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# Development of a Cell Co-Culture Model to Mimic Cardiac Ischemia/Reperfusion In Vitro

--Manuscript Draft--

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| **Article Type:** | Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE62913R3 |
| **Full Title:** | Development of a Cell Co-Culture Model to Mimic Cardiac Ischemia/Reperfusion In Vitro |
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| **Additional Information:** | |
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**TITLE:**

# Development of a Cell Co-Culture Model to Mimic Cardiac Ischemia/Reperfusion *In Vitro*

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**SUMMARY:**

Spatial distance is a key parameter in assessing hypoxia/reoxygenation injury in a co-culture model of separate endothelial and cardiomyocyte cell layers, suggesting, for the first time, that optimizing the co-culture spatial environment is necessary to provide a favorable *in vitro* model for testing the role of endothelial cells in cardiomyocyte protection.

**ABSTRACT:**

Ischemic heart disease is the leading cause of death and disability worldwide. Reperfusion causes additional injury beyond ischemia. Endothelial cells (ECs) can protect cardiomyocytes (CMs) from reperfusion injury through cell-cell interactions. Co-cultures can help investigate the role of cell-cell interactions. A mixed co-culture is the simplest approach but is limited as isolated treatments and downstream analyses of single cell types are not feasible. To investigate whether ECs can dose-dependently attenuate CM cell damage and whether this protection can be further optimized by varying the contact distance between the two cell lines, we used Mouse Primary Coronary Artery Endothelial Cells and Adult Mouse Cardiomyocytes to test three types of cell culture inserts which varied in their inter-cell layer distance at 0.5, 1.0, and 2.0 mm, respectively. In CMs-only, cellular injury as assessed by lactate dehydrogenase (LDH) release increased significantly during hypoxia and further upon reoxygenation when the distance was 2.0 mm compared to 0.5 and 1.0 mm. When ECs and CMs were in nearly direct contact (0.5 mm), there was only a mild attenuation of the reoxygenation injury of CMs following hypoxia. This attenuation was significantly increased when the spatial distance was 1.0 mm. With 2.0 mm distance, ECs attenuated CM injury during both hypoxia and hypoxia/reoxygenation, indicating that sufficient culture distancing is necessary for ECs to crosstalk with CMs, so that secreted signal molecules can circulate and fully stimulate protective pathways. Our findings suggest, for the first time, that optimizing the EC/CM co-culture spatial environment is necessary to provide a

favorable *in vitro* model for testing the role of ECs in CM-protection against simulated ischemia/reperfusion injury. The goal of this report is to provide a step-by-step approach for investigators to use this important model to their advantage.

**INTRODUCTION:**

Ischemic heart disease is the leading cause of death and disability worldwide[1,2]. However, the treatment process of reperfusion can itself cause cardiomyocyte death, known as myocardial ischemia/reperfusion (IR) injury, for which there is still no effective remedy[3]. Endothelial cells (ECs) have been suggested to protect cardiomyocytes (CMs) through the secretion of paracrine signals, as well as cell-to-cell interactions[4].

Cell co-culture models have been used extensively to investigate the role of autocrine and/or paracrine cell-cell interactions on cell function and differentiation. Among co-culture models, mixed co-culture is the simplest, where two different types of cells are in direct contact within a single culture compartment at a desired cell ratio[5]. However, separate treatments between cell types and downstream analysis of a single cell type are not readily feasible given the mixed population.

Previous studies indicated that hypoxic and ischemic insults cause significant damage to the integrity of cell membrane as measured by the release of lactate dehydrogenase (LDH). This injury is worsened upon reoxygenation, mimicking reperfusion injury[6–8]. The goal of the current protocol was to test the hypotheses that the presence of ECs can dose-dependently attenuate cell membrane leakage of CMs caused by hypoxia and reoxygenation (HR) and that the protective effect of ECs can be optimized by varying the contact distance between the two cell lines. Thus, we employed three types of cell culture inserts and Mouse Primary Coronary Artery Endothelial Cells and Adult Mouse Cardiomyocytes. The inserts, branded by Corning, Merck Millipore, and Greiner Bio-One allowed us to create three different cell culture crosstalk conditions with inter-cell line distances of 0.5, 1.0, and 2.0 mm, respectively. 100,000 ECs were plated per insert in each case.

In addition, in order to determine whether the density of ECs in co-culture contributes to HR injury attenuation in this model, we studied the dose-response relationship between EC concentration and LDH release by CMs. ECs were plated at 25,00050,000 and 100,000 per insert, respectively, in the 2.0 mm insert.

This report provides a step-by-step approach for investigators to use this important model to their advantage.

**PROTOCOL:**

## 1. Experimental preparation/plating

1.1. Maintain CMs and ECs according to the manufacturer's instructions.

1.1.1. Thaw both cell lines when they arrive from vendors. Plate in T25 flasks after being washed with fresh media. It is recommended to purchase each cell culture media from the same vendors the cells were purchased from. The next day, refresh the cells with media and use when confluent.

