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TITLE:

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

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gustation, tongue, adult stem cells, *in vitro*, LGR5, FACS, renewal

SUMMARY:

The protocol presents a method for culturing and processing lingual organoids derived from taste stem cells isolated from the posterior taste papilla of adult mice.

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 $Lgr5^{EGFP\text{-}IRES\text{-}CreERT2}$ 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 tongue[1]. Each taste bud is composed of 50–100 short-lived, 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[2–4]. In the mouse CVP, $LGR5^+$ stem cells along the basal lamina produce all TRC types as well as non-taste epithelial cells[5]. 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 types[6]. The rapid turnover of TRCs renders the taste system susceptible to disruption by medical treatments, including radiation and certain drug therapies[7–13]. 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[14–16]. 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 2014[18]. 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 gel[19]. 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[17,18,20–22]; 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 of 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. $Lgr5^{EGFP\text{-}IRES\text{-}CreERT2}$ 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**).

1.1. Euthanize the mice with CO_2 asphyxiation according to IACUC regulations, followed by an approved secondary method such as bilateral thoracotomy, cervical dislocation, decapitation, or exsanguination.

1.2. 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 Ca^{2+} and Mg^{2+}.

1.3. 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.

1.4. 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 Ca^{2+}/Mg^{2+}-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 of 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.

1.5. Incubate the tongue in sterile Ca^{2+}/Mg^{2+}-free dPBS at room temperature for precisely 33 min.

1.6. Make small cuts in the epithelium bilaterally and just anterior of 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.**

2.1. Add the dissociation enzyme cocktail (final concentration: 2 mg/mL type-I Collagenase, 2mg/mL Elastase, and 5 mg/mL Dispase II in Ca^{2+}/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.

2.2. 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.

2.2.1. Spin the supernatant for 5 min at 370 x *g* and 4 °C to pellet cells.

2.2.2. 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 Ca^{2+}/Mg^{2+}-free PBS (50 µL per CVP)). Store on ice.

2.3. 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.

2.4. 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.

2.4.1. Spin the tubes with dissociated cells for 5 min at 370 x *g* and 4 °C to pellet cells.

2.4.2. Remove the supernatant and resuspend cell pellets in FACS Buffer (100 µL per CVP). Store on ice.

2.5. Pass the cells through a 30 µm nylon mesh filter and add DAPI ($\lambda_{emission}$ = 450 nm) to cell mixtures prior to FACS. Isolate Lgr5-GFP^{+} cells via FACS using the green fluorescent protein channel ($\lambda_{excitation}$ = 488 nm; $\lambda_{emission}$ = 530 nm). Sort the cells using a 100 µm nozzle into a fresh FBS-coated 1.5 mL microcentrifuge tube containing 300 µL of Ca^{2+}/Mg^{2+} free dPBS. Place the cells on ice until plating.

3. Plating of Lgr5-EGFP cells

3.1. Determine the volume of LGR5^{+} cell suspension received from the flow cytometer.

3.2. 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.

3.3. Spin the tube for 5 min at 370 x *g* and 4 °C to pellet cells (pellet may not be visible). Remove the supernatant and place the tube on ice.

3.4. 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.

3.5. 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-spondin[23]. 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.**

4.1. Two days after plating, remove WENRAS + Y27632 media from each well using a 1 mL pipette or via vacuum aspiration, ensuring no cross-contamination between conditions. Add 300 µL of WENRAS media down the side of the well to not disrupt the matrix gel. Return the plate to the incubator.

4.2. 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

5.1. Harvesting organoids for RNA

5.1.1. Place a 48-well plate on ice for 30 min to depolymerize the matrix gel.

5.1.2. 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.

5.1.3. 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.

5.1.4. Remove the remaining media and resuspend the organoids in 350 µL lysis buffer + β-mercaptoethanol (βME) (10 µL βME per 1 mL lysis buffer). Place the samples on ice for immediate RNA extraction or store at -80 °C.

5.2. Quantitative RT-PCR analysis

5.2.1. Measure RNA quantity via spectrophotometer. Reverse-transcribe RNA using a cDNA Synthesis Kit.

5.2.2. 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

6.1. Harvesting and fixing the organoids

6.1.1. Place a 48-well plate on ice for 30 min to loosen the matrix gel.

6.1.2. Remove the organoid media and add 400 µL of ice-cold PBS to each well. Then, remove PBS and add 400 µL of ice-cold Cell Recovery Solution to each well. Rock gently at 4 °C for 30 min.

6.1.3. 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.

6.1.4. Rinse each well with 300 µL PBS + 1% BSA and transfer any remaining organoids to the corresponding tubes. Remove Cell Recovery Solution/PBS + BSA from each tube. Add 400 µL of ice-cold PBS, then repeat with another ice-cold PBS rinse.

6.1.5. Remove PBS and fix organoids with 300 µL ice-cold 4% PFA (in 0.1 M PB) for 20 min, incubating at room temperature. Remove PFA and rinse organoids with 400 µL ice-cold PBS.

6.1.6. Remove PBS; then, add 400 µL PBS + 1% BSA. Store at 4 °C.

6.2. Immunofluorescence staining

6.2.1. Rinse organoids in 500 µL 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.

6.2.2. Add the primary antibody solution (primary antibodies diluted in blocking solution) and rock gently for 3 nights at 4 °C.

6.2.3. 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.

6.2.4. 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.

6.2.5. Wash the organoids 3x for 20 min with 0.1 M PB, rocking gently and covered at room temperature.

6.3. Slide mounting of organoids for inverted confocal microscopy.

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

6.3.1. Create a ~1 mm thick 22 x 22 mm square perimeter of non-toxic modeling clay on a microscope slide.

6.3.2. 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.

6.3.3. 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 interest[24]. 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 out[25]. 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[17–22]. 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 TGFß signaling inhibitor) and SB202190 (a p38 MAPKinase signaling inhibitor) are used to promote organoid growth[26]. 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 lineages[5]. 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 expression[27] 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 epithelium[1]. 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*.

FIGURE AND TABLE LEGENDS:

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.

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.

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.

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.

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 µm (**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: ±SD.

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: ± SD.

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.

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 µm for A, B, and C. Nuclear marker DAPI (blue, right column).

Table 1: Primer sequences used for quantitative RT-PCR.

Supplementary Table 1: Comparison of published lingual organoid culture media components.

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 $LGR5^+$ cells[17]. 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 experimentation[28]. 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 µL 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 work[29].

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 biology[32]. 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[22,33–35]. 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].

ACKNOWLEDGMENTS:

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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.

DISCLOSURES:

The authors have nothing to disclose.

