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Title: A Hypoxia-Reoxygenation Injury Model in Self-Assembling Human Cardioids

Authors and Affiliations:

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar. **No**

- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

- 3. Filming location:** Will the filming need to take place in multiple locations? **No**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 19

Number of Shots: 56

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen. Please answer in *stand-alone full sentence with sufficient context.***

Answers will be slightly edited and only 5 answers are chosen in accordance with the journal guidelines

INTRODUCTION:

REQUIRED: ~~Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Lauren Parker:** Our research focuses on finding new ways to help the heart regenerate after injury. For this, we generate self-assembling cardiac organoids and perform hypoxia-reoxygenation studies.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the research contexts or systems in which this protocol can be applied.**~~

- 1.2. **Lauren Parker:** This protocol can be applied to study injury response, therapeutic interventions to promote regeneration, and can be used for patient-specific disease modeling.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~**What does this protocol allow researchers to study or measure?**~~

- 1.3. **Lauren Parker:** This protocol allows researchers to mimic myocardial infarction in iPSC-derived human cardiac organoids.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the most important consideration or challenge when performing this protocol.**~~

- 1.4. **Lauren Parker:** The greatest technical challenge in ensuring reproducible injury is the complete removal of existing media.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Lauren Parker:** Our future studies aim at identifying factors that can promote tissue regeneration in human cardiac organoid models.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Differentiation of iPSC Derived Cardiac Mesoderm Cells to Human Cardiac Organoids (hCOs)

Demonstrator: Lauren Parker

2.1. To begin, add Accutase to cover the human induced pluripotent stem cell-derived cardiac mesoderm cells [1] and place the plate in an incubator at 37 degrees Celsius for 5 minutes [2]. Using a pipette, add an equal volume of D2 cardiac mesoderm media to each well [3] and gently pipette up and down to release the adherent cells [4]. Transfer the cell suspension to a 15-milliliter conical tube [5].

2.1.1. Talent adding Accutase to the culture plate to fully cover the cells.

2.1.2. Talent placing the plate into an incubator set to 37 degrees Celsius.

2.1.3. Close-up of talent adding D2 cardiac mesoderm media to each well.

Author's NOTE: Steps 2.1.3-2.1.5 were combined into one shot

2.1.4. Talent gently pipetting up and down in the well to detach and dissociate the cells.

2.1.5. Talent transferring the cell suspension into a 15 milliliter conical tube.

2.2. Using a centrifuge, pellet the cells at 300 *g* for 3 minutes at room temperature [1]. Resuspend the pellet in 1 milliliter of D2 cardiac mesoderm specification media containing 5 micromolar IWP2 and 50 micrograms per milliliter ascorbic acid in RB minus [2].

2.2.1. Talent placing the 15 milliliter conical tube into the centrifuge.

2.2.2. Talent resuspending the cell pellet in fresh specification media by gentle pipetting.

2.3. Transfer 10 microliters of the cell suspension into a microtube and add 10 microliters of Trypan blue [1]. Count the cells using the preferred counting method [2]. Then, dilute the cells to a concentration of 100000 cells per milliliter in D2 cardiac mesoderm specification media supplemented with 2 micromolar thiazovivin [3].

2.3.1. Talent adding 10 microliters of Trypan blue to the cell tube.

2.3.2. Talent placing the sample in a cell counter.

2.3.3. Talent diluting the cell suspension with specification media containing thiazovivin to achieve the desired concentration.

2.4. Using a multichannel pipette, dispense 100 microliters into each well of an ultra-low attachment 96-well plate to achieve a final concentration of 10000 cells per well [2]. Spin the plate at 500 *g* for 5 minutes at room temperature to induce human cardiac organoid formation in suspension culture [3]. Observe the formation of a large, well-circumscribed cell pellet in each well [4-TXT].

2.4.1. Talent pouring the prepared cell suspension into a reagent reservoir.

2.4.2. Talent using a multichannel pipette to dispense 100 microliters into each well of a 96 well ultra low attachment plate.

2.4.3. Talent placing the 96 well plate into the centrifuge.

2.4.4. LAB MEDIA: 05-10-2025-d2-3d-ipsc-4x.png. **TXT: Repeat the centrifugation step if required**

2.5. After 24 hours, prepare 10 milliliters of D3 media containing 5 micromolar IWP2 and 50 micrograms per milliliter ascorbic acid in RB minus per plate [1]. Using a multichannel pipette, add 100 microliters of D3 media to each well without removing any existing media [2].

