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Title: Generation, Maintenance, and Characterization of Human Pluripotent Stem Cell-Derived Intestinal and Colonic Organoids

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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.

   Leica S9D

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**

Current Protocol Length

Number of Steps: 19
Number of Shots: 46

Introduction

NOTE: This script follows our old format, so we do not have the interview questions.

1.1. **Jorge Munera:** The following protocol describes the generation of human intestinal and colonic organoids from human pluripotent stem cells. This technique will allow the user to elucidate pathways involved in intestinal patterning **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *B-roll: 2.20.1.*

1.2. **Jorge Munera:** This technique allows the user to generate region-specific organoids that are isogenic **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *B-roll: 2.16.1.*

1.3. **Jorge Munera:** This protocol could provide insight into the factors that regulate the establishment and maintenance of intestinal patterning **[1]**.

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

Note: Place the interview statements 4.1 and 4.2 here to conform to our new style.

NOTE: This section has been removed to meet our current style.
~~**Introduction of the Demonstrator on Camera**~~

~~1.4. **Jorge Munera:** Demonstrating the procedure will be Na Qu, a Senior research specialist from my laboratory **[1] [2]**.~~

~~1.4.1. INTERVIEW: Author saying the above.~~

~~1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.~~

Protocol

2. Generation of Human Intestinal and Colonic Organoids

2.1. Begin by placing an extracellular matrix- or ECM *(E-C-M)*-coated 24-well plate inside a biosafety cabinet for 30 minutes to allow it to reach room temperature **[1]**. Place mTeSR1 *(pronounce 'm-teaser-1')* complete medium, cell detachment solution, and advanced DMEM inside a 37 degrees Celsius water bath and allow them to warm up for 30 minutes **[2]**.

2.1.1. WIDE: Establishing shot of the talent placing the ECM-coated plate inside the biosafety cabinet.

2.1.2. Talent placing mTeSR1 complete medium, cell detachment solution, and advanced DMEM inside the water bath.

2.2. ~~Verify that hPSCs are at least 85 percent confluent with minimal differentiation **[1]** and remove any differentiated cells if necessary **[2]**.~~ Prepare the plating medium in a 50-milliliter conical tube by adding 13 milliliters of mTeSR1 and 13 microliters of 10 millimolar Y-27632 *(Y-2-7-6-3-2)* Rho-associated protein kinase or ROCK *(Rock)* inhibitor **[1]**. NOTE: VO is struck through for the removed shots.

~~2.2.1. SCOPE: Talent verifying the confluency of the hPSCs.~~ Note: The author asked to skip the shot.

~~2.2.2. SCOPE: Talent removing the differentiated cells.~~ Note: The author asked to skip the shot.

2.2.3. Talent preparing the plating medium.

2.3. To collect cells from the 6-well plate, aspirate the medium from 3 to 4 wells **[1]** and wash once with 2 milliliters of advanced DMEM per well **[2]**. Aspirate the advanced DMEM **[3]** and dispense 1 milliliter of the cell dissociation solution into each well **[4]**.

2.3.1. Talent aspirating medium from 3-4 wells.

2.3.2. Talent washing the wells with advanced DMEM.

2.3.3. Talent aspirating the advanced DMEM.

2.3.4. Talent dispensing the cell dissociation solution.

2.4. Incubate the plate for 5 to 7 minutes inside a 5 percent carbon dioxide, 37 degrees Celsius incubator **[1].** ~~and check under the microscope that the cells are in suspension **[2].**~~ Dissociate any clumps of cells by pipetting up and down 4 to 5 times using a 5-milliliter pipette to prepare the cell suspension **[2]**. NOTE: VO is struck through for the removed shot and slightly edited.

2.4.1. Talent placing the plate in the incubator.

~~2.4.2. SCOPE: Talent checking if the cells are in suspension.~~ Note: The author asked to skip the shot.

2.4.3. Talent dissociating the clumps of cells by pipetting.

2.5. Add 2 milliliters of advanced DMEM into each well **[1]**, gently pipette up and down 4 to 5 times **[2]**, and transfer to a 15-milliliter conical tube **[3]**. Spin down the cells at 300 × *g* for 3 minutes at room temperature **[4]**.

