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Title: 3D Culturing of organoids from the intestinal villi epithelium undergoing dedifferentiation.

Authors and Affiliations:

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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: 20
Number of Shots: 26

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Kylee Wrath:** This method can confirm the organoid-forming capacity of dedifferentiating villi epithelium that acquire stem cell markers in vivo.

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

1.2. **Kylee Wrath:** The collection of villi is performed via scraping, rather than the EDTA chelation method, preventing the complete loss of the underlying mesenchyme that may provide the niche signals, if required, for organoid initiation.

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

1.3. **Kylee Wrath:** This method is applicable in epithelial tissues where differentiated and proliferative compartments are physically delineated, and the dedifferentiating cells expresses stem cell markers in vivo.

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

1.4. **Kylee Wrath:** Some factors in this protocol need to be determined empirically, including the optimal stage to harvest villi and the optimal pressure for villi scraping, which will take time and practice.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.2, 3.2.1, 4.3.1 and 4.5.1*

Ethics Title Card

1.5. All conducted mouse experiments had the approval of the Institutional Animal Care and Use Committee at Stevens Institute of Technology.

Protocol

2. Mice

2.1. Induce the Smad4 knock out and β *('beta')*-catenin gain of function mutation in mutant mice with intraperitoneal injection of tamoxifen in corn oil for four consecutive days **[1-TXT]**. After euthanizing the mouse, spray the abdomen with 70% ethanol to prevent mouse fur from getting into the peritoneal cavity **[2]**.

2.1.1. WIDE: Talent injecting solution into a mouse. **TEXT: Mutant Mice: Smad4$^{f/f}$; Catnb$^{lox(ex3)}$/$^{+}$; Villin-Cre^{ERT2}**

2.1.2. Talent spraying ethanol on mouse

2.2. Open the abdominal cavity with dissection scissors to expose the intestine **[1]** and isolate the intestine using scissors and forceps **[2]**.

2.2.1. Talent opening the abdominal cavity

2.2.2. Talent isolating the intestine with the aid of scissors and forceps

3. Duodenum Isolation and Preparation

3.1. Dissect out the proximal half of the duodenum **[1]**, then flush the duodenum with 5 milliliters of ice-cold PBS in a 10-milliliter syringe to clear the luminal content **[2]**.

3.1.1. Talent dissecting out the proximal duodenum

3.1.2. Talent flushing the duodenum with PBS

3.2. Open the duodenum longitudinally with an angled scissor **[1]** and lay the duodenum flat on a 15-centimeter Petri dish on ice with the lumen of the duodenum facing the operator **[2]**.

3.2.1. Talent opening the duodenum with scissor *Videographer: This step is important!*

3.2.2. Duodenum in a Petri dish kept on ice *Videographer: This step is important!*

4. Villi Isolation by Scraping

4.1. Prior to beginning the scraping, place a 70-micrometer mesh strainer in one of the wells of a 6-well tissue culture plate **[1]**. Fill all the wells with 4 milliliters of 1x PBS and place the plate on ice **[2]**.

4.1.1. Talent placing strainer in one of the well in a tissue culture plate

4.1.2. Talent filling all the wells with PBS and keeping it on ice

4.2. Scrape the villi using two microscopic glass slides, one to hold the duodenum down and the other to scrape **[1]**.

4.2.1. Talent scraping the villi using two glass slides *Videographer: This step is important!*

4.3. Scrape the luminal side of the duodenum superficially twice to remove the mucus without removing the villi **[1]**.

4.3.1. Talent scraping the luminal side of the duodenum

4.4. Then, scrape the duodenum two more times to collect the villi on the slides without tethering the crypts **[1]**.

4.4.1. Talent scraping the duodenum

4.5. Use a 1-milliliter transfer pipet containing PBS to transfer the villi to a 70-micrometer mesh strainer in the 6-well dish. Collect the villi after every scrape **[1]**.

4.5.1. Talent transferring villi to the strainer *Videographer: This step is important!*

4.6. To remove loose crypts, wash the villi collected in the 70-micrometer strainer by transferring the strainer through a series of wells in the 6-well dish containing 4 milliliters of cold PBS per well **[1]**.

4.6.1. Talent transferring the strainer through series of wells *Videographer: This step is important!*

4.7. Using a p1000 pipet, transfer the villi suspension in about 3 milliliters of PBS from the 70-micrometer strainer to a new 15-milliliter tube on ice **[1]**.

