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

Lauren A. Shechtman^{#1}, Christina M. Piarowski^{#1}, Jennifer K. Scott¹, Erin J. Golden¹, Dany Gaillard¹, Linda A. Barlow¹

¹Department of Cell and Developmental Biology and the Rocky Mountain Taste and Smell Center, University of Colorado Anschutz Medical Campus

[#] These authors contributed equally to this work.

Abstract

The sense of taste is mediated by taste buds on the tongue, which are composed of rapidly renewing taste receptor cells (TRCs). This continual turnover is powered by local progenitor cells and renders taste function prone to disruption by a multitude of medical treatments, which in turn severely impacts the quality of life. Thus, studying this process in the context of drug treatment is vital to understanding if and how taste progenitor function and TRC production are affected. Given the ethical concerns and limited availability of human taste tissue, mouse models, which have a taste system similar to humans, are commonly used. Compared to *in vivo* methods, which are time-consuming, expensive, and not amenable to high throughput studies, murine lingual organoids can enable experiments to be run rapidly with many replicates and fewer mice. Here, previously published protocols have been adapted and a standardized method for generating taste organoids from taste progenitor cells isolated from the circumvallate papilla (CVP) of adult mice is presented. Taste progenitor cells in the CVP express LGR5 and can be isolated via EGFP fluorescence-activated cell sorting (FACS) from mice carrying an *Lgr5*^{EGFP-IRES-CreERT2} allele. Sorted cells are plated onto a matrix gel-based 3D culture system and cultured for 12 days. Organoids expand for the first 6 days of the culture period via proliferation and then enter a differentiation phase, during which they generate all three taste cell types along with non-taste epithelial cells. Organoids can be harvested upon maturation at day 12 or at any time during the growth process for RNA expression and immunohistochemical analysis. Standardizing culture methods for production of lingual organoids from adult stem cells will improve reproducibility and advance lingual organoids as a powerful drug screening tool in the fight to help patients experiencing taste dysfunction.

Introduction

In rodents, lingual taste buds are housed in fungiform papillae distributed anteriorly, bilateral foliate papillae posteriorly, as well as a single circumvallate papilla (CVP) at the posterodorsal midline of the tongue¹. Each taste bud is composed of 50-100 short-lived,

Corresponding Authors: Dany Gaillard, dany.gaillard@cuanschutz.edu; Linda A. Barlow, linda.barlow@cuanschutz.edu.

Disclosures

The authors have nothing to disclose.

rapidly renewing taste receptor cells (TRCs), which include type I glial-like support cells, type II cells that detect sweet, bitter, and umami, and type III cells that detect sour^{2, 3, 4}. In the mouse CVP, LGR5⁺ stem cells along the basal lamina produce all TRC types as well as non-taste epithelial cells⁵. When renewing the taste lineage, LGR5 daughter cells are first specified as post-mitotic taste precursor cells (type IV cells) that enter a taste bud and are capable of differentiating into any of the three TRC types⁶. The rapid turnover of TRCs renders the taste system susceptible to disruption by medical treatments, including radiation and certain drug therapies^{7, 8, 9, 10, 11, 12, 13}. Thus, studying the taste system in the context of taste stem cell regulation and TRC differentiation is vital for understanding how to mitigate or prevent taste dysfunction.

Mice are a traditional model for *in vivo* studies in taste science since they have a taste system organized similarly to humans^{14, 15, 16}. However, mice are not ideal for high throughput studies, as they are expensive to maintain and time-consuming to work with. To overcome this, *in vitro* organoid culture methods have been developed in recent years. Taste organoids can be generated from native CVP tissue, a process in which organoids bud off from isolated mouse CVP epithelium cultured *ex vivo*¹⁷. These organoids display a multilayered epithelium consistent with the *in vivo* taste system. A more efficient way to generate organoids that does not require *ex vivo* CVP culture was developed by Ren et al. in 2014¹⁸. Adapting methods and culture media first developed to grow intestinal organoids, they isolated single Lgr5-GFP⁺ progenitor cells from mouse CVP and plated them in matrix gel¹⁹. These single cells generated lingual organoids that proliferate during the first 6 days of culture, begin to differentiate around day 8, and by the end of the culture period contain non-taste epithelial cells and all three TRC types^{18, 20}. To date, multiple studies utilizing the lingual organoid model system have been published^{17, 18, 20, 21, 22}; however, methods and culture conditions used to generate these organoids vary across publications (Supplementary Table 1). Thus, these methods have been adjusted and optimized here to present a detailed standardized protocol for the culture of lingual organoids derived from LGR5⁺ progenitors of adult mouse CVP.

Lingual organoids provide a unique model for studying the cell biological processes driving taste cell development and renewal. As the applications of lingual organoids expand and more labs move toward utilizing *in vitro* organoid models, it is important that the field strives to develop and adopt standardized protocols to improve reproducibility. Establishing lingual organoids as a standard tool within taste science would enable high throughput studies that tease apart how single stem cells generate the differentiated cells of the adult taste system. Additionally, lingual organoids could be employed to rapidly screen drugs for potential impacts on taste homeostasis, which could then be investigated more thoroughly in animal models. This approach ultimately will enhance efforts to devise therapies that improve the quality of life for future drug recipients.

Protocol

All the animal procedures were performed in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals, Animal Welfare Act, and Public Health Service Policy, and were approved by the Institutional Animal Care and Use

Committee (IACUC) at the University of Colorado Anschutz Medical Campus. Lgr5^{EGFP-IRES-CreERT2} mice used in this protocol are from The Jackson Laboratory, Stock No. 008875.

NOTE: The following steps should be completed before beginning to ensure smooth and timely progression of the protocol: set water bath to 37 °C, set centrifuge to 4 °C, make injection and dissociation enzyme solutions from the 10 mg/mL Dispase, Collagenase, and Elastase stock solutions (see Table of Materials), remove matrix gel from -20 °C freezer (~750 µL needed for a 48-well plate) and thaw by submerging the vial in ice for at least 3-4 h, pre-coat microcentrifuge tubes in undiluted FBS by rocking gently at room temperature for at least 30 min (two 2 mL tubes for tissue collection, two 1.5 mL tubes for dissociated cells, and one 1.5 mL tube for collection of single cells from cell sorter; remove excess FBS before use).

