 days old produce a good yield of cells to generate CSs.

Generation of human CSs

Ⓞ **Timing:** 10 days

For a complete procedure on generation of Human CSs including plating, collection of iCMs, HCFs, and HCAECs please refer to (Sharma and Gentile, 2021).

25. To plate HCFs and HCAECs, thaw commercially available cryovials 37°C.
26. Then, transfer cell suspensions from the cryovials to separate T25 culture flasks.

Note: For maximum collection of cells, rinse cryovials twice with 1 mL of culture medium from the same 15 mL tube.

27. Add 7 mL of each HCFG and HMECG media to HCFs and HCAECs respectively. Incubate cells at 37°C with 5% CO₂.
28. After 18 h, rinse cells with sterile PBS to remove freezing the medium and dead cells.
29. Then, replace PBS with 8 mL of appropriate culture medium in each culture flask and incubate at 37°C, 5% CO₂.
30. Examine cellular expansion and viability regularly and replace media every other day until cells reach 80%–90% confluency.
31. Then, collect one iCMs cryovial and place it in a water bath at 37°C for 4 min.
32. Then, transfer the iCMs from the cryovial to a 50 mL centrifuge tube using a 1 mL pipette tip.
33. Rinse the empty iCMs cryovial with 1 mL of room temperature plating medium to recover residual cells. Transfer this 1 mL suspension drop-wise over 90 s to the 50 mL centrifuge tube containing the iCMs cell suspension.

Note: Gently swirl the tube while adding the medium to mix the solution completely and to decrease the osmotic shock on the thawed cells.

34. Now, slowly add 8 mL of room temperature Plating Medium to the 50 mL centrifuge tube. Add the first 1 mL dropwise over 30–60 s. Then, add the remaining volume over the next 30 s.

Note: Gently swirl the centrifuge tube while adding the Plating Medium. Gently mix the contents of the 50 mL centrifuge tube by inverting 2–3 times (avoiding vigorous shaking or vortexing).

35. Then, count the cells immediately with a hemocytometer and check the viable cell density (cells/mL).
36. Then, take the fibronectin pre-coated T25 flasks and aspirate the solution without letting the flasks dry.
37. Then, transfer seeding volume of iCMs (1.6 × 10⁶ viable iCMs in 8 mL room temperature Plating Medium) in the fibronectin pre-coated T25 flasks.
38. Place iCMs in the incubator for 48 h at 37°C, 5% CO₂.
39. 2 days later, wash off dead iCMs by gently pipetting the Plating Medium up and down 5 times.
 - a. Aspirate the Plating Medium and replace with the 8 mL of pre-warmed Maintenance Medium.

- b. Place the flasks back in the 5% CO₂ incubator.
40. Replace the Maintenance Medium every other day until 80%–80% confluency is reached.
41. Once ready, collect the confluent HCAECs, HCFs and iCMs. First collect HCAECs and HCFs and then iCMs. (See [troubleshooting 2](#)).

△ CRITICAL: All the 3 cell types need to be ready at the same time, therefore plate iCMs a few days prior to HCFs and HCAECs using the seeding density mentioned above. The HCAECs and HCFs should be used according to numbers iCMs collected. The left over HCAECs and HCFs were either plated again or frozen again. With the seeding volume of iCMs used in the study, approximately 200 CSs made of 30,000 cardiac cells can be successfully generated.

42. Remove culture medium from HCFs and HCAECs tissue flasks and rinse them with 3 mL PBS for T25 flask.
 - a. Then remove PBS and add 3 mL of 0.25% trypsin EDTA solution to each T25 flask.
 - b. Incubate for 5 min at 37°C, 5% CO₂.
 - c. Neutralize the trypsin EDTA solution with 5 mL of culture medium immediately.
 - d. Transfer cell suspensions to a 15 mL Falcon tube and centrifuge cells at 300 × g for 4 min.
43. Now remove the supernatant carefully from each tube and add 1 mL HCS medium to each cell pellet and resuspend them.
44. Keep the cell suspension on ice and count cells as done in step 20.
45. Now take the iCMs tissue flasks and remove Maintenance Medium from them.
 - a. Rinse with 3 mL of PBS and add 1 mL of 0.25% trypsin EDTA solution to each T25 flask.
 - b. Incubate at 37°C, 5% CO₂.