1.1.2. Maintain the cell culture incubator at 37 °C with 21% $O_2$, 5% $CO_2$, 74% $N_2$ and keep it humidified.

NOTE: The Mouse Primary Coronary Artery Endothelial Cells used in this protocol are isolated from coronary arteries of C57BL/6 mice, and Adult Mouse Cardiomyocytes are isolated from adult C57BL/6J mouse hearts and commercially obtained (see **Table of Materials**).

1.2. Plate CMs under sterile conditions onto the bottom of 24-well plates (inferior layer). 24 h later, plate ECs into the insert (or superior portion) of the co-culture well. 24 h after EC plating, place EC inserts onto CM plate bases, starting the co-culture period. Allow the cells to co-culture for at least 12–24 h before use. These steps are described in detail below.

1.2.1. Estimate CM cell line confluency under a light microscope. When cells appear to be 90%–100% confluent in culture flasks, proceed with experimental plating.

1.2.2. Remove the media from the flasks containing confluent cell cultures and trypsinize with 3–5 mL of Trypsin/Ethylenediaminetetraacetic acid (EDTA) for T25 flasks. Agitate the flask gently, incubate at 37 °C for 2–5 min, and then assess the enzymatic progress under a light microscope. Once the cells start to round up, use a cell scraper to detach the cells from the surface.

1.2.3. After detachment, inactivate the trypsin solution by adding the trypsin/cell solution to a 50 mL tube containing 10 mL of media for T25 flasks. Centrifuge the cell suspension at 120 x *g* for 2 min to obtain a soft cell pellet. Remove the supernatant and resuspend the pellet in 5 mL of media.

1.2.4. To perform cell counting and confirm cell viability, mix an aliquot of 10 µL of resuspended cells and 10 µL of trypan blue dye. Count the living cells using a cell counter. Dilute the cells with fresh regular media to achieve the desired seeding densities. Volumes are calculated depending on the desired seed density and cell concentration of stock solutions. All plating should be performed under sterile conditions.

1.2.5. Plate CMs at seeding densities of 300,000 per well onto the bottom of a 24-well plate pre-coated with extracellular matrix. Maintain the cells at 37 °C with 5% $CO_2$ overnight.

1.2.6. After 24 h, plate ECs into inserts at an optimal plating density of 100,000 per insert (culture area is 33.6 $mm^2$), (**Figure 1**). After 24 h of EC plating, place EC inserts inside the CM wells, initiating co-culture. Allow cells to co-culture for 12–24 h before performing the experiments.

1.2.7. Ensure that, by the time experiments are performed, both CMs and ECs have reached 80%–90% confluency.

## 2. Hypoxia/reoxygenation to simulate ischemia/reperfusion injury *In Vitro*

NOTE: The following steps need to be performed as described, do not pause in-between.

2.1. Prepare the hypoxic media by pouring ~25 mL of media in a 50 mL conical tube. Air-seal the top with a silicone membrane and use a sterilized pipette to punch a hole. Create another hole, this time leaving the pipette submerged approximately 2/3 into the media.

2.2. Flush the media with hypoxic gas (0.0125% $O_2$, 5% $CO_2$, 94.99% $N_2$) for 5 min at a flow rate of 30 L/min. This minimizes $O_2$ dissolved in the media when hypoxia starts (step 2.5).

2.3. Discard the media of the 24-well plates or culture inserts containing attached cells and wash with 100 µL/well of 10% Phosphate Buffered Saline (PBS) very gently. Afterwards, add 500 µL of the freshly prepared hypoxic media to each well of the plates or inserts.

NOTE: Hypoxia in the media is interrupted as briefly as possible during this step before true hypoxia for cells and media starts in step 2.5.

2.3.1. In the normoxic control group, replace the original media with fresh normal media containing glucose and serum.

2.4. Humidify the hypoxia chamber by placing a Petri-dish filled with sterile water within the chamber. Next, place the plates comprising the hypoxic groups into the chamber.

2.5. Flush the hypoxia chamber with hypoxic gas (0.0125% $O_2$, 5% $CO_2$, 94.99% $N_2$) for 5 min at a flow of 30 L/min. Place the chamber in the 37 °C incubator for 24 h.

2.6. After hypoxia, cultivate CMs and ECs under normal conditions. To do so, discard the old media of the plates/inserts, replace it with normal media (containing glucose and serum; 500 µL of each well/insert) and store it in the incubator under normal culture conditions (21% $O_2$, 5% $CO_2$, 74% $N_2$, 37 °C) for 2 h to mimic reperfusion.

2.7. To control for any effects of media replacement, change the media of normoxic cells at the same time as the hypoxic media is changed.

NOTE: **Figure 2** provides a schematic overview of this protocol.

## 3. Endpoint assessment

3.1. Transfer the cell culture media from the 24-well plate into a 96-well plate. Use 200 µL of media from each well of the 24-well plate and equally distribute into four wells of the 96-well

plate, accordingly. Use one plate each for normoxia versus hypoxia versus HR.

3.2. Determine the degree of cellular injury, e.g., by measuring absorbance for LDH using a cytotoxicity assay kit following the manufacturer's instructions. Perform the assay in a 96-well plate as instructed in the assay protocol.

## 4. Statistics

4.1. Display the parametric data as mean/standard deviation and non-parametric data as box plots with median/interquartile range. Adequate parametric versus non-parametric multiple comparisons tests with acceptable post-hoc tests to decrease the chance for a type-1 error should be performed. Significance is typically set at an alpha of 0.05 (two-tailed).

