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Title: Generation of a Mouse Prostate Organoid-Based Model for Studying Host-Pathogen Interactions

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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? **Yes**

If **Yes**, can you record movies/images using your own microscope camera? **No**

Shot numbers: 1.7.1, 1.9.1, 1.9.3

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: 21

Number of Shots: 54

Introduction

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

INTRODUCTION:

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

- 1.1. **Carmen Aguilar:** This research focuses on understanding how bacterial pathogens infect the prostate epithelium and which host pathogen interactions determine infection outcome.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Carmen Aguilar:** Existing in vitro models poorly capture prostate epithelial diversity; this model provides a physiologically relevant system reflecting epithelial cell types.

CONCLUSION:

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

- 1.3. **Maria Guedes:** This protocol allows researchers to study bacterial infection dynamics and host-pathogen interactions in a model that preserves epithelial cellular diversity.

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

- 1.4. **Maria Guedes:** Future studies can use this model to investigate diverse prostate diseases, including infections, cancer biology, and test potential therapeutic compounds.

NOTE: The shot numbers for interviews were not added in the shotlist

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at University of Würzburg

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

NOTE: The shotlist prepared by the writer had the protocol number starting from 1. Not changing it at postshoot stage as we don't know how the slating was done.

1. ~~Generation of Prostate Organoid-Based Model~~ 3D Prostate Organoid Disruption into Single Cells

NOTE: The title was changed at the postshoot stage

Demonstrator: Maria Guedes

- 1.1. To begin, obtain three-dimensional murine prostate organoids cultured on drops of extracellular matrix in a 24-well plate [1]. Aspirate the medium from the desired number of wells [2] and add 500 microliters of fresh culture medium to each well [3-TXT]. **NOTE:** VO altered at the postshoot stage

- 1.1.1. WIDE: Show the entire experimental set up with the talent ready to aspirate the medium.
 - 1.1.2. Talent aspirating culture medium from wells of a 24-well plate containing 3D organoids.
 - 1.1.3. Talent pipetting 500 µL of addMEM/F12+/- into each well of the 24-well plate.
TXT: Culture medium: addMEM/F12+/-

- 1.2. Using a P1000 (*P-1000*) pipette, mechanically disrupt the extracellular matrix drop in each well [1]. Collect this suspension in a 15 milliliter conical tube, with no more than two wells collected per tube [2].

- 1.2.1. Talent disrupting the extracellular matrix drop in a well using a P1000 pipette.
 - 1.2.2. Talent transferring the disrupted organoid suspension into a 15 mL conical tube.

- 1.3. Add fresh culture medium to bring the volume to 10 milliliters [1]. Gently invert the tube 3 to 4 times to mix [2] and centrifuge at 450 *g* for 5 minutes at 4 degrees Celsius [3].

- 1.3.1. Talent adding addMEM/F12 +/- into the conical tube to reach 10 mL.
Videographer: Please ensure that the label shows addMEM/F12 +/- on the culture medium bottle and is visible.
 - 1.3.2. Talent gently inverting the conical tube several times to mix the suspension.
 - 1.3.3. Talent placing the tube into a centrifuge.

- 1.4. Aspirate the supernatant [1]. Resuspend the pellet in 2 milliliters of enzyme solution prewarmed to 37 degrees Celsius [2-TXT] and Incubate the suspension in a water bath at 37 degrees Celsius for 15 minutes [3]. Using a P1000 pipette, mix the cell suspension every 5 minutes during incubation [4].
 - 1.4.1. Talent aspirating the supernatant from the conical tube.
 - 1.4.2. Talent resuspending the pellet in prewarmed enzyme solution.
TXT: TrypLE 149 Express Enzyme supplemented with 10 μ M RHOKi
 - 1.4.3. Talent placing the tube containing the cell suspension into a 37 °C water bath.
 - 1.4.4. Shot of the talent mixing the suspension in the tube using a P1000 pipette.
- 1.5. After incubation, add fresh culture medium to reach a final volume of 10 milliliters [1]. Gently invert the tube 3 to 4 times to mix [2]. Centrifuge at 450 *g* for 5 minutes at 4 degrees Celsius [3].
 - 1.5.1. Talent pipetting addMEM/F12 +/- into the tube to reach 10 mL.
Videographer: Please ensure that the label shows addMEM/F12 +/- on the culture medium bottle and is visible.
 - 1.5.2. Talent gently inverting the tube to mix the suspension.
 - 1.5.3. Talent placing the tube into the centrifuge.

