FINAL SCRIPT: APPROVED FOR FILMING

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Title: Modeling Hypoxia/Reoxygenation Injury in Proximal Tubular Epithelial Cells

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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? **Yes**

If **Yes**, how far apart are the locations? 14 km

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: 17
Number of Shots: 30

Introduction

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

INTRODUCTION:

1.1. **Mariano Marin-Blazquez:** Our research focuses on modeling renal ischemia–reperfusion injury in proximal tubular cells to understand damage mechanisms and explore therapeutic strategies.

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

1.2. **Mariano Marin-Blazquez:** Reproducing the complex ischemic environment of the kidney in vitro remains difficult, especially cold ischemia and immune interactions.

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

CONCLUSION:

1.3. **Mariano Marin-Blazquez:** There is a lack of standardized, cell-based models to study kidney ischemia–reperfusion injury under controlled metabolic conditions.

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

1.4. **Mariano Marin-Blazquez:** Our protocol is simple, cost-effective, and reproducible, requiring only standard laboratory equipment and a hypoxia incubator.

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

1.5. **Mariano Marin-Blazquez:** Identifying novel therapeutic targets and validating protective compounds using both in vitro and in vivo kidney 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. Hypoxia/Reoxygenation Procedure and RT-qPCR

Institution: Universidad Católica de Murcia (UCAM)

Video Editor: Here, instead of Demonstrator, we have added the Institution name and this is an exception that has been approved.

2.1. To begin, remove the culture medium from the IM-PTECs *(I-M-P-T-E-Cees)* to refresh the cells **[1-TXT]**. Add 2 milliliters of fresh L3 medium without interferon gamma into the wells of a 6-well plate **[2-TXT]**. Place the cells in a hypoxia incubator set at 1 percent oxygen and 5 percent carbon dioxide at 37 degrees Celsius for 48 hours **[3]**.

2.1.1. WIDE: Talent aspirating the spent culture medium from the IM-PTEC wells. **TXT: IM-PTECs: Immortalized Murine Proximal Tubular Epithelial Cells**

2.1.2. Talent dispensing fresh L3 medium without interferon gamma into each well of the culture plate. **TXT: Add 0.1 mL/well for 96-well plates**

2.1.3. Talent placing the culture plates inside the hypoxia incubator.

2.2. After incubation, remove the IM-PTEC plates from the hypoxia incubator **[1]** and replace the medium with fresh L3 medium without interferon gamma **[2]**. Place the cells into a standard cell culture incubator under normal oxygen conditions and 5 percent carbon dioxide at 37 degrees Celsius for 24 hours **[3]**.

2.2.1. Talent removing the IM-PTEC culture plates from the hypoxia incubator.

2.2.2. Talent adding fresh L3 medium without interferon gamma.

2.2.3. Talent placing the refreshed plates in the incubator and closing the door to start incubation.

2.3. To perform RT-qPCR *(R-T-Q-P-C-R)*, prepare the primer sets by mixing them to a final concentration of 10 micromolar **[1-TXT]**.

2.3.1. Talent pipetting and mixing primer solutions in a microcentrifuge tube. **TXT: RT-qPCR: Real-Time Quantitative Polymerase Chain Reaction**

2.4. Prepare the reaction mix by combining the required reagents along with primers and nuclease-free water per well **[1-TXT]**. Mix gently to ensure homogeneity **[2]**.

2.4.1. Talent pipetting reagents into a microcentrifuge tube labeled RT-qPCR mix. **TXT: 5 µL RT-qPCR Master Mix; 0.4 µL 10 µM forward primer; 0.4 µL 10 µM reverse**

primer; 2.2 µL nuclease-free H_2O

2.4.2. Talent gently flicking the tube to mix the solution uniformly.

2.5. Using a pipette, dispense 8 microliters of the RT-qPCR mix into each well of a 384-well PCR plate **[1]** and add 2 microliters of the complementary DNA sample to each well, allowing a small droplet to remain on the edge of the well **[2]**.

2.5.1. Talent dispensing 8 microliters of RT-qPCR mix into each well of the PCR plate.

2.5.2. Shot of pipetting 2 microliters of complementary DNA sample into the same wells, showing a drop on the edge of each well.

