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Title: Generation of 3D Collagen-Embedded A549/MRC-5 Alveolar Organoids using A Mechanical Cell-Stretching Bioreactor

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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? **YES**
- 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: 29

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. **Emmanuel Osei:** Our research focuses on generating physiologically relevant models of the airways, which we can use to study multicellular interactions in health and disease.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

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

- 1.2. **Emmanuel Osei:** Common in vitro airway models lack structural and mechanical complexity; this protocol generates alveolar co-culture organoids subjected to pathological mechanical strain.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 5.3.1*

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

- 1.3. **Emmanuel Osei:** This protocol enables investigation of how mechanical strain shapes cellular dynamics in lung disease, which is driven by abnormal breathing patterns.
 - 1.3.1. 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?~~

- 1.4. **Emmanuel Osei:** We present a culture and characterization protocol enabling assessment of organoid morphology during growth and inflammatory and cellular marker expression after strain.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: Figures 1 and 3*

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

- 1.5. **Emmanuel Osei:** Future studies can incorporate patient-derived cells, immune components, and environmental exposures to investigate personalized disease mechanisms and mechanobiological therapeutic targets
- 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

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

2. Establishing and Characterizing the Maturation of a 3D Alveolar Epithelial-fibroblast Organoid Model

Demonstrator: Tony Guo

- 2.1. To begin, prepare a mixture of 2 parts basement matrix and 3 parts Advanced DMEM [1]. Add 300 microliters of this solution to each well of a 24-well plate, ensuring the bottom of each well is evenly coated [2]. Place the plate in a humidified incubator at 37 degrees Celsius for 1 hour to allow the matrix to solidify [3].
 - 2.1.1. Talent preparing the 2:3 basement matrix and advanced Dulbecco's Modified Eagle Medium mixture.
 - 2.1.2. Talent adding 300 microliters of the solution to each well of a 24-well plate.
 - 2.1.3. Talent placing the 24-well plate in the humidified incubator.
- 2.2. For organoid culture, prepare a 5% basement matrix solution by mixing 60 microliters basement matrix with 1,140 microliters complete DMEM [1].
 - 2.2.1. Talent diluting 60 microliters of basement matrix in 1,140 microliters of complete Dulbecco's Modified Eagle Medium for each well.
- 2.3. During solidification of the basement matrix mixture, mix 300,000 A549 (*A-Five-Four-Nine*) cells with 30,000 MRC-5 (*M-R-C-Five*) cells in 200 microliters of the previously prepared 5% basement matrix solution [1].
 - 2.3.1. Talent mixing 300,000 A549 cells and 30,000 MRC-5 cells in 200 microliters of the previously prepared 5% basement matrix solution.
- 2.4. Once the solution has solidified, pipette 200 microliters of the A549 and MRC-5 mixture onto the gelled matrix [1]. Return the 24-well plate to the incubator at 37 degrees Celsius and 5% carbon dioxide for 21 days to allow alveolar organoid growth and differentiation [2].
 - 2.4.1. Talent pipetting 200 microliters of the A549 and MRC-5 mixture onto the gelled matrix.
 - 2.4.2. Talent placing the 24-well plate in the incubator.

2.5. Then, visualize the cultures under a light microscope for the next 48 hours. Confirm that the cells are aggregating during this time [1].

2.5.1. SCOPE: Show cells aggregating in culture.

2.6. Replace the 5% basement matrix and DMEM solution every 48 hours by gently tilting the plate to collect the old medium along the well wall [1]. Then aspirate the old solution and add 200 microliters of fresh 5% Matrigel medium [2].

2.6.1. Talent gently tilting the plate so the old solution collects along the well wall.

NOTE: 2.6.1 and 2.6.2 are combined in a single shot

2.6.2. Talent aspirating the old solution from the well wall and adding the fresh medium to the well.

2.7. At 3, 9, and 21 days of maturation, image the organoids at 10 times objective magnification. Capture four representative images at each time point [2].

2.7.1. LAB MEDIA: Figure 1

3. Harvesting 3D Alveolar Epithelial-fibroblast Organoids

3.1. After 21 days of maturation, aspirate the supernatant from each well [1]. Then wash the cultures gently with approximately 2 milliliters of ice-cold PBS [2].

3.1.1. WIDE: Talent aspirating the supernatant from a 24-well plate containing organoid cultures. **NOTE: 3.1.1 and 3.1.2 are combined in a single shot**

3.1.2. Talent gently adding ice-cold phosphate-buffered saline to wash the wells.

3.2. Add 2 milliliters of cold organoid harvesting solution to each well and incubate at 2 to 8 degrees Celsius for 30 to 90 minutes with moderate shaking [1].

3.2.1. Talent adding cold organoid harvesting solution to the wells of the plate.

3.3. After confirming dissociation under the brightfield microscope, using a cell scraper, gently dislodge the organoid and basement matrix hydrogel to accelerate the process [1]. Pool the contents of all wells into a 50-milliliter conical tube placed on ice [2].

3.3.1. Talent gently dislodging the organoid–basement matrix hydrogel with a cell scraper.

3.3.2. Talent transferring the contents of multiple wells into a 50 millilitre conical tube on ice.

3.4. Then, centrifuge the tube at 500 *g* for 5 minutes at 2 to 8 degrees Celsius [1]. Once the supernatant is aspirated, wash the organoid pellet twice with approximately 2 millilitres of ice-cold PBS using centrifugation [2].

3.4.1. Talent placing the 50-millilitre conical tube into a centrifuge.

3.4.2. Talent adding ice-cold phosphate-buffered saline to wash the organoid pellet.

4. Establishing 3D Collagen-embedded Alveolar-Fibroblast Organoids and Mechanical Strain Application

4.1. To prepare a working solution of collagen I gel, combine PBS, 11.4 millimolar sodium hydroxide, 2 milligrams per milliliter collagen I, and sterile water [1-TXT]. Then, add 1 milliliter of the prepared collagen I solution to the foam anchors on the periphery of the plate [2-TXT].

4.1.1. Talent preparing the collagen I working solution by combining phosphate-buffered saline, sodium hydroxide, and collagen I. **TXT: Working concentration: 2 mg/mL collagen I gel**

4.1.2. Talent adding 1 millilitre of the collagen I solution onto the foam anchors around the periphery of the plate.

4.2. Resuspend the organoid pellet in 6 milliliters of 2 milligrams per milliliter rat tail collagen [1]. Pipette 1 milliliter of the suspension into the center of a circular foam 6-well plate, ensuring the organoid–collagen mixture integrates with the surrounding organoid-free collagen [2].

4.2.1. Talent resuspending the organoid pellet in rat tail collagen. **NOTE: 4.2.1 and 4.2.2 are combined in a single shot**

4.2.2. Talent pipetting the organoid suspension into the center of the circular foam 6-well plate.

4.3. To polymerize the collagen I, place the plate in an incubator at 37 degrees Celsius with 5 percent carbon dioxide for 1 hour [1]. Confirm polymerization once the gel appears cloudy [2].

4.3.1. Talent placing the circular foam plate into the incubator.

4.3.2. Close-up of the cloudy collagen gel after polymerization.

4.4. Next, add 2 milliliters of complete DMEM on top of the three-dimensional collagen-

embedded A549 and MRC-5 organoids [1-TXT].

4.4.1. Talent adding complete Dulbecco's Modified Eagle Medium on top of the collagen-embedded organoids.

4.5. After incubation for 24 hours, replace the culture medium with DMEM containing 1 percent fetal bovine serum [1]. Incubate overnight prior to mechanical strain exposure [2].

4.5.1. Talent aspirating the existing medium and adding Dulbecco's Modified Eagle Medium containing 1 percent fetal bovine serum.

4.5.2. Talent placing the plate back into the incubator for overnight incubation.

5. Cell Stretching Bioreactor Setup and Exposure to Mechanical Strain

5.1. Insert the loading stations into each well of the baseplate [1]. Ensure stations have six 25-millimeter loading posts that deform the tissue train membrane and apply equibiaxial strain to the 3D culture [2].

5.1.1. WIDE: Talent inserting loading stations into the wells of the baseplate.

5.1.2. Close-up of loading posts positioned within the baseplate wells.

5.2. Apply a thin layer of silicone grease to the top of the loading posts [1]. Place red gaskets onto the bottom of each 6-well plate [2] and insert the prepared plates into the baseplate on top of the loading posts [3].

5.2.1. Talent applying a thin layer of silicone grease to the top of the loading posts.

5.2.2. Talent positioning red gaskets onto the bottom of a 6-well plate. **NOTE: 5.2.2. and 5.2.3. are combined in a single shot**

5.2.3. Talent placing the prepared 6-well plate into the baseplate so it sits on top of the loading posts.

5.3. Now, cover the assembly with a Plexiglas sheet [1] and add approximately 5 to 10 pounds of weight on top to ensure a tight seal [2].

5.3.1. Talent placing a Plexiglas sheet over the assembled baseplate. **NOTE: 5.3.1 and 5.3.2 are combined in a single shot**

5.3.2. Talent placing weights on top of the Plexiglas sheet.

5.4. Then, move the apparatus into a humidified incubator at 37 degrees Celsius with 5

percent carbon dioxide [1]. Connect the quick-disconnect fittings to the baseplate [2].

5.4.1. Talent placing the apparatus inside the incubator.

5.4.2. Talent connecting the quick-disconnect fittings to the baseplate.

5.5. Turn on the air compressor and open the outflow valve from the air compressor [1].

5.5.1. Talent turning on the air compressor and opening the outflow valve on the air compressor.

5.6. Next, turn on the vacuum pressure pump [1] and open the pressure reservoir valve connected to the vacuum tank outflow with a pressure of 90 kilopascal [2].

5.6.1. Talent turning on the vacuum pressure pump. **NOTE: 5.6.1 and 5.6.2 are combined in a single shot**

5.6.2. Talent opening the pressure reservoir valve connected to the vacuum tank outflow.

5.7. Open the inflow valve from the air compressor located near the bioreactor controller [1]. Adjust the pressure so it does not exceed 15 pounds per square inch at the vent port [2].

5.7.1. Talent opening the inflow valve from the air compressor near the bioreactor controller. **NOTE: 5.7.1 and 5.7.2 are combined in a single shot**

5.7.2. Close-up of the pressure gauge at the vent port showing pressure adjusted to no more than 15 pounds per square inch.

5.8. Now, launch the onboard software [1]. Create a new regimen and set the cyclic mechanical strain parameters with a minimum elongation of 0 percent, a maximum elongation of 18 percent, and a frequency of 0.4 hertz [2]. Start the protocol for 24 hours [3].

5.8.1. SCREEN: JOVE-69447-FX6000_screenshot_1.mp4: 00:00-00:06

5.8.2. SCREEN: JOVE-69447-FX6000_screenshot_100:06-00:09

5.8.3. SCREEN: JOVE-69447-FX6000_screenshot_1 00:09-00:13

5.9. During the strain session, observe the plate and membrane to ensure visible movement [1]. Confirm that the membrane is deforming around the loading posts [2].

5.9.1. Talent observing the plate during the strain session. **NOTE: 5.9.1 and 5.9.2 are**

combined in a single shot

5.9.2. Close-up of the flexible membrane deforming around the loading posts.

5.10. Next, check the water trap periodically for moisture accumulation [1]. Observe the computer screen to monitor run time, progress, and morphology of the strain regimen [2].

5.10.1. Talent inspecting the water trap for moisture. NOTE: 5.10.1 and 5.10.2 are combined in a single shot

5.10.2. SCREEN: Display the software interface showing run time and regimen progress.

5.11. After completion of the strain regimen, disconnect the baseplate assembly [1]. Return the strained cultures to the incubator for 24 hours before downstream characterization analyses [2].

5.11.1. Talent disconnecting the baseplate assembly from the system. NOTE: 5.11.1 and 5.11.2 are combined in a single shot

5.11.2. Talent placing the culture plate into the incubator.

5.12. Now, close or turn off the air compressor inflow valve, pressure reservoir valve, vacuum pressure pump, and air compressor outflow valve [1]. Finally, turn off the air compressor [2].

5.12.1. Talent closing the air compressor outflow valve. NOTE: 5.12.1 and 5.12.2 are combined in a single shot

5.12.2. Talent turning off the air compressor.

5.13. Then, collect the supernatant after 24 hours of incubation following mechanical strain exposure [1]. After immunostaining, image the organoids for morphological characterization [2].

5.13.1. Talent collecting supernatant from the culture plate into a tube.

5.13.2. Talent at the confocal microscope checking the acquired images.

Results

6. Results

6.1. The average number of organoids per well significantly decreased on day 9 [1] and day 21 [2] compared to day 3 [3].

6.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the bar labeled “Day 9”.*

6.1.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the bar labeled “Day 21”.*

6.1.3. LAB MEDIA: Figure 2A. *Video editor: Highlight the bar labeled “Day 3”.*

6.2. The average organoid area increased significantly on day 9 [1] and day 21 [2] compared to day 3 [3].

6.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the bar labeled “Day 9”.*

6.2.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the bar labeled “Day 21”.*

6.2.3. LAB MEDIA: Figure 2B. *Video editor: Highlight the bar labeled “Day 3”.*

6.3. The average organoid diameter increased significantly on day 9 [1] and day 21 [2] compared to day 3 [3].

6.3.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the bar labeled “Day 9”.*

6.3.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the bar labeled “Day 21”.*

6.3.3. LAB MEDIA: Figure 2C. *Video editor: Highlight the bar labeled “Day 3”.*

6.4. Under strained conditions, lung fibroblasts developed F-actin–positive cytoplasmic projections [1] that disrupted the typical spherical morphology of the organoids [2].

6.4.1. LAB MEDIA: Figure 3. *Video editor: Zoom in on the green filament-like extensions projecting outward from the organoid in Figure 3B.*

6.4.2. LAB MEDIA: Figure 3B. *Video editor: Show the overall organoid shape and emphasize that it is no longer round.*

6.5. F-actin fluorescence intensity was significantly higher in strained organoids compared to control organoids [1]. However, ZO-1 (*Zo-One*) fluorescence intensity was significantly reduced in strained organoids compared to control organoids [2].

6.5.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the bar labeled “Strain”, which is*

taller than the bar labeled “Control”.

6.5.2. LAB MEDIA: Figure 3D. *Video editor: Highlight the bar labeled “Strain”, which is lower than the bar labeled “Control”.*

1. Organoid
Pronunciation link: <https://www.merriam-webster.com/dictionary/organoid>
IPA: /ˈɔːr.gəˌnoɪd/
Phonetic Spelling: or·guh·noyd
2. Alveolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/alveolar>
IPA: /ælˈviː.ə.lə/
Phonetic Spelling: al·vee·uh·ler
3. Differentiation
Pronunciation link: <https://www.merriam-webster.com/dictionary/differentiation>
IPA: /ˌdɪf.ə.ren.ʃiˈeɪ.ʃən/
Phonetic Spelling: dif·uh·ren·shee·ay·shuhn
4. Magnification
Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>
IPA: /ˌmæɡ.nə.fɪˈkeɪ.ʃən/
Phonetic Spelling: mag·nuh·fih·kay·shuhn
5. Epithelial
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
IPA: /ˌep.əˈθiː.li.əl/
Phonetic Spelling: ep·uh·thee·lee·uhl
6. Fibroblast
Pronunciation link: <https://www.merriam-webster.com/dictionary/fibroblast>
IPA: /ˈfaɪ.broʊ.blæst/
Phonetic Spelling: fy·broh·blast
7. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /dɪˌsoʊ.siˈeɪ.ʃən/
Phonetic Spelling: dih·soh·see·ay·shuhn
8. Hydrogel
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrogel>
IPA: /ˈhaɪ.droʊ.dʒel/
Phonetic Spelling: hy·droh·jel

9. Polymerize
Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerize>
IPA: /pəˈlɪm.əˌraɪz/
Phonetic Spelling: puh·lim·uh·ryze
10. Equibiaxial
Pronunciation link: No confirmed link found
IPA: /ˌiː.kwɪˈbɑɪ.æk.si.əl/
Phonetic Spelling: ee·kwi·by·ak·see·uhl
11. Plexiglas
Pronunciation link: <https://www.merriam-webster.com/dictionary/Plexiglas>
IPA: /ˈplek.siˌglæs/
Phonetic Spelling: plek·see·glas
12. Kilopascal
Pronunciation link: <https://www.merriam-webster.com/dictionary/kilopascal>
IPA: /ˈkɪl.ɒs.pæs.kəl/
Phonetic Spelling: kil·oh·pas·kuhl
13. Elongation
Pronunciation link: <https://www.merriam-webster.com/dictionary/elongation>
IPA: /ɪˌlɔːŋˈɡeɪ.ʃən/
Phonetic Spelling: ee·long·gay·shuhn
14. Morphology
Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>
IPA: /mɔːrˈfɑːlə.dʒi/
Phonetic Spelling: mor·fol·uh·jee
15. Immunostaining
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunostaining>
IPA: /ɪˌmjuː.nəʊˈsteɪ.nɪŋ/
Phonetic Spelling: ih·myoo·noh·stay·ning
16. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈres.əns/
Phonetic Spelling: floor·eh·suhns