Note: Check iCMs every minute until they detach from the flask.

46. Then, immediately neutralize the trypsin EDTA solution with 4 mL of HCS medium. Then, transfer cell suspension to a 15 mL Falcon tube and centrifuge them at 300 × g for 5 min.
47. Carefully remove the supernatant from each tube and add 1 mL of HCS medium to the cell pellet.
 - a. Resuspend and place it on ice.
 - b. Count the cells following step 20.
48. Mix iCMs, HCFs and HCAECs in 2:1:1 ratio by plating 10,000 iCMs, 5,000 HCFs and 5,000 HCAECs per HDC containing 20 μL of HCS medium. Adjust to the final volume for the total number of CSs.
49. Then, follow steps 22–24 ([Figure 1](#)).

Note: Mouse CSs are easily and rapidly formed compared to human CSs where all cell types have to be plated individually first and mixed together to form CSs. Also, being made from neonatal cardiac cells mouse CSs have high reproducibility. In order to obtain a good ratio of CSs (whether human or mouse) all the steps should be strictly followed. A regular check on formation of CSs is must.

Establishing and imaging ischemia reperfusion (I/R) in 3D human and mice CSs using EVOS™ microscope system

⌚ **Timing:** 3 days

In vitro modeling of the I/R injury was achieved by culturing CSs at varying oxygen concentrations while visualizing changes in intracellular oxygen concentrations in EVOS™ Microscope system using Image-iT (a reversible hypoxic fluorescent dye sensible to changes in intracellular oxygen levels ([Figure 2](#))). Both EVOS™ FL Auto and M7000 microscope systems can be used for time lapse operations. EVOS™ M7000 is an upgraded version of EVOS™ FL Auto system, and the look of screen and naming

