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Title: Culture Methods to Study Apical-Specific Interactions Using Intestinal Organoid Models

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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. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ■ Interviewees self-record interview statements. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations? **No, just adjacent rooms in the same building.**

Current Protocol Length

Number of Steps: 19
Number of Shots: 45

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Martin Stahl:** These protocols aim to provide robust and reproducible model systems to perform orientation-specific studies of the intestinal epithelium.

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

1.2. **Fisal Elstone:** Apical out organoids can be generated in high numbers and hold promise for high throughput screening. Monolayers are easier to be manipulated and offer access to both apical and basal sides.

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

Protocol

2. Inversion of Intestinal Organoid Polarity

2.1. Make sure that the size of the organoids is 150 to 250 micrometers in diameter prior to beginning the inversion protocol **[1-TXT]**. Carefully remove and discard the medium from each well containing organoids, without disrupting the ECM (Pronounce: spell E-C-M) dome **[2].**

2.1.1. WIDE: Establishing shot of talent taking culture plate out of the incubator. **TEXT: Day 3 – 5**

2.1.2. Talent removing the medium from each well containing organoids.

2.2. Add 1 milliliter of ice-cold dissociation solution to each well and incubate the plate at room temperature for 1 minute **[1].**

2.2.1. Talent adding ice-cold dissociation solution in each well.

2.3. Carefully dislodge the domes by pipetting slowly with a coated tip, taking care to not disrupt and fragment the organoids **[1].** Transfer the organoid suspension to a plate treated with anti-adherent solution **[2]** and place the plate on a shaker at 4 degrees Celsius for 30 minutes **[3-TXT].** *Videographer: This step is difficult and important!*

2.3.1. Talent dislodging the coated dome with the coated tip.

2.3.2. Talent transferring the organoid suspension to anti-adherent solution treated plate.

2.3.3. Talent placing the plate on a shaker. **TEXT: 70 rpm**

2.4. After 30 minutes, remove the plate **[1]** and gently pipette the solution up and down using a 1-milliliter pipette tip coated with anti-adherent solution **[2].** Place the plate on the shaker at 4 degrees Celsius for another 30 minutes **[3].**

2.4.1. Talent removing the plate from the shaker.

2.4.2. Talent pipetting the solution using anti-adherent solution coated tip.

2.4.3. Talent placing the plate on a gyro shaker.

2.5. Remove the plate and let the organoids settle by gravity for 1 to 2 minutes at room temperature **[1]**. ~~Observe the plate under the microscope to verify organoid~~

~~sedimentation **[2].**~~ After the organoids settle, remove as much of the dissociation solution as possible **[3]** and wash them by adding 1.5 milliliters of DMEM F-12 **[4].**

2.5.1. Talent removing the plate from shaker.

~~2.5.2. SCOPE: Organoid sedimentation.~~

2.5.3. Talent removing maximum dissociation solution.

2.5.4. Talent adding DMEM/F-12 solution for washing.

2.6. Allow the organoids to sediment and remove the supernatant **[1]**, then repeat the wash **[2].**

2.6.1. Talent removing the supernatant from the sedimented organoids. NOTE: In Take 1, focus improved at about 50 seconds

2.6.2. Talent adding DMEM/F-12 for washing.

2.7. Remove as much of the DMEM F-12 as possible **[1]** and add 0.5 milliliters of Intestinal Organoid Expansion Medium **[2].** Incubate overnight at 37 degrees Celsius and 5% carbon dioxide **[3].**

2.7.1. Talent removing maximum DMEM/F-12 medium.

2.7.2. Talent adding intestinal organoid expansion medium. NOTE: Do not use take 1, wrong reagent in the shot

2.7.3. Talent incubating the plate overnight.

2.8. On the following day, perform a partial-medium change by tilting the plate at a 25- to 30-degree angle **[1]** and removing medium along the wall of the well, taking care to not remove suspended organoids **[2-TXT].** *Videographer: This step is important!*

2.8.1. Talent tilting the plate. NOTE: Combined shot with 2.8.2

2.8.2. Talent removing the medium. **TEXT: Remove 0.4 mL of medium**

2.9. Add 0.4 milliliters of Intestinal Organoid Expansion medium **[1]** and incubate at 37 degrees Celsius and 5% carbon dioxide for 3 days **[2].** *Videographer: This step is important!*