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623

Figure 1

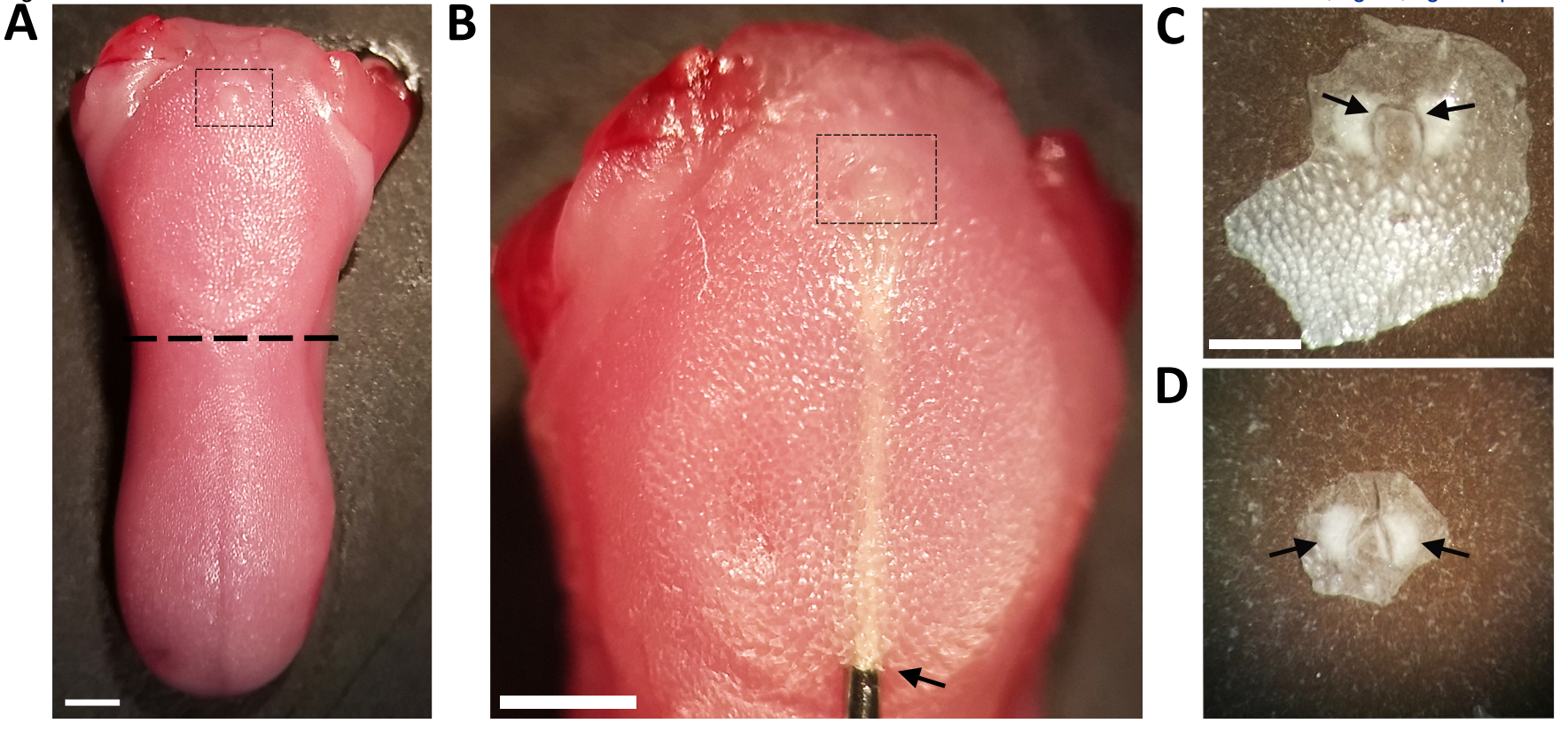

Figure 2

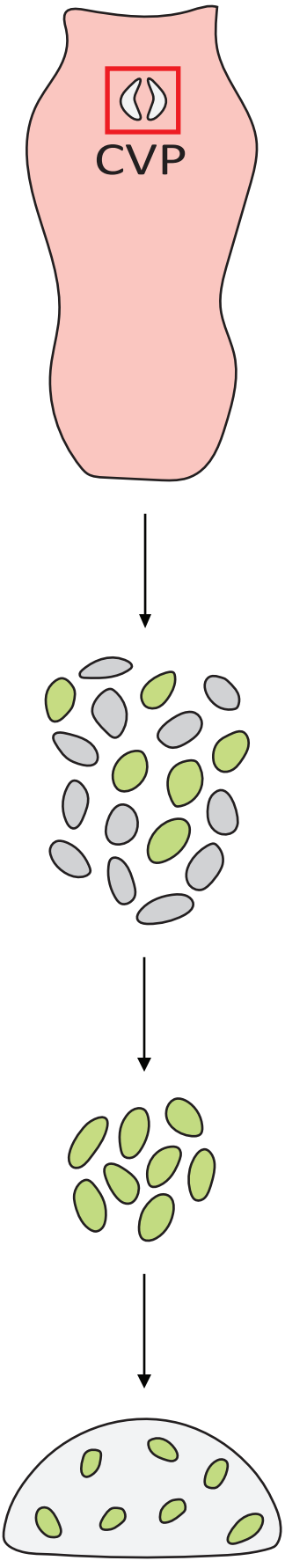

Figure 3

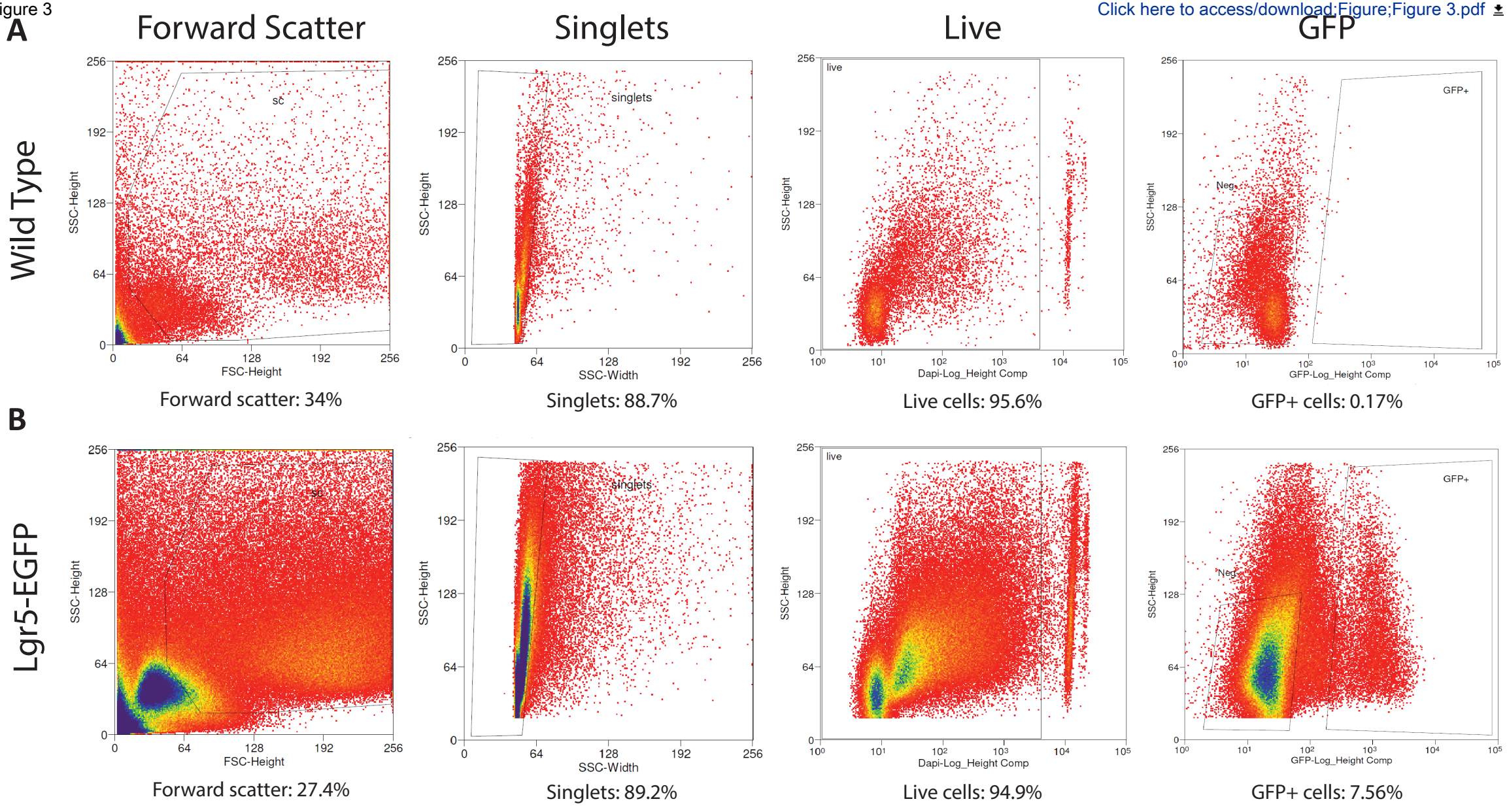