4.2. For all the experiments carried out in the current study, perform at least three well replicates within one experiment. There were three to six repetitions of each experiment used for statistical analysis.

## REPRESENTATIVE RESULTS:

All three types of inserts (A, B, C) used in this experiment have the same pore size of 0.4 µm. The only difference among them is the insert-to-base height, which allows distances between the two co-cultured cell layers to be 0.5, 1.0 and 2.0 mm, respectively, (**Figure 3**) and that they are from different vendors (for details see **Table of Materials**).

To establish an *in vitro* co-culture model with separate layers of two cell lines undergoing HR to simulate IR injury, we examined the cell membrane integrity of CMs co-cultured with or without ECs. Utilizing a separate insert for ECs that can be placed at varied distances above the CM layer allowed us to also assess the differential effects of cell layer distance on the severity of membrane damage in the CMs. In a separate set of experiments, we varied the density of ECs and thus the ratio of ECs to CMs. In addition, to differentiate simulated ischemia from simulated IR injury, experiments were carried out under conditions of 1) normoxia, 2) hypoxia only, and 3) HR. For this model, we used 24 h hypoxia, followed by 2 h reoxygenation.

### Variable distances

### 0.5 mm distance between the cell layers (Insert A)

When CMs were cultured alone, hypoxia led to a significantly increased LDH release compared to normoxia, consistent with our previous studies (**Figure 4A**)[6]. However, LDH was only mildly further increased upon 2 h reoxygenation compared to the hypoxia-only group. When ECs and CMs were co-cultured together at a 0.5 mm distance, the increase in LDH release by hypoxia only was not attenuated indicating the ECs did not show any protective effect (compared to CMs alone group under the hypoxia-only conditions). However, ECs exerted a mild but significant protection on CMs during HR.

### 1.0 mm distance between the cell layers (Insert B)

The same experiment was performed as above, except that the distance between the two cell layers was increased to 1.0 mm (**Figure 4B**). We found that the LDH release was also significantly increased in CMs-only by hypoxia-only. However, unlike in the 0.5 mm insert, the LDH increase in CMs-only was potentiated by HR over hypoxia-only. Moreover, this potentiation was more inhibited and almost abolished by the presence of the ECs during reoxygenation compared to the CMs-only group.

**2.0 mm distance between the cell layers (Insert C)**

When co-culture inserts that create a distance of 2.0 mm between the two cell lines (**Figure 4C**) were used, we also found a significant increase of LDH release by CMs-only during hypoxia, to the same degree as in the 0.5 mm- and the 1.0 mm-experiments. Interestingly, however, the additional increase in LDH release by reoxygenation was even more pronounced than in the 1.0 mm experiments. Both of these increases were attenuated, though not abolished, by the presence of ECs, indicating that different to using 0.5 or 1.0 mm inserts, ECs significantly protected CMs from injury under both hypoxia-only and HR conditions.

**Variable EC intensities**

In a different set of experiments, the concentration of ECs plated in 2.0 mm inserts was titrated to 25,000, 50,000 and 100,000 cells per insert, respectively. LDH release was measured after 24 h hypoxia followed by 2 h reoxygenation. Our results showed that increasing EC intensity in the co-culture led to a dose-dependent attenuation of LDH release caused by HR (**Figure 5**); no such effect was seen under normoxic conditions.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Schematic of insert inside the well.** The inserts with the ECs (up to 100,000 cells per insert) are transferred to the 24-well plates with the CMs (300,000 per well) on their bottom.

**Figure 2: Experimental design.** Cells are cultured under normal oxygen conditions (95% $O_2$), and then split into two groups: a normoxic control group will continue to be cultured under normal conditions for another 24 h, whereas the hypoxia group will be cultured in a hypoxia chamber for 24 h with only 0.01% $O_2$ and no glucose. After these 24 h interventions, both groups of cells are refreshed with media and cultured continually in a 95% $O_2$ environment for another 2 h, the reoxygenation phase, before eventually conducting the endpoint assay(s), e.g., LDH analysis.

**Figure 3: Images and schematics of the three different inserts.** The distance between the endothelial cells in the insert and the cardiomyocytes at the bottom of the well can be varied by using different inserts. All three types of inserts used here have the same pore size of 0.4 µm. The only difference among them is the insert-to-base height, which allows the distances between the two co-cultured cell layers to be 0.5 (**A**), 1.0 (**B**), and 2.0 mm (**C**), respectively.

**Figure 4: Comparison of three types of culture inserts in assessing the release of lactate dehydrogenase (LDH; in absorbance units [au]) under normoxic (left), hypoxia-only (center), and hypoxia/reoxygenation conditions (right) for cardiomyocytes alone (CM; white), endothelial cells alone (EC; black), and co-cultures of CM with EC (check pattern).** (**A**) 0.5 mm

distance, (**B**) 1.0 mm distance, (**C**) 2.0 mm distance between the two different cell layers. ECs were plated at a density of 100,000 cells per insert, CMs at a density of 300,000 cells per well. Data are mean ± standard deviation, n = 4 per group. Statistics: ANOVA followed by Student-Newman-Keuls post-hoc test. $P < 0.05$ (two-tailed) indicated by horizontal bars between each compared pair.