EVOS™ Software Screenshots

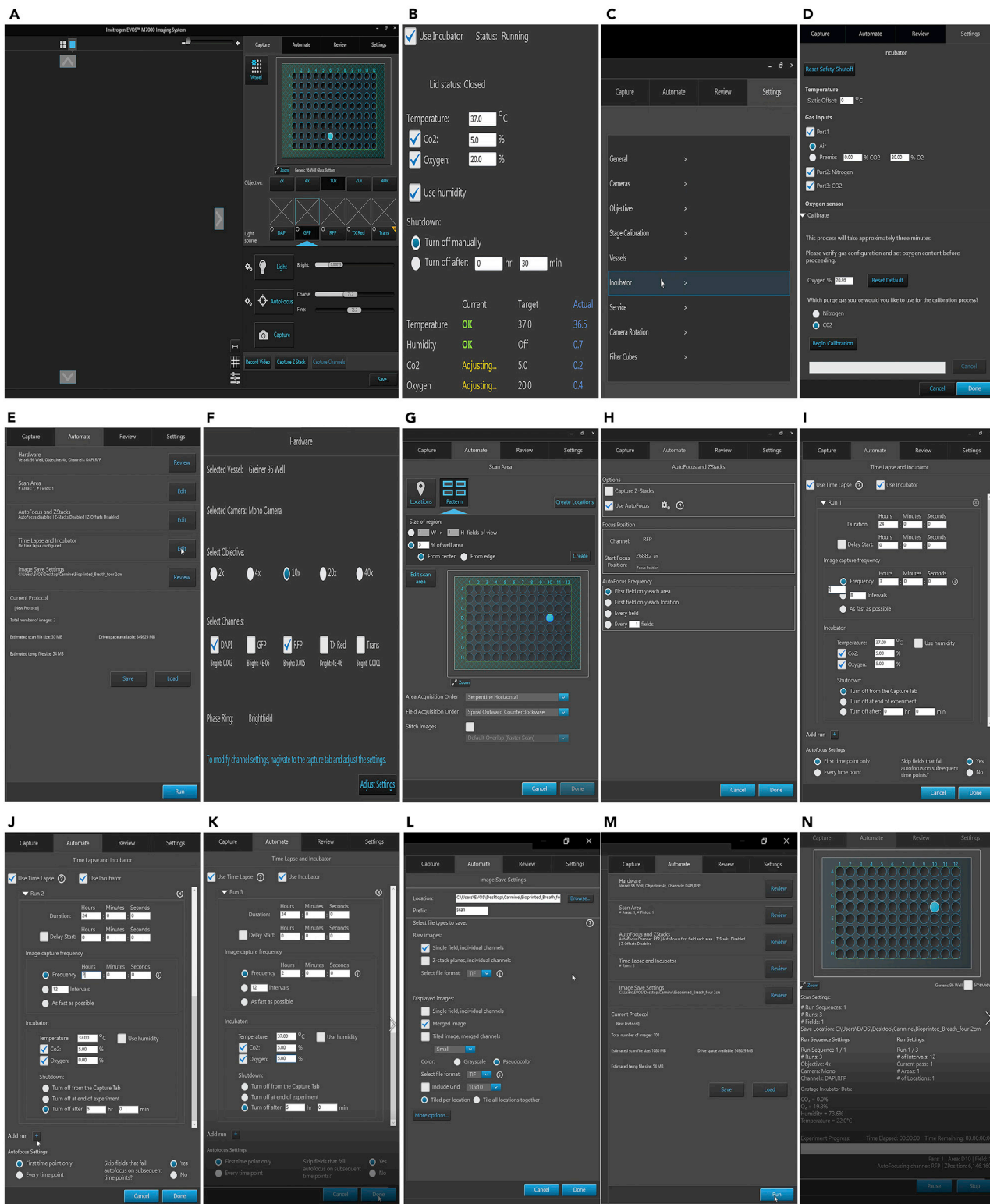


Figure 2. Setting up hypoxic/normoxic conditions
(A–N) EVOS™ screen snapshots for steps used to set up the I/R injury protocol.

of tabs is bit different. Here we have mentioned names and steps according to EVOS M7000 software.

50. Collect CSs with a 1 mL pipette tip into a 50 mL tube on ice and centrifuge the tube at $300 \times g$ for 5 min. For detailed video on collection of spheroids please refer to (Sharma and Gentile, 2021) (See troubleshooting 3).
51. Remove the supernatant from the tube containing CSs and resuspend the CSs pellet with 1.99 mL suitable medium (complete DMEM for mCSs and HCS medium for hCSs).

Note: The pellet obtained must be kept on ice until use.

52. Take two 96 well plates and mark one as control and other as test.
53. Add 250 μ L of CS suspension (approximately 10 CSs/ well) into 4 wells of control plate and cover it. Place the plate in the 5% CO₂ tissue culture incubator.

Note: In order to have same number of CSs in each well, exact number of CSs should be collected in separate Falcon tubes and centrifuged.

54. Add 10 μ L of 10 μ M Image-iT solution to the left-over 0.99 mL CS suspension. Then, add 2 drops of Nucblue (Hoechst) stain to it.
55. Now, add 250 μ L of this CS suspension into 4 wells of test plate and cover it. Incubate it for 20 min in the 5% CO₂ tissue culture incubator.
56. Turn on the EVOS™ imaging system and the connected computer and monitor. Start the EVOS™ microscope software (Figure 2A).
57. On the capture tab, click the Incubator button to display the Incubator control panel.
 - a. Select use incubator, then enter the target values for temperature, CO₂, and O₂ and humidity.
 - b. Select the turn off manually shutdown option and click apply and then done (Figure 2B).
58. In the settings tab, click Incubator to open the incubator configuration panel (Figures 2C and 2D).
59. Select the gas inputs for the EVOS™ onstage incubator as follows:
 - a. Port 1 – Air.
 - a. Port 2 – N₂.
 - c. Port 3 – CO₂.
60. Then click done and turn on the regulators on the gas tanks.

Note: Use the industrial grade gas tanks and regulators.

61. Then set the flow on the regulators as follows:
 - a. Air: 40–50 psi.
 - b. CO₂: 40–50 psi.
 - c. N₂: 40–50 psi.

Note: Do not exceed pressure beyond 50 psi.

62. Then expand the Calibrate panel under O₂ Sensor. Select the purge gas source for your calibration (N₂ or CO₂).
63. Click begin calibration (Figure 2D).