4.7.1. Talent transferring the villi suspension to the tube on ice

4.8. Use a 0.1% BSA coated blunt-ended p200 pipet tip to transfer a 50-microliter volume of the villi suspension onto a glass slide **[1]**. Count the number of villi in the 50-microliter droplet under 4x magnification to determine the concentration of villi in the PBS suspension and to confirm the absence of tethered crypts **[2]**.

4.8.1. Talent using blunt end tip to transfer villi suspension on glass slide

4.8.2. Talent observing villi under microscope

5. Plating of Villi on BME-R1 Matrix

5.1. To plate the villi, use a 0.1% BSA coated p200 blunt-ended pipet tip to transfer the villi to a microcentrifuge tube for a plating density of 6 villi per well in 12.5 microliters of BME-R1 matrix **[1]**.

5.1.1. Talent transferring the villi to microfuge tube

5.2. Spin down the villi for 2 minutes at 200 times g at 4 degrees Celsius **[1]**, remove the supernatant, and repeat the centrifugation to remove any residual PBS **[2]**.

5.2.1. Talent putting the tubes for centrifugation

5.2.2. Talent removing the supernatant

5.3. In a laminar flow-hood, resuspend the villi pellet gently in the required amount of cold BME-R1 thawed on ice **[1]**.

5.3.1. Talent resuspending the villi pellet

5.4. Using a p20 pipet, plate 12.5 microliters of the villi in BME-R1 matrix per well of a 96-well U-bottom plate pre-warmed to 37 degrees Celsius **[1]**.

5.4.1. Talent plating villi in pre-warmed plate

5.5. Incubate the plate in a tissue culture incubator at 37 degrees Celsius for 15 minutes to allow solidification of the BME-R1 matrix **[1]**.

5.5.1. Talent incubating the plate in incubator

5.6. To each well, add 125 microliters of pre-warmed ENR media supplemented as described in the text manuscript **[1-TXT]**.

5.6.1. Talent adding pre-warmed media to the well **TEXT: ENR: Epidermal growth factor -Noggin- R-spondin1**

5.7. Incubate the plated villi in a tissue culture incubator at 37 degrees Celsius with 5% Carbon dioxide and change the media every other day. Discard any well in which organoids appear before two days **[1]**.

5.7.1. Talent changing media in tissue culture plate

Results

6. Results: Organoid Formation from the Dedifferentiating Villi

6.1. There is a difference in appearance of the organoids emerging from the crypts **[1]** versus the dedifferentiated villi epithelium from the same mouse intestine **[2]**. The crypt-derived organoids appear overnight as spherical structures with well-defined borders **[3]**.

6.1.1. LAB MEDIA: Figure 2B and 2C *Video editor: Please highlight the upper panels of 2B and 2C*

6.1.2. LAB MEDIA: Figure 2B and 2C *Video editor: Please highlight the lower panels of 2B and 2C*

6.1.3. LAB MEDIA: Figure 2 *Video editor: Please highlight the lower panel of 2C*

6.2. The kinetics and morphological appearance of the organoids initiating from villi was examined, which appear irregularly shaped at first and take two to five days before they can be seen under a microscope **[1].**

6.2.1. LAB MEDIA: Figure 3

6.3. Organoid initiation from two different villi is shown. The villi with organoid-forming potential appears dense, possibly due to the retention of the underlying mesenchyme. Organoid initiation from the villus is apparent at day two from one of the villi **[1]**, while in the other villus the organoid appears at day four **[2]**.

6.3.1. LAB MEDIA: Figure 3 *Video editor: Please highlight the broken box area in first row and the solid arrow in the second row in the Day-2 column*

6.3.2. LAB MEDIA: Figure 3 *Video editor: Please highlight the solid (unbroken) box area in first row and the arrow in the third or last row in the Day-4 column*

Conclusion

7. Conclusion Interview Statements

7.1. **Kylee Wrath:** It is crucial to take the proper steps to avoid crypt contamination when harvesting the villi and growing the resulting organoids.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.1, 4.6.1, 4.8.1, 4.8.2, 5.7.1*

7.2. **Kylee Wrath:** Phenotypic differences between the organoids emerging from the crypts and villi of the same mutant were observed using this procedure. Further experimentation can be carried out to look for molecular differences between the two.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 2*

7.3. **Kylee Wrath:** This protocol can be used to study the differences between the organoids arising from endogenous crypts versus the ectopic crypts arising from dedifferentiation. Thus, implications of dedifferentiation could be addressed.

7.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3*