2.6. After all samples have been added, tap the plate gently until all droplets fall completely into the wells **[1]** and seal the plate firmly with its membrane **[2]**. Spin the 384-well PCR plate to remove air bubbles and ensure even distribution **[3]**.

2.6.1. Talent gently tapping the PCR plate to ensure liquid settles into each well.

2.6.2. Talent sealing the 384-well plate with a transparent adhesive membrane.

2.6.3. Talent placing the plate into a centrifuge and initiating the spin cycle.

2.7. Now, place the sealed plate into the RT-qPCR System **[1]**. Set the program **[2]** and start the measurement **[3]**.

2.7.1. Talent loading the sealed 384-well plate into the RT-qPCR machine.

2.7.2. TEXT ON PLAIN BACKGROUND:

- 98 °C for 30 s
- 40 cycles of 98 °C for 10 s
- 60 °C for 30 s
- Melting curve at 95 °C.

2.7.3. Talent clicking Start to initiate the RT-qPCR run.

3. Mitochondrial Respiratory Function Assessment

Institution: Servicio de Cultivo de Tejidos (ACTI)

Video Editor: Here, instead of Demonstrator, we have added Institution name and this is an exception that has been approved.

3.1. Turn on the oxygen consumption rate and extracellular acidification rate analyzer to let it warm up for at least 5 hours before starting the experiment **[1]**.

3.1.1. Talent switching on the OCR/ECAR analyzer.

3.2. Hydrate a sensor cartridge overnight at 37 degrees Celsius in a non–carbon dioxide incubator **[1]**.

3.2.1. Talent placing the sensor cartridge into a hydration plate and transferring it into an incubator set at 37 degrees Celsius.

3.3. On the next day, prepare the assay medium for one 96-well cell culture microplate by adding Dulbecco's Modified Eagle Medium with appropriate supplements **[1-TXT]** and mixing gently until homogeneous **[2]**.

3.3.1. Talent pipetting reagents into a sterile tube labeled "Assay Medium." **TXT: DMEM medium pH 7.4: 33.4 mL; 1 M Glucose: 0.875 mL; 100 mM Pyruvate: 0.35 mL; 200 mM: Glutamine: 0.35 mL**

3.3.2. Talent gently inverting or pipetting the tube to mix the solution evenly.

3.4. Next, prepare the inhibitor solutions by mixing the required components **[1]**.

3.4.1. LAB MEDIA: Table 3.

3.5. Then, remove the hydrated sensor cartridge from the incubator **[1]**. Using a multichannel pipette and a loading guide, load 25 microliters of each inhibitor into its corresponding port **[2]**.

3.5.1. Talent taking out the assembled sensor cartridge and placing it on a sterile bench.

3.5.2. Talent using a multichannel pipette to dispense 25 microliters of inhibitor into a port.

3.6. Now, aspirate the growth medium from the cell culture microplate **[1]** and add 175 microliters of assay medium tempered at 37 degrees Celsius into each well using a multichannel pipette **[2]**.

3.6.1. Talent removing the old growth medium from the 96-well plate using a multichannel pipette.

3.6.2. Talent dispensing 175 microliters of the pre-warmed assay medium into each well.

3.7. Place the cell culture microplate in a 37-degrees Celsius non–carbon dioxide incubator and incubate for 45 to 60 minutes before starting the assay **[1]**.

3.7.1. Talent placing the plate inside the non–carbon dioxide incubator and closing the door.

3.8. After incubation, insert the calibration plate containing the loaded sensor cartridge into the analyzer for calibration **[1]**.

3.8.1. Talent loading the calibration plate with the sensor cartridge into the analyzer.

3.9. Once calibration is complete, place the cell culture microplate into the analyzer for measurement **[1]**.

3.9.1. Talent inserting the prepared 96-well cell culture plate into the analyzer.

3.10. After the measurement run, remove and save the cell culture microplate for subsequent quantification of total protein for data normalization **[1]**.