Note: The system will automatically calibrate the O₂ sensor for the proper functioning of the EVOS™ onstage incubator. The entire calibration process takes approximately 3 min.

64. Then after 20 min of incubation with dyes, place the test plate on the onstage incubator.
 - a. On the capture tab, click the Vessel button.

- b. Select the sample vessel as 96 well plate.
- c. Select monochrome option in camera field in settings tab.
65. Then in the automate tab, click on hardware option.
 - a. Select 4× objective and Dpi and RFP imaging channels.
 - b. Press done to proceed (Figures 2E and 2F).
66. Then click on scan area.
 - a. Click the edit button to open the scan area panel.
 - b. In this panel click the pattern button to open the scan pattern tool to define the areas and fields to be captured.
 - c. Then press create tab.
 - d. Then select serpentine horizontal and spiral outward counterclockwise options for area acquisition order and field acquisition order fields respectively.
 - e. Press done to proceed (Figure 2G).
67. In the same automate tab, click on autofocus and z-stacks.
 - a. Under options select use autofocus frequency.
 - b. Under autofocus frequency, select first field only each area (Figure 2H).
68. Then move to time lapse and incubator option in automate tab.
 - a. Click the edit button to open the time lapse and incubator panel.
 - b. Select use time Lapse and incubator.
 - c. Under Run 1, add duration as 24 h and image capture frequency as 3 h.
 - d. Under incubator, select temperature as 37°C, CO₂ at 5% and O₂ at 5% options.
 - e. Under shutdown, select turn off from the capture tab. Then press add run (Figure 2I).

Note: The image capture frequency is a choice.

69. Under Run 2, add duration as 20 h and image capture frequency as 2 h.
 - a. Under incubator, select temperature as 37°C, CO₂ at 5% and O₂ at 0% options.
 - b. Under shutdown, select turn off from the capture tab. Then press add run (Figure 2J).
70. Under Run 3, add duration as 17 h and image capture frequency as 2 h.
 - a. Under incubator, select temperature as 37°C, CO₂ at 5% and O₂ at 5% options.
 - b. Under shutdown, select turn off from the capture tab.
 - c. Under autofocus settings select first time point only field.
 - d. Then click done (Figure 2K).

Note: The gases will stabilize within 1–3 min after changing conditions.

71. Then in the automate tab go to image save settings.
 - a. Select location by browsing the folder where images will be stored.
 - b. Then select file name prefix and add scan.
 - c. In raw images section select single field, individual channels.
 - d. In displayed images select merged images.
 - e. In color select pseudocolor.
 - f. For file formats select TIFF and tile per location options.
 - g. Then press done (Figure 2L). (See [troubleshooting 4](#)).
72. Then click on run (Figure 2M).
73. Then the experiment will start (Figure 2N).
74. On the third day, half an hour before the time lapse finishes, replace the media in the control plate with appropriate media containing 10 μM Image-iT and 2 drops of Hoechst. Incubate for 20 min.
75. Turn off all the gas regulators after the time lapse finishes and take out the plate.
 - a. Then image the CSs in the control plate manually.

Note: In order to ensure the proper functioning of imaging system, start the EVOS™ system at least an hour before the start of each experiment, check the level of gases to be used (enough for a 3-day experiment) and calibrate the oxygen sensor with either nitrogen or carbon dioxide every time you use the incubation chamber.

Evaluation of cell death and viability

⌚ Timing: 1 day

76. Prepare the control and test 96 well plates as mentioned in step in 53. Then place the control plate in the 5% CO₂ tissue culture incubator.
77. Place the test plate in EVOS™ onstage incubator and then follow the steps 56–75.

Note: There is no need to select Dapi and RFP channels as there are no stains in these CSs. Therefore, for images use phase channel.

78. On the 3rd day, prepare 1 mL solution of appropriate medium using LIVE/DEAD Viability/Cytotoxicity Kit reagents (ethidium homodimer and calcein-AM) and Hoechst stain.
 - a. Use 4 μM of ethidium homodimer and 2 μM calcein-AM with 2 drops of Hoechst to prepare the solution of medium.

