FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 62300
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Project Page Link: https://www.jove.com/account/file-uploader?src=19001548

Title: Generation and Culture of Lingual Organoids Derived from Adult Mouse Taste Stem Cells

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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

   Please list the make and model of your microscope.

   Zeiss Stemi SV6 or Olympus SZX12 (has an Olympus U-CMAD-2 camera adapter)

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?

   ☒ 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: 23
Number of Shots: 48

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Lauren Shechtman:** Organoids serve as a powerful *in vitro* technology for drug screening and understanding biological processes. Thus, generating organoids that model the tongue is crucial for studying taste cell development and regeneration.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 4.6.2.*

1.2. **Christina Piarowski:** Traditional *in vivo* taste studies can be expensive and time consuming. This organoid protocol offers a standardized, reproducible alternative that minimizes those challenges while allowing for higher throughput experiments.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 4.7.1.*

Introduction of Demonstrator on Camera

1.3. **Christina Piarowski:** Demonstrating part of the procedure will be Jennifer K. Scott, a Professional Research Assistant from our laboratory.

1.3.1. INTERVIEW: Author saying the above.

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

Ethics Title Card

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

Protocol

2. Isolation of CVP Epithelium

2.1. After euthanizing an adult mouse **[1-TXT]**, use large sterile dissection scissors to cut the cheeks and break the jaw **[2]**. Then, lift the tongue and cut the lingual frenulum to separate the tongue from the floor of the oral cavity **[3]**.

2.1.1. WIDE: Establishing shot of talent at the biosafety cabinet with the euthanized mouse in view. **TEXT: Lgr5$^{EGFP\text{-}IRES\text{-}CreERT2}$ mouse**

2.1.2. Talent cutting the cheeks and breaking the jaw.

2.1.3. Talent lifting the tongue and cutting the lingual frenulum.

2.2. Cut the tongue out and collect it in sterile ice-cold dPBS *(D-P-B-S)* with calcium and magnesium **[1]**.

2.2.1. Talent cutting the tongue out and putting it in dPBS.

2.3. Under a dissecting microscope **[1]**, remove and discard the anterior tongue by cutting just anterior of the intermolar eminence with a razor blade **[2]**, then use a delicate task wipe to remove any hair and excess liquid from the posterior tongue **[3]**.

2.3.1. Talent placing the tongue under the microscope.

2.3.2. SCOPE: Talent removing the anterior tongue.

2.3.3. SCOPE: Talent removing hair and excess liquid from the posterior tongue.

2.4. Next, fill a 1-milliliter syringe with 200 to 300 microliters of injection enzyme solution **[1-TXT]** and insert a 30-gauge half-inch needle just above the intermolar eminence until just anterior of the circumvallate papilla, or CVP **[2]**. *Videographer: This step is important!*

2.4.1. Talent filling the syringe with the injection enzyme solution. **TEXT: See manuscript for injection enzyme solution preparation**

2.4.2. SCOPE: Talent inserting the needle.

2.5. Inject the enzyme solution underneath and at the lateral edges of the CVP between the epithelium and the underlying tissues. Withdraw the syringe slowly and continuously from the tongue as the solution is being injected **[1]**.

2.5.1. SCOPE: Talent injecting the solution and slowly withdrawing the syringe.

2.6. Incubate the tongue in sterile calcium-magnesium-free dPBS at room temperature for precisely 33 minutes **[1]**.

2.6.1. Talent adding the tongue in dPBS.

2.7. Using extra fine dissection scissors, make small cuts in the epithelium bilaterally and just anterior of the CVP **[1]**, then gently peel the epithelium by lifting it with fine forceps **[2]**. *Videographer: This step is important!*

2.7.1. SCOPE: Talent making cuts in the epithelium.

2.7.2. SCOPE: Talent peeling the epithelium.

2.8. Once the trench epithelium is free of the underlying connective tissue **[1]**, place it in a 2-milliliter microcentrifuge tube pre-coated with FBS **[2]**.

2.8.1. SCOPE: Talent removing the underlying connective tissue from the trench epithelium.

2.8.2. Talent placing the trench epithelium in a 2 mL tube.

3. Dissociation of CVP Epithelium

3.1. Add the dissociation enzyme cocktail to the tubes containing the peeled CVP epithelia **[1-TXT]** and incubate them in a 37-degree Celsius water bath for 45 minutes **[2]** with brief vortexing every 15 minutes **[3]**. During the last 15 minutes of incubation, prewarm 0.25% Trypsin-EDTA *(E-D-T-A)* in the water bath **[4]**.