2.5.1. Talent adding advanced DMEM into the wells.

2.5.2. Talent pipetting up and down.

2.5.3. Talent transferring the medium into the conical tube.

2.5.4. Talent spinning down the cells.

2.6. Aspirate the medium from the tube without aspirating the cell pellet **[1]** and add 6 milliliters of the prepared medium of mTeSR1 plus ROCK inhibitor **[2]**. Gently resuspend the cells by pipetting up and down 3 to 4 times **[3]**.

2.6.1. Talent aspirating the medium from the tube.

2.6.2. Talent adding the prepared medium into the tube.

2.6.3. Talent resuspending the cells.

2.7. Then, transfer the suspension to the rest of the mTeSR1 and ROCK inhibitor medium inside the 50-milliliter tube **[1]**. Resuspend vigorously 4 to 5 times and count the cells using a hemacytometer **[2].** Aspirate the ECM from the 24-well plate just before plating the cells **[3]**.

2.7.1. Transferring the suspension to the 50 mL tube.

2.7.2. Talent pipetting cells into the hemocytometer.

2.7.3. Talent aspirating the ECM from the 24-well plate.

2.8. Resuspend the cells again by pipetting up and down 2 to 3 times and dispense 0.5 milliliters of the cell suspension in each well **[1]**. Gently rock the plate 3 times clockwise, 3 times counterclockwise, 3 times forward and back, and 3 times side to side to evenly disperse the cells **[2]**.

2.8.1. Talent dispensing the cell suspension into wells.

2.8.2. Talent rocking the plate in different directions.

2.9. Transfer the plate to a 37 degrees Celsius, 5 percent carbon dioxide incubator and incubate for 24 hours **[1]**. After 24 hours, aspirate the spent medium, add 0.5 milliliters per well of mTeSR1, and incubate again for 24 hours under the same conditions **[2]**.

2.9.1. Talent placing the plate inside the incubator.

2.9.2. Talent adding mTeSR1 into the wells.

2.10. In a 15-milliliter conical tube, add 13 milliliters of Activin Day 1 medium, 13 microliters of 100 micrograms per milliliter Activin A, and 1.95 microliters of 100 micrograms per milliliter BMP4 *(B-M-P-Four)* **[1]**. Warm the medium in a 37 degrees Celsius water bath **[2]**.

2.10.1. Talent adding Activin Day 1 medium, Activin A, and BMP4.

2.10.2. Talent warming the medium in the water bath.

2.11. Aspirate the mTeSR1 medium from the 24-well plate and add 0.5 mL of Activin Day 1 medium per well **[1]**. Place the plate in a 37 degrees Celsius, 5 percent carbon dioxide incubator and incubate for 24 hours. Check the cells after 24 hours **[2]**.

2.11.1. Talent adding Activin Day 1 medium per well.

2.11.2. Talent placing the plate for incubation.

2.12. Prepare Activin Day 2 complete medium by adding 12.5 microliters of 100 micrograms per milliliter Activin A into 12.5 milliliters of Activin Day 2 medium in a 15-milliliter conical tube and place the tube in a 37 degrees Celsius water bath **[1]**. Remove the 24-well differentiation plate from the carbon dioxide incubator and remove the spent medium **[2]**.

2.12.1. Talent preparing the Activin Day 2 complete medium.

2.12.2. Talent removing the spent medium.

2.13. Dispense 0.5 milliliters of prewarmed Activin Day 2 medium per well and place the plate back inside the carbon dioxide incubator for 24 hours **[1]**.

2.13.1. Talent dispensing the prewarmed Activin Day 2 medium into the wells.

2.14. Prepare Activin Day 3 complete medium by adding 12.5 microliters of 100 micrograms per milliliter Activin A into 12.5 milliliters of Activin Day 3 medium in a 15-milliliter conical tube. Place the tube in a 37 degrees Celsius water bath **[1]**. Remove the spent medium and dispense 0.5 mL of Activin Day 3 medium per well **[2]**.

2.14.1. Talent preparing the Activin Day 3 complete medium.

2.14.2. Talent dispensing the prewarmed Activin Day 3 medium into the wells.

2.15. To begin with differentiation of definitive endoderm into mid-hindgut spheroids, add 25 milliliters of mid-hindgut induction medium with FGF4 *(F-G-F-Four)* in a 50-milliliter conical tube **[1]** and place it in a 37 degrees Celsius water bath for 30 minutes **[2]**.

