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Title: Generation of Murine Primary Colon Epithelial Monolayers from Intestinal Crypts

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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 wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 16
Number of Shots: 43

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Miguel Quiros:** Our protocol provides a fast, reproducible, and reliable method to generate direct primary 2D intestinal epithelial monolayer on different surfaces with minimal debris.

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

1.2. **Chithra Muraleedharan:** The main advantages of this protocol are the low consumption of media and low-cost maintenance of cell culture. It's a quick method to perform functional tests and fast downstream applications.

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

OPTIONAL:

1.3. **Darius Feier:** This protocol will provide insight into the study of wound repair, permeability barrier and transepithelial migration of different cell types. Also, this model can be useful in host-pathogen interaction, damaged epithelia and drug discovery.

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

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at University of Michigan.

Protocol

2. Preparation of Plates, Chamber Slides and Cell Culture Membrane Inserts

2.1. To begin, add 200 microliters of coating solution to each well of a 48-well plate and chamber slide **[1-TXT]**. Pre-incubate both in a 5% carbon dioxide incubator at 37 degrees Celsius for 2 hours **[2]**.

2.1.1. Talent adding coating solution to a well. **TEXT: Coating solution: laminin (1:40 dilution) and collagen (1:30 dilution) in cold DPBS with Ca^{2+} and Mg^{2+}**

2.1.2. Talent placing well plates in the incubator. *Videographer: Obtain multiple usable shots this will be reused in 4.1.4.*

2.2. Coat 0.4-micrometer cell membrane inserts with 200 microliters of collagen solution **[1]**. Incubate the plate with the membrane inserts at 4 degrees Celsius for 30 minutes **[2],** then transfer it to a 5% carbon dioxide incubator at 37 degrees Celsius for 2 hours **[3]**.

2.2.1. Talent adding collagen solution to cell membrane inserts.

2.2.2. Talent placing membrane inserts in the refrigerator.

2.2.3. Talent placing membrane inserts in the incubator. *Videographer: Obtain multiple usable shots as this will be reused in 4.3.3.*

3. Crypt Isolation

3.1. Disinfect a euthanized mouse with 70% ethanol **[1-TXT]**, then dissect out the colon from rectum to cecum with the help of scissors and forceps **[2]**. Gently flush out the feces with ice cold PBS in a 10-milliliter syringe fitted with a 20-gauge feeding tube **[3]**.

3.1.1. Talent wiping the mouse with the ethanol. **TEXT: Use C57Bl/6 mice, 8-12 weeks old**

3.1.2. Talent dissecting colon from the mice.

3.1.3. Talent cleaning the colon.

3.2. Remove the proximal colon **[1]** and slide the distal colon gently onto the 20-gauge feeding tube **[2]**. Tie the colon at the end of the tube with 4-0 silk suture thread **[3]**. *Videographer: This step is important!*

3.2.1. Talent removing proximal colon.

3.2.2. Talent sliding the colon onto the feeding tube.

3.2.3. Talent tying colon to the tube with a thread.

3.3. Invert the colon inside out over the tied end and tie the other end with thread **[1]**, then cut the colon below the tip of the feeding tube using surgical scissors **[2]**. *Videographer: This step is important!*

3.3.1. Talent inverting the colon and tying the end.

3.3.2. Talent cutting the colon off the feeding tube.

3.4. Gently open the untied end of the inverted colon onto the tip of a 1.25-milliliter repeat syringe using its plunger **[1].** Slide the untied end of the inverted colon onto the syringe **[2]** and tie it tightly with a thread **[3]**. *Videographer: This step is important!*

3.4.1. Talent is opening the end of colon with syringe.

3.4.2. Talent sliding colon onto the syringe.

3.4.3. Talent tying colon to the tube with a thread.

3.5. Insert the plunger into the syringe and inflate the colon until it looks turgent with no visible wrinkles **[1]**. Place the inflated colon sausage in a 15-milliliter tube containing 5 milliliters of cell recovery solution on ice for 20 minutes **[2]**. Inflate and deflate the colon once every 5 minutes **[3-TXT]**. *Videographer: This step is important!*

3.5.1. Talent inflating the colon.

3.5.2. Talent placing the tube containing colon tissue on ice.

3.5.3. Talent inflating and deflating the colon. **TEXT: The colon sausage must remain inflated during the incubation**

3.6. Tie off the inflated colon below the tip of the repeat syringe using 4-0 silk suture thread **[1]**. Cut the colon sausage off the repeat syringe **[2]** and place it in a 15-milliliter tube containing 10 milliliters of 50 millimolar EDTA for 40 minutes **[3]**. Rotate the tube at 4 degrees Celsius **[4]**.