**Figure 5: Concentration of co-cultured endothelial cells** (EC; 25: 25,000 cells per insert; 50: 50,000 cells per insert; 100: 100,000 cells per insert) dose-dependently affected protection of cardiomyocytes (CM) against hypoxia/reoxygenation injury (right) compared to normoxic conditions (left) as evidenced by release of lactate dehydrogenase (LDH; in absorbance units [au]). ECs had no effect on LDH release under normoxic conditions. Data are mean ± standard deviation, n = 4 per group. Statistics: ANOVA followed by Student-Newman-Keuls post-hoc test. $P < 0.05$ (two-tailed) indicated by horizontal bars between each compared pair.

## DISCUSSION:

### Critical steps in the protocol

Cell co-culture models have been used to study cellular mechanisms of cardioprotection. How to create two separate layers with a meaningful distance between them is, thus, crucial for the development of a suitable co-culture model. A challenge in studying simulated ischemia/reperfusion (IR), i.e., HR, injury is that not only ischemia (hypoxia) itself but also reperfusion (reoxygenation) aggravates cellular dysfunction. Therefore, a realistic model needs to reflect these characteristics by, for example, demonstrating sufficiently increased injury by reoxygenation following hypoxia as opposed to hypoxia alone, but still allowing attenuation of injury with cell-protective drugs or strategies such as, in our case, the presence of ECs. Interestingly, the distance between the two cell layers appears to play an important role in developing a suitable co-culture model. We were able to demonstrate that a 1.0 mm and a 2.0 mm distance between ECs and CMs allowed the expected HR injury response, but, more importantly, that only 2.0 mm yielded a consistently favorable outcome for cellular injury protection by the presence of ECs under both hypoxic and HR conditions.

### Modifications and troubleshooting of the method

Cell-cell interactions in co-culture models are affected by multiple variables such as the number of distinct co-cultured populations, the degree of similarity of the cell types, the degree of physical separation between them, the difference between population local environments, the volume of cultures and timing consideration of the co-culture[9–12]. There exist trade-offs between these factors. These interactions are strongly affected by the environment, which in turn is determined by the protocol and set-up. It is critical to develop experimental models that are replicable, measurable, and comparable. Our approach was to control and limit the number of variables that may disrupt the consistence of the data obtained from different experiments conducted at different times through plating an accurate number of cells, using the same ratio of two co-cultured cell lines, the same volumes of the cell culture media and assay reagents, etc., with the exception of the three different cell culture inserts.

### Limitations of the method

Our model works well with ECs and CMs, likely owed to the ubiquitous presence of ECs in the cardiovascular system. How well other types of cell lines of potential interest interact with CMs, neurons, or other cells of interest to be studied *in vitro* is yet to be evaluated as different cell lines could interact differently in the co-culture environment. One difference between the co-cultured cell populations could be their ecological relationship, e.g., if they are natural competitors or cooperators. Also, as in all other *in vitro* cell models, the generated cellular damage in the experiment might not represent the actual pathophysiological state of injury *in vivo*. Furthermore, we acknowledge that the tested distances between ECs and CMs of 0.5, 1.0 and 2.0 mm do not reproduce their spacing *in vivo*; for the given *in vitro* model; however, our results describe the implications of optimal distancing between two cell layers to be studied by scientists who would like to take advantage of this technique.

**The significance of the method with respect to existing/alternative methods**

The existing/alternative co-culture methods include direct mixing of cell lines or creating separated environment with a degree of separation such as using transwell plates[13], microfluidic platforms[14] or three-dimensional scaffolds[15], among which transwell plates are the least expensive and easiest available. Despite natural limitations, co-cultures are highly relevant for drug research because they provide a more representative *in vivo*-like tissue model and allow for high-throughput testing and in-depth monitoring of drug effects on cell-cell interactions[16]. Our model was accurately calibrated using well-defined spatial distances between ECs and CMs, instead of a complete and uncontrolled mixture of two different types of cells. Moreover, this model allows separate treatments and analyses individual types of cells, which is impossible to achieve in a mixed co-culture, not to mention *in vivo*. Because IR injury involves separate HR processes, this model is well suited to studying the cellular mechanisms of ischemia/hypoxia vs reperfusion/reoxygenation damage separately. Our data suggest that it may be a highly valuable and efficient cell co-culture model to be used in simulated IR injury studies. Our observations clearly indicate that an optimal spatial distance, in our case 2.0 mm, is necessary for ECs to crosstalk with CMs in this specific co-culture model, so that potentially secreted signaling molecules by the ECs can circulate effectively to illicit protection of CMs against injury by hypoxia-only and/or HR.

**Importance and potential applications of the methods in specific research areas**

Our model indicates the significance of combining mouse coronary artery ECs with mouse CMs to improve the investigation of protective measures against HR injury; that varying the spatial distance of co-cultures demonstrates an effective way to better understand the spatial conditions affecting the protective action of ECs on CM injury caused by HR; and that the density of ECs or the EC-to-CM ratio is positively correlated to the extent of protection against HR injury.