2.5.1. Talent preparing D3 media in a sterile container and measuring 10 milliliters.

2.5.2. Talent using a multichannel pipette to add 100 microliters of D3 media into each well of the plate without aspirating existing media.

2.6. The next day, remove 100 microliters of D3 media from each well [1] and replace it with 100 microliters of D4 media containing 5 micromolar IWP2 in RB minus [2]. ~~Then, replace the media again with 100 microliters of fresh D4 media [3].~~

2.6.1. Talent aspirating 100 microliters of media from each well of the plate.

2.6.2. Talent adding 100 microliters of D4 media into each well.

2.6.3. ~~Talent performing a second replacement by adding 100 microliters of fresh D4 media to each well.~~

Author's NOTE: Step 2.6.3 should be deleted.

3. Fixation and Whole-Mount Immunostaining of Human Cardiac Organoids

3.1. Using a 1000 microliter pipette with the tip cut off, transfer the human cardiac organoids to a 1.7-milliliter microcentrifuge tube [1]. Allow the organoids to sink to the bottom of the tube [2] and gently remove the excess media [3]. Add phosphate-

buffered saline to wash the organoids once [4], wait a few seconds for them to sink, and gently remove the excess PBS [5].

3.1.1. Talent using a 1000 microliter pipette with the tip cut off to transfer human cardiac organoids into a 1.7 milliliter microcentrifuge tube.

3.1.2. Close-up of organoids settling at the bottom of the microcentrifuge tube.

3.1.3. Talent carefully aspirating excess media without disturbing the organoids.

3.1.4. Talent adding PBS into the tube to wash the organoids.

3.1.5. Close-up of organoids at the bottom as talent gently removes excess PBS.

3.2. Next, add 4 percent paraformaldehyde to fix the organoids [1] and place the tube at room temperature for 30 minutes to allow fixation [2].

3.2.1. Talent adding 4 percent paraformaldehyde into the microcentrifuge tube containing the organoids.

3.2.2. Talent placing the tube upright in a rack on the benchtop for fixation at room temperature.

3.3. To begin whole mount immunostaining, use a 1000 microliter pipette with the tip cut off to transfer the desired number of fixed organoids to a 1.7-milliliter microcentrifuge tube [1]. Remove the fixative solution [2] and wash the organoids with PBS containing 0.1 percent Tween 20 [3].

3.3.1. Talent transferring fixed organoids into a clean 1.7 milliliter microcentrifuge tube.

3.3.2. Talent gently aspirating and discarding the fixative solution from the tube.

3.3.3. Talent adding PBS with 0.1 percent Tween 20 to wash the organoids.

3.4. Prepare the primary antibody solution by diluting the antibody to the desired concentration in staining solution [1]. Using a pipette, add 50 to 100 microliters of the primary antibody solution to each microcentrifuge tube, ensuring the organoids are fully covered [2-TXT]. After washing, incubate the sample with secondary antibody diluted in staining solution for 24 hours at 4 degrees Celsius on a shaking platform [3-TXT].

3.4.1. Close-up of talent preparing the staining solution and adding primary antibody to achieve the desired concentration.

3.4.2. Talent pipetting 50 to 100 microliters of primary antibody solution into the tube to fully cover the organoids. **TXT: Wash 3 × 1 h in PBS-T at RT**

Talent placing the sample on a shaking platform set to 4 degrees Celsius. **TXT: Wash 3 × 1 h in PBS-T at RT; Overnight PBS-T wash at 4 °C**

3.5. Next, using a cut pipette tip, transfer the organoids onto a positively charged glass slide [1]. Gently remove excess PBS with Tween 20 [2] and add a few drops of mounting media to cover the organoids [3]. Carefully place a coverslip over the organoids to flatten them [4-TXT].

3.5.1. Talent transferring stained organoids onto a positively charged glass slide.

3.5.2. Close-up of talent carefully removing excess buffer from around the organoids on the slide.

3.5.3. Talent adding mounting media drops to fully cover the organoids.

3.5.4. Talent gently lowering a coverslip onto the organoids to flatten them evenly. **TXT: Perform fluorescence microscopy**

4. Embedding the Cardiac Organoids for Cryosectioning

4.1. Add a small drop of optimal cutting temperature compound to the mold, just enough to cover the organoids [1]. Using fine tweezers or a pipette tip, gently reposition the organoids in the compound so they are not clumped and are away from the edges of the mold [2]. Position the organoids as close to the bottom of the mold as possible to maximize the number present in a single cryosection [3].