Figure 4

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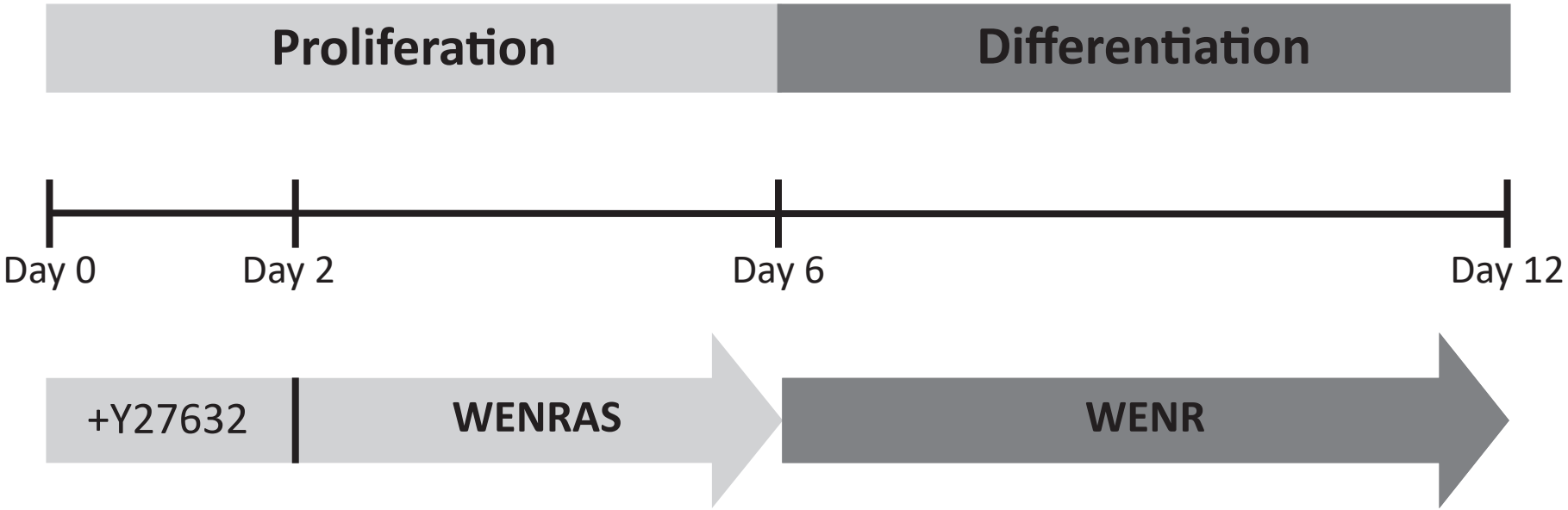

Media Components: **W**nt3a, **E**GF, **N**oggin, **R**-Spondin, **A**8301, **S**B202190