**ACKNOWLEDGMENTS:**

This work was supported, in part, by the US Department of Veterans Affairs Biomedical Laboratory R&D Service (I01 BX003482) and by institutional funds to M.L.R.

**DISCLOSURES:**

The authors declare no conflicts of interest.

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Figure 1



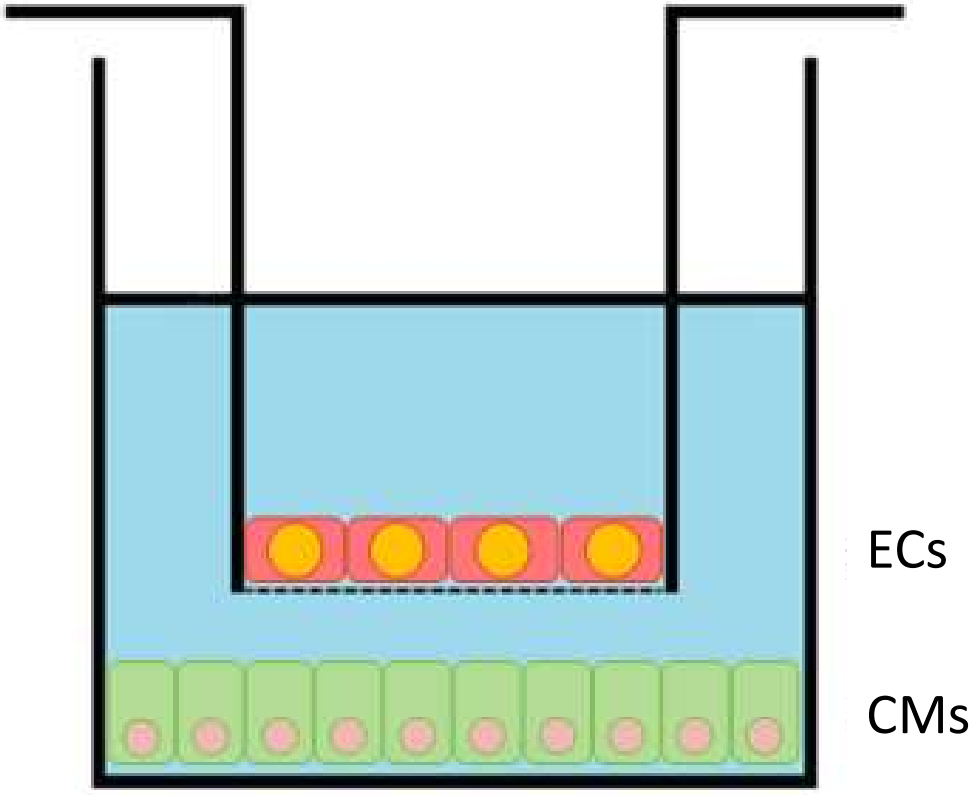
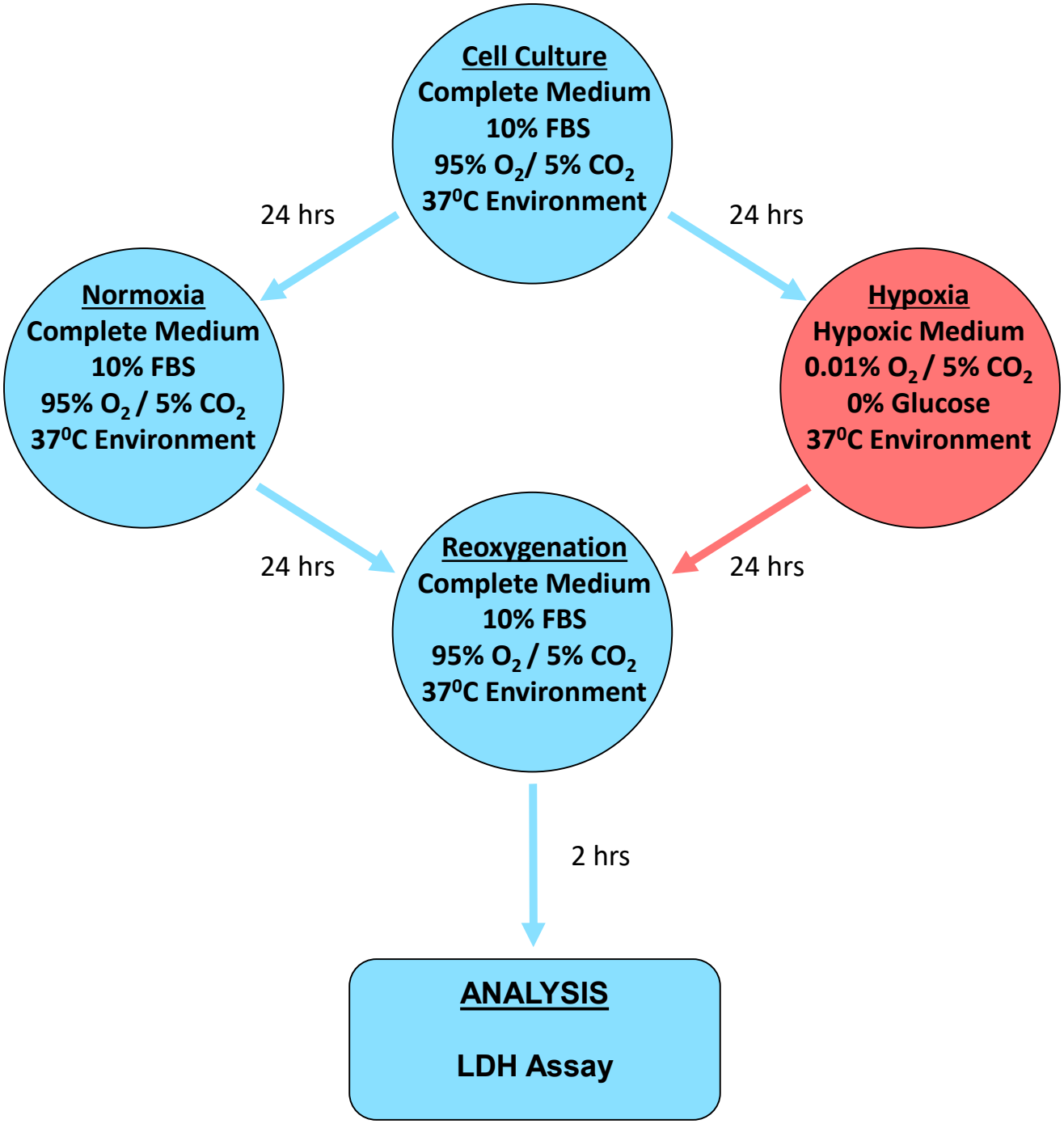