1. Isolation of CVP epithelium

NOTE: To obtain enough LGR5⁺ cells for a full 48-well plate, collect three Lgr5-EGFP CVPs in the same tube and process simultaneously. Importantly, harvest and process the CVP of at least one wild type littermate in parallel in a separate tube and utilize it as a gating control to set FACS parameters (see Representative Results).

1. Euthanize the mice with CO₂ asphyxiation according to IACUC regulations, followed by an approved secondary method such as bilateral thoracotomy, cervical dislocation, decapitation, or exsanguination.
2. Use large sterile dissection scissors to cut the cheeks and break the jaw. Lift the tongue and cut the lingual frenulum to separate the tongue from the floor of the oral cavity. Cut out the tongue and collect it in sterile ice-cold Dulbecco's phosphate-buffered saline (dPBS) with Ca²⁺ and Mg²⁺.
3. Remove and discard the anterior tongue by cutting just anterior of the intermolar eminence with a razor blade (Figure 1A, **dashed line**). Use a delicate task wipe to remove any hair and excess liquid from the posterior tongue.
4. Fill 1 mL syringe with 200-300 µL of injection enzyme solution (final concentration: 2 mg/mL type-I Collagenase and 5 mg/mL Dispase II in Ca²⁺/Mg²⁺-containing dPBS, diluted from 10 mg/mL stock solutions) and insert a 30 G x ½ needle just above the intermolar eminence (Figure 1B, **black arrow**) until just anterior to the CVP (Figure 1B, **black box**). Inject the enzyme solution underneath and at the lateral edges of the CVP between the epithelium and the underlying tissues (lamina propria, muscle). Withdraw the syringe slowly and continuously from the tongue as the injection is performed.
5. Incubate the tongue in sterile Ca²⁺/Mg²⁺-free dPBS at room temperature for precisely 33 min.
6. Make small cuts in the epithelium bilaterally and just anterior to the CVP using extra fine dissection scissors, and gently peel the epithelium by lifting it with fine forceps. Once the trench epithelium is free of the underlying connective tissue,

place it in an empty 2 mL microcentrifuge tube pre-coated with FBS. Do the epithelial trimming before or after detaching the CVP epithelium (Figure 1C,D).

2. Dissociation of CVP epithelium

NOTE: Dissociation of the CVP epithelium and plating are represented graphically in Figure 2.

1. Add the dissociation enzyme cocktail (final concentration: 2 mg/mL type-I Collagenase, 2 mg/mL Elastase, and 5 mg/mL Dispase II in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing dPBS, diluted from 10 mg/mL stock solutions) to tubes containing peeled CVP epithelia (200 μL per CVP). Incubate in a 37 °C water bath for 45 min. Vortex briefly every 15 min.

NOTE: Prewarm 0.25% Trypsin-EDTA in 37 °C water bath during the last 15 min of enzyme cocktail incubation.

2. Following incubation, vortex (three pulses) then triturate with a glass Pasteur pipette for 1 min. After tissue pieces settle, pipette the supernatant containing first collection of dissociated cells, into new FBS-coated 1.5 mL microcentrifuge tubes corresponding to the genotype. Process the remaining tissue pieces further as described in step 2.3. below.
 1. Spin the supernatant for 5 min at 370 x *g* and 4 °C to pellet cells.
 2. Remove the resulting supernatant and resuspend the cell pellet in Fluorescence-Activated Cell Sorting (FACS) Buffer (1 mM EDTA, 25 mM HEPES (pH 7.0) and 1% FBS in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (50 μL per CVP)). Store on ice.
3. While carrying out steps 2.2.1 and 2.2.2, dissociate the remaining tissue pieces from step 2.2 by adding pre-warmed 0.25% trypsin-EDTA (200 μL per CVP) to the original 2 mL microcentrifuge tubes and incubate in a 37 °C water bath for 30 min. Vortex briefly every 10 min.
4. Following incubation, vortex the tube containing tissue pieces (three pulses) then triturate with a glass Pasteur pipette for 1 min. After tissue pieces settle, pipette the supernatant into the 1.5 mL microcentrifuge tubes containing cells from step 2.2.2. Discard the tubes containing the remaining tissue pieces.
 1. Spin the tubes with dissociated cells for 5 min at 370 x *g* and 4 °C to pellet cells.
 2. Remove the supernatant and resuspend cell pellets in FACS Buffer (100 μL per CVP). Store on ice.
5. Pass the cells through a 30 μm nylon mesh filter and add DAPI ($\lambda_{\text{emission}} = 450$ nm) to cell mixtures prior to FACS. Isolate Lgr5-GFP⁺ cells via FACS using the green fluorescent protein channel ($\lambda_{\text{excitation}} = 488$ nm; $\lambda_{\text{emission}} = 530$ nm). Sort the cells using a 100 μm nozzle into a fresh FBS-coated 1.5 mL microcentrifuge tube containing 300 μL of $\text{Ca}^{2+}/\text{Mg}^{2+}$ free dPBS. Place the cells on ice until plating.

3. Plating of Lgr5-EGFP cells

1. Determine the volume of LGR5⁺ cell suspension received from the flow cytometer.
2. Based on the number of cells obtained from the sorter, calculate the number of cells per μL . Then, determine the volume needed to obtain the desired number of cells for plating (we use 200 cells per well of a 48-well plate) and transfer that total volume of suspended cells into a new microcentrifuge tube.
3. Spin the tube for 5 min at $370 \times g$ and 4°C to pellet cells (pellet may not be visible). Remove the supernatant and place the tube on ice.
4. Gently resuspend the cell pellet in the appropriate amount of matrix gel (15 μL per well for 48-well plates); pipette up and down gently to thoroughly distribute cells in matrix gel. Place 15 μL of matrix gel/cell mixture in the center of each well. Keep the microcentrifuge tube on ice in a 50 mL conical tube during plating to prevent matrix gel from gelling. Continue to mix matrix gel/cell mixture throughout plating by pipetting up and down every three wells to ensure an even distribution of cells across wells.
5. Place the plate in the incubator (37°C , 5% CO_2 , ~95% humidity) for 10 min to allow matrix gel gelling. Then, add 300 μL of room temperature WENRAS + Y27632 media to each well and return the plate to the incubator.