Plating Single-Cell Suspension onto a 2D Culture Surface

NOTE: Treat this as a title card for a separate section (added at postshoot stage)

- 1.6. Aspirate the supernatant without disturbing the pellet [1]. Resuspend the pellet in culture medium supplemented with 10 micromolar RHOK (*R-H-O-K*) inhibitor [2-TXT].
 - 1.6.1. Talent carefully aspirating the supernatant while leaving the pellet undisturbed.
 - 1.6.2. Talent resuspending the pellet in culture medium supplemented with RHOKi.
TXT: Use 300 μ L of medium/well of organoids processed initially
- 1.7. Using a Neubauer counting chamber [1] count the cells in the suspension [2].
 - 1.7.1. SCOPE: Shot of the cells in the Neubauer counting chamber.
 - 1.7.2. Talent counting cells in the suspension under a microscope.
- 1.8. Dilute the suspension in culture medium supplemented with 10 micromolar RHOK inhibitor [1-TXT]. Then, seed 200 microliters of the suspension per well for a 48-well plate [2] and 300 microliters per well for chambered micro-slides [3]. Incubate the

plates in a cell culture incubator at 37 degrees Celsius and 5 percent carbon dioxide [4].

1.8.1. Talent diluting the cell suspension with culture medium in a conical tube. **TXT: Final concentration: 62,500 cells/mL**

1.8.2. Talent dispensing the 200 µL of cell suspension into wells.

1.8.3. Talent dispensing the 300 µL of cell suspension into wells.

1.8.4. Talent placing the plate into a cell culture incubator.

1.9. After 1 day, check if most single cells have attached to the two dimensional culture surface [1]. Replace the culture medium every 2 to 3 days [2] and continue culture until confluency is reached within 7 days [3].

1.9.1. SCOPE: Shot of attached cells spread across the culture surface in the well.

1.9.2. Shot of talent aspirating old medium and adding fresh culture medium to the well.

1.9.3. SCOPE: Shot of a confluent monolayer of cells after 7 days of culture.

1.10. For inducing differentiation to enrich luminal cells, add 10 nanomolar 5 alpha dihydrotestosterone or DHT (*D-H-T*) to the cell suspension during seeding [1-TXT]

1.10.1. Talent adding DHT to the cell suspension during seeding.

TXT: Use medium with 10 nM DHT for all subsequent changes

2. ~~Bacterial Infection with~~ Preparation of Uropathogenic *Escherichia coli* (UPEC) for Infecting the Organoids

NOTE: The title was changed at the postshoot stage

2.1. TEXT ON A PLAIN BACKGROUND:

Caution: While handling BSL-2 infectious pathogens:

- Wear gloves and appropriate eye and face protection
- Keep the lab coat fully buttoned
- Perform all procedures inside a certified biological safety cabinet
- Decontaminate work surfaces after handling biological materials
- Wash hands thoroughly after all procedures
- Collect BSL-2 waste in designated biohazard containers
- Decontaminate all waste materials before disposal

2.2. Streak the uropathogenic *Escherichia coli* strain or UPEC (*U-P-E-C*) onto an ampicillin-supplemented LB (*L-B*) agar plate [1-TXT]. Incubate the plate at 37 degrees Celsius for 16 to 24 hours [2].