Figure 2





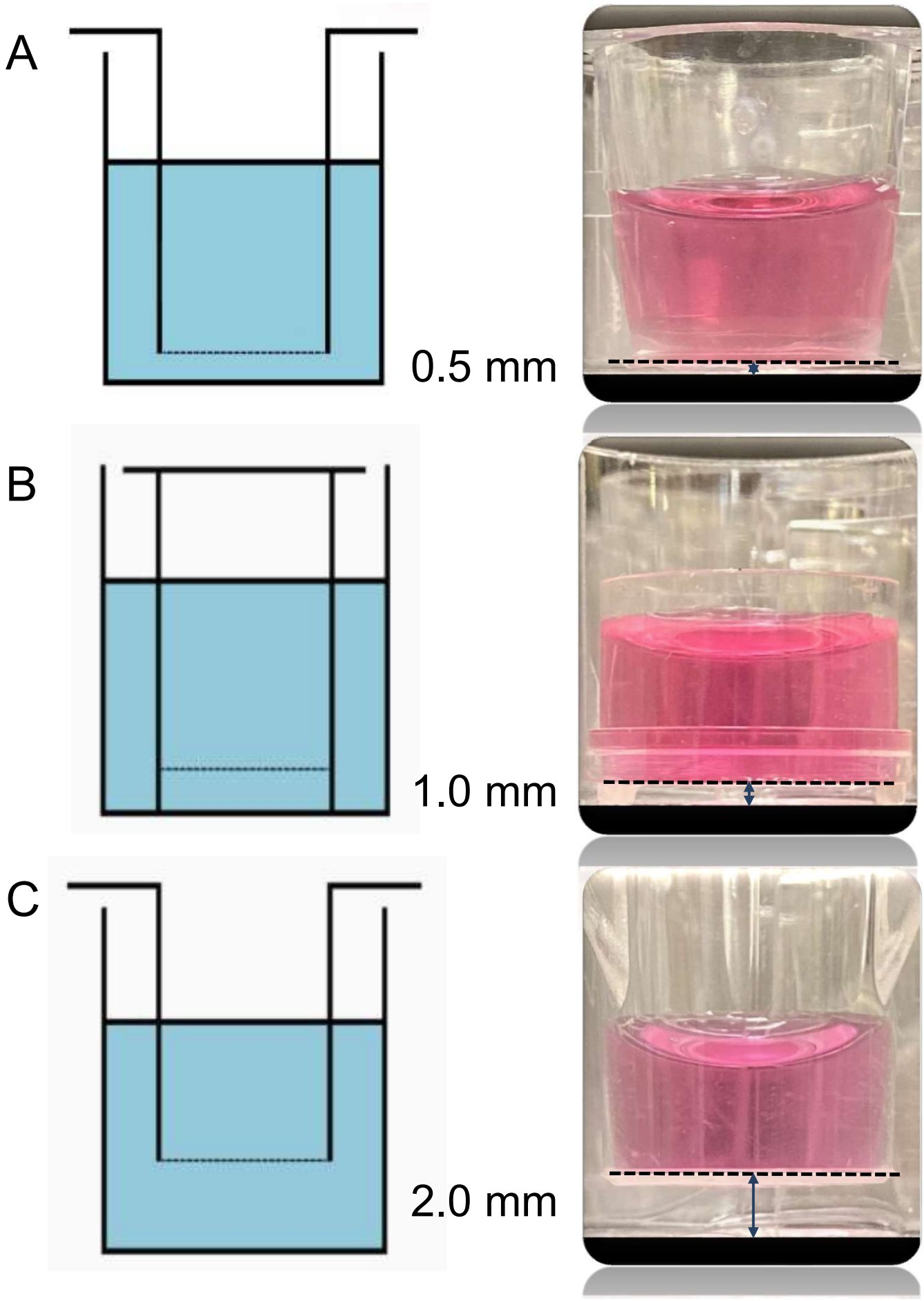


Figure 4