Figure 5

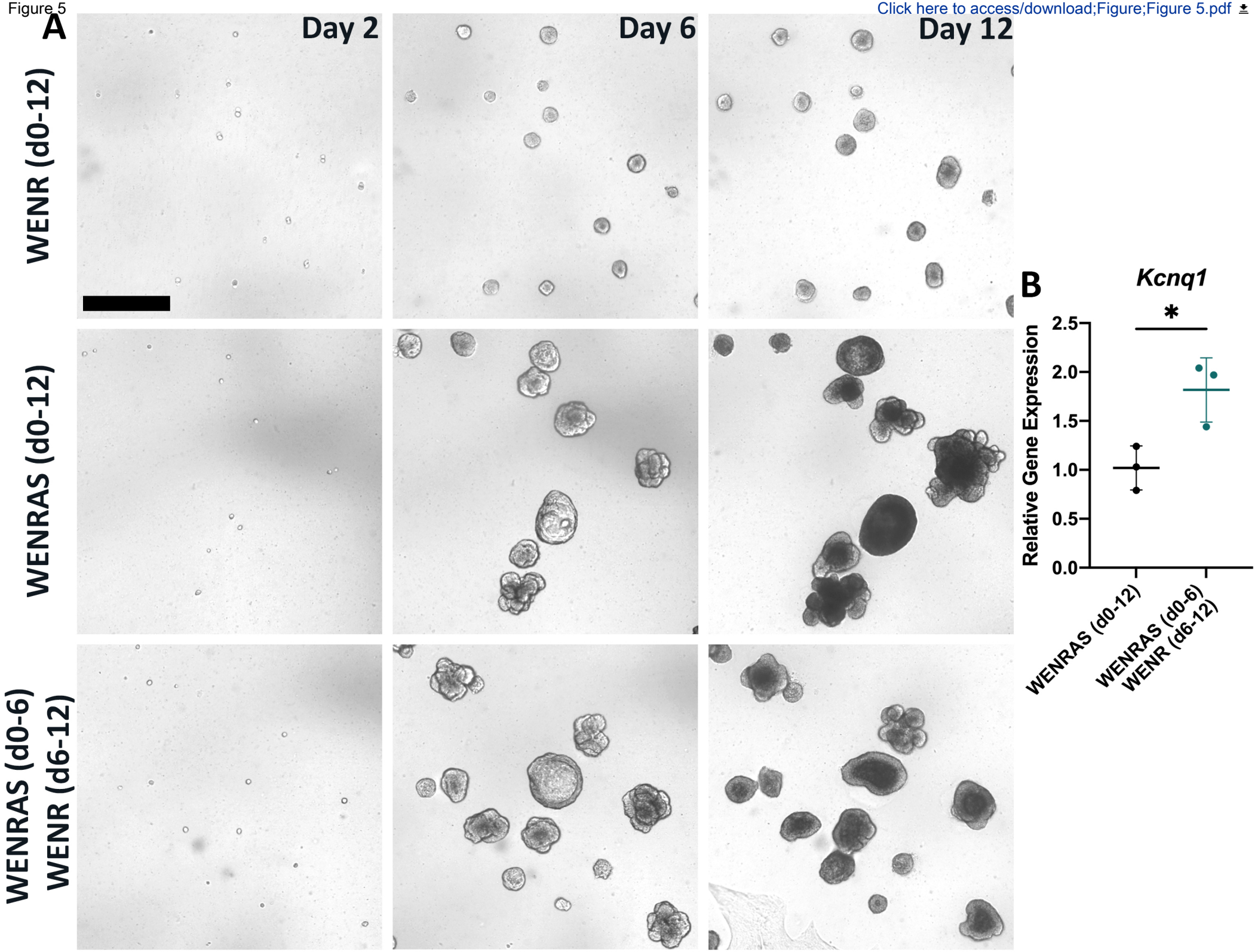

Figure 6

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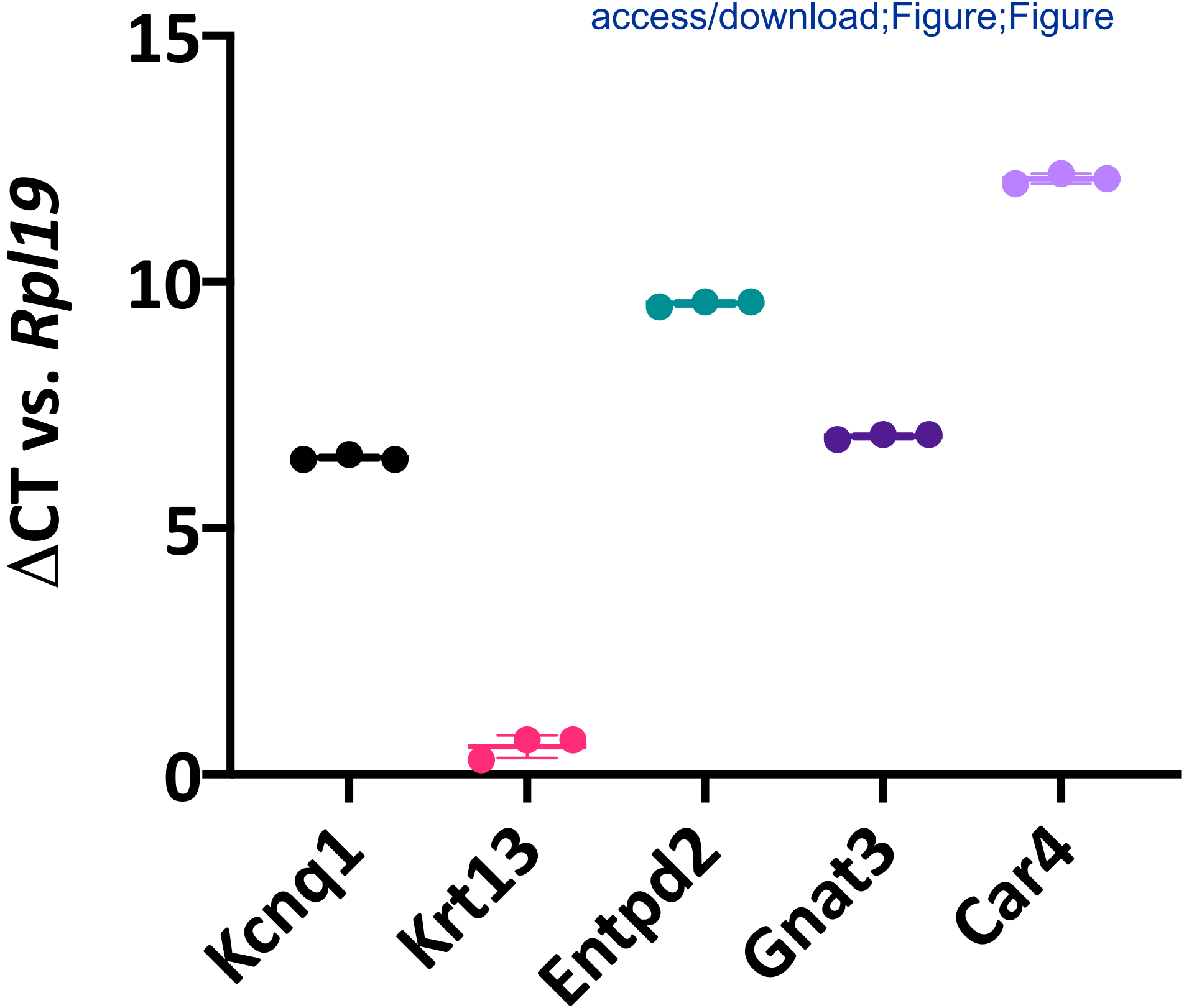