4. Organoid maintenance

NOTE: Organoids are grown in conventional organoid media (WENR) comprising recombinant EGF and 50% conditioned media containing Wnt3a, Noggin, and R-spondin²³. Drugs A8301 and SB202190 are added for the first 6 days of the culture period to optimize growth (WENRAS media) (Figure 5), then removed to promote differentiation (WENR media)²⁰. Y27632 is added for the first 2 days of culture to promote survival. Media conditions relative to the culture timeline are presented in Figure 4.

1. Two days after plating, remove WENRAS + Y27632 media from each well using a 1 mL pipette, ensuring no cross-contamination. Add 300 μL of WENRAS media down the side of the well to not disrupt the matrix gel. Return the plate to the incubator.
2. Change the media every 2 days, using the appropriate media for the culture stage (Figure 4). Maintain organoids until day 12, when the organoids are ready to harvest.

5. RNA processing

1. Harvesting organoids for RNA
 1. Place 48-well plate on ice for 30 min to depolymerize the matrix gel.
 2. Using a 1 mL pipette, pull up the organoid media; then, as the media is returned to the well, use the tip of the pipette to scratch and further break up the matrix gel. Transfer the contents to a 1.5 mL

microcentrifuge tube, pooling contents of three wells in one tube. Centrifuge the tubes for 5 min at 300 x *g* at room temperature.

3. Remove as much media supernatant as possible without removing any organoids; then, spin down tubes again for 5 min at 300 x *g* at room temperature.
4. Remove the remaining media and resuspend the organoids in 350 μ L lysis buffer + β -mercaptoethanol (β ME) (10 μ L β ME per 1 mL lysis buffer). Place the samples on ice for immediate RNA extraction or store at -80°C .

2. Quantitative RT-PCR analysis

1. Measure RNA quantity via spectrophotometer. Reverse-transcribe RNA using a cDNA Synthesis Kit.
2. Mix cDNA equivalent to 5 ng RNA with 200 nM pre-validated forward and reverse primers (Table 1) and fluorescent PCR Master Mix. Run the qRT-PCR reaction for 40 cycles at: 95°C for 15 s, then 60°C for 60 s.

6. Immunohistochemistry

1. Harvesting and fixing the organoids

1. Place 48-well plate on ice for 30 min to loosen the matrix gel.
2. Remove the organoid media and add 400 μ L of ice-cold PBS to each well. Then, remove PBS and add 400 μ L of ice-cold Cell Recovery Solution to each well. Rock gently at 4°C for 30 min.
3. Coat a 1 mL pipet tip with 1% BSA in PBS, and gently pipet contents of the well up and down to break up the matrix gel. Transfer the organoids to a 1.5 mL microcentrifuge tube placed on ice.
4. Rinse each well with 300 μ L PBS + 1% BSA and transfer any remaining organoids to the corresponding tubes. Remove Cell Recovery Solution/PBS + BSA from each tube. Add 400 μ L of ice-cold PBS, then repeat with another ice-cold PBS rinse.
5. Remove PBS and fix organoids with 300 μ L ice-cold 4% PFA (in 0.1 M PB) for 20 min, incubating at room temperature. Remove PFA and rinse organoids with 400 μ L ice-cold PBS.
6. Remove PBS; then, add 400 μ L PBS + 1% BSA. Store at 4°C .

2. Immunofluorescence staining

1. Rinse organoids in 500 μ L PBS + 1% BSA. Then, incubate organoids in blocking solution (5% normal goat or donkey serum, 1% bovine serum albumin, 0.3% Triton X 100 in 1x PBS pH 7.3) for 2 h, rocking gently at room temperature.

2. Add the primary antibody solution (primary antibodies diluted in blocking solution) and rock gently for 3 nights at 4 °C.
 3. Wash organoids 4x for 1 h with 500 µL PBS + 0.2% Triton, rocking gently at room temperature. Add secondary antibody solution (secondary antibodies diluted in blocking solution) and rock organoids overnight, protected from light, at 4 °C.
 4. Wash organoids 3x for 1 h with 500 µL PBS + 0.2% Triton, protected from light and rocking gently at room temperature. Incubate with DAPI diluted 1:10,000 in 0.1 M PB for 20 min, rocking and covered at room temperature.
 5. Wash the organoids 3x for 20 min with 0.1 M PB, rocking gently and covered at room temperature.
3. Slide mounting of organoids for inverted confocal microscopy.

NOTE: Step-by-step pictures of the slide mounting process are shown in Figure 7.

1. Create a ~1 mm thick 22 x 22 mm square perimeter of non-toxic modeling clay on a microscope slide.
2. Remove 0.1 M PB from microcentrifuge tube, and gently resuspend organoids in 100 µL mounting medium of choice; then, transfer to center of the clay square.
3. Fill the clay square until the mounting medium is almost to the top. Then, place 22 x 22 mm square coverslip over clay and press down firmly on the sides of the coverslip to seal. Let it cure according to the manufacturer's instructions (here, room temperature for 1-2 days) and store at 4 °C.