3.1.1. Talent adding the dissociation enzyme cocktail. **TEXT: See manuscript for dissociation enzyme cocktail preparation**

3.1.2. Talent incubating the tubes in the water bath.

3.1.3. Talent vortexing the tubes.

3.1.4. Talent prewarming Trypsin-EDTA.

3.2. Following incubation, vortex the tubes **[1]** and triturate with a glass Pasteur pipette for 1 minute **[2]**. After tissue pieces settle, pipette the supernatant containing the first collection of dissociated cells into new FBS-coated 1.5-milliliter microcentrifuge tubes **[3]**. *Videographer: This step is important!*

3.2.1. Talent vortexing the tubes.

3.2.2. Talent triturating the samples with a glass Pasteur pipette.

3.2.3. Talent transferring the supernatant into new tubes.

3.3. Spin the supernatant for 5 minutes at 370 x *g* and 4 degrees Celsius to pellet the cells **[1].** Remove the resulting supernatant **[2]**, then resuspend the cell pellet in FACS buffer and keep it on ice **[3]**. *Videographer: This step is important!*

3.3.1. Talent putting the tubes in a centrifuge.

3.3.2. Talent removing supernatant.

3.3.3. Talent resuspending the cell pellet and keeping it on ice.

3.4. To dissociate the remaining tissue pieces in the original 2-milliliter microcentrifuge tubes, add pre-warmed 0.25% trypsin-EDTA **[1]** and incubate at 37 degrees Celsius for 30 minutes **[2]** with brief vortexing every 10 minutes **[3]**. Then, triturate the tissue pieces with a glass Pasteur pipette for 1 minute **[4]**.

3.4.1. Talent adding trypsin-EDTA.

3.4.2. Talent incubating the tubes.

3.4.3. Talent vortexing the tubes.

3.4.4. Talent triturating the tissue pieces with a glass Pasteur pipette.

3.5. After the tissue pieces settle, pipette the supernatant into the 1.5-milliter microcentrifuge tubes containing the previously collected dissociated cells in FACS buffer **[1-TXT]**.

3.5.1. Talent pipetting the supernatant into 1.5 mL tubes. **TEXT: Discard the remaining tissue pieces**

3.6. Spin the tubes with the dissociated cells **[1]**. After removing the supernatant, resuspend the cell pellets in FACS Buffer and keep them on ice **[2]**.

3.6.1. Talent putting the tubes in a centrifuge.

3.6.2. Talent resuspending the cell pellets and keeping them on ice.

3.7. Pass the cells through a 30-micrometer nylon mesh filter **[1]** and add DAPI *(dappy)* **[2]**.

3.7.1. Talent passing the cells through a filter.

3.7.2. Talent adding DAPI.

3.8. Then, isolate the Lgr5-GFP$^+$ *(L-G-R-five-G-F-P-positive)* cells via FACS using the green fluorescent protein channel as described in the text manuscript **[1]**.

3.8.1. LAB MEDIA: Figure 3B. *Video Editor: Emphasize the GFP+ cells in the right-most panel*

4. Plating of Lgr5-EGFP Cells and Organoid Maintenance

4.1. Transfer the desired number of LGR5-positive suspended cells into a new microcentrifuge tube **[1-TXT]**

4.1.1. WIDE: Talent transferring the cells to a new tube. **TEXT: 200 cells per well of a 48-well plate**

4.2. Spin the tube for 5 minutes at 370 x *g* and 4 degrees Celsius to pellet the cells **[1]**. Remove the supernatant and place the tube on ice **[2]**.

4.2.1. Talent placing the tube in the centrifuge.

4.2.2. Talent removing the supernatant and placing the tube on ice.

4.3. Gently resuspend the cell pellet in the appropriate amount of matrix gel with gentle pipetting **[1]**, then keep the microcentrifuge tube on ice in a 50-milliliter conical tube to prevent the matrix gel from gelling **[2]**. *Videographer: This step is important!*

4.3.1. Talent resuspending the cell pellet in the matrix gel.

4.3.2. Talent keeping the microcentrifuge tube in a 50 mL tube on ice.

4.4. Add 15 microliters of the matrix gel and cell mixture in the center of each well of a 48-well plate **[1]**. To ensure an even distribution of cells, mix the matrix gel and cell mixture by pipetting up and down after plating every three wells **[2]**. *Videographer: This step is important!*

4.4.1. Talent placing gel-cell mixture in the well.

4.4.2. Talent mixing the matrix gel/cell mixture, then adding it into the well.

4.5. Place the plate in an incubator at 37 degrees Celsius, 5% carbon dioxide and 95% humidity for 10 minutes to allow gelling of the matrix gel **[1]**. Then, add 300 microliters of room temperature WENRAS *(when-R-A-S)* media supplemented with the rock inhibitor, Y27632 *(Y-two-seven-six-three-two)*, to each well and return the plate to the incubator **[2]**.