Figure 7

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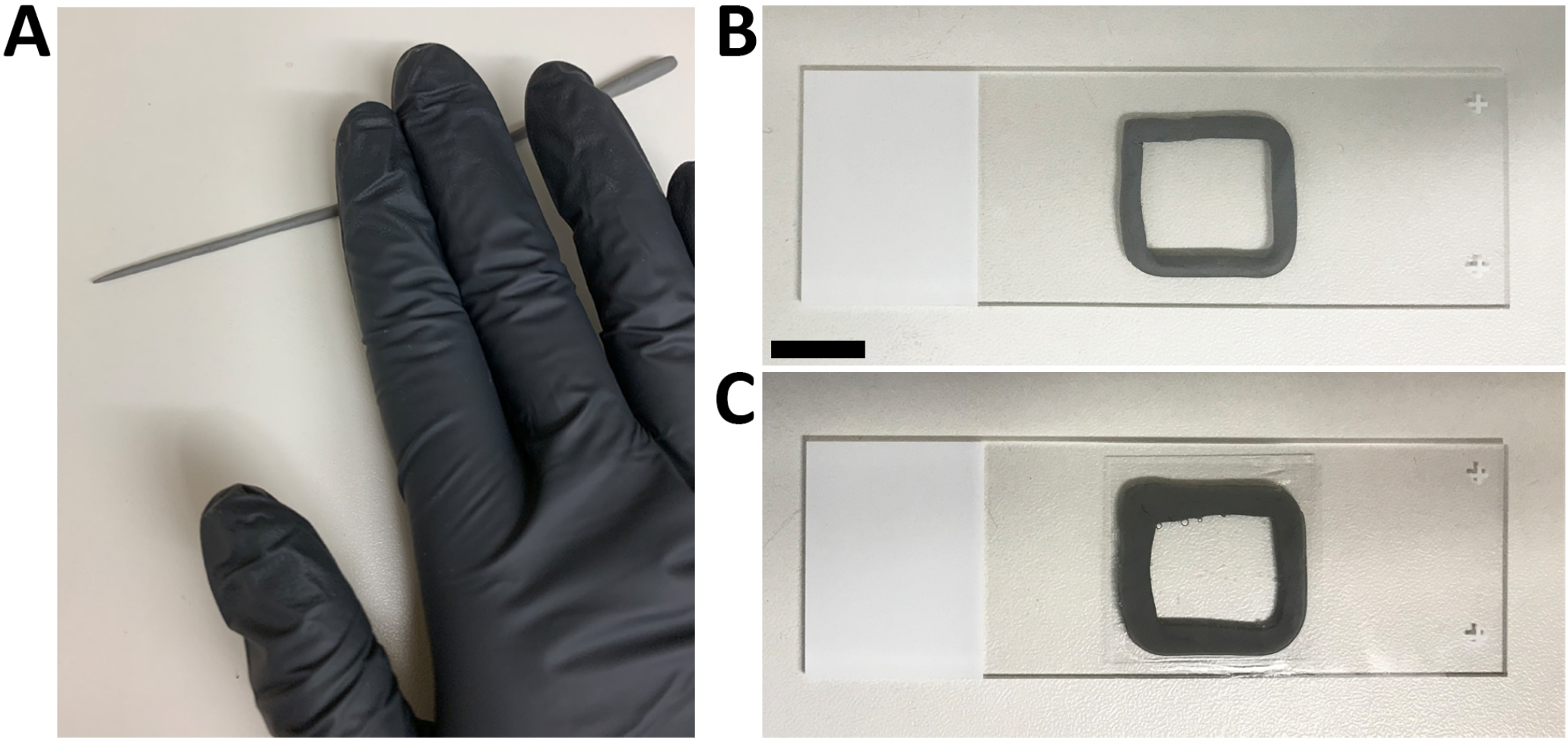
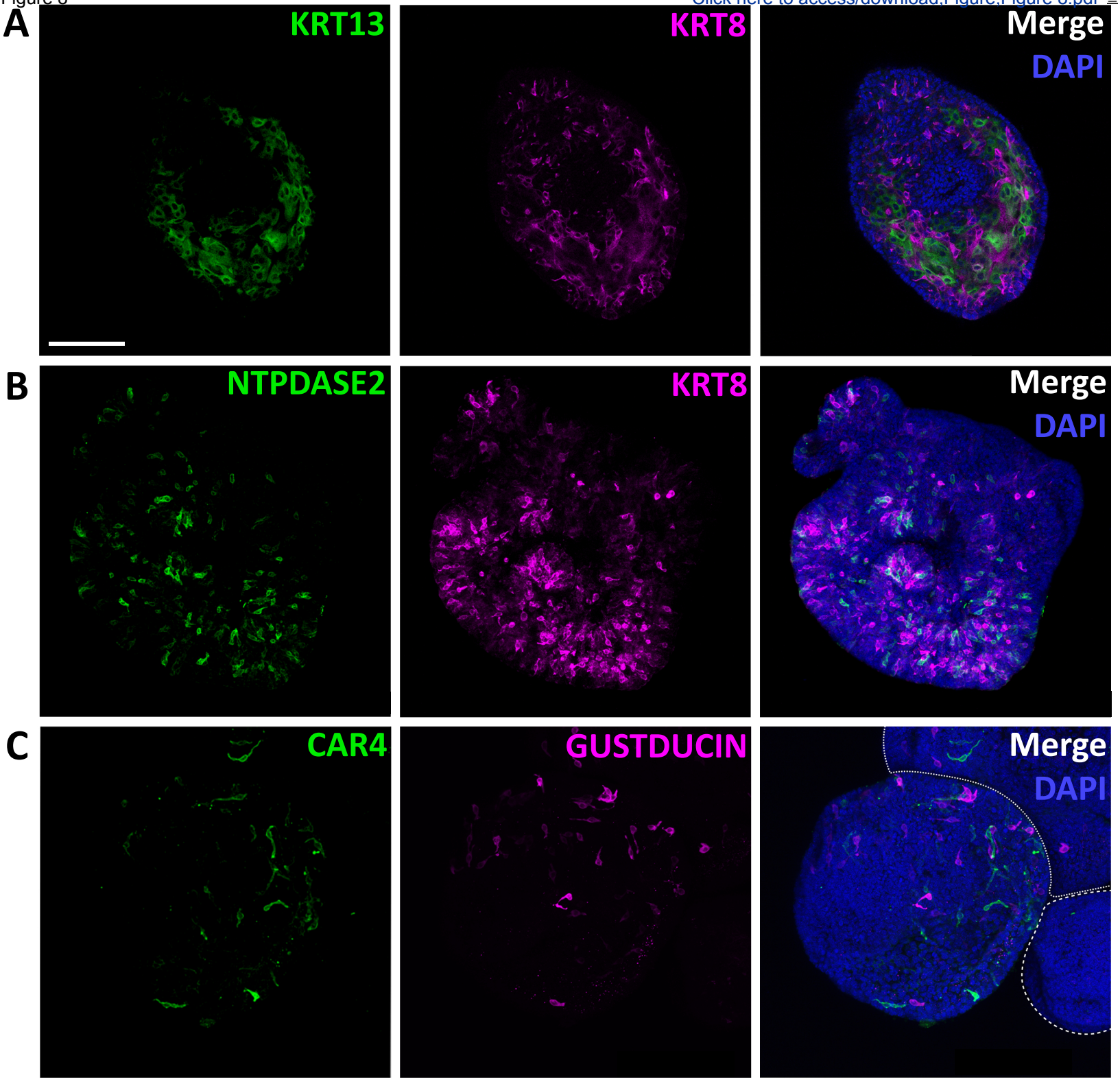


Figure 8

Click here to access/download:Figure:Figure 8.pdf



Gene Name	Forward primer (5'-3')
Car4	CTGCTAGGACAAAGGTGAACC
Entpd2	GACAAGGAAAATGACACAGGTATCGTGG
Gnat3	ATCCAGGAATCCAAGCCTGC
Kcnq1	TTTGTTCATCCCCATCTCAG
Krt13	TCATCTCGGTTTGTCACTGGA
Rpl19	GGTCTGGTTGGATCCCAATG

Reverse primer (5'-3')

CTCCACTGTGTGTTGATTGTTCT

GTTCAAGACATTCAACCAGACTC

TGGTTTTCACCCGGGAATGT

GTTGCTGGGTAGGAAGAG

TGATCTTCTCGTTGCCAGAGAG

CCCGGGAATGGACAGTCA

Assession Number

NM_007607.2

NM_009849.2

NM_001081143.1

NM_008434.2

NM_010662.2

NM_009078.2

Name of Material/ Equipment	Company	Catalog Number
Antibodies		
Alexa Fluor 546 Donkey anti Goat IgG	Molecular Probes	A11056, RRID: AB_142628
Alexa Fluor 546 Goat anti Rabbit IgG	Molecular Probes	A11010, RRID:AB_2534077
Alexa Fluor 568 Goat anti Guinea pig IgG	Invitrogen	A11075, RRID:AB_2534119
Alexa Fluor 647 Donkey anti Rabbit IgG	Molecular Probes	A31573, RRID:AB_2536183
Alexa Fluor 647 Goat anti Rat IgG	Molecular Probes	A21247, RRID:AB_141778
DAPI (for FACS)	Thermo Fischer	62247
DAPI (for immunohistochemistry)	Invitrogen	D3571, RRID:AB_2307445
Goat anti-CAR4	R&D Systems	AF2414, RRID:AB_2070332
Guinea pig anti-KRT13	Acris Antibodies	BP5076, RRID:AB_979608
Rabbit anti-GUSTDUCIN	Santa Cruz Biotechnology Inc.	sc-395, RRID:AB_673678
Rabbit anti-NTPDASE2	CHUQ	mN2-36LI6, RRID:AB_2800455
Rat anti-KRT8	DSHB	TROMA-IS, RRID: AB_531826
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 Jackeed CO2 Incubator	Thermo Scientific	4110
Incucyte	Sartorius	Model: S3
MoFlo XDP100	Cytomation Inc	Model: S13211997
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
Water bath	Precision	51220073
Media		