Representative Results

Mice have one CVP, located posteriorly on the tongue, from which LGR5⁺ stem cells can be isolated (Figure 1A, **black box**). Injection of an enzyme solution under and around the CVP (Figure 1B) results in slight swelling of the epithelium and digestion of the connective tissue. Sufficient digestion is achieved following a 33 min incubation, which allows easy separation of the CVP epithelium from the underlying tissue. When attempting to peel the CVP epithelium, cuts should be made at a sufficient distance from the CVP to ensure trenches are not disrupted or damaged (Figure 1C). This also enables one to grip the epithelium using forceps without damaging the CVP. Trimming the epithelium surrounding the CVP removes non-target cells and increases the efficiency of the following steps by decreasing the tissue mass being manipulated (Figure 1D). It is important to check that the two trenches (Figure 1C, **black arrows**) are present before adding CVP epithelium to the microcentrifuge tube; successful peeling of the CVP includes part of the Von Ebner's glands and ducts (Figure 1D, **black arrows**). If the peeled epithelium does not contain these two

opaque structures, the trench epithelium is most likely ruptured due to incomplete digestion of the connective tissue.

To establish proper FACS settings to collect Lgr5-EGFP cells, cells from dissociated CVPs are separately obtained from wild type and Lgr5-EGFP mice. Wild type CVP cells are analyzed first to establish gating parameters, a process in which populations of cells are categorized within the scatterplot output by characteristics of interest²⁴. Here, four parameters were used to ultimately identify cells for plating. The first parameter, forward scatter, filters out particles and debris based on the detected surface area. This parameter removes ~70% of all detected events during the sort (Figure 3). The width parameter further filters events based on size to ensure selection of single cells (singlets). Approximately 90% of events are singlets (Figure 3). The nuclear marker DAPI is taken up by dead but not live cells and thus allows dead cells to be sorted out²⁵. This protocol optimizes cell viability, as over 90% of events are live cells (Figure 3). Lastly, GFP gating parameters are set above the autofluorescence level of wild type cells. Wild type cells are not collected; they are used solely as a gating control for GFP fluorescence. With gating parameters determined from the wild type sample, cells from the Lgr5-EGFP sample are then run through the flow cytometer to be sorted for collection. Gates can be adjusted at the beginning of the Lgr5-EGFP sort to accommodate clear clustering of certain cell populations but should not be significantly changed mid-sort. It was found that the dissociation of three pooled Lgr5-EGFP CVPs yields ~500,000 cells, including an average of 10,000 GFP⁺ cells.

Proper media and culture conditions are vital for optimal growth and differentiation of organoids. Previous studies utilizing lingual organoids modeled their media components after those from the intestinal organoid field, including Wnt3a, EGF, Noggin, and R-spondin^{17, 18, 19, 20, 21, 22}. However, when lingual organoids are cultured using a similar method (WENR media), organoids do not grow efficiently (Figure 5A). In human intestinal organoids, drugs A8301 (a TGF β signaling inhibitor) and SB202190 (a p38 MAPKinase signaling inhibitor) are used to promote organoid growth²⁶. Indeed, adding these inhibitors (WENRAS media) induces robust growth of lingual organoids (Figure 5A). Interestingly, removing these inhibitors from the media after 6 days results in higher expression of general TRC marker *Kcnq1*, suggesting A8301 and SB202190 hinder taste cell differentiation (Figure 5B). Thus, optimal growth and differentiation are obtained by culturing organoids in WENRAS media from days 0-6 and WENR media from days 6-12 (Figure 4, Figure 5), respectively.

Organoid cell type composition can be characterized by qRT-PCR or immunohistochemistry. Mature organoids contain both taste cells, marked by *Kcnq1* and KRT8, and non-taste epithelial cells, marked by *Krt13*/KRT13 (Figure 6, 8A). This suggests isolated LGR5⁺ cells have a similar potency *in vitro* as they do *in vivo* since, in the adult tongue, Lgr5-GFP⁺ cells produce both taste and non-taste lineages⁵. Further, *Krt13* is expressed at higher levels than all 3 TRC markers (Figure 6), suggesting organoids are predominately composed of non-taste epithelial cells. In fact, relative quantification of gene expression²⁷ indicates *Krt13* is expressed 50x higher than general TRC marker *Kcnq1* (Student's *t*-test, *p* = 0.0004). This is expected, as the tongue has a similar proportion of taste versus non-taste epithelium¹. Organoids express all three TRC types (Figure 6, 8B,C). Type I cells (marked by *Entpd2*)

and bitter type II cells (marked by *Gnat3*) are highly expressed in taste organoids, while sour sensing type III cells (marked by *Car4*) are less common (Figure 6). TRCs are randomly distributed in organoids (Figure 8) rather than in discrete taste bud structures observed *in vivo*.

Discussion

Reported here is an efficient and readily repeatable method for culturing, maintaining, and processing lingual organoids derived from adult mouse taste stem cells. It was found that using three CVPs from 8 to 20-week-old *Lgr5*-EGFP mice is sufficient to obtain ~10,000 GFP⁺ cells for experimental use, resulting in 50 wells plated at a density of 200 cells per well in 48-well plates. Removal of CVP trench epithelia is optimized by injecting the lingual epithelium with freshly made Dispase II and type-I Collagenase solution, followed by a 33 min incubation. However, a shorter incubation time, old enzyme aliquots, different lots, or using enzymes from different manufacturers can result in incomplete trench removal. Conversely, a longer incubation time causes over-digestion of the tissue, resulting in loss of taste tissue integrity. Following peeling, further enrichment for CVP trenches was done by trimming away the epithelium surrounding the CVP. It is critical that all microcentrifuge tubes used during the dissociation and collection process are coated with FBS to prevent tissue and cells from sticking to the plastic walls of the tubes, which significantly reduces recovery of isolated cells (data not shown). Using a light coat of FBS and removing excess liquid from the tubes prior to use minimizes possible inhibitory effects on Trypsin or other enzymes.

Lingual organoids have been grown successfully from dissociated CVP tissue without prior sorting of LGR5⁺ cells¹⁷. Although this method results in the formation of organoids, we have found that a smaller proportion of these organoids contain taste cells compared to those grown from isolated progenitors (data not shown). Flow sorting for *Lgr5*-EGFP⁺ cells enriches for taste-competent progenitors, resulting in a higher proportion of taste-replete organoids. Currently, we collect all cells above the threshold of GFP autofluorescence (Figure 3); however, it is possible that GFP brightness levels are associated with variable organoid forming efficiency or taste competency. This hypothesis has not yet been tested but is a promising avenue for future work as it could allow further enrichment of taste cell-containing organoids.

