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Generation and Characterization of Murine Oral Mucosal Organoid Cultures

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TITLE:

Generation and Characterization of Murine Oral Mucosal Organoid Cultures

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KEYWORDS:

organoids, oral mucosa, tongue epithelium, adult stem cells, gene expression, whole-mount imaging

SUMMARY:

We present a method for the generation and characterization of oral mucosal organoid cultures derived from the tongue epithelium of adult mice.

ABSTRACT:

The mucous lining covering the inside of our mouth, the oral mucosa, is a highly compartmentalized tissue and can be subdivided into the buccal mucosa, gingiva, lips, palate, and tongue. Its uppermost layer, the oral epithelium, is maintained by adult stem cells throughout life. Proliferation and differentiation of adult epithelial stem cells have been intensively studied using *in vivo* mouse models as well as two-dimensional (2D) feeder-cell based *in vitro* models. Complementary to these methods is organoid technology, where adult stem cells are embedded into an extracellular matrix (ECM)-rich hydrogel and provided with a culture medium containing a defined cocktail of growth factors. Under these conditions, adult stem cells proliferate and spontaneously form three-dimensional (3D) cell clusters, the so-called organoids. Organoid cultures were initially established from murine small intestinal epithelial stem cells. However, the method has since been adapted for other epithelial stem cell types. Here, we describe a protocol for the generation and characterization of murine oral mucosal organoid cultures. Primary epithelial cells are isolated from murine tongue tissue, embedded into an ECM hydrogel, and cultured in a medium containing: epidermal growth factor (EGF), R-spondin, and fibroblast growth factor (FGF) 10. Within 7 to 14 days of initial seeding, the resulting organoids can be passaged for further expansion and cryopreservation. We additionally present strategies for the characterization of established organoid cultures via 3D whole-mount imaging and gene-expression analysis. This protocol may serve as a tool to investigate oral epithelial stem cell

behavior *ex vivo* in a reductionist manner.

INTRODUCTION:

The oral mucosa is the mucous lining covering the inside of our mouth. It functions as the entrance of the alimentary tract and is involved in the initiation of the digestive process[1,2]. In addition, the oral mucosa acts as our body's barrier to the outer environment providing protection from physical, chemical and biological insults[1]. Based on the function and histology, the oral mucosa in mammals can be divided into three types: masticatory mucosa (including the hard palate and gingiva), the lining mucosa (functioning as the surface of the soft palate, the ventral surface of the tongue and the buccal surface), and the specialized mucosa (covering the dorsal surface of the tongue)[2]. All oral mucosal tissues consist of two layers: the surface stratified squamous epithelium and the underlying lamina propria[1]. The oral epithelial keratinocyte is the main cell type of the epithelium, which is also the location of intra-epithelial immune cells such as Langerhans cells[1]. The stromal compartment, the lamina propria, comprises of the different cell types such as fibroblasts, endothelial cells, neuronal cells, and immune cells[1]. As in all stratified epithelia, stem and progenitor cells reside in the basal layer of the oral epithelium[1]. These specialized cells have the ability to replace lost tissue through cell divisions and, therefore, feed the cellular turnover throughout adult life[3]. In contrast to other epithelia such as the intestinal epithelium[4] or skin epidermis[5], the oral epithelia remain poorly understood. However, recent studies uncovered different genes such as *Krt14, Lrig1*, *Sox2*, *Bmi1*, and *Gli1* that mark oral epithelial stem and progenitor cells in mice[1,6–8]. As the oral epithelium is the origin of oral carcinomas and a critical player in mucosal inflammation, wounding, and regeneration[1], a better understanding of its basic cell biology is paramount for potential new therapeutic approaches and drug discoveries.

Animal models have been widely used for basic studies on the oral mucosal epithelium[1]. For example, the aforementioned markers of oral epithelial stem and progenitor cells have largely been defined using genetic lineage tracing mouse models[1,6–9]. However, *ex vivo* approaches using cultured cells of human or murine origin have also been broadly used[10]. Conventionally, such cell culture work has been performed using cell lines derived from oral squamous cell carcinoma (OSCCs) or cell lines generated from (spontaneously or genetically) immortalized primary cells[10]. These 2D cell culture methods have limitations with critical implications on investigating the adult homoeostasis: (1) cell immortalization is accompanied with a large degree of genetic instability, (2) limited capacity to differentiate, (3) requirement for feeder cells, and (4) a largely undefined growth medium containing serum[11]. Collectively, these gold standard *in vitro* methods did not allow long-term cultures of epithelial stem cells without limiting their capacity to proliferate and differentiate as well as transforming their wild-type genome.