Note: Calcein-AM stains live cells by interacting with intracellular esterases and produces green fluorescence. Ethidium homodimer-1 highlights dead cells in red fluorescence showing loss of plasma membrane integrity.

79. After the time lapse finishes, take out the test and control plate and add 100 μL of above prepared medium into each well with CSs.
80. After 2 h, measure the fluorescence into each well at 645 nm for ethidium homodimer and at 530 nm for calcein-AM, respectively.
81. Use Graphpad PRISM for (or an equivalent software for statistical analysis) data analysis and statistics of the fluorescence measurements.

Note: To view the video-based procedure, please refer to ([Sharma and Gentile, 2021](#)).

Preparation of CSs for confocal microscopy

⌚ Timing: 3 days

82. Prepare the control and test 96 well plates as mentioned in step in 53. Then place the control plate in the 5% CO₂ tissue culture incubator.
83. Place the test plate in EVOS™ onstage incubator and then follow the steps 56–75.

Note: There is no need to select Dapi and RFP channels as there are no stains in these CSs. Therefore, for images please use phase channel.

84. On the 3rd day, after time lapse finishes, fix CSs in 4% paraformaldehyde (PFA) for 1 h at room temperature. (See [troubleshooting 5](#)).
85. Remove PFA and rinse three times with PBS containing 0.01% sodium azide (PBSA) (3 × 30 min).

Note: PFA and sodium azide is a hazardous chemical, therefore, use fume hood while working with sodium azide solution.

86. Remove PBSA and Add 200 μ L of PBSA containing 0.02% Triton-X-100 to each well for 30 min using a shaker.
87. Add 200 μ L of 3% bovine serum albumin in PBSA solution for 60 min (overnight) at room temperature.
88. Prepare solution containing 10 μ g/mL of primary mouse anti-human antibodies against CD31 diluted in blocking solution.
89. Add 100 μ L of primary antibody solution to each well and incubate overnight at 4°C.
90. Rinse the plate three times with PBSA for 20 min at room temperature on a rocking plate.
91. Prepare a solution containing Hoechst DNA stain and 10 μ g/mL of Cy3- conjugated secondary donkey anti-mouse antibody diluted in blocking solution. Add 100 μ L of secondary antibody solution containing Hoechst stain to each well and incubate overnight at 4°C.
92. Rinse the plate three times for 20 min with PBSA at room temperature on a rocking plate.
93. Add 100 μ L of Vectashield mounting medium to each well.
94. Image CSs under a laser scanning confocal microscope.
 - a. Perform optical sectioning along the Z axis.
 - b. First select objective lens as 10 \times , then under locate tab select channels as Dapi, Green and red.
 - c. Then turn off reflecting light.
95. After that go to z-stacks tab under live acquisition tab, use the software recommended settings for slicing and resolution as follows:
 - a. Interval: 0.43.
 - b. Slices: 10.
 - c. Optimal: 0.43 μ M.
96. Then select the first and last values by moving the knob.
97. Then, stop live acquisition mode and press start experiment.
98. Collapse images into a single focal plane using ImageJ software.
99. Use the z-stacks of confocal images in Imaris 9.2.6 software for 3D rendering analysis.
100. Use drag and drop method to open image to the Imaris program icon.
101. Then click on surpass tab.
 - a. Select 3D view, add frame and volume.
 - b. In the surpass tree each tab comes with setting options.
 - c. Select channels in volume settings, add colors and threshold values to different channels.
102. Click on snapshot for desired image and save it.

Evaluation of contraction frequency and fractional shortening

⌚ Timing: 1 day

103. Prepare the control and test 96 well plates as mentioned in step in 53. Then place the control plate in the 5% CO₂ tissue culture incubator.
104. Place the test plate in EVOS™ onstage incubator and then follow the steps 56–75.

Note: There is no need to select Dapi and RFP channels as there are no stains in these CSs. Therefore, for images use phase channel.

105. On the 3rd day, after time lapse finishes, take out test and control plate.
106. Place the single CSs using a suitable dish on the stage of Olympus microscope and record the contractile movements of each CS using Olympus Cellsens software.
107. Then manually count the percentage of fractional shortening and contractional frequency by analyzing the recorded videos using the given formulas.
 - a. Contraction frequency: Count number of contractions per minute.
 - b. Fractional shortening percentage: $\frac{\text{Total length} - \text{length of contraction}}{\text{total length}} \times 100$.
108. Use GraphPad Prism software for data analysis and statistics.