It is well known that plating density affects the efficiency of organoid differentiation, and we recommend that optimal plating density be determined prior to experimentation²⁸. Size and depth of the wells, as well as matrix gel volume, should be considered when establishing plating density. Based on our preliminary work (data not shown), we find that in a 48-well plate, 15 μ L of matrix gel and a plating density of 200 cells per well allows efficient organoid expansion and differentiation of all three TRC types. We have found that lingual organoids can be cultured successfully and reproducibly in Reduced Growth Factor Basement Membrane Extract (RGF BME), a synthetic and less expensive matrix gel alternative. Other alternative matrices may also support lingual organoid culture, but further testing is required to investigate this possibility.

During plating, collected cells should be thoroughly but gently resuspended in matrix gel by frequently pipetting up and down to keep the cell suspension homogeneous. The tube containing the cell-matrix gel mixture should also be kept on ice during the entire plating process to prevent matrix gel from gelling on the sides of the tube. These measures ensure an even distribution of cells across wells, yielding more reproducible results when hand-plating cells. Notably, recent developments in microfluidic technologies provide high throughput options for cell dispensing and is a promising tool for future work²⁹.

To assess gene expression in cultured organoids, it was found to be necessary to pool at least three wells for each biological replicate to obtain sufficient RNA and consistency across replicates (Figure 6). It was also determined that immediately lysing harvested organoids according to specific manufacturer's instructions optimizes the quality and quantity of extracted RNA. While not tested here, other storage options such as flash-freezing or resuspension in storage reagents may also preserve RNA yield and quality.

Performing immunohistochemistry on organoids can be challenging, as primary and secondary antibodies must penetrate any residual matrix gel and the entire organoid epithelium. In previous reports, organoids were fixed while still suspended in matrix gel, which subsequently required extremely high concentrations of primary antibody solution to reveal protein expression^{17, 18}. Similar to other reports, removing organoids from matrix gel using Cell Recovery Solution prior to fixation was found to increase the efficiency of staining without the need for a high antibody concentration^{30, 31}; however, the detection of some taste cell markers, e.g., CAR4, requires a higher concentration of antibody. Further, incubating organoids in primary antisera for 3 nights increases the probability of antibody binding. However, this method may also increase background fluorescence if organoids are insufficiently washed following immunostaining. Importantly, diluted primary antibody solution can be saved and successfully re-used one or more times if stored for less than a week (data not shown). For optimal imaging, organoids are mounted by placing a coverslip on top of a clay perimeter of modeling clay to preserve their 3D structure. Placing the cover glass directly on the slide compresses and breaks organoids, preventing proper analysis.

Organoid culture is a highly applicable, cost-efficient technique. Some applications include, but are not limited to, disease modeling and drug screening and the study of stem cell and developmental biology³². Therefore, it is crucial to standardize organoid models to allow reproducibility across laboratories. In the future, it would be useful to develop a standardized method for passaging lingual organoids that guarantees that taste stem cell potency can be propagated over serial passages, thereby reducing the need for additional animals to generate more organoids. Importantly, while lingual organoids are composed of both taste and non-taste epithelial cells, the organization of these cells differs compared to the *in vivo* taste system. Discrepancies between organoids and taste epithelium *in vivo* may be due to the media used for organoid culture and/or because organoids lack interaction with the taste bud microenvironment, including important signals from gustatory nerves and the lamina propria that are required for taste bud formation and maintenance^{22, 33, 34, 35}. Future work to incorporate nerves or vasculature into lingual organoid culture, a strategy currently being adopted in other organoid systems, could allow more accurate modeling of the *in vivo* taste epithelium^{36, 37}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Peter Dempsey and Monica Brown (University of Colorado Anschutz Medical Campus Organoid and Tissue Modeling Shared Resource) for providing WNR conditioned media and valuable discussions. We also thank the University of Colorado Cancer Center Cell Technologies and Flow Cytometry Shared Resources, especially Dmitry Baturin, for cell sorting expertise. This work was funded by: NIH/NIDCD R01 DC012383, DC012383-S1, DC012383-S2, and NIH/NCI R21 CA236480 to LAB, and R21DC016131 and R21DC016131-02S1 to DG, and F32 DC015958 to EJG.

References

1. Barlow LA, Progress and renewal in gustation: new insights into taste bud development. *Development*. 142, 3620–3629 (2015). [PubMed: 26534983]
2. Liman ER, Zhang YV, Montell C, Peripheral coding of taste. *Neuron*. 81 (5), 984–1000 (2014). [PubMed: 24607224]
3. Finger TE, Silver WL, The neurobiology of taste and smell. 287–314, John Wiley-Liss and Sons Inc, New York, US (2000).
4. Barlow LA, Klein OD, Developing and regenerating a sense of taste. *Current Topics in Developmental Biology*. 111, 401–419 (2015). [PubMed: 25662267]
5. Yee KK, et al. Lgr5-EGFP marks taste bud stem/progenitor cells in posterior tongue. *Stem Cells* (Dayton, Ohio). 31 (5), 992–1000 (2013).
6. Miura H, Scott JK, Harada S, Barlow LA, Sonic hedgehog-expressing basal cells are general post-mitotic precursors of functional taste receptor cells. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*. 243 (10), 1286–1297 (2014). [PubMed: 24590958]
7. Deshpande TS, et al. Radiation-related alterations of taste function in patients with head and neck cancer: a systematic review. *Current Treatment Options in Oncology*. 19 (12), 72 (2018). [PubMed: 30411162]
8. Nolden AA, Hwang LD, Boltong A, Reed DR, Chemosensory changes from cancer treatment and their effects on patients' food behavior: A scoping review. *Nutrients*. 11 (10), 2285 (2019).
9. Doty RL, Shah M, Bromley SM, Drug-induced taste disorders. *Drug Safety*. 31 (3), 199–215 (2008). [PubMed: 18302445]
10. Kumari A, et al. Recovery of taste organs and sensory function after severe loss from Hedgehog/Smoothed inhibition with cancer drug sonidegib. *Proceedings of the National Academy of Sciences of the United States of America*. 114 (48), E10369–E10378 (2017). [PubMed: 29133390]
11. Ermilov AN, et al. Maintenance of taste organs is strictly dependent on epithelial hedgehog/gli signaling. *PLoS Genetics*. 12 (11), e1006442 (2016). [PubMed: 27893742]
12. Gaillard D, Shechtman LA, Millar SE, Barlow LA, Fractionated head and neck irradiation impacts taste progenitors, differentiated taste cells, and Wnt/ β -catenin signaling in adult mice. *Scientific Reports*. 9, 17934 (2019). [PubMed: 31784592]
13. Nguyen HM, Reyland ME, Barlow LA, Mechanisms of taste bud cell loss after head and neck irradiation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 32 (10), 3474–3484 (2012). [PubMed: 22399770]
14. Ohla K, et al. Recognizing taste: Coding patterns along the neural axis in mammals. *Chemical Senses*. 44 (4), 237–247 (2019). [PubMed: 30788507]
15. Chaudhari N, Roper SD, The cell biology of taste. *The Journal of Cell Biology*. 190, 285–296 (2010). [PubMed: 20696704]
16. Breslin PA, Spector AC, Mammalian taste perception. *Current Biology: CB*. 18 (4), R148–155 (2008). [PubMed: 18302913]