2.9.1. Talent adding Intestinal organoid expansion medium.

2.9.2. Talent incubating the plate.

2.10. If aggregates have formed, use a 1-milliliter pipette with a tip coated with anti-adherent solution to shear the aggregates by pipetting up and down 20 times while pressing the end of the tip into the bottom of the plate **[1].**

2.10.1. Talent shearing the aggregates using a pipette tip.

3. Dissociating intestinal organoids for monolayer generation and culture

3.1. Add 1 milliliter of pre-warmed 0.05% Trypsin-EDTA *(E-D-T-A)* to resuspend organoids **[1]** and mix thoroughly to ensure an even suspension **[2]**. Add up to an additional 1 milliliter of Trypsin-EDTA for a large number of cells, or if a significant amount of ECM (Pronounce: spell E-C-M) remains **[3].**

3.1.1. Talent adding 0.05% Trypsin-EDTA to resuspend the organoids.

3.1.2. Talent mixing the suspension.

3.1.3. Talent adding more 0.05% Trypsin-EDTA for large number of cells.

3.2. Incubate at 37 degrees Celsius for 5 to 10 minutes **[1]**, then mix thoroughly with a 1 milliliter pipette to disrupt the organoids so that they are completely dissociated into single cells or small fragments **[2].**

3.2.1. Talent incubating the tube.

3.2.2. Talent mixing the suspension to disrupt the organoids using pipette.

3.3. To completely dissociate fragments or whole organoids, continue the incubation with Trypsin-EDTA **[1]** at 37 degrees Celsius for another 3 to 5 minutes **[2].**

3.3.1. Talent adding Trypsin-EDTA for whole organoids.

3.3.2. Talent incubating the tube.

3.4. Once the organoids are sufficiently dissociated, add an equal volume of DMEM F-12 **[1-TXT]** and pipette up and down to mix thoroughly **[2]**. Inactivate the Trypsin-EDTA by adding 10% FBS *(F-B-S)* to the cells **[3].**

3.4.1. Talent adding DMEM/F-12. **TEXT: 1 mL DMEM/F-12 per mL Trypsin-EDTA**

3.4.2. Talent mixing the solution by pipetting up and down.

3.4.3. Talent adding 10% FBS.

3.5. Centrifuge fragments at 200 times *g* for 5 minutes at 2 to 8 degrees Celsius **[1].** If the dissociated organoids fail to pellet, mix the cells thoroughly by pipetting up and down

[2] and centrifuge them again **[3].** Carefully remove as much supernatant as possible, leaving only the cell pellet **[4].**

3.5.1. Talent centrifuging the tube.

3.5.2. Talent mixing the cells by pipetting up and down.

3.5.3. Talent centrifuging the tube.

3.5.4. Talent discarding the supernatant.

3.6. Resuspend the cells in 100 microliters of Intestinal Organoid Differentiation medium for each well to be seeded, adjusting the volume appropriately for larger or smaller well sizes **[1].**

3.6.1. Talent adding Intestinal organoid differentiation medium in the tube.

3.7. Remove the coated plates from the incubator **[1]** and remove the excess basement membrane matrix solution from each well **[2].**

3.7.1. Talent removing the coated plates from the incubator.

3.7.2. Talent removing excess membrane matrix solution from each well.

3.8. Add 100 microliters of the cell suspension to the upper well of each cell culture insert **[1]** and 500 microliters of Intestinal Organoid Differentiation medium to the lower well **[1B],** then incubate at 37 degrees Celsius and 5% carbon dioxide **[2]**. Replace the medium in both the upper and lower wells every 2 to 3 days **[3]**

3.8.1. Talent adding intestinal organoid differentiation medium to the cell culture insert.

3.8.1B ADDED SHOT: Talent adding medium to the well. NOTE: Slated incorrectly as 3.8.3 take 1

3.8.2. Talent placing the plate in the incubator.

3.8.3. Talent removing media from the well and the insert

3.9. To establish an ALI culture, remove medium from the upper and lower wells **[1-TXT]** and add fresh Intestinal Organoid Differentiation medium to the lower well, leaving the upper well empty **[2].**