Gene expression analysis via mRNA isolation and RT2 qPCR analysis

⌚ Timing: 1 day

MRNA isolation and qPCR analyses were performed using miRNeasy Mini kit and a cataloged cardiovascular disease array from Qiagen following instruction manuals supplied with the kits. The genes included the array are all relevant to cardiac diseases. For more details, please refer to (Sharma et al., 2022).

109. Prepare the two 96 well plates as prepared in step in 53. Then place the control plate in the 5% CO₂ tissue culture incubator.
110. Place the test plate in EVOSTM onstage incubator and then follow the steps 56–75.

Note: There is no need to select Dapi and RFP channels as there are no stains in these CSs. Therefore, for images use phase channel.

111. On the 3rd day, after time lapse finishes, take out test and control plate.
112. Collect the CSs in 50 mL Falcon tube and centrifuge at 300 × g at 40°C.
 - a. Carefully remove all supernatant by aspiration.
 - b. Then add 700 μL QIAzol Lysis Reagent.
 - c. Vortex for 1 min to mix and place it at 25°C for 5 min to promote dissociation of nucleoprotein complexes.
113. Add 140 μL chloroform to the tube containing the homogenate and cap it securely.
 - a. Shake the tube vigorously for 15 s and place the tube on the benchtop at room temperature for 2–3 min.
 - b. Then, centrifuge the tube for 15 min at 12,000 × g at 4°C.
114. Transfer the upper aqueous phase to a new collection tube and to this add 525 μL of 100% ethanol and mix thoroughly by pipetting up and down several times.
115. Pipet up to 700 μL of the sample, including any precipitate that may have formed, into a RNeasy Mini spin column in a 2 mL collection tube and close the lid gently and centrifuge at ≥8,000 × g for 15 s at room temperature.
116. Discard the flowthrough.
 - a. Reuse the collection tube.
 - b. Repeat step 115 with the remaining sample.
 - c. Discard the flow-through.
117. Add 500 μL Buffer RPE onto the RNeasy Mini spin column.
 - a. Close the lid gently.
 - b. Centrifuge for 15 s at ≥8,000 × g to wash the column.
 - c. Discard the flowthrough.
118. Add another 500 μL Buffer RPE to the RNeasy Mini spin column.
 - a. Close the lid gently.
 - b. Centrifuge for 2 min at ≥8,000 × g to dry the RNeasy Mini spin column membrane.
119. Transfer the RNeasy Mini spin column to a new 1.5 mL collection tube.
 - a. Add 30–50 μL RNase-free water directly onto the RNeasy Mini spin column membrane.
 - b. Close the lid gently and centrifuge for 1 min at ≥8,000 × g (≥10,000 rpm) to elute the RNA.
120. Repeat the step 119 if RNA yield is >30 μg. Put the RNA on ice.

Note: The average RNA yield range for a 10 mg mouse heart tissue is 5–25 μg and for 1 × 10⁶ cardiac cells is 10–15 μg. It is essential to use the correct amount of starting material to obtain optimal RNA yield and purity.

121. Now, thaw the reagents of the RT2 First Strand Kit.

122. Prepare the genomic DNA elimination mix as shown below.

Reagents	Amount
RNA	25 ng-5 µg
Buffer GE	2 µL
RNase-free water	As required
Total Volume	10 µL

- a. Mix gently by pipetting up and down.
- b. Centrifuge briefly.
- c. Incubate the genomic DNA elimination mix for 5 min at 42°C.
- d. Place immediately on ice for at least 1 min.

123. Prepare 10 µL of the reverse-transcription mix as shown below.

Reagents	Amount
5x Buffer BC3	4 µL
Control P2	1 µL
RE3 Reverse Transcriptase Mix	2 µL
RNase-free water	3 µL

124. Add 10 µL reverse-transcription mix to each tube containing 10 µL genomic DNA elimination mix.

- a. Mix gently by pipetting up and down.
- b. Incubate at 42°C for exactly 15 min.
- c. Then immediately stop the reaction by incubating at 95°C for 5 min.