A83 01	Sigma	SML0788-5MG
Advanced DMEM/F12	Gibco	12634-010
B27 Supplement	Gibco	17504044
Gentamicin	Gibco	15750-060
Glutamax	Gibco	35050061
HEPES	Gibco	15630080
Murine EGF	Peprotech	315-09-1MG
Murine Noggin	Peprotech	250-38
N-acetyl-L-cysteine	Sigma	A9165
Nicotinamide	Sigma	N0636-100g
Pen/Strep	Gibco	15140-122
Primocin	InvivoGen	ant-pm-1
SB202190	R&D Systems	1264
WRN Conditioned media		
Y27632 dihydochloride 10ug	APExBIO	A3008-10

Other

1 ml TB Syringe	BD Syringe	309659
2-Mercaptoethanol, min. 98%	Sigma	M3148-25ML
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
Cultrex RGF BME, Type 2, Pathclear	R&D Systems	3533-005-02
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 Tryspin 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
Power SYBR 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

	Comments/Description
1:2000	
1:2000	
1:2000	
1:2000	
1:2000	
1:10000	
1:50	
1:250	
1:250	
1:300	
1:100	

184 L, Polished Stainless Steel
Cancer Center Cell Technologies Shared Resource, University of Colorado Anschutz Medical Campus
Gates Center Flow Cytometry Core, University of Colorado Anschutz Medical Campus

Stock concentration 10 mM, final concentration 500 nM

Stock concentration 50X, final concentration 1X
Stock concentration 1000X, final concentration 1X
Stock concentration 100X, final concentration 1X
Stock concentration 100X, final concentration 1X
Stock concentration 500 µg/mL, final concentration 50 ng/mL
Stock concentration 50 µg/mL, final concentration 25 ng/mL
Stock concentration 0.5 M, final concentration 1 mM
Stock concentration 1 M, final concentration 1 mM
Stock concentration 100X, final concentration 1X
Stock concentration 500X, final concentration 1X
Stock concentration 10 mM, final concentration 0.4 µM

Received from Dempsey Lab (AMC Organoid and Tissue Modeling Share Resource). Derived from L-WRN (ATCC® CRL-3276™) cells
Stock concentration 10 mM, final concentration 10 µM

β-mercaptoethanol

Matrigel

22x22mm

March 8, 2021

Dear Dr. Nguyen,

Thank you for your valuable feedback on our JoVE methods manuscript. The reviewers' comments help clarify our protocol and improve the manuscript. We have revised the manuscript accordingly, and address each point below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thoroughly proofread the manuscript.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. E.g.: Matrigel, Eppendorf, Kim Wipe, Qiagen

All commercial language has been removed.

3. Line 56: "type-I/ type-II.." instead of "type I/ type II".

Conventional nomenclature in the field references 'type I/type II' cells without the hyphen, thus we prefer to use this format to be consistent with the accepted format.

4. Line 112-113: Please do not highlight euthanasia steps for filming. Also specify what "approved secondary method" means.

We removed highlighting of the euthanasia steps for filming. We also specified secondary methods approved by the University of Colorado IACUC: Bilateral thoracotomy, cervical dislocation, decapitation or exsanguination.

5. Abbreviate liters to L to avoid confusion. E.g.: 10 mL, 8 µL

This has been corrected throughout the manuscript.

6. Italicize g in centrifugation speeds. E.g.: 1,000 x g

This has been corrected throughout the manuscript.

7. Please do not use "&" in the references. Ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

The format of the reference list is now compliant.

8. Please sort the Materials Table alphabetically by the name of the material.

We sorted the Materials Table alphabetically by the name of the material within each section. If preferred, we can remove the sections and order all materials alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript summary:

In their manuscript, Shechtman et al. describe a protocol for the generation and culture of murine epithelial organoids derived from Lgr5+ taste bud stem cells. The authors first describe a procedure to isolate and dissociate circumvallate taste papilla (CVP) epithelium. The resulting single-cell preparation was then flow sorted to purify Lgr5+ cells that were embedded in Matrigel and provided with growth medium. Within 12 days, generated organoids can be harvested and used for analysis via quantitative PCR (qPCR) and immunohistochemistry.

Major concerns:

- Introduction: Please provide a rationale for the growth medium components used, in particular the niche factors (i.e. growth factors/cytokines).

The main components used in the lingual organoid growth medium (Wnt3a, EGF, Noggin and R-spondin) are adapted from conventional culture media of intestinal organoids. Although previous publications using the lingual organoid model system consistently used these four components, other media components and their proportions within the media varied (presented in new Supplementary Figure). When we modeled our medium based on the four consistent ingredients (WENR media), organoid growth was minimal (new Figure 5, WENR condition). Thus, we adapted this protocol by adding drugs A8301, and SB202190, which resulted in robust organoid growth (Figure 5, WENRAS condition). We have clarified our rationale for adding A8301 and SB202190 to our media with the addition of a new Figure 5 and within the representative results section. Within the introduction and the representative results section, we also cite the intestinal organoid papers in which the main factors, as well as A8301 and SB202190, were first used (and are listed below).

Sato, T. and H. Clevers, *Primary mouse small intestinal epithelial cell cultures.* Methods Mol Biol, 2013. **945**: p. 319-28.

Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772, doi:10.1053/j.gastro.2011.07.050 (2011).

- Protocol: Did the authors test Matrigel-alternatives such as Basement Membrane Extract (BME) or Geltrex Matrix? If so, please comment on whether these alternatives give similar results.

As indicated in the materials list, we use Reduced Growth Factor Basement Membrane Extract (RGF BME), Type 2, Pathclear (R&D Systems) as a less expensive substitute for Matrigel. We have not tested more Matrigel alternatives. In the discussion we now acknowledge this limitation and suggest future testing of these alternative culture matrices.

Can organoids also be generated without prior flow sorting of Lgr5+ cells by simply embedding the single-cell preparation harvested from the digestion of the CVP epithelium? Or does this increase the chance of having contamination with non-taste bud epithelial cells? Please comment.

Lingual organoids can be generated without prior flow sorting of $Lgr5^{+}$ cells. This has previously been shown in the reference below. In our hands however, this yields a smaller proportion of organoids with taste bud cells, as suggested by the reviewer. Flow sorting for Lgr5-GFP^{+} cells enriches for progenitors that are taste competent. We have added this point to the discussion.

Aihara, E. *et al.* Characterization of stem/progenitor cell cycle using murine circumvallate papilla taste bud organoid. *Sci Rep* **5**, 17185, doi:10.1038/srep17185 (2015)

- Step 1.1: Please give possible examples for secondary methods.

We added examples of secondary methods approved by the University of Colorado IACUC.

Step 2.1: Please specify the type of collagenase used here.

The enzyme used is type-I collagenase from *Clostridium histolyticum* and is intended for general use. We clarified this in the materials section.

- Part 5: Please provide sufficient details for the qPCR procedure, including assay kits and primers used. I would suggest to expand the protocol on this here after step 5.4.

We added the qPCR procedure following step 5.4 as suggested. Primer sequences were added in Table 1.

- Steps 6.1.2 and 6.2.2.: Please define "low speed".

We acknowledge that “low speed” is not specific. However, the manufacturer of the rocker we use does not provide a specific speed. Thus, we changed all “low speed” descriptions to “rock gently” to avoid confusion.