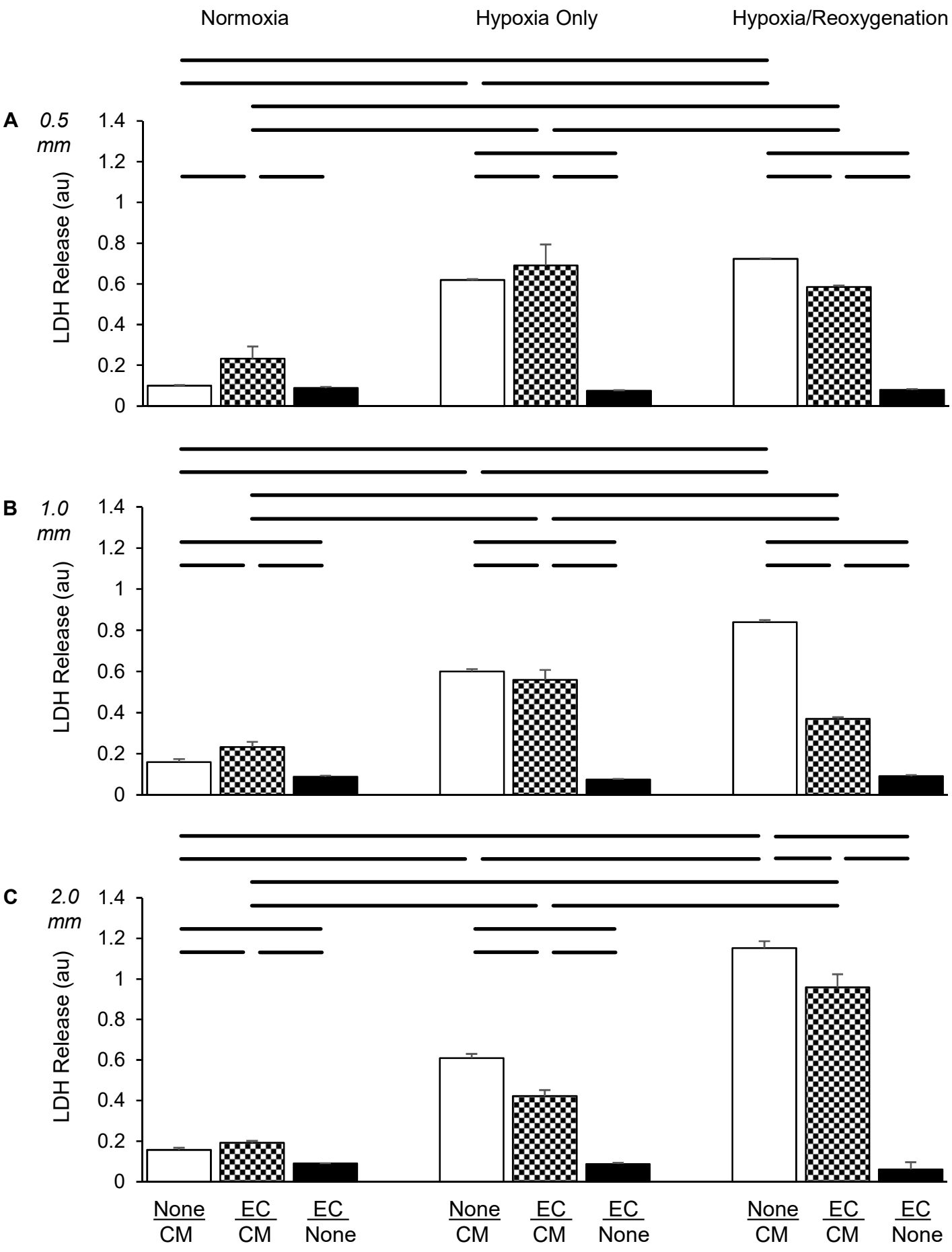
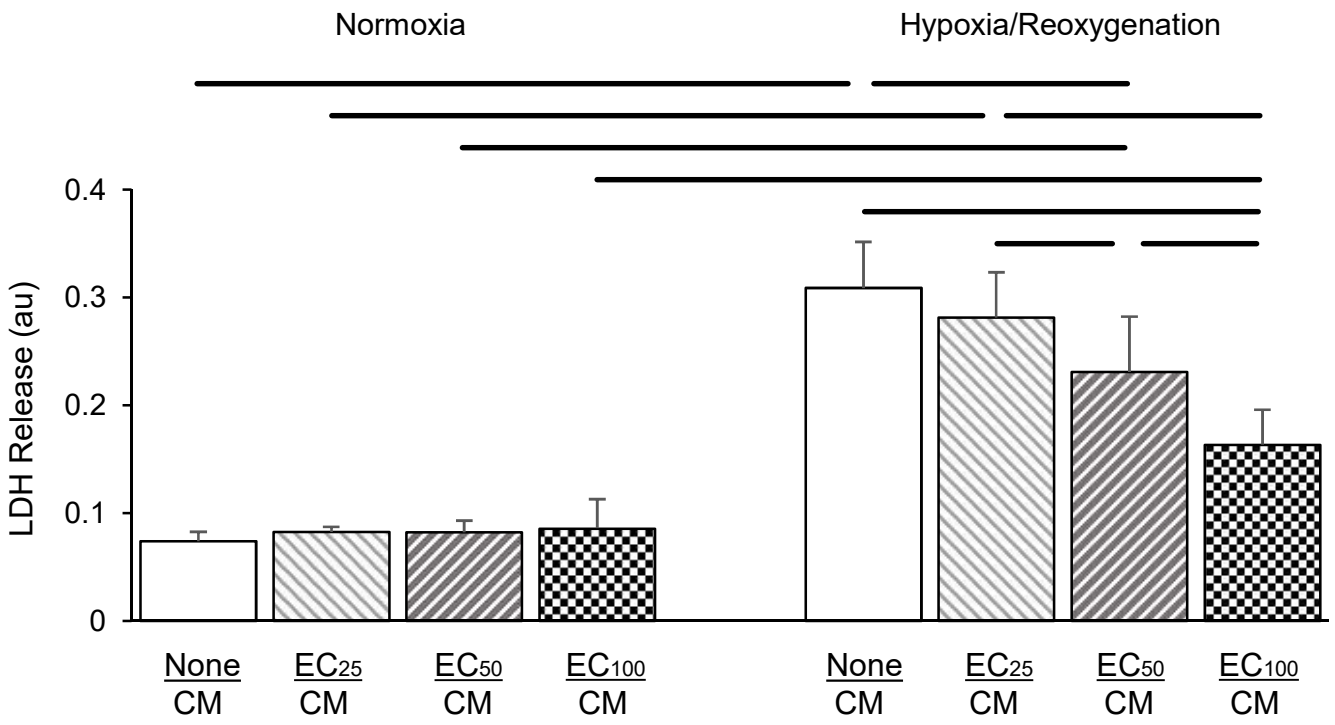