125. Add 91 µL RNase-free water to each reaction.

- a. Mix by pipetting up and down several times.
- b. Place the reactions on ice.

126. Briefly centrifuge the RT2 SYBR Green Mastermix (10–15 s) to bring the contents to the bottom of the tube.

127. Prepare the PCR components mix in a wide mouth 5 mL tube for each as shown below.

Reagents	Amount
RT2 SYBR Green Mastermix	650 µL
cDNA synthesis reaction	102 µL
RNase-free water	548 µL

128. Dispense the 4 samples of PCR components mix into the cardiovascular disease RT2 Profiler PCR Array employing E format and the using the plate covers supplied with the kit.

129. Now seal the RT2 Profiler PCR Array with Optical Adhesive Film.

- a. Centrifuge for 1 min at 1,000 g at room temperature (15°C–25°C) to remove bubbles.
- b. Visually inspect the plate from underneath to ensure no bubbles are present in the wells.

130. Set PCR cycling program on a Bio-Rad Cycler.

131. After the PCR cycling finishes, export the CT values for all wells to a blank Excel spreadsheet for Data Analysis using Qiagen Geneglobe Web-based software.

132. Use the Fold change values obtained from QIAGEN web software to do Principal component analysis using Prism software.

EXPECTED OUTCOMES

Neonatal mouse cardiac cells or human cardiac cells may take up to 10 days to form contracting CSs. We recommend to precisely follow the protocol for high yield of CSs. Further, changes in oxygen concentration bring out the changes in fluorescence of CSs stained with reversible hypoxic fluorescent dye. Increased fluorescence should be expected and observed in CSs when exposed to hypoxic

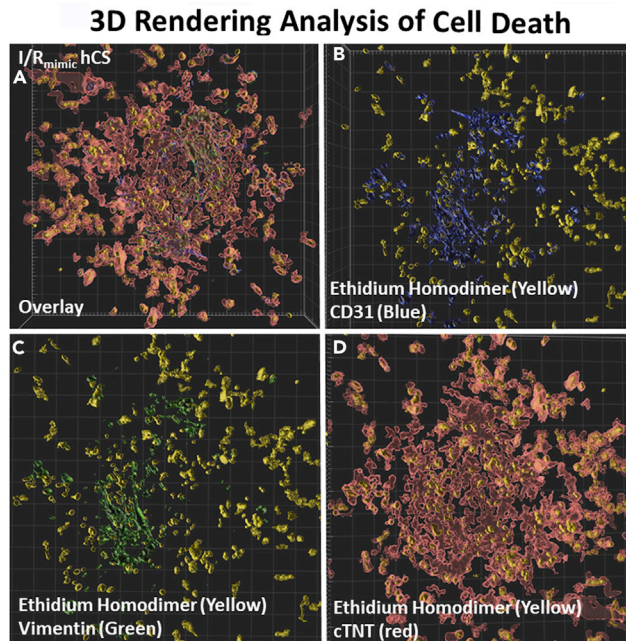


Figure 3. 3D cell death analyses of confocal images of an I/R CS using Imaris software

3D rendering analyses of confocal images of an I/R CS stained with ethidium homodimer (yellow), and with antibodies against CD31 for endothelial cells (blue), cTNT for cardiomyocytes (red) and vimentin for fibroblasts (green). (A–D) (A) shows overlay of (B) highlighting all the dead cardiomyocytes, (C) highlighting dead endothelial cells and (D) highlighting dead fibroblasts, respectively. Scale Bar: 100 μ M. Reused from (Sharma et al., 2022).

conditions (0% O₂) for 2 h following the exposure to 5% O₂ (physiological concentration of O₂) (Keeley and Mann, 2019) for 24 h. A decrease in fluorescence intensity was observed after switching the O₂ concentration back to physiological levels (5% O₂) for 24 h. The statistical analysis of Image-iT fluorescence confirmed the significant difference between normoxic, hypoxic and reoxygenation conditions at the T₀, T₂₀ and T₃₇ time intervals respectively.