17. Aihara E, et al. Characterization of stem/progenitor cell cycle using murine circumvallate papilla taste bud organoid. *Scientific Reports*. 5, 17185 (2015). [PubMed: 26597788]
18. Ren W, et al. Single Lgr5- or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 111 (46), 16401–16406 (2014). [PubMed: 25368147]
19. Sato T, Clevers H, Primary mouse small intestinal epithelial cell cultures. *Methods in Molecular Biology (Clifton, N.J.)*. 945, 319–328 (2013).
20. Ren W, et al. Transcriptome analyses of taste organoids reveal multiple pathways involved in taste cell generation. *Scientific Reports*. 7, 4004 (2017). [PubMed: 28638111]
21. Feng S, Achoute L, Margolskee RF, Jiang P, Wang H, Lipopolysaccharide-induced inflammatory cytokine expression in taste organoids. *Chemical Senses*. 45 (3), 187–194 (2020). [PubMed: 31993633]
22. Lin X, et al. R-spondin substitutes for neuronal input for taste cell regeneration in adult mice. *Proceedings of the National Academy of Sciences of the United States of America*. 118 (2), e2001833118 (2021). [PubMed: 33443181]
23. Miyoshi H, Stappenbeck TS, In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. *Nature Protocols*. 8 (12), 2471–2482 (2013). [PubMed: 24232249]
24. Staats J, Divekar A, McCoy JP, Maecker HT, Immunophenotyping: *Methods and Protocols*. 81–104, Springer, New York, US (2019).
25. Perfetto SP, et al. Amine-reactive dyes for dead cell discrimination in fixed samples. *Current Protocols in Cytometry*. Chapter 9, Unit-9.34 (2010).
26. Sato T, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. 141 (5), 1762–1772 (2011). [PubMed: 21889923]
27. Livak KJ, Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 25 (4), 402–408 (2001). [PubMed: 11846609]
28. Morizane R, Bonventre JV, Generation of nephron progenitor cells and kidney organoids from human pluripotent stem cells. *Nature Protocols*. 12 (1), 195–207 (2017). [PubMed: 28005067]
29. Ekert JE, et al. Recommended guidelines for developing, qualifying, and implementing complex in vitro models (CIVMs) for drug discovery. *SLAS Discovery: Advancing Life Sciences R & D*. 25 (10), 1174–1190 (2020). [PubMed: 32495689]
30. Dekkers JF, et al. High-resolution 3D imaging of fixed and cleared organoids. *Nature Protocols*. 14 (6), 1756–1771 (2019). [PubMed: 31053799]
31. Fujii M, et al. Human intestinal organoids maintain self-renewal capacity and cellular diversity in niche-inspired culture condition. *Cell Stem Cell*. 23 (6), 787–793.e6 (2018). [PubMed: 30526881]
32. Clevers H, Modeling development and disease with organoids. *Cell*. 165 (7), 1586–1597 (2016). [PubMed: 27315476]
33. Castillo-Azofeifa D, et al. Sonic hedgehog from both nerves and epithelium is a key trophic factor for taste bud maintenance. *Development (Cambridge, England)*. 144 (17), 3054–3065 (2017).
34. Vintschgau M. v., Hönigschmied J, Nervus glossopharyngeus und schmeckbecher. *Archiv für die gesamte Physiologie des Menschen und der Tiere*. 14, 443–448 (1877).
35. Liu HX, et al. Multiple Shh signaling centers participate in fungiform papilla and taste bud formation and maintenance. *Developmental Biology*. 382 (1), 82–97 (2013). [PubMed: 23916850]
36. Workman MJ, et al. Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system. *Nature Medicine*. 23 (1), 49–59 (2017).
37. Koning M, van den Berg CW, Rabelink TJ, Stem cell-derived kidney organoids: engineering the vasculature. *Cell and Molecular Life Sciences: CMLS*. 77 (12), 2257–2273 (2020).

Figure 1: Dissected tongue and peeled CVP epithelium.

(A) The tongue is dissected out, and the anterior tongue is removed by cutting just anterior of the intermolar eminence (dashed line), leaving the posterior tongue, which includes the CVP (black box) (B) A needle is inserted just anterior to the intermolar eminence (black arrow), and enzyme mixture is injected below and to the lateral edges of the CVP (black box). (C) Untrimmed peeled epithelium surrounding the CVP trenches (black arrows) and (D) trimmed peeled CVP epithelium. Von Ebner's glands and ducts (D, black arrows) are visible after successful peeling of the trenches. Scale bar: 1 mm. [Please click here to view a larger version of this figure.](#)

Figure 2: Workflow of lingual organoid generation.