- 2.2.1. Talent streaking bacteria across a Luria Bertani agar plate using an inoculating loop. **TXT: 100 µg/mL ampicillin**
 - 2.2.2. Talent placing the agar plate into an incubator.
- 2.3. Using a sterile loop, inoculate 3 milliliters of ampicillin-supplemented LB broth with a single bacterial colony [1-TXT]. Incubate the culture statically at 37 degrees Celsius for 12 to 16 hours [2].
 - 2.3.1. Talent picking a single colony from the agar plate and inoculating a tube containing LB broth. **TXT: 100 µg/mL ampicillin**
 - 2.3.2. Shot of the talent placing the inoculated tube into an incubator.
- 2.4. Vortex the overnight bacterial culture [1]. Measure optical density of the culture at 600 nanometers [2]. Then, calculate the volume of culture needed to achieve a starting optical density of 0.05 in 3 milliliters of broth [3]. Add the calculated volume of bacterial culture to fresh ampicillin-supplemented LB broth [4]. Incubate statically at 37 degrees Celsius for 24 hours [5].
 - 2.4.1. Talent vortexing the bacterial culture tube.
 - 2.4.2. Talent placing a sample in a spectrophotometer to measure OD₆₀₀.
 - 2.4.3. Shot of the talent calculating the required inoculation volume on a laboratory notebook or calculator.
 - 2.4.4. Talent pipetting the calculated volume of bacterial culture into fresh Luria Bertani broth.
 - 2.4.5. Talent placing the culture tube into an incubator.
- 2.5. After incubation, vortex the bacterial culture [1]. Measure the optical density at 600 nanometers [2]. Transfer the volume corresponding to optical density 1 from the culture into a tube [3]. Centrifuge at 12,000 g for 2 minutes at room temperature [4]. Aspirate and discard the supernatant [5].
 - 2.5.1. Talent vortexing the bacterial culture tube.
 - 2.5.2. Talent measuring OD₆₀₀ using a spectrophotometer.
 - 2.5.3. Talent pipetting the calculated volume of bacterial culture into a centrifuge tube.
 - 2.5.4. Talent placing the tube into a centrifuge.
 - 2.5.5. Talent aspirating the supernatant and discarding it.
- 2.6. Resuspend the bacterial pellet in 1 milliliter of Primocin-free organoid culture medium without DHT [1]. Then, dilute the suspension with mouse prostate organoid-culture medium to achieve a multiplicity of infection of 100 [2].

- 2.6.1. Talent resuspending the bacterial pellet in Primocin-free organoid culture medium without DHT.
- 2.6.2. Talent diluting the bacterial suspension to the calculated multiplicity of infection.

Infecting the Prepared Prostate-Organoid Model with Uropathogenic *Escherichia coli* (UPEC)

NOTE: Treat this as a title card for a separate section (added at postshoot stage)

- 2.7. Aspirate the culture medium from the prepared organoid-based model [1]. Add 200 microliters of culture medium containing bacteria to each well of a 48-well plate [2]. Incubate for 1 hour in a cell culture incubator at 37 degrees Celsius with 5 percent carbon dioxide [3].
 - 2.7.1. Talent aspirating medium from wells containing the organoid-based cell culture.
 - 2.7.2. Talent pipetting bacterial suspension into the wells.
 - 2.7.3. Talent placing the culture plate into a cell culture incubator.
- 2.8. Aspirate the bacteria-containing medium from the wells [1] and wash the wells three times with fresh culture medium to remove extracellular bacteria [2].
 - 2.8.1. Shot of the talent aspirating the bacteria-containing medium from the wells.
 - 2.8.2. Shot of the talent washing the wells using addMEM/F12+/+ medium.
Videographer: Please ensure that the label shows addMEM/F12 +/- on the culture medium bottle and is visible.
- 2.9. Next, add culture medium containing 50 micrograms per milliliter gentamicin [1] and incubate for 30 minutes [2].
 - 2.9.1. Talent adding gentamicin-containing medium into the wells.
 - 2.9.2. Talent placing the culture plate into the incubator.
- 2.10. Aspirate the medium [1]. Add fresh culture medium supplemented with 10 micrograms per milliliter gentamicin for the remaining infection time [2].
 - 2.10.1. Talent aspirating medium from the wells.
 - 2.10.2. Talent adding fresh medium supplemented with gentamicin into the wells.
- 2.11. Use a confocal microscope to image the infected cells after fluorescence staining [1] and quantify infected cells using a flow cytometer [2].