- **Representative Results: Could the authors please provide some bright-field images of the organoids during different lengths in culture (e.g. showing the growth progression from single cells to fully grown organoids)?**

Bright field images of lingual organoids have been published by the reference below. In addition to citing this paper, we have added a new Figure 5 comprising lingual organoids imaged at progressive days during culture.

Ren, W. *et al.* Single Lgr5- or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo. *Proc Natl Acad Sci U S A* **111**, 16401-16406, doi:10.1073/pnas.1409064111 (2014)

- Figure 3: Ideally, the number of analysed cells purified from wild-type mice should be similar to those purified from Lgr5-EGFP-IRES-CreERT2 mice. Could the authors please amend the provided data accordingly? Is the EGFP brightness relevant for the organoid-forming efficiency? This is because the GFP gate in panel B seems to also contain cells with medium brightness for EGFP. Please indicate percentages for the cells/events recorded in the gates (preferably with ranges and error bars obtained by biological replication).

Epithelial cells from wild type (WT) mice are used only to set the baseline fluorescence above which Lgr5-GFP+ cells are subsequently gated and thereby collected. WT cells are not collected nor cultured, and this small set of cells serves only as a gating control. We have clarified this distinction in the text of the representative results section.

We have also considered the hypothesis that Lgr5-GFP brightness may affect organoid potential but have yet to test it. We mention this now in the discussion.

We have amended Figure 3 as requested to include percentages for the cells/events recorded.

- Figure 5: Quantitative PCR (qPCR) data graphically presented are usually transformed as 2-deltaCt (see PMID: 18546601). Could the authors please amend the graphics accordingly?

While the $2^{-DeltaDeltaCt}$ method is generally used to compare the relative expression of a gene of interest between conditions, the goal of Figure 5 (now Figure 6) is to show the absolute level of amplification for each of the represented genes. Per the reviewer's suggestion, we now include the relative expression of non-taste marker *Krt13* and general taste bud marker *Kcnq1* in the text of the representative results section, by calculating the fold change between *Krt13* and *Kcnq1* expression using the Livak and Schmittgen $2^{-DeltaDeltaCt}$ method (Doi: 10.1006/meth.2001.1262), to support the hypothesis that organoids are predominantly composed of non-taste epithelium.

A control showing the gene expression typical for CVP epithelium is missing here. Could the author please perform qPCR for these markers on RNA isolated from CVP epithelium here?

Multiple housekeeping genes have previously been tested in our lab to attempt to compare organoids and CVP tissue. However, all genes tested while consistent within a tissue type, were highly variable between CVP tissue and organoids (>2Ct). Therefore, gene expression between CVP and organoids cannot be reliably compared in this manuscript (e.g. 10.1023/b:bile.0000019559.84305.47).

Krt13 expression is very minimal suggesting that the organoids are predominantly composed of taste bud cells. Could the authors please emphasize this observation more in their revised manuscript?

A low deltaCt value reflects high gene expression levels, which is supported by the fold change between *Krt13* and *Kcnq1* expression calculated with the $2^{-DeltaDeltaCt}$ method (See above). Higher expression of *Krt13* vs. *Kcnq1* suggests that organoids are predominantly composed of non-taste epithelium. We emphasize this observation in the representative results.

Does each biological replicate represent one mouse? Or if pooled from several mice, was the entire experiment including CVP isolation and digestion independently repeated for each biological replicate?

Each organoid experiment comprises pooled FACS sorted cells from 3 Lgr5-GFP^{+} mice which are then plated at 200 cells/well into 48 well plates. For PCR, each biological replicate represents organoids from three pooled wells.

Minor concerns:

- Please correct "LGR5-EGFP" to "Lgr5-EGFP" (Lgr5 as gene name in italics), as this is not a fusion protein of LGR5 and EGFP but EGFP expression is controlled by the Lgr5 promotor.

This has been corrected.

- Please provide a reference for the (validation of) WRN conditioned.

WRN conditioned medium from L-WRN cells has been shown to support 3D culture of intestinal stem cells and spheroids by the following reference:

Miyoshi H, Stappenbeck TS. In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. Nat Protoc. 2013 Dec;8(12):2471-82. doi: 10.1038/nprot.2013.153. Epub 2013 Nov 14. PMID: 24232249; PMCID: PMC3969856.

We provided this reference in the Note section, under the **Maintenance** section of the protocol.

Reviewer #2:

Manuscript Summary:

This manuscript addresses an important technical issue in the culture methods for production of lingual organoids for adult mice. The detailed description of the method for producing organoids in this manuscript seems to contribute to the development of cell biology research of taste bud cell in the future. Could you please answer several questions about technical problems.

Minor Concerns:

Line 135: **Are there any problems in the step of dissociation of CVP epithelium using Eppendorf tube "pre-coat with FBS" Because my laboratory use FBS as inhibitor of trypsin-EDTA.**

We do not notice an issue using FBS coated tubes, as most of the FBS is removed prior to adding anything to the tube. The film of FBS coating the tubes is enough to prevent sticking of cells to the sides of the Eppendorf tube, but not enough to inhibit trypsin-EDTA. We now address this concern in the “Isolation of CVP epithelium and Lgr5-EGFP progenitors” section of the discussion.

Line 141: In this manuscript describes Collagenase, Elastase and Dispase as "Dissociation enzyme cocktail", is it a mistake of 0.25% Typsin-EDTA?

The dissociation enzyme cocktail containing Collagenase, Elastase and Dispase is used to first treat the peeled CVP epithelium (step 2.1). After this initial digestion, 0.25% Trypsin-EDTA is added to continue digestion of the remaining CVP tissue into single cells (step 2.3). We have made this distinction clearer in the manuscript.

Line 202: Could you please describe the purpose of adding the drugs (A8301 and SB202190) to the culture medium.

Adding the drugs A8301 and SB202190 to the culture medium promotes growth, as discussed in (Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772, doi:10.1053/j.gastro.2011.07.050 (2011)). To further address this question, our new figure 5 shows the growth of organoids tracked from day 2 to day 12 of culture. We show that organoids grown in conventional organoid medium (WENR) for 12 days grow much less efficiently, but with addition of A8301 and SB202190 (WENRAS), larger organoids result. Additionally, qPCR data show that organoids grown in WENRAS (day 0-12) have lower expression of the taste cell marker *Kcnq1*. Previous work from the Jiang lab showed that markers of taste cell differentiation are detected in organoids beginning at ~day 8 of culture (Ren, W. *et al.* Transcriptome analyses of taste organoids reveal multiple pathways involved in taste cell generation. *Sci Rep* **7**, 4004, doi:10.1038/s41598-017-04099-5 (2017)), and thus we reasoned that WENRAS promotion of growth may repress taste cell differentiation. Thus, we used WENRAS during the growth phase (day 0-6) and then switched to WENR during the differentiation phase (day 6-12). Consistent with our hypothesis, organoids grown in WENRAS (day 0-6), then switched WENR (day 6-12) express *Kcnq1* at a significantly higher level. This rationale has now been clarified in the text.

Line 372: Your description looks like that only one CVP epithelium from LGR5 mice could obtain 50 wells in a 48-well plate. Is it correct? Because in our laboratory three CVP use for 1well in a 24-well plate using wild-type mice.