3.6.1. Talent tying inflated colon with thread. *Videographer: This step is difficult and important!*

3.6.2. Talent cutting the colon sausage.

3.6.3. Talent placing the colon in the tube.

3.6.4. Talent putting the tube on the rotator in an incubator.

3.7. Replace the EDTA solution with 5 milliliters of shake buffer **[1]** and shake the sausage manually in vertical position for 2 minutes **[2].** *Videographer: This step is difficult and important!*

3.7.1. Talent adding buffer in the tube.

3.7.2. Talent shaking the colon sausage vigorously. *Videographer: Obtain multiple usable shots as this will be reused in 3.8.2.*

3.8. Decant the shaking solution into a new 15-milliliter tube **[1]** and repeat the shaking step until a total of 10 milliliters of crypts in shaking buffer is collected **[2]**.

3.8.1. Talent collecting shaking solution in a fresh tube.

3.8.2. *Use 3.7.2.*

3.9. Count the number of crypts in 20 microliters of the suspension under a microscope **[1]** and dilute the sample to obtain the concentration of 5 crypts per microliter **[2]**. Centrifuge the crypts sample at 400 x *g* for 10 minutes at 4 degrees Celsius **[3]**.

3.9.1. Talent observing crypts suspension under the microscope.

3.9.2. Talent diluting the crypts suspension.

3.9.3. Talent placing the tube in centrifuge and closing the lid.

3.10. In the meantime, remove the 48-well plate and membrane inserts from the incubator and place it in the biosafety cabinet **[1]**. Aspirate the coating solution using a P200 pipette **[2]** and leave the plate with the lid slightly offset until cells are ready to be plated **[3].**

3.10.1. Talent removing well plates from an incubator.

3.10.2. Talent aspirating the coating solution.

3.10.3. Plates with the lid slightly offset, sitting on the lab bench.

4. Culturing 2D Monolayer

4.1. After centrifugation of the crypts suspension, remove the shaking buffer **[1]** and re-suspend the intact pellet in 3 milliliters of LWRN complete media **[2]**. Add 200 microliters of crypts to each well of the pre-coated 48-well plate and chamber slide **[3]** and incubate both in a 5% carbon dioxide incubator at 37 degrees Celsius **[4].**

4.1.1. Talent removing buffer from the tube.

4.1.2. Talent resuspending the pellet in the media.

4.1.3. Talent adding crypts to the well plate.

4.1.4. *Use 2.1.2.*

4.2. On the next day, aspirate the media and add fresh media **[1].** Generally, the cells become confluent in 24 to 48 hours **[2]**.

4.2.1. Talent aspirating the media.

4.2.2. LABMEDIA: Figure 3 *Video Editor: Just show the figure on the right (48 hours after plating).*

4.3. For cell culture membrane inserts, add 200 microliters of crypts to top of the inserts **[1]** and 600 microliters of complete L-WRN media to the bottom **[2]**. On the next day, aspirate the media and add fresh media only to the top chamber **[3]**. Incubate the plate in a 5% carbon dioxide incubator at 37 degrees Celsius **[4]**.

4.3.1. Talent adding crypts to top of the inserts.

4.3.2. Talent adding media to the bottom of membrane inserts.

4.3.3. Talent aspirating the media.

4.3.4. *Use 2.2.3.*

4.4. Measure transepithelial electrical resistance every day using an Epithelial Volt-Ohm meter **[1-TXT]**.

4.4.1. Talent measuring TEER. **TEXT: TEER reading more than 300 Ω.cm^2 indicates confluency**

Results

5. Results: Reliability of The Primary Epithelial Colon Monolayer Cultures

5.1. After isolating the crypts from a murine colon and verifying the concentration **[1]**, the crypts suspension was concentrated to 5 crypts per microliter **[2]**.

5.1.1. LAB MEDIA: Figure 2 A

5.1.2. LAB MEDIA: Figure 2 B

5.2. 48-well plate wells were subjected to a scratch-wound assay upon reaching the cell confluence **[1]**. After 24 hours, wound repair was observed, indicating that the culture was healthy and viable **[2]**.

5.2.1. LAB MEDIA: Figure 4 A

5.2.2. LAB MEDIA: Figure 4 B

5.3. Higher TEER values were achieved when media was changed from LWRN to differentiation media **[1]**. Also, a decrease of ISC markers and an increase in differentiation markers were observed, indicating that the differentiated monolayers were generated successfully **[2]**.

5.3.1. LAB MEDIA: Figure 5 A.

5.3.2. LAB MEDIA: Figure 5 B, C.

5.4. In addition, the appearance of sub-types of differentiated epithelial cells grown in different conditions was shown by immunofluorescence **[1]**.

5.4.1. LAB MEDIA: Figure 5 D.

Conclusion

6. Conclusion Interview Statements

6.1. **Miguel Quiros:** When attempting this protocol, it is important to make sure that the colon is not ruptured at any point during the prep and stays inflated to release crypts in an extremely clean preparation.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.5.1 – 3.6.1*

6.2. **Darius Feier:** Once a 2D IEC monolayer is formed, we can perform immunofluorescence, scratch wound and permeability assays, or other downstream applications. This will provide knowledge in wound healing, migration of different cell types and epithelial repair and damage studies.

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