4.1.1. Close-up of talent dispensing a small drop of optimal cutting temperature compound into the mold containing organoids.

4.1.2. Talent using fine tweezers or a pipette tip to gently separate and reposition organoids within the compound.

4.1.3. Close-up showing organoids positioned evenly near the bottom of the mold.

4.2. Place the mold into a Styrofoam cooler containing a layer of dry ice to freeze the initial layer of optimal cutting temperature compound [1]. Wait a few minutes for the compound to harden [2] and then move the mold to the benchtop [3]. Add additional optimal cutting temperature compound until the mold is filled [4]. Allow the first layer to fully melt before returning the mold to the dry ice to complete freezing [5-TXT].

4.2.1. Talent placing the mold into a Styrofoam cooler with dry ice at the bottom.

4.2.2. Close-up of the compound solidifying around the organoids.

4.2.3. Talent transferring the mold from the dry ice to the benchtop.

- 4.2.4. Talent adding more optimal cutting temperature compound to fill the mold completely.
- 4.2.5. Talent returning the mold to the dry ice for final freezing. **TXT: Perform cryosectioning to obtain 6 μ m sections**

5. Inducing Hypoxia-Reoxygenation Injury in Human Cardiac Organoids

- 5.1. Using a pipette, transfer 5 milliliters of hypoxia media per 96 well human cardiac organoid plate into a cell culture flask [1]. Place the flask inside a hypoxia chamber [2] and flush the chamber with anoxic gas composed of 5 percent carbon dioxide and 95 percent nitrogen for 3 minutes [3]. Place the chamber in an incubator at 37 degrees Celsius for at least 1 hour before initiating hypoxia [4].
 - 5.1.1. Talent pipetting 5 milliliters of hypoxia media into a cell culture flask.
 - 5.1.2. Talent placing the flask inside the hypoxia chamber and sealing it.
 - 5.1.3. Close-up of the chamber being flushed with the anoxic gas mixture for 3 minutes.
 - 5.1.4. Talent placing the hypoxia chamber into an incubator set to 37 degrees Celsius.
- 5.2. Using a multichannel pipette, remove approximately 150 microliters of media from all wells of the human cardiac organoid plate [1]. Then, using a 200 microliter pipette, carefully remove the remaining media from each individual well without disturbing the organoids [2].
 - 5.2.1. Talent aspirating approximately 150 microliters of media from all wells using a multichannel pipette.
 - 5.2.2. Close-up of talent using a 200-microliter pipette to gently remove residual media from a single well while leaving the organoid intact.
- 5.3. Add 50 microliters of hypoxia media to each well [1].
 - 5.3.1. Talent dispensing 50 microliters of hypoxia media into each well of the 96 well plate.
- 5.4. Place the human cardiac organoid plate into the hypoxia chamber [1] and flush the chamber with anoxic gas composed of 5 percent carbon dioxide and 95 percent nitrogen for 3 minutes [2]. Place the hypoxia chamber in an incubator at 37 degrees Celsius for 6 hours [3].

- 5.4.1. Talent placing the 96 well plate into the hypoxia chamber and sealing it.
- 5.4.2. Close-up of the chamber being flushed with the anoxic gas mixture.
- 5.4.3. Talent placing the hypoxia chamber into the incubator set to 37 degrees Celsius.

- 5.5. To initiate reoxygenation, remove the human cardiac organoids from the hypoxia chamber [1]. Using a multichannel pipette, add 150 microliters of pre-warmed RB plus media to each well [2].
 - 5.5.1. Talent removing the plate from the hypoxia chamber and opening the chamber.
 - 5.5.2. Talent dispensing 150 microliters of pre warmed RB plus media into each well.

- 5.6. Finally, place the plate in an incubator at 37 degrees Celsius and allow at least 3 hours of reoxygenation before performing downstream assays [1]. Harvest media and organoids to perform downstream analyses including LDH release assay, calcium imaging, and calcium transient analysis to assess cell injury and functional activity [2].
 - 5.6.1. Talent placing the 96 well plate into the incubator for the reoxygenation period.
 - 5.6.2. *Reuse 2.1.5 and 2.2.1*

Results

6. Results

6.1. After 6 hours of hypoxia and 24 hours of reperfusion, extracellular lactate dehydrogenase activity was significantly increased in injured human cardiac organoids compared to uninjured controls [1]

6.1.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the bar labeled “Injured”* .