Organoid technology has emerged as a tool to establish cultures of near native epithelial tissue *in vitro*[11]. In their 2009 study, Sato et al. described the first epithelial organoid culture system[12]. Their method was based on embedding individual small intestinal stem cells marked by the Wnt/β-catenin target gene *Lgr5*[13] into a 3D extra-cellular matrix (ECM)-rich hydrogel[12]. By providing a defined cocktail of growth factors important for stemness, the seeded adult epithelial stem cells were able proliferate to their capacity in culture[12]. Eventually, cell clusters formed out

of the actively cycling stem cells containing all major intestinal epithelial cell types[12], effectively resembling the tissue-of-origin[11]. In contrast to conventional 2D cultures, organoid technology allowed long-term maintenance of murine intestinal epithelial stem cells under feeder-free conditions with a serum-free and fully defined medium[10,11]. In addition, the method does not significantly alter the genetic makeup or phenotype of the cultured stem cells. Furthermore, long-term culture retained the stem cell's capacity to proliferate and differentiate without a requirement for cell immortalization. Within just over a decade, this early epithelial organoid culture system was amended to grow adult stem cells from many other epithelial tissues such as colon (large intestine)[12,14,15], endometrium[16], liver[17,18], lungs[19,20], mammary glands[21], ovaries[22], pancreas[23,24], skin epidermis[25], and stomach[26]. While most protocols used adult epithelial stem cells derived from mammals such as humans[11,27], mice[11], cats[28], dogs[29], and pigs[30], it has even been possible to generate epithelial organoids from snake venom glands[31]. Organoid technology has become a widely used stem cell culture method with a high degree of versatility[11]. As epithelial organoids remain largely genetically[32,33] and phenotypically stable they are excellent models for gene editing[34,35] to study the gene function[36] or tumorigenesis[27,37–40]. In addition, organoid cultures can be transplanted into mice[37,41] and are used to study host-microbe interactions[42] (including pathogenic infections[43–45]). Furthermore, organoid-based co-cultures with cells of the microenvironment such as immune cells[46–48] and fibroblasts[49,50] have been described. In the context of disease, organoids have been used for generations of living tissue biobanks[21,22,51] as well as testing drugs[27] for efficacy[52,53] and toxicity[54].

In this protocol, we describe an optimized methodology for the establishment and maintenance of oral mucosal organoid cultures from murine tongue epithelium. It is based on previous reports describing the isolation of the tongue epithelium using enzymatic digestion[55] and the derivation of epithelial organoids from mouse and human oral mucosa[52,53]. The growth medium for murine oral mucosal organoids contains critical factors maintaining the stem cell state. R-spondin activates the Wnt/β-catenin signaling cascade[5], while epidermal growth factor (EGF) and fibroblast growth factor (FGF) 10 are cytokines and ligands of receptor tyrosine kinases that stimulate several signaling pathways such as the MAPK/ERK pathway and the PI3K/AKT/mTOR pathway[25]. We further describe in detail how the organoid cultures can be characterized by gene and protein expression analysis and compared with the tissue-of-origin.

PROTOCOL:

All methods described here were performed in compliance with European Union and German legislation on animal experimentation.

NOTE: Prepare working place, including sterile surgical instruments (fine forceps, fine scissors, and scalpels) and Petri dishes filled with cold PBSO. Thaw BME overnight and keep it at 4 °C or on ice until usage. Pre-warm cell culture plates in an incubator overnight before starting the cell isolation. All materials are provided in **Table 1** (**Table of Materials**).

1 Establishment of murine oral mucosal organoid culture

1.1 Dissection of murine tongue

1.1.1 Euthanize the mouse according to the institutional guidelines and respective national and intergovernmental legislation.

NOTE: For this protocol, mice were euthanized by CO_2 exposure, as cervical dislocation may lead to instability of the head, which results in difficulties with organ harvesting.

1.1.2 Lay the mouse on its back and fixate it by pinning the paws to a suitable underlay.

1.1.3 Disinfect the mouse by spraying it with 70% EtOH until it is completely wet.

1.1.4 Cut the skin with scissors, first vertically along the trachea from the sternum to the lip, and then horizontally from the trachea toward the clavicula on both sides. Every incision is around 2 cm long.

1.1.5 Pull aside the fur to uncover the jaw.

1.1.6 Cut through the jaw muscles till the back of the oral cavity.

1.1.7 Open the oral cavity as far as possible by pulling the lower and upper jaw in opposite directions using two forceps, which results in the dislocation of the lower jaw.

1.1.8 Use blunt forceps to grab the tongue and remove as much of the tongue as possible by cutting vertically in the back.

1.1.9 Place the tongue in cold PBS free of Mg^{2+} and Ca^{2+} (PBSO).

1.1.10 Cut the tongue horizontally to separate the dorsal and ventral tongue mucosa.

NOTE: The dorsal tongue mucosa is the upper side of the tongue, and the ventral tongue is the lower side of the tongue that is in contact with the oral floor. Both mucosae can be discriminated by their morphology, as the dorsal tongue mucosa is visibly roughened, while the ventral tongue mucosa has a smooth surface. The ventral tongue covers a smaller area than the dorsal tongue (~ 5 x 2 mm ventral tongue; ~ 8 x 3 mm dorsal tongue).

1.1.11 Optional: Fix one part of the tongue in either fixative (e.g., 4% paraformaldehyde) or optimal cutting temperature (OCT) medium for cryopreservation.

1.1.12 Optional: Snap-freeze fragments for RNA or protein isolation at -80 °C.

1.2 Digestion for separation of epithelium and lamina propria

1.2.1 Prepare a fresh enzymatic cocktail containing 1 mg/mL collagenase A and 2 mg/mL Dispase II in PBSO and warm-up solution to 37 °C before use.

1.2.2 Inject at least 500 µL of the enzymatic cocktail into the subepithelial space from the posterior cut end of the tongue using a 26 G needle.

1.2.3 Insert the needle deep into the tissue perforating the lamina propria and the underlying muscle carefully remaining parallel to the epithelium.

1.2.4 Inject the cocktail while slowly retracting the needle.

NOTE: A lightening in color and visible expansion of the tongue tissue, confirms a sufficient injection of the digestion enzymes. Also, an induction of a transparent phase underneath the epithelium indicates a proper injection.

1.2.5 Repeat the injection up to five times.

1.2.6 Transfer the tissue into a 2 mL microcentrifuge tube containing the same enzymatic cocktail.

1.2.7 Incubate the sample for 1 h at 37 °C on a shaker (at 300 rpm).

1.2.8 Transfer the tissue into a Petri dish containing PBSO.

1.2.9 Grab the muscle and the tip of the tongue with tweezers and carefully pull the muscle away from the epithelium. If resistance is encountered, probably at the posterior cutting end, lift the epithelium with blunt tweezers.

1.2.10 Wash the separated epithelium with PBSO and proceed to the desired application. For the establishment of organoids proceed to step 1.3.1 and for tissue whole-mount preparations proceed to step 4.1.1.

1.3 Establishing murine oral mucosal organoids from primary tissue

1.3.1 Cut the epithelium into small pieces of around 2 x 2 mm in size.

1.3.2 Digest the tissue in 1 mL of 0.125% trypsin added to PBSO at 37 °C.

NOTE: Digestion should not exceed 30 min.

1.3.3 Check the digestion regularly by shaking every 10 min.

1.3.4 When the mixture becomes cloudy (depending on the amount of tissue) or when a mixture of cell clumps is observed, extend the disruption by vortexing for 5 s and pipetting up and down 10–20 times.

1.3.5 Wash once by topping up with 10 mL of Advanced DMEM/F12+++ medium.

1.3.6 Directly filter the cell suspension using a 70 µm cell-strainer.

1.3.7 Centrifuge at 350 x *g* for 5 min at 4 °C.

1.3.8 Discard the supernatant and resuspend the cells in 1 mL of Advanced DMEM/F12+++ medium for counting.

1.3.9 Count the cells using a Neubauer counting chamber or an equivalent method.

1.3.10 Centrifuge at 350 x *g* for 5 min at 4 °C and aspirate the supernatant.

1.3.11 Resuspend the pellet in BME (keep BME on ice to prevent solidification). Calculate the amount of BME, depending on the cell number (approximately 10,000 cells/40 µL of BME). If the medium cannot be aspirated completely, carefully remove the rest of it using a 100 µL pipette.

NOTE: The concentration of BME should not be less than 70%, as this can lead to insufficient solidification.

1.3.12 Plate the cells on the bottom of pre-heated cell culture (suspension) plates in 10 µL droplets using a P100 pipette.

1.3.13 Place the culture plate upside down into the incubator for 30 min–1 h to let the BME solidify.

1.3.14 Prepare the required amount of murine oral mucosal organoid medium freshly adding ROCK inhibitor and Primocin (see **Tables of Materials** and **Table 1**).

1.3.15 After solidification, add the pre-warmed medium to the cell droplets by carefully pipetting against the wall of the wells to avoid droplet detachment.

1.3.16 Incubate the plates in a humidified incubator at 37 °C and 5% CO_2.

1.3.17 Change the medium every 2–3 days. ROCK inhibitor and Primocin stay in the culture medium for the first two passages.

2 Passaging, cryopreservation and thawing of murine oral mucosal organoids

2.1 Passaging of murine oral mucosal organoid cultures

2.1.1 Murine oral mucosal organoids can be passaged for the first time between 10 and 12 days after initial plating.

2.1.2 For splitting, resuspend the BME droplets in the medium with a P1000 pipette and transfer them to a 15 mL conical tube containing 2 mL of ice-cold PBSO.

2.1.3 Top up the volume with 5 mL of ice-cold Advanced DMEM/F12+++ medium.

2.1.4 Centrifuge organoids at 300 x *g* for 5 min at 4 °C.

2.1.5 Aspirate the supernatant and digest the organoids using 0.125% trypsin in PBSO.

2.1.6 Resuspend the pellet in 1 mL of 0.125% trypsin solution and incubate suspension at 37 °C until organoids break in pieces. Check the digestion every 2 min.

2.1.7 Resuspend the cell suspension thoroughly by pipetting up and down 20–30 times using a P1000 pipette and repeat harsh resuspension with a P200 pipette.

2.1.8 Wash the cells with 10 mL of Advanced DMEM/F12+++ medium.

2.1.9 Optional: Directly filter the cell suspension using a 70 µm cell-strainer to generate a homogeneous cell suspension.

2.1.10 Centrifuge the cell suspension at 350 x *g* for 5 min at 4 °C.

2.1.11 Aspirate the supernatant and resuspend the pellet in BME and proceed with the organoids as described in steps 1.3.8–1.3.17.

2.2 Cryopreservation and thawing of murine oral mucosal organoid cultures

2.2.1 For cryopreservation, let murine oral mucosal organoids grow for 3–5 days after passaging.

2.2.2 Detach organoids from the culture plates as described in steps 2.1.2–2.1.4.

2.2.3 After centrifugation, resuspend the organoids in 1 mL of freezing medium (Advanced DMEM/F12+++ medium containing 10% FCS and 10% DMSO) and transfer the cell suspension into 2 mL cryovials.

2.2.4 Place the cells in the desired freezing containers at -80 °C for up to 24 h. For long-term storage, keep the cells below -120 °C, for example, in a liquid nitrogen tank.

2.2.5 Thaw the cryopreserved cells at 37 °C and quickly transfer the cell suspension to a canonical tube containing 9 mL of pre-warmed Advanced DMEM/F12+++ medium.

2.2.6 Centrifuge the cell suspension at 350 x *g* and 4 °C for 5 min.

2.2.7 Discard the supernatant and proceed with the organoids as described in steps 1.3.8–1.3.17.

3 Gene expression analysis of murine oral mucosal tissue and organoids

3.1 RNA extraction from murine oral mucosal organoids and native tissue

3.1.1 Harvest the murine oral mucosal organoids as described in steps 2.1.2–2.1.4.

3.1.2 Discard the supernatant and wash the organoids in cold PBSO.

3.1.3 Centrifuge the organoids at 300 x *g* and 4 °C for 5 min and discard the supernatant.

3.1.4 To isolate RNA from native tissue, use the separated epithelium (see step 1.2.10).

3.1.5 Cut the tissue in small pieces of 2 mm x 2 mm.

3.1.6 For RNA isolation, use an established method or kit: Resuspend organoids or tissue pieces in, respectively, 350 or 700 µL lysis buffer.

3.1.7 Harshly vortex lysed organoids for at least 10 s and lyse the tissue for at least 30 s.

3.1.8 Place the solution at -80 °C for at least 2 h.

3.1.9 Thaw the cell lysate on ice and proceed with RNA isolation according to the manufacturer's instructions.

3.2 cDNA synthesis by reverse transcription reaction

3.2.1 Measure the RNA concentration and calculate the volume for a total RNA input of 0.1–1 µg.

3.2.2 For cDNA synthesis, use a cDNA Synthesis Kit following the manufacturer's instructions. For this experiment, the following formulation was used: 4 µL of 5x reaction mix, 1 µL of Reverse Transcriptase, x µL of 0.1–1 µg of total RNA and x µL of nuclease-free water up to 20 µL of the final volume.

3.2.3 Perform reverse transcription in a three-step cycler program with the following program: 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

3.2.4 Store cDNA at -20 °C.

3.3 Gene expression analysis by quantitative real-time PCR

3.3.1 For the quantitative real-time PCR, perform all the reactions in technical duplicates.

3.3.2 Prepare a mix of 5 µL of qPCR Supermix, 1 µL of reverse primer (400 nM), 1 µL of forward primer (400 nM), 1 µL of cDNA (10–20 ng/well) and 2 µL of nuclease-free water.

3.3.3 For amplification, use the standard settings as follows: polymerase activation and DNA denaturation at 95 °C for 30 s, denaturation at 95 °C for 5–10 s, annealing/extension and plate read at 60 °C for 60 s for 40 cycles. Perform melt curve analysis at 65–95 °C with 0.5 °C increments at 2–5 s/step (or use the instrument's default settings).

3.3.4 Analyze data using desired methods such as the ΔCt or $\Delta\Delta$Ct methods[56] or using an analysis software provided by the manufacturer of the thermocycler following the given instructions.

4 Protein expression analysis of murine oral mucosal tissue and organoids

NOTE: Whole-mount staining of tongue epithelium was performed in a 24-well plate, transferring the tissue with forceps from well to well in each step.

4.1 Fixation of murine oral mucosal tissue and organoid cultures

4.1.1 For tissue whole-mount staining proceed from step 1.2.10 and continue with step 4.1.5.

4.1.2 For organoid staining, harvest organoids as described in step 2.1.2 and continue with step 4.1.3.

4.1.3 Top up the cell suspension with 10 mL of PBSO.

4.1.4 Centrifuge the cell suspension at 350 x *g* for 5 min at 4 °C and discard the supernatant.

4.1.5 Fix the epithelium or organoids in 4% paraformaldehyde for 30 min at room temperature (21 °C).

4.1.6 Wash the samples once in PBSO. Centrifuge the organoids at 350 x *g* for 5 min at 4 °C and discard the supernatant.

4.2 Whole-mount staining of murine oral mucosal tissue and organoid cultures

4.2.1 Unmask the epitopes by incubating the samples in 0.2% Triton X-100 solution for 20 min at room temperature.

4.2.2 Transfer the samples into the blocking solution (5% donkey serum in PBSO) and incubate for 1 h at room temperature.

4.2.3 Dilute the antibodies in blocking solution and incubate the samples in antibody solution overnight at 4 °C.

4.2.4 Wash the cells or tissue three times (5 min each) with washing buffer containing 0.1% Tween-20 and 1% PBSO in ddH2O.

4.2.5 Dilute the secondary antibodies 1:400 in PBSO.

4.2.6 Incubate the samples in secondary antibody solution for 3 h at room temperature.

4.2.7 Repeat washing step 4.2.4. For tissue samples, proceed with step 4.2.8. For organoid samples, proceed with step 4.2.9.

4.2.8 Place the epithelium on a slide with the basal side (side that was attached to the lamina propria) facing up. Mount the epithelium in an aqueous mountant with DAPI and a cover slip. Proceed with step 4.2.11.

4.2.9 For organoid samples, resuspend the stained organoids in any suitable gel matrix that solidifies at room temperature.

4.2.10 Quickly pipette droplets into a 96-well glass bottom plate (5 µL/well). Place the plate on ice and let the gel matrix solidify for 15 min. Mount the organoids in an aqueous mountant with DAPI by adding 100 µL per well.

4.2.11 Store the stained samples at 4 °C protected from light until image analysis.

REPRESENTATIVE RESULTS:

This protocol describes the separation of the tongue epithelium from the underlying lamina propria and muscle using an enzymatic cocktail (**Figure 1**). The separated epithelium can further be used for organoid generation as well as harvested for different types of gene and protein analyses. Likewise, the digested layer of lamina propria and muscle may be used for procedures of choice.

For organoid cultures, the tongue epithelium is further digested into small clumps of cells (about 2–3 cells) using trypsin solution. A complete digestion into single cells is not advisable, as this can negatively impact organoid outgrowth[52,53]. The resulting cell suspension is seeded into the basement membrane hydrogel (**Figure 2A**), which is then let to solidify to form a 3D ECM-rich scaffold supporting organoid growth. Within 7 to 14 days, oral mucosal organoids derived from

murine tongue epithelium grow into larger compact organoids with a keratinizing inner core (**Figure 2A**).

Established organoid cultures can be maintained by passaging. Using 0.125% trypsin solution, organoids can be dissociated into smaller cell clumps ideally containing 2–3 cells, which are again embedded into the basement membrane hydrogel (**Figure 2B**). Over a period of up to 14 days, organoids will regrow into larger organoids with the keratinizing inner core appearing within about 3–5 days (**Figure 2B**).

Gene expression analysis of murine oral mucosal organoid cultures and reference tongue epithelium demonstrated that organoids do faithfully recapitulate the expression of the key markers of the mucosal epithelium (**Figure 3A**) such as the proliferation marker *Mki67* (**Figure 3B**), the basal layer markers *p63* and *Krt5* (**Figure 3C,D**), the general epithelial marker *Cdh1* (encoding E-cadherin) (**Figure 3E**) as well as the terminal differentiation marker *Ivl* (encoding Involucrin) (**Figure 3F**). No statistically significant differences in expression of the marker genes tested were found between the oral mucosal epithelium and the mucosal organoid cultures (**Figure 3B–F**).

Whole-mount preparations of tongue epithelium and oral mucosal organoids can be used to validate the proper cell composition within established organoid cultures. **Figure 4** shows expression of keratin 14 (KRT14) in the basal layer of the tongue epithelium as well as in the outermost layer of the oral mucosal organoids, which corresponds to the epithelial basal layer. In addition, expression of the proliferation marker KI-67 is restricted to the KRT14-positive basal layer in both tissues and organoids (**Figure 4A,B**). In contrast, all the cells in both tongue epithelium and organoids show expression of the epithelial marker E-cadherin (ECAD; **Figure 4A,B**), which marks adherens junctions connecting the epithelial cells.

FIGURE AND TABLE LEGENDS:

Figure 1: Digestion of the tongue and separation of the epithelium from the lamina propria. (**A**) A micrograph of the intact tongue before digestion. (**B**) Micrographs of the tongue epithelium and lamina propria (including the muscle layer) following digestion and separation. Scale bars: 5 mm.

Figure 2: Organoid establishment and passaging. (**A**) Representative brightfield images of the cell clumps directly following initial plating (left panel) and of the organoids growing in culture between 1 to 10 days following plating (three panels on the right-hand side). (**B**) Representative brightfield images of the passaged organoids directly after plating (left panel) and growing organoids between 3 to 10 days following passaging (three panels on the right-hand side). Scale bars: 250 μm.

Figure 3: Gene expression analysis of tongue epithelium and oral mucosal organoids. (**A**) Schematic highlighting the different layers of the stratified and keratinizing tongue epithelium. BL, basal layer; CE, cornified envelope with dead keratinocytes; SL, suprabasal layers with viable keratinocytes (including the spinous layer and granular layer). (**B–F**) Gene expression analysis of

marker genes: proliferation marker *Mki67* usually restricted to the basal layer (**B**), basal layer markers *p63* (**C**) and *Krt5* (**D**), epithelial marker *Cdh1* (encoding E-cadherin; **E**) and differentiation marker *Ivl* (encoding Involucrin; **F**). Dark-shaded columns show data from the primary epithelium of the ventral and dorsal tongue and light-shaded columns show data from the organoid cultures established from the respective epithelia. Data were calculated following the ΔCt method and are presented as mean average from 3–4 biological replicates relative to the housekeeping gene *Actb*. Error bars indicate the standard error of the mean. No statistically significant differences were found (n.s., not significant; $p > 0.05$) using 2-way ANOVA with Tukey's multiple comparisons test.

Figure 4: Protein expression analysis of tongue epithelium and oral mucosal organoids. Whole-mount staining of ventral tongue epithelium (**A**) and oral mucosal organoids derived from primary ventral tongue epithelium (**B**). DAPI in blue marks DNA, keratin 14 (KRT14) expression is shown in green, E-cadherin (ECAD) expression is shown in cyan and KI-67 expression is shown in red. Scale bars: 100 μm (A) and 50 μm (B).

Table 1: Medium composition.

DISCUSSION:

Tissue digestion

The collagenase digestion helps in separating the epithelium from the underlying lamina propria and muscle tissue. This step allows for a better comparison of the primary tissue with the subsequently generated oral mucosal organoids. As overdigestion with enzymes impacts the organoid-forming capacity of the adult epithelial stem cells, we advise to perform the collagenase incubation for no longer than 1 h and the trypsin digestion no longer than 30 min. Upon collagenase digestion, the epithelium is very fragile. It is therefore important to take extra care when separating the epithelium to avoid ripping the epithelium apart. When checking for proper separation under the microscope, it should be assessed whether the connective tissue (typically whiteish in appearance) detached from the tongue epithelium. In addition, the specialized epithelium of the dorsal tongue and lining epithelium of the ventral tongue are connected via a transition zone that covers the lateral side of the tongue. When further working with the epithelium, it is therefore important to note that the epithelia of the dorsal and ventral tongue cannot be completely separated.

Organoid appearance and handling

In line with murine skin epidermal organoids[25], oral mucosal organoids grow in a compact manner without the cystic spheroid phenotype described for other epithelial organoid cultures[11,12,14,15,19,20]. When organoids start to attach to the bottom of the cell culture plate, a re-embedding of the organoids will be necessary, as otherwise the cells will start growing as a monolayer sheet at the bottom of the plate. These cells might be difficult to recover and may have changed in characteristics due to 2D culture. Therefore, we strongly recommend using suspension culture plates for murine oral mucosal organoid cultures.

The digestion time for passaging organoids is dependent on the size and compactness of the organoids. We recommend a short digestion time (<5 min) for cultures with mostly smaller organoids. Cultures with larger organoids should be digested for no longer than 10 min, as an overdigestion negatively impacts cell viability and organoid outgrowth. Dispersing the organoids by shaking during the digestion procedure increases the efficiency of the organoid dissociation. Depending on the assay, it may be necessary to have a culture of similarly sized organoids. To achieve a more homogenous culture, we recommend passing the cell clusters through a 70 μm cell strainer upon digestion, as the dissociated cell clusters otherwise clump back together in culture very quickly. As the basal-like cells are located at the very outer layer of the organoids, these cells are likely to be the first ones to be dissociated upon digestion. Thus, big clumps of differentiated and keratinized cells within the newly seeded cell suspension after splitting, might interfere with proper organoid formation. It is of great importance to pre-warm the medium to 37 °C, as the drops might dissociate otherwise. Due to the compact structure of murine oral mucosal organoids, microinjection might be difficult to perform.

Gene expression

Using the whole tongue as the input for the RNA isolation may lead to significant differences in marker gene expression between tissue and organoids. In addition, we observed decreased RNA quality when isolating RNA from the whole tongue as the input, which is likely due to the muscle tissue. As was observed in other organoid cultures such as those established from murine skin[25], we advise to collect RNA also from later passages (starting at passage 5). Organoid cultures before passaging may still contain non-epithelial cell types such as lymphocytes or fibroblast, causing artefacts in the analysis.[25]

Protein expression

Depending on the organoid size and tissue thickness, we recommend an additional step for staining the DNA with DAPI for whole-mount staining. Furthermore, the separated tongue epithelium as well as established organoids may also be used for western blot, electron microscopy, or conventional histology based on published studies[53].

Possible applications

In line with the already well-established organoid protocols, oral mucosal organoids are amenable to a wide range of downstream applications[11]. So far, murine, and human tissue-derived oral mucosal organoid cultures have been used for biobanking, testing efficacy[52,53],. and toxicity[54] of anti-cancer drugs[27] or radiotherapy[52,53]. Oral mucosal organoids can also be infected with viruses such as herpes simplex virus and human papilloma virus[53]. To assess the capacity to form metastasis, organoids derived from oral cancer may also be transplanted into mice[53]. Based on robust protocols[34,35], cultures of oral mucosal organoids may further be used for different gene-editing methods, including CRISPR-Cas9-based approaches.

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DISCLOSURES:

K.K. is named the inventor on a patent pending that is related to organoid technology.

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692

Figure 1

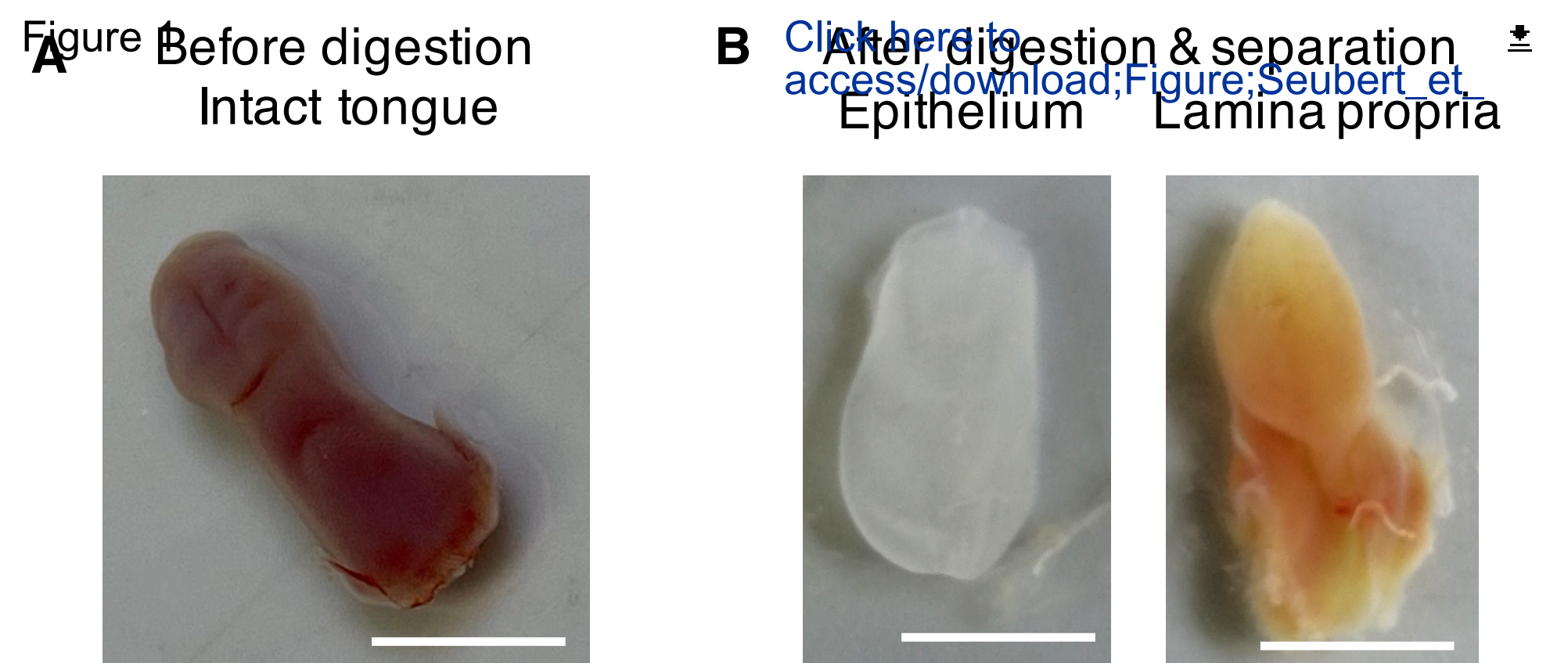


Figure 2