3.10.1. Talent removing the plate from the analyzer.

Results

4. Results

4.1. Forty-eight hours of hypoxia followed by twenty-four hours of reoxygenation produced the greatest increase in caspase-3 activity **[1]** while maintaining cell viability comparable to that of normoxic controls **[2]**.

4.1.1. LAB MEDIA: Supplementary Figure 1. *Video editor: Highlight the bar showing 48H 24R in graph A*.

4.1.2. LAB MEDIA: Supplementary Figure 1. *Video editor: Highlight the bar for H/R in B graph*.

4.2. After hypoxia and reoxygenation, there was a significant increase in the expression of the kidney injury markers Kim-1 *(Kim-1)* and Ngal *(N-G-A-L)* **[1]**, as well as in the expression of markers of renal partial epithelial-to-mesenchymal transition and fibrosis, Acta2 *(act-uh-2)* and Col1a1 *(col-1-A-1)* **[2]**.

4.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the bars labelled "H/R" for Kim-1 and Ngal*

4.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight the bar labelled "H/R" for Acta2 and Col1a1*

4.3. Mitochondrial respiration analysis showed that, after the ischemic challenge, all respiration parameters—including basal respiration **[1]**, ATP production **[2]**, proton leak **[3]**, maximal respiration **[4]**, and spare capacity were reduced, indicating hindered mitochondrial metabolism **[5]**.

4.3.1. LAB MEDIA: Figure 3. *Video editor: Focus on the "Basal respiration" line for H/R* .

4.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight the "ATP production" line for H/R* .

4.3.3. LAB MEDIA: Figure 3. *Video editor: Show the "Proton leak" region under the pink H/R line*.

4.3.4. LAB MEDIA: Figure 3. *Video editor: Highlight the "Maximal respiration" line for H/R*.

4.3.5. LAB MEDIA: Figure 3. *Video editor: Indicate the "Spare capacity" line for H/R*.

- Hypoxia

Pronunciation link: https://www.merriam-webster.com/dictionary/hypoxia
IPA: /haɪˈpɑksiə/
Phonetic Spelling: hy-POK-see-uh

- Incubator

Pronunciation link: https://www.merriam-webster.com/dictionary/incubator
IPA: /ˈɪnkjəˌbeɪtər/
Phonetic Spelling: IN-kyoo-bay-ter

- Interferon

Pronunciation link: https://www.merriam-webster.com/dictionary/interferon
IPA: /ˌɪntəˈfɪərɒn/ or /ˌɪntərˈfɪərɒn/ (US)
Phonetic Spelling: in-ter-FEE-ron

- Microliter

Pronunciation link: https://www.merriam-webster.com/dictionary/microliter
IPA: /ˈmaɪkroʊˌliːtər/
Phonetic Spelling: MY-kroh-lee-ter

- Nuclease

Pronunciation link: https://www.merriam-webster.com/dictionary/nuclease
IPA: /ˈnuːˌkleɪs/ or /nuːˈkleɪs/
Phonetic Spelling: NOO-klays

- Polymerase

Pronunciation link: https://www.merriam-webster.com/dictionary/polymerase
IPA: /ˈpɑːləməˌreɪz/
Phonetic Spelling: PAH-luh-muh-rayz

- Quantitative

Pronunciation link: https://www.merriam-webster.com/dictionary/quantitative
IPA: /ˈkwɑntɪˌteɪtɪv/
Phonetic Spelling: KWANT-i-tay-tiv

- Primer

Pronunciation link: https://www.merriam-webster.com/dictionary/primer
IPA: /ˈpraɪmər/
Phonetic Spelling: PRY-mer

- Pipette

Pronunciation link: https://www.merriam-webster.com/dictionary/pipette
IPA: /ˌpɪˈpɛt/ or /pəˈpɛt/
Phonetic Spelling: pi-PET or puh-PET

- Microcentrifuge

Pronunciation link: No confirmed link found (specialized term)
IPA (approx): /ˌmaɪkroʊˈsɛntrɪˌfjuːdʒ/
Phonetic Spelling: MY-kroh-SEN-tri-fyo͞oj

- Centrifuge

Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːdʒ/
Phonetic Spelling: SEN-truh-fyo͞oj