The tongue is removed from *Lgr5*-EGFP mice. The CVP trench epithelia (red box) are peeled from the underlying connective tissue and dissociated into single cells. GFP⁺ cells are isolated and plated in matrix gel at a density of 200 cells per well in a 48-well plate. [Please click here to view a larger version of this figure.](#)

Figure 3: Gating for Fluorescence-Activated Cell Sorting.

(A) The control (wild type CVP cells) run determines the FACS gates that eliminate debris and broken cells via forward scatter, identifies singlets via side scatter width, separates DAPI^{neg} live from DAPI⁺ dead cells, and establishes the autofluorescence level of wild type cells. (B) Previously determined gates are applied to the experimental run (Lgr5^{EGFP}-IRES-CreERT2 CVP cells) to isolate debris-free, single, live, Lgr5-EGFP⁺ cells.

[Please click here to view a larger version of this figure.](#)

Figure 4: Lingual organoid culture timeline and required media.

ROCK inhibitor Y27632 is added to the media for the first 48 h of culture to promote cell survival. During the proliferation phase, organoids are fed conditioned medium containing A8301 and SB202190 (WENRAS) to optimize growth. These drugs are withheld from media (WENR) starting at day 6 to promote differentiation. [Please click here to view a larger version of this figure.](#)

Figure 5: Drugs A8301 and SB202190 affect organoid growth and differentiation.

(A) Brightfield images of organoids grown in either WENR media (day 0-12), WENRAS media (day 0-12), or WENRAS (day 0-6), then WENR (day 6-12). Images were captured at day 2, day 6, and day 12 of culture using live imaging software. Scale bar: 400 μ m (B) Relative gene expression of a global TRC marker *Kcnq1* is significantly reduced when drugs A8301 and SB202190 are present during organoid differentiation. Each point represents one biological replicate, which included three pooled wells. Mean change in relative gene expression (horizontal line) was calculated by averaging three biological replicates. Error bars: $\pm$ SD. [Please click here to view a larger version of this figure.](#)

Figure 6: Lingual organoids express canonical taste cell markers.

Change in cycle threshold (Ct) value of global TRC marker *Kcnq1*, non-taste epithelial marker cytokeratin 13 (*Krt13*), type I TRC marker *Entpd2*, type II bitter TRC marker *Gnat3*, and type III sour TRC marker *Car4*, compared to housekeeping gene *Rpl19*. Each point represents one biological replicate, which included three pooled wells from a 48-well plate. Mean change in Ct value (horizontal line) for each marker was calculated by averaging three biological replicates. Unpaired Student's *t*-test, **p* < 0.05. Error bars: $\pm$ SD. [Please click here to view a larger version of this figure.](#)

Figure 7: Mounting of lingual organoids for inverted confocal microscopy.

(A) An ~1 mm thick string of non-toxic modeling clay is created. (B) The clay is sculpted as an ~20 mm x 20 mm square in the center of a 24 mm x 75 mm microscope slide. (C) A 22 mm x 22 mm square coverslip seals organoids suspended in mounting medium. Scale bar: 10 mm. [Please click here to view a larger version of this figure.](#)

Figure 8: Immunolabeling of intact lingual organoids.

Confocal images of fixed, immunostained organoids. (A) Optical section of an organoid stained for non-taste epithelial marker KRT13 (green) and general TRC marker KRT8 (magenta). (B) Maximum projection of a confocal z-stack of one organoid stained for type I glial-like cell marker NTPDase2 (green) and KRT8 (magenta). (C) Maximum projection of a confocal z-stack of two partially shown organoids (white-dashed outlines) and one complete organoid stained for type III sour detecting cell marker CAR4 (green), and bitter detecting type II cell marker GUSTDUCIN (magenta). Scale bar: 100 μ m for A, B, and C.

Nuclear marker DAPI (blue, right column). [Please click here to view a larger version of this figure.](#)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1:

Primer sequences used for quantitative RT-PCR.

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')	Assession Number
<i>Cnr4</i>	CTGCTAGGACAAAGGTGAACC	CTCCACTGTGTGTTGATTGTCT	NM_007607.2
<i>Enpd2</i>	GACAAGGAAATGACACAGGTATCGTGG	GTTCAGACATTCACCAGACTC	NM_009849.2
<i>Gnat3</i>	ATCCAGGAATCCAAGCCTGC	TGGTTTTCACCCGGGAATGT	MM_001081143.1
<i>Kenq1</i>	TTTGTTTCATCCCATCTCAG	GTTGCTGGGTAGGAAGAG	NM_008434.2
<i>Krt13</i>	TCATCTCGGTTTGTCACTGGA	TGATCTTCTCGTTGCCAGAGAG	NM_010662.2
<i>Rpl19</i>	GGTCTGGTTGGATCCCAATG	CCCGGGAATGGACAGTCA	NM_009078.2

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Antibodies			
Alexa Fluor 546 Donkey anti Goat IgG	Molecular Probes	A11056, RRID: AB_142628	1:2000
Alexa Fluor 546 Goat anti Rabbit IgG	Molecular Probes	A11010, RRID:AB_2534077	1:2000
Alexa Fluor 568 Goat anti Guinea pig IgG	Invitrogen	A11075, RRID:AB_2534119	1:2000
Alexa Fluor 647 Donkey anti Rabbit IgG	Molecular Probes	A31573, RRID:AB_2536183	1:2000
Alexa Fluor 647 Goat anti Rat IgG	Molecular Probes	A21247, RRID:AB_141778	1:2000
DAPI (for FACS)	Thermo Fischer	62247	
DAPI (for immunohistochemistry)	Invitrogen	D3571, RRID:AB_2307445	1:10000
Goat anti-CAR4	R&D Systems	AF2414, RRID:AB_2070332	1:50
Guinea pig anti-KRT13	Acris Antibodies	BP5076, RRID:AB_979608	1:250
Rabbit anti-GUSTDUCIN	Santa Cruz Biotechnology Inc.	sc-395, RRID:AB_673678	1:250
Rabbit anti-NTPDASE2	CHUQ	mN2-36LI6, RRID:AB_2800455	1:300
Rat anti-KRT8	DSHB	TROMA-IS, RRID: AB_531826	1:100
Equipment			
2D rocker	Benchmark Scientific Inc.	BR2000	
3D Rotator	Lab-Line Instruments	4630	
Big-Digit Timer/Stopwatch	Fisher Scientific	S407992	
Centrifuge	Eppendorf	5415D	
CO2 tank	Airgas	CD USP50	
FormaTM Series 3 Water Jacketed CO2 Incubator	Thermo Scientific	4110	184 L, Polished Stainless Steel
Incucyte	Sartorius	Model: S3	Cancer Center Cell Technologies Shared Resource, University of Colorado Anschutz Medical Campus
MoFlo XDP100	Cytomation Inc	Model: S13211997	Gates Center Flow Cytometry Core, University of Colorado Anschutz Medical Campus
Orbital Shaker	New Brunswick Scientific	Excella E1	
Real-Time PCR System	Applied Biosystems	4376600	
Refrigerated Centrifuge	Eppendorf	5417R	
Spectrophotometer	Thermo Scientific	ND-1000	
Stereomicroscope	Zeiss	Stemi SV6	
Thermal Cycler	Bio-Rad	580BR	
Vortex	Fisher Scientific	12-812	

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Water bath	Precision	51220073	
Media			
A83 01	Sigma	SML0788-5MG	Stock concentration 10 mM, final concentration 500 nM
Advanced DMEM/F12	Gibco	12634-010	
B27 Supplement	Gibco	17504044	Stock concentration 50X, final concentration 1X
Gentamicin	Gibco	15750-060	Stock concentration 1000X, final concentration 1X
Glutamax	Gibco	35050061	Stock concentration 100X, final concentration 1X
HEPES	Gibco	15630080	Stock concentration 100X, final concentration 1X
Murine EGF	Peptotech	315-09-1MG	Stock concentration 500 µg/mL, final concentration 50 ng/mL
Murine Noggin	Peptotech	250-38	Stock concentration 50 µg/mL, final concentration 25 ng/mL
N-acetyl-L-cysteine	Sigma	A9165	Stock concentration 0.5 M, final concentration 1 mM
Nicotinamide	Sigma	N0636-100g	Stock concentration 1 M, final concentration 1 mM
Pen/Strep	Gibco	15140-122	Stock concentration 100X, final concentration 1X
Primocin	InvivoGen	ant-pm-1	Stock concentration 500X, final concentration 1X
SB202190	R&D Systems	1264	Stock concentration 10 mM, final concentration 0.4 µM
WRN Conditioned media			Received from Dempsey Lab (AMC Organoid and Tissue Modeling Share Resource). Derived from L-WRN (ATCC® CRL-3276™) cells
Y27632 dihydrochloride 10ug	APExBIO	A3008-10	Stock concentration 10 mM, final concentration 10 µM
Other			
1 ml TB Syringe	BD Syringe	309659	
2-Mercaptoethanol, min. 98%	Sigma	M3148-25ML	β-mercaptoethanol
2.0 mL Microcentrifuge Tubes	USA Scientific	1420-2700	
48-well plates	Thermo Scientific	150687	
5 3/4 inch Pasteur Pipets	Fisherbrand	12-678-8A	
Albumin from bovine serum (BSA)	Sigma Life Science	A9647-100G	
Buffer RLT Lysis buffer	QIAGEN	1015750	
Cell Recovery Solution	Corning	354253	
Cohan-Vannas Spring Scissors	Fine Science Tools	15000-02	
Collagenase from Clostridium histolyticum, type I	Sigma Life Science	C0130-1G	

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Cultrex RGF BME, Type 2, Pathclear	R&D Systems	3533-005-02	Matrigel
Dispase II (neutral protease, grade II)	Sigma-Aldrich (Roche)	4942078001	
Disposable Filters	Sysmex	04-0042-2316	
Dulbecco's Phosphate Buffered Saline pH 7.4 (1X) (Ca ²⁺ & Mg ²⁺ free)	Gibco	10010-023	
Dulbecco's Phosphate Buffered Saline with Ca ²⁺ & Mg ²⁺	Sigma Life Sciences	D8662-500ML	
Dumont #5 Forceps	Fine Science Tools	11252-30	
EDTA, 0.5M (pH 8.0)	Promega	V4231	
Elastase Lyophilized	Worthington Biochemical	LS002292	
Extra Fine Bonn Scissors	Fine Science Tools	14084-08	
Fetal Bovine Serum (FBS)	Gibco	26140-079	
Fluoromount G	SouthernBiotech	0100-01	
HEPES Solution	Sigma Life Science	H3537-100ML	
HyClone Trypsin 0.25% + EDTA	Thermo Scientific	25200-056	
iScript cDNA Synthesis Kit	Bio-Rad	1706691	
Modeling Clay, Gray	Sargent Art	22-4084	
Needle	BD Syringe	305106	
Normal Donkey Serum	Jackson ImmunoResearch	017-000-121	
Normal Goat Serum	Jackson ImmunoResearch	005-000-121	
Paraformaldehyde	Sigma-Aldrich	158127	
PowerSYBR Green PCR Master Mix	Applied Biosystems	4367659	
RNeasy Micro Kit	QIAGEN	74004	
Safe-Lock Tubes 1.5 mL	Eppendorf	022363204	
Sodium Chloride	Fisher Chemical	7647-14-5	
Sodium Phosphate dibasic anhydrous	Fisher Chemical	7558-79-4	
Sodium Phosphate monobasic anhydrous	Fisher Bioreagents	7558-80-7	
SuperFrost Plus Microscope Slides	Fisher Scientific	12-550-15	
Surgical Scissors - Sharp	Fine Science Tools	14002-14	
Triton X-100	Sigma Life Science	T8787-100ML	
VWR micro cover glass	VWR	48366067	22x22mm