2.15.1. Talent adding the mid-hindgut induction medium with FGF4 to the conical tube.

2.15.2. Talent placing the tube in the water bath.

2.16. To prepare the complete mid-hindgut induction medium, add 7.5 microliters of CHIR99021 (pronounce 'Kay-er-99021') after the medium is warm **[1]**. Remove the spent medium, dispense 0.5 milliliters of mid-hindgut induction medium per well, and incubate at 37 degrees Celsius, 5 percent carbon dioxide for 24 hours **[2]**.

2.16.1. Talent adding CHIR99021 to the medium.

2.16.2. Talent dispensing the mid-hindgut induction medium into the wells.

2.17. After condensation of the cells within the monolayer occurred the next day, replace the spent medium with a fresh mid-hindgut induction medium and place the plate back for incubation for 24 hours **[1]**.

2.17.1. Talent replacing the spent medium with a fresh mid-hindgut induction medium.

2.18. To avoid discarding the floating spheroids while changing the medium, transfer the old medium into a 15-milliliter tube **[1]** and centrifuge at 300 × *g* for 1 minute **[2].** Resuspend the spheroids in 12.5 milliliters of fresh mid-hindgut induction medium **[3]**,

add 0.5 milliliters per well into the same 24-well plate, and incubate for 24 hours in the incubator **[4].**

2.18.1. Talent transferring the old medium into the 15 mL tube.

2.18.2. Talent centrifuging the tube.

2.18.3. Talent resuspending the spheroids. *Videographer: This step is important!*

2.18.4. Talent adding 0.5 mL medium to each well. *Videographer: This step is important!*

2.19. On day 4 of hindgut induction, harvest floating spheroids from the plate wells by collecting the medium into a 15-milliliter tube **[1]** followed by centrifugation at 300 × *g* for 1 minute **[2]**.

2.19.1. Talent harvesting the floating spheroids. *Videographer: This step is important!*

2.19.2. Talent centrifuging the tube.

Results

3. Results

3.1. The efficiency of definitive endoderm induction was assessed by performing immunofluorescence staining for FOXA2 *(Fox-A-Two)* and SOX17 *(Socks-Seventeen)* **[1]**.

3.1.1. LAB MEDIA: Figure 2F, 2G, and 2H.

3.2. The expression of mRNA *(M-R-N-A)* of the anterior HOX *(hox)* factor *HOXD3 (hox-D-Three)* was found to be the highest in NOGGIN *(noggin)*-treated human intestinal organoids or HIOs *(H-I-Ohs)* **[1]**, less in epithelial growth factor- or EGF *(E-G-F)*-treated HIOs, and lowest in BMP-treated human colonic organoids or HCOs *(H-C-Ohs)* **[2].**

3.2.1. LAB MEDIA: Figure 4. *Video editor: Emphasize the orange bar labeled 'HOXD3'.*

3.2.2. LAB MEDIA: Figure 4. *Video editor: Emphasize the green bar labeled 'HOXD3'.*

3.3. Conversely, *HOXA13 (hox-A-Thirteen)* and *HOXD13 (hox-D-Thirteen)* mRNA expressions were found to be low in HIOs **[1]** and high in HCOs **[2]**.

3.3.1. LAB MEDIA: Figure 4. *Video editor: Emphasize the orange bars labeled 'HOXA 13' and 'HOXD 13'.*

3.3.2. LAB MEDIA: Figure 4. *Video editor: Emphasize the green bars labeled 'HOXA 13' and 'HOXD 13'.*

3.4. Immunofluorescence staining was performed for SATB2 *(Sat-B-2)*, CDX2 *(C-D-X-2)*, and CDH1 *(C-D-H-1)* to determine if the HCO epithelium was properly patterned **[1]**.

3.4.1. LAB MEDIA: Figure 5.

Conclusion

4. Conclusion Interview Statements

NOTE: To conform to our new style, move statements 4.1 and 4.2 after the introduction statement 1.3.

4.1. **Jorge Munera:** Differentiation of human pluripotent stem cells into definitive endoderm requires serum starvation of cells. Therefore, careful dispensing of media is necessary to prevent disruption of the monolayer **[1]**.

4.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.13.1., 2.14.2.*

4.2. **Jorge Munera:** HIOs and HCOs can be transplanted into the kidney capsule of mice which allows them to mature allowing the interrogation of mechanisms of intestinal maturation **[1]**.

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