- Multichannel

Pronunciation link: https://www.merriam-webster.com/dictionary/multichannel
IPA: /ˌmʌltiˈtʃænəl/
Phonetic Spelling: MUL-tee-CHAN-uhl

- Epithelial

Pronunciation link: https://www.merriam-webster.com/dictionary/epithelial
IPA: /ˌɛpɪˈθiːliəl/
Phonetic Spelling: ep-ih-THEE-lee-uhl

- Murine

Pronunciation link: https://www.merriam-webster.com/dictionary/murine
IPA: /ˈmʊəriːn/ or /ˈmjʊrɪn/
Phonetic Spelling: MUR-een

- Immortalized

Pronunciation link: https://www.merriam-webster.com/dictionary/immortalize (base verb)
IPA (as adjective): /ɪˈmɔrtəlˌaɪzd/
Phonetic Spelling: ih-MOR-tuh-lyzed

- Proximal

Pronunciation link: https://www.merriam-webster.com/dictionary/proximal
IPA: /ˈprɑksɪməl/
Phonetic Spelling: PRAHK-si-muhl

- Tubular

Pronunciation link: https://www.merriam-webster.com/dictionary/tubular
IPA: /ˈtuːbjələr/ or /ˈtjuːbjələr/ (US)
Phonetic Spelling: TOO-byoo-lur

- Medium

Pronunciation link: https://www.merriam-webster.com/dictionary/medium
IPA: /ˈmiːdiəm/
Phonetic Spelling: MEE-dee-um

- Reoxygenation

Pronunciation link: No confirmed link found
IPA (approx): /ˌriˌɑksiːdʒəˈneɪʃən/
Phonetic Spelling: ree-ahk-see-juh-NAY-shun

- Viability

Pronunciation link: https://www.merriam-webster.com/dictionary/viability
IPA: /ˌvaɪəˈbɪlɪti/
Phonetic Spelling: vy-uh-BIL-ih-tee

- Normoxic

Pronunciation link: No confirmed link found
IPA (approx): /nɔrˈmɒksɪk/ or /nɔrˈmɑːksɪk/
Phonetic Spelling: nor-MAHK-sik

- Mitochondrial

Pronunciation link: https://www.merriam-webster.com/dictionary/mitochondrial
IPA: /ˌmaɪtoʊˈkɑndriəl/
Phonetic Spelling: my-toh-KON-dree-uhl

- Extracellular

Pronunciation link: https://www.merriam-webster.com/dictionary/extracellular
IPA: /ˌɛkstrəˈsɛljələr/
Phonetic Spelling: ex-truh-SEL-yoo-lur

- Acidification

Pronunciation link: https://www.merriam-webster.com/dictionary/acidification
IPA: /əˌsɪdɪfɪˈkeɪʃən/
Phonetic Spelling: uh-sid-ih-fi-KAY-shun

- Analyzer

Pronunciation link: https://www.merriam-webster.com/dictionary/analyzer
IPA: /ˈænəˌlaɪzər/
Phonetic Spelling: AN-uh-LYE-zer

- Basal

Pronunciation link: https://www.merriam-webster.com/dictionary/basal
IPA: /ˈbeɪsəl/
Phonetic Spelling: BAY-suhl

- Ischemic

Pronunciation link: https://www.merriam-webster.com/dictionary/ischemic
IPA: /ɪsˈkiːmɪk/ or /aɪsˈkiːmɪk/ (US)
Phonetic Spelling: is-KEE-mik or eye-S-KEE-mik

- Caspase

Pronunciation link: https://www.merriam-webster.com/dictionary/caspase
IPA: /ˈkæspeɪs/
Phonetic Spelling: KAS-pays

- Fibrosis

Pronunciation link: https://www.merriam-webster.com/dictionary/fibrosis
IPA: /faɪˈbroʊsɪs/
Phonetic Spelling: fy-BROH-sis

- Normalization

Pronunciation link: https://www.merriam-webster.com/dictionary/normalization
IPA: /ˌnɔrmələˈzeɪʃən/ or /ˌnɔrməlɪˈzeɪʃən/ (US)
Phonetic Spelling: nor-muh-li-ZAY-shun