Figure 5





Revised Table of Materials

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**Table of Materials**

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We thank the editor for the additional questions, comments and suggestions of our re-revised manuscript submission R2. We have re-re-revised the manuscript according to all suggestions (all additions in the text are highlighted using the "Track Changes" function) including minor formatting changes for consistency. The following response addresses the questions, comments and suggestions in a point-by-point manner:

**Editorial and production comments:**

*Former A1: Affiliations should be in order as per our journal's style. So Dr Zhu's affiliation will be 1.*
Response: Due to the laboratory's funding by the VA, it is expected to name this affiliation first. We have checked with VA leadership: they are willing to grant this request. The corresponding changes have been made.

*Former A2: Unfortunately, the production quality of the video is not of publication grade. For example, there is a lot of pillar boxing and handheld recording., etc. We therefore kindly request you to switch to JoVE produced video. You have two options in this case: 1. JoVE produced video: In this case, after the manuscript is accepted, we will write the script, we will send a videographer to your lab to film the procedure and then we will produce the video for you. 2. APF: This is our hybrid filming option where upon manuscript acceptance we will generate the script for you. You will film as per the script generated and send us all the raw files straight out of the camera. We will then produce the video for you. Please let me know how you would like to proceed. I have highlighted the steps that can be used for generating the script. Please review and edit/approve.*
Response: Based on the email communication with Benjamin Werth (9/10 to 9/13/2021), we agree to have the submission changed to a JoVE-produced video. The filming address is the same as was provided in the original submission's front page: Department of Anesthesiology, Vanderbilt University Medical Center, 1161 21st Avenue South, T4217 MCN, Nashville, TN 37232-2520, USA. The yellow selection appears reasonable.

VANDERBILT UNIVERSITY MEDICAL CENTER

*Matthias Riess, M.D., Ph.D., FASA*
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September 22, 2021

**Submission of Re-Revised Manuscript 62913R3**

Dear Members of the JoVE Editorial Board:

On behalf of my co-authors Zhu Li, Matthew J.W. Hampton, Matthew B. Barajas and myself, I am submitting a third revision of the original article entitled "*Development of a Cell Co-Culture Model to Mimic Cardiac Ischemia/Reperfusion in Vitro*" for publication in JoVE's special methods collection "Current and novel experimental methods in ischemia/reperfusion research" that I am guest-editing.

We have re-re-revised the manuscript according to all suggestions (all additions in the text are highlighted using the "Track Changes" function). Since the last revision of the accompanying video still has not been deemed acceptable, we agree to switching this submission to a 'JoVE-produced video' as agreed by email with Benjamin Werth (communications 9/10 to 9/13/2021).

Our manuscript has not been submitted for publication nor has it been published in whole or in part elsewhere. I attest to the fact that all authors listed on the title page significantly contributed to and have read the manuscript, attest to the validity and legitimacy of the data and their interpretation, and agree to its submission in the current form. I acknowledge that all authors have read the Instructions for Authors and agree with its contents. Copyright transfer and – if needed – the signatures of all authors will be provided prior to publication of the accepted manuscript.

Please do not hesitate to contact us if further information is needed. We are looking forward to hearing from you.

Respectfully yours,

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