4.5.1. Talent placing the plate in the incubator.

4.5.2. Talent adding media to each well.

4.6. Two days after plating, remove the media from each well using a 1-milliliter pipette or via vacuum aspiration, ensuring no cross-contamination between conditions **[1]**. Add 300 microliters of WENRAS media down the side of the well, taking care to not disrupt the matrix gel **[2]**, and return the plate to the incubator **[3]**.

4.6.1. Talent removing media.

4.6.2. Talent adding WENRAS media.

4.6.3. Talent placing the plate in the incubator.

4.7. Change the media every 2 days using the appropriate media for the culture stage. Maintain the organoids until day 12, when they are ready to harvest **[1]**.
Videographer: This step is important!

4.7.1. Talent changing the media.

Results

5. Results: Growth and Differentiation of the Lingual Organoids

5.1. When lingual organoids are cultured **[1]** using WENR *(W-E-N-R)* media, they do not grow efficiently **[2]**. However, after adding A8301 *(A-eight-three-zero-one)*, a TGF-beta signaling inhibitor, and SB202190, a p38 MAPKinase *(map-kinase)* signaling inhibitor, robust growth is observed **[3]**.

5.1.1. LAB MEDIA: Figure 5A.

5.1.2. LAB MEDIA: Figure 5A. *Video Editor: Emphasize the top row*

5.1.3. LAB MEDIA: Figure 5A. *Video Editor: Emphasize the middle row*

5.2. Interestingly, removing these inhibitors from the media after 6 days results in higher expression of general taste receptor cell marker Kcnq1 *(K-C-N-Q-1)*, suggesting that A8301 and SB202190 hinder taste cell differentiation **[1]**.

5.2.1. LAB MEDIA: Figure 5B. *Video Editor: Emphasize the Kcnq1 gene expression for WENRAS+WENR (teal dots)*

5.3. Thus, optimal growth and differentiation are obtained by culturing organoids in WENRAS media from days 0 to 6 **[1]** and WENR media from days 6 to 12 **[2]**.

5.3.1. LAB MEDIA: Figure 4. *Video Editor: Emphasize the arrow for WENRAS*

5.3.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize the arrow for WENR*

5.4. Mature organoids contain both, taste cells marked by Keratin-8 **[1]** and non-taste epithelial cells marked by Keratin-13 **[2].** Further, Keratin-13 is expressed at higher levels than all 3 taste receptor cell markers, suggesting that organoids are predominately composed of non-taste epithelial cells **[3]**.

5.4.1. LAB MEDIA: Figure 8A. *Video Editor: Emphasize the KRT8 panel*

5.4.2. LAB MEDIA: Figure 8A. *Video Editor: Emphasize the KRT13 panel*

5.4.3. LAB MEDIA: Figure 6. *Video Editor: Emphasize the Krt13 ΔCt values (pink dots)*

5.5. The organoids express all taste receptor cell types **[1]**. Type I *('one')* cells, marked by Entpd2 *(E-N-T-P-D-two)*, and bitter type II *('two')* cells, marked by Gnat3 *(G-nat-three)*, are highly expressed in taste organoids **[2]**, while sour sensing type III *('three')* cells, marked by Car4 *(car-four)*, are less common **[3]**.

5.5.1. LAB MEDIA: Figure 8B and 8C.

5.5.2. LAB MEDIA: Figure 6. *Video Editor: Emphasize the Entpd2 (teal dots) and Gnat3 ΔCt values (blue dots) when mentioned in the VO*

5.5.3. LAB MEDIA: Figure 6. *Video Editor: Emphasize the Car4 ΔCt values (purple dots)*

5.6. The taste receptor cells are randomly distributed in the organoids rather than in discrete taste bud structures observed *in vivo*.

5.6.1. LAB MEDIA: Figure 8B and 8C.

Conclusion

6. Conclusion Interview Statements

6.1. **Jennifer Scott:** Make sure to include some tissue posterior to the CVP when dissecting the tongue from the oral cavity. Additionally, make sure both trenches pop out and are obtained when peeling the epithelium.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.2.1, 2.7.2.*