Epithelial cells from three Lgr5-GFP^{+} CVPs are processed and sorted together, and the pooled sample is used to fill a 48-well plate at 200 cells/well. We have clarified this in the manuscript as well as in the “note” in the “Isolation of CVP epithelium” section of the protocol and at the end of the FACS paragraph of representative results.

Reviewer #3:

Manuscript Summary:

Shechtman and Piarowski et al. attempted to adapt previous published protocol (Ren et al. PNAS 2014) and presented a standardized method for generating taste organoid from taste progenitor cells isolated from CVP of adult mice that carried LRG5EGFP-IRES-CREERT2 allele.

Major Concerns:

However, by referring to study by Ren et al., I cannot see any difference between the studies, in terms of the protocol used to generate the taste bud organoid. The author should clarify clearly what have been adapted to ease the reader who wish to establish the taste bud organoid from this study. Apart from that, what is the difference between this current study compared to Aihara et al. (Sci Rep 2015), that characterize progenitor cell cycle using murine CVP taste bud organoid.

> To address this concern in the revised manuscript, we clarified that, to date, each cited taste organoid publication used different culture conditions. To illustrate some of the differences, we included a new supplementary table comparing the culture media used in those studies and ours. In addition, we added a more detailed description of the changes we made to the culture media and culture protocol (e.g WENR vs WENRAS), supported with a rationale and representative data.

It seems like the protocol to generate the taste bud organoid is not uncommon.

> The goal of method articles in JoVE is to provide written step-by-step instructions along with a video tutorial for a technique of interest that has already been published or is original. This format provides more accurate descriptions of the procedures than a standard publication format and maximizes reproducibility. This manuscript follows JoVE editorial policies: "JoVE does publish methods and techniques that have been used to generate data previously published in results-driven journals. In all cases, JoVE requires original manuscript text that does not overlap with any other article, either previously published or at any stage in the publication process. If an author uses representative results or figures from a prior publication, the author must obtain permission from the original publisher and include appropriate citation. JoVE encourages authors to publish the most recent version of their method, especially if the method has been improved upon following original use." (https://www.jove.com/authors/editorial-policies#step-3).

Plus, the authors also indicate the potential application of taste bud organoid as a platform for drug screening tool. However, there is no evidence from this paper that this generated organoid can be facilitated as a platform for drug screening. It would be great if the authors can demonstrate the ability of the established organoid by screening for some potential drugs to address this basic application of the organoid.

Here we provide a detailed, step-by-step, protocol for consistently generating organoids from adult mouse lingual stem cells. Developing this approach for drug screening is a future application of this technology, but beyond the scope of this JoVE article. The advantages of organoid cultures, including quick generation in multi-well plates, as well as references to other organoid models are described in the manuscript to support the perspective of using taste organoids as a drug screening tool.

Minor Concerns:
Overall, the authors have carefully described the protocol step by step however, I still have few comments which needs to be addressed.
Comments:
1) The authors failed to tell the reader which protocol has been modified. Optimizing the culture media component is not sufficient to claim the protocol as modified.

When a reagent, here culture media, is altered, the protocol is inherently different because the experimental outcome may be changed. As described in the manuscript, the change in media composition after 6 days is done to promote differentiation following optimal growth (Please see new figures 4 and 5). In addition, culture media is not the only difference compared to previously published procedures (e.g. cell dissociation, plating density, etc.). Also, please refer to editorial policies cited above and at https://www.jove.com/authors/editorial-policies.

2) It is unclear how the authors prevent contamination from the tongue that existed from the mouse even though the culture media consist of primocin and other antibacterial/fungus agents. Did the authors sterilize the tongue by dipping in ethanol or did they use sterile PBS?

In order to prevent contamination, we use sterile PBS to collect the tongues. We amended the line to say we collect in ice-cold **sterile** dPBS with Ca^{+} and Mg^{+}.

3) The description of the entire part of the qPCR technique in this protocol is missing.

We added the qPCR procedure following step 5.4 as suggested.

4) Under section 4. , Maintenance. The author should remove the EGF from the 50% conditioned media derived from CRL3276 as it only consists of Wnt3a, R-spondin and Noggin.

The reviewer is correct that EGF is not in the media derived from CRL3276 cells. To avoid confusion, the wording of this section was changed to make clear that WENR and WENRAS media comprise 50% conditioned media plus recombinant EGF added prior to culture.

5) The mounting of organoid for inverted confocal microscope is always challenging. It would be great if the author can show the similar pattern as depicted in figure 1 for these steps especially how the authors create a 1mm thick 22x22 mm square perimeter of non-toxic modeling clay in the microscopic slide.

We agree that properly mounting the organoids for inverted confocal microscopy is challenging yet important for capturing high quality images. In order to help the reader visualize the method developed by our lab, we added a new figure 7 as requested showing progressive steps in the slide mounting process.

6) Which in vivo study is being referred in line 321-324 to serve as the evidence for showing the discrepancies between organoid and taste epithelium in this study? Regardless of the findings, the authors have no backup to claim that the discrepancy is due to lack of interaction between organoids and the taste bud microenvironment.

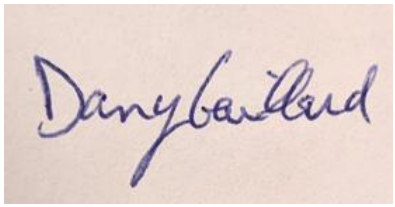
We are simply speculating on this possibility, as taste cells have been shown to interact with the lamina propria and gustatory nerves *in vivo*. We cited specific studies that show the importance of these interactions. Additionally, we moved this speculation to the discussion section.

7) Please tidy up the arrangement of the table of materials as the last few pages is not aligned with the first page.

Done.

Given these extensive revisions and improvements, including new figures and a carefully edited text, we hope that our manuscript now is acceptable for publication at JoVE.

Sincerely,



Linda A. Barlow, PhD
Professor

Dany Gaillard, PhD
Instructor

Ren et al, 2014

Wnt-conditioned media (unknown)

Noggin (100ng/mL)

R-spondin-1 (200ng/mL)

DMEM/F12

EGF (50ng/mL)

N-acetylcysteine (1mM)

B27 Supplement (2%)

N2 Supplement (1%)

Y27632 (10µM)

Jagged-1 (1µM)

Aihara et al, 2015

Wnt-conditioned media (50%)
Noggin (100ng/mL)
R-spondin (10%)
DMEM/F12
EGF (50ng/mL)
Penicillin/Streptomycin (100U/mL)
B27 Supplement (1X)
N2 Supplement (1X)
Glutamax (2mM)
HEPES (10mM)

Ren et al, 2017

Wnt3a Conditioned Media (50%)
Noggin (10%)
R-spondin conditioned media (20%)
DMEM/F12 (20%)
EGF (50ng/mL)
Penicillin/Streptomycin (1X)
B27 Supplement (2%)
N2 Supplement (1%)
Y27632 (10µM)
Commercial Noggin (10ng/mL)

Feng et al, 2020

Wnt3a
R-spondin
Noggin
DMEM/F12
EGF
B27 Supplement
N2 Supplement

Lin et al, 2021

Wnt3a Conditioned Media (50%)
Noggin (10%)
R-spondin conditioned media (20%)
DMEM/F12 (20%)
EGF (50ng/mL)
Penicillin/Streptomycin (1X)
B27 Supplement (2%)
N2 Supplement (1%)
Y27632 (10µM)