- 2.11.1. Representative shot placing the chambered μ -slides in a laser scanning confocal microscope or a fluorescence microscope.
- 2.11.2. Representative shot of the talent loading a sample tube containing infected cells into the flow cytometer sample port.

Results

3. Results

- 3.1. In the control condition without dihydrotestosterone, most cells were cytokeratin 5 positive basal cells, with only a few showing low CD24a (C-D-24-a) luminal expression [1]. Supplementing with 1 nanomolar DHT produced mixed basal - luminal populations, though CD24a stayed low [2].
- 3.1.1. LAB MEDIA: Figure 2B. *Video editor: Add an arrow to show magenta-stained cells in the Day 7 image when 'CK5-positive basal cells' are mentioned. Likewise, add an arrow to show the green-stained cells in the Day 7 image when 'low CD24a luminal expression' is mentioned.*
- 3.1.2. LAB MEDIA: Figure 2C. *Video editor: Add arrows to point towards the magenta and green stained cells in the Day 7 image when 'mixed basal - luminal populations' is mentioned. Then, remove both arrows and add an arrow to show the green-stained cells in the Day 7 image when 'CD24a stayed low' is mentioned.*
- 3.2. Increasing DHT to 10 nanomolar promoted differentiation into luminal cells while maintaining some basal cells after 7 days of culture [1]. This correlated with the quantification studies showing a decrease in cytokeratin 5 positive basal cells [2] and an increase in CD24a positive luminal cells with increasing DHT concentration over time [3].
- 3.2.1. LAB MEDIA: Figure 2D. *Video editor: Add an arrow to show the green-stained cells in the Day 7 image when 'differentiation into luminal cells' is mentioned and then add an arrow to show magenta-stained cells in the Day 7 image when 'maintaining some basal cells' are mentioned.*
- 3.2.2. LAB MEDIA: Figure 2E. *Video editor: Please highlight the 3 bars for Day 7*
- 3.2.3. LAB MEDIA: Figure 2F. *Video editor: Please highlight the 3 bars for Day 7*
- 3.3. At 0 hours post-infection, UPEC adhered to epithelial cells in high numbers [1]. After gentamicin treatment at 1 hour, the number of UPEC-positive cells dropped sharply [2]. At 24 hours, infected cell numbers were similar to those at 1 hour [3]. Confocal imaging at 24 hours showed intracellular bacteria continued to replicate, forming

community-like clusters [4].

- 3.3.1. LAB MEDIA: Figure 4C. *Video editor: Highlight the bar labeled 0 hpi*
- 3.3.2. LAB MEDIA: Figure 4C. *Video editor: Highlight the bar labeled 1 hpi*
- 3.3.3. LAB MEDIA: Figure 4C. *Video editor: Highlight the bars labeled 24 hpi first and then emphasize the bars for both 24 hpi and 1 hpi*
- 3.3.4. LAB MEDIA: Figure 4B. *Video editor: Highlight the panel labeled 24 hpi (right side, top and bottom squares) and emphasize the red clusters.*