6.2. The hypoxia-treated cardiac organoids exhibited TUNEL (*tunel*) positive cardiomyocyte nuclei [1] and the percentage of TUNEL positive cardiomyocyte nuclei was significantly higher [2].

6.2.1. LAB MEDIA: Figure 4B. *Video editor: Zoom in on the image labeled “6h H/R 24hpi” and focus on the regions pointed by white arrow heads*

6.2.2. LAB MEDIA: Figure 4C. *Video editor: Highlight the bar labeled “Injured”*

6.3. A time-dependent increase in scar formation was observed with increasing duration of hypoxia prior to reoxygenation [1].

6.3.1. LAB MEDIA: Figure 6. *Video editor: Highlight the each row serially.*

6.4. At 24 hours post-reperfusion, 69 percent of uninjured human cardiac organoids were beating [1], whereas none of the injured organoids were beating [2].

6.4.1. LAB MEDIA: Figure 7A. *Video editor: Highlight the blue ‘beating’ portion of the “Uninjured” bar.*

6.4.2. LAB MEDIA: Figure 7A. *Video editor: Highlight the “Injured” bar.*

6.5. Uninjured human cardiac organoids exhibited synchronized calcium transients across five regions of interest, with peaks occurring at the same time and with similar waveforms [1].

6.5.1. LAB MEDIA: Figure 7B. *Video editor: Show the colored traces*

6.6. Injured human cardiac organoids did not exhibit coordinated contractions and instead displayed irregular spontaneous calcium transients across regions of interest [1].

6.6.1. LAB MEDIA: Figure 7C. *Video editor: Show the colored traces.*

1. Accutase
Pronunciation link: <https://www.howtopronounce.com/accutase> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /əkj'ʊ:.re.ɪs/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: aky·oo·re·is ([howtopronounce.com](https://www.howtopronounce.com))
2. pluripotent
Pronunciation link: <https://www.howtopronounce.com/pluripotent> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /pl'ʊ.ɪ.p..outənt/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: plu·rip·ohtuhnt ([howtopronounce.com](https://www.howtopronounce.com))
3. thiazovivin
Pronunciation link: <https://www.howtopronounce.com/thiazovivin> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /θ'aɪ...ə.z.ɑ:vɪvɪn/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: theye·uh·zahvivin ([howtopronounce.com](https://www.howtopronounce.com))
4. organoids
Pronunciation link: <https://www.howtopronounce.com/organoids> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /ɔ:.ɪg.æn....oʊaɪdɪ':z/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: aw·rgan·oheyedeez ([howtopronounce.com](https://www.howtopronounce.com))
5. hypoxia
Pronunciation link: <https://www.howtopronounce.com/hypoxia> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /haɪ.. 'p.ɑ:.ksɪə/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: heye·pah·kseeuh ([howtopronounce.com](https://www.howtopronounce.com))
6. reoxygenation
Pronunciation link: <https://www.howtopronounce.com/reoxygenation> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /ɪ.ɑ:.k.sɪ.dʒ..ə'neɪʃən/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: ri·ahk·si·juh·nayshuhn ([howtopronounce.com](https://www.howtopronounce.com))
7. cardiomyocyte
Pronunciation link: <https://www.howtopronounce.com/cardiomyocyte> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /kɑ:.ɪd.ɪ.'...oʊ.mɪəsɪt/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: kah·rdi·oh·mearseyet ([howtopronounce.com](https://www.howtopronounce.com))
8. cryosectioning
Pronunciation link: <https://www.howtopronounce.com/cryosectioning> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /kɹaɪ'....oʊ.skʃənɪŋ/ ([howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: kreye·oh·skshuhning ([howtopronounce.com](https://www.howtopronounce.com))
9. paraformaldehyde
Pronunciation link: <https://www.howtopronounce.com/paraformaldehyde> ([howtopronounce.com](https://www.howtopronounce.com))
IPA: /pæ.ɪ.ə.fo:.ɪm'.æɪ.dɪhaɪd/ ([zh.howtopronounce.com](https://www.howtopronounce.com))
Phonetic Spelling: pa·ruh·fo·rmal·diheyed ([zh.howtopronounce.com](https://www.howtopronounce.com))

10. ascorbic acid

Pronunciation link: <https://www.howtopronounce.com/ascorbic-acid-2>
([howtopronounce.com](https://www.howtopronounce.com))

IPA: /əsk'ɒːɪb.ɪk. æsɪd/ ([howtopronounce.com](https://www.howtopronounce.com))

Phonetic Spelling: asko·rbik·asid ([howtopronounce.com](https://www.howtopronounce.com))