Cell damage occurred following I/R injury was analyzed by measuring the toxicity ratios by measuring dead and live cells fluorescence. A statistically significant increase in toxicity ratios observed between control and I/R CSs. To confirm the effect of I/R injury for each cell type 3D rendering analyses was done using the confocal images of CSs stained with ethidium homodimer, cell type-specific antibodies against CD31, cTNT and vimentin (staining cardiac endothelial cells, myocytes and fibroblasts, respectively). The results thus obtained confirmed that the cardiomyocytes are more susceptible to I/R injury than endothelial cells and fibroblasts (Figure 3). Cell death also resulted in significant decrease in contraction frequency and fractional shortening. Finally, a detailed analyses on the regulation of cardiovascular disease genes during the I/R injury confirmed that our I/R injury model recapitulates almost all the features typical of *in vivo* I/R injury.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism software to calculate mean \pm standard deviation (SD) and perform appropriate unpaired t-test according to distribution and sample variance. One-way ANOVA with Tukey post-hoc test was used for comparisons of multiple groups. Significance was set to $p < 0.05$. For single gene expression analysis, fold changes were calculated as $2^{(-\text{Avg. } \Delta\Delta\text{Ct})}$ and analyzed based on the online software from QIAGEN. A minimum of $n=3$ biological replicates was used per group.

LIMITATIONS

Our method to generate CSs is highly reproducible compared to other existing models. Our *in vitro* I/R model is pioneer model that studies the I/R injury typical of MI using the 3 major cell types found in a heart. Despite of several advantages, it presents a few limitations. First, the absence of blood, blood-derived cells, and their effects on the myocardium (such as, platelets, macrophages, and other blood cells). These are all critical for the release of vasoconstrictive and proinflammatory agents typical of the myocardial I/R injury but are absent in our model (Sharma et al., 2022; Ziegler et al., 2018). We used a closed chamber, which did not allow us measure changes in pH, calcium concentration and the nutrients over time. Despite these limitations, our I/R *in vitro* model closely recapitulates pathophysiological oxygen concentration changes observed in *in vivo* conditions. This protocol has the potential to be used for drug discovery of novel therapies with better translation of findings from the bench to the bedside, with the hope to find better treatments to both prevent and treat cardiovascular disease in patients (Sharma and Gentile, 2021).

TROUBLESHOOTING

Problem 1 (generation of CSs)

How to avoid drying of CSs in the incubator?

Potential solution

CSs are highly susceptible to drying. Pipetting 1.5 mL PBS around the sides of the HDC plate will help to avoid drying of CSs. We recommend checking the PBS levels too while replacing media every other day. If necessary, refill the sides of plates with PBS again.

Problem 2 (generation of CSs)

What if you do not have enough number of cells to make CSs?

Potential solution

If you do not have enough cells to generate a 384 well plate of CSs then plate according to the number of cells you have using 2:1:1 ratio for iCMs, HCAECs and HCFs.

Problem 3 (analyses of CSs)

How to prevent sticking of CSs and cell death while collection?

Potential solution

Cut the tip of pipette around 0.2 cm from the edge before its use with a sterile sharp scalpel or a scissor to prevent sticking of CSs during their collection. In order to reduce damage to cells during procedures, CS pellets must be kept on ice until use.

Problem 4 (analyses of CSs)

How to obtain good images that could be easily analyzed?

Potential solution

We recommend using manual adjusting of focus if autofocus does not gives good photos plus always save images in TIFF format with 8-bit configuration.

Problem 5 (analyses of CSs)

How to avoid damage and better image the stained CSs?

Potential solution

Always stain the CSs in dark room and cover the plate with aluminum foil until use.

RESOURCE AVAILABILITY

Lead contact

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Materials availability

This study did not generate new unique reagents.

Data and code availability

Source data for figures in the paper are available online (Sharma et al., 2022), DOI: <https://doi.org/10.1088/1758-5090/ac47d8>.

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AUTHOR CONTRIBUTIONS

Conceptualization: P.S. and C.G.; methodology: P.S.; investigation: P.S. and C.L.C.M.; writing: P.S.; editing: P.S., C.L.C.M., and C.G.; supervision: C.G.; funding acquisition: P.S. and C.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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