- 1. Organoid
Pronunciation link: <https://www.merriam-webster.com/dictionary/organoid>
IPA: /'ɔ:rgə,nɔɪd/
Phonetic Spelling: or·guh·noyd
- 2. Murine
Pronunciation link: <https://www.merriam-webster.com/dictionary/murine>
IPA: /'mjʊr.ən/
Phonetic Spelling: myoor·ine
- 3. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /'æs.pə'reɪt/
Phonetic Spelling: as·puh·rayt
- 4. Extracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/extracellular>
IPA: /,ɛkstrə'seljələɹ/
Phonetic Spelling: ek·struh·sel·yuh·ler
- 5. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sentrə,fjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
- 6. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /suː'pɜːrnə,tænt/
Phonetic Spelling: soo·per·nuh·tant
- 7. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /,ri:sə'spend/
Phonetic Spelling: ree·suh·spend

8. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'ɪŋkjə,bert/
Phonetic Spelling: ing·kyuh·bayt
9. Neubauer
Pronunciation link: <https://www.howtopronounce.com/neubauer>
IPA: /'nɔɪ,bəʊər/
Phonetic Spelling: noy·bow·er
10. Confluency
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluency>
IPA: /kən'flu:ənsi/
Phonetic Spelling: kuhn·floo·uhn·see
11. Dihydrotestosterone
Pronunciation link: <https://www.merriam-webster.com/dictionary/dihydrotestosterone>
IPA: /,daɪ,hædʁoʊtə'stə:stə,roʊn/
Phonetic Spelling: dye·hy·droh·tes·tos·ter·ohn
12. Uropathogenic
Pronunciation link: <https://www.merriam-webster.com/dictionary/uropathogenic>
IPA: /,jʊərə,pæθə'dʒɛnɪk/
Phonetic Spelling: yoor·uh·path·uh·jen·ik
13. Escherichia
Pronunciation link: <https://www.merriam-webster.com/dictionary/Escherichia>
IPA: /,ɛʃə'rɪkiə/
Phonetic Spelling: esh·uh·rik·ee·uh
14. Ampicillin
Pronunciation link: <https://www.merriam-webster.com/dictionary/ampicillin>
IPA: /,æmpə'sɪlɪn/
Phonetic Spelling: am·puh·sil·in
15. Inoculate
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculate>
IPA: /ɪ'nɑ:kjə,lɛɪt/
Phonetic Spelling: ih·nok·yuh·layt
16. Vortex
Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
IPA: /'vɔ:r,teks/
Phonetic Spelling: vor·teks
17. Spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /,spektroʊfə'tɑ:mətər/
Phonetic Spelling: spek·troh·fuh·tom·uh·ter
18. Multiplicity
Pronunciation link: <https://www.merriam-webster.com/dictionary/multiplicity>
IPA: /,mʌltə'plɪsɪti/
Phonetic Spelling: mul·tuh·plis·uh·tee

19. Gentamicin
Pronunciation link: <https://www.merriam-webster.com/dictionary/gentamicin>
IPA: /ˌdʒentəˈmaɪsɪn/
Phonetic Spelling: jen·tuh·my·sin
20. Cytometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometer>
IPA: /saɪˈtɑːmɪtər/
Phonetic Spelling: sy·tom·uh·ter
21. Cytokeratin
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytokeratin>
IPA: /ˌsaɪtoʊˈkerətɪn/
Phonetic Spelling: sy·toh·ker·uh·tin
22. Luminal
Pronunciation link: <https://www.merriam-webster.com/dictionary/luminal>
IPA: /ˈluːmɪnəl/
Phonetic Spelling: loo·muh·nuhl
23. Differentiation
Pronunciation link: <https://www.merriam-webster.com/dictionary/differentiation>
IPA: /ˌdɪfəˈrenʃiˈeɪʃən/
Phonetic Spelling: dif·uh·ren·shee·ay·shuhn
24. Epithelial
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
IPA: /ˌɛpəˈθiːliəl/
Phonetic Spelling: ep·uh·thee·lee·uhl
25. Intracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/intracellular>
IPA: /ˌɪntrəˈseljələr/
Phonetic Spelling: in·truh·sel·yuh·ler