3.9.1. Talent removing the medium form upper and lower wells. **TEXT: ALI- Air-Liquid Interface**

3.9.2. Talent adding fresh Intestinal organoid differentiation medium to the lower well.

Results

4. Analysis and Characterization of the Morphology, Cell Polarity, and Differentiation Markers of the Intestinal Organoids

4.1. The intestinal organoids cultured with Intestinal Organoid Expansion Medium exhibited a cystic morphology **[1]**. When ECM is removed, some organoids tend to aggregate during the first 3 days **[2]** and need to be sheared to increase the organoid number **[3].**

4.1.1. LAB MEDIA: Figure 1A.

4.1.2. LAB MEDIA: Figure 1B. *Video editor focus on the left panel.*

4.1.3. LAB MEDIA: Figure 1B. *Video editor focus on the right panel.*

4.2. Intestinal organoids cultured in ECM continued to expand **[1]** and exhibit spontaneous formation of secondary budding structures **[2].**

4.2.1. LAB MEDIA: Figure 1C. *Video editor focus on the left panel.*

4.2.2. LAB MEDIA: Figure 1C. *Video editor focus on the right panel.*

4.3. Organoids maintained for 5 days in the absence of extracellular matrix exhibited elongated **[1]**, cystic **[2]**, and irregular **[3]** forms**.**

4.3.1. LAB MEDIA: Figure 1D. *Video editor focus on the right panel and show Supplementary Figure 1A in the inset.*

4.3.2. LAB MEDIA: Supplementary Figure 1B.

4.3.3. LAB MEDIA: Supplementary Figure 1C.

4.4. The expression of apical markers, such as VILLIN *(pronounce 'villin')* **[1]** and ZO-1 *(pronounce 'ZO-1')* **[2]**, was detected at the outer side of the epithelium that was exposed to the medium **[3]**.

4.4.1. LAB MEDIA: Figure 2A and B. *Video editor emphasize the green stained cells in A.*

4.4.2. LAB MEDIA: Figure 2A and B. *Video editor emphasize the green stained cells in B.*

4.4.3. LAB MEDIA: Figure 2A and B.

4.5. ECM-embedded organoids stained for nuclei, VILLIN **[1]**, and ZO-1 **[2]**, demonstrating an apicobasal polarity where the apical side was facing the lumen of the organoid **[3]**.

4.5.1. LAB MEDIA: Figure 2C and D. *Video editor emphasize the green stained cells in C.*

4.5.2. LAB MEDIA: Figure 2C and D. *Video editor emphasize the green stained cells in D.*

4.5.3. LAB MEDIA: Figure 2C and D.

4.6. Once the monolayer was established, cells formed tight junctions and a confluent layer **[2].** The confluent layer also orients its VILLIN-containing brush border toward the apical side of the epithelium **[3]**, forming ZO-1 multi-protein complexes in between cells **[4].**

~~4.6.1. LAB MEDIA: Figure 3B. *Video editor focus on the cobblestone appearing in left panel figure.*~~

4.6.2. LAB MEDIA: Figure 3B. *Video editor focus on the cobblestone appearing in left panel figure.*

4.6.3. LAB MEDIA: Figure 4B. *Video editor focus on the green stained region.*

4.6.4. LAB MEDIA: Figure 4B. *Video editor focus on the red stained region.*

4.7. Transition to an ALI culture induced further differentiation **[1]** with more prominent goblet cells **[2]**, which was visualized by staining for the secreted mucin protein, MUC2 *(Miuk-two)* **[3]**.

4.7.1. LAB MEDIA: Figure 3C.

4.7.2. LAB MEDIA: Figure 4A. *Video editor focus on the green stained cells.*

4.7.3. LAB MEDIA: Figure 4C and 4D. *Video editor focus on the green stained region.*

Conclusion

5. Conclusion Interview Statements

5.1. **Georgios Stroulios:** It is crucial for the polarity inversion to remove all ECM without disrupting the organoids. Media changes should be done with care to avoid removing suspended organoids.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3, 2.8 and 2.9.*

5.2. **Martin Stahl:** Ensuring that there are sufficient single cells for the establishment of the monolayer culture is a major determinant of its quality.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.*

5.3. **Fisal Elstone:** Apical-specific functions, such as nutrient absorption or host-pathogen interactions, can now be studied in relevant systems that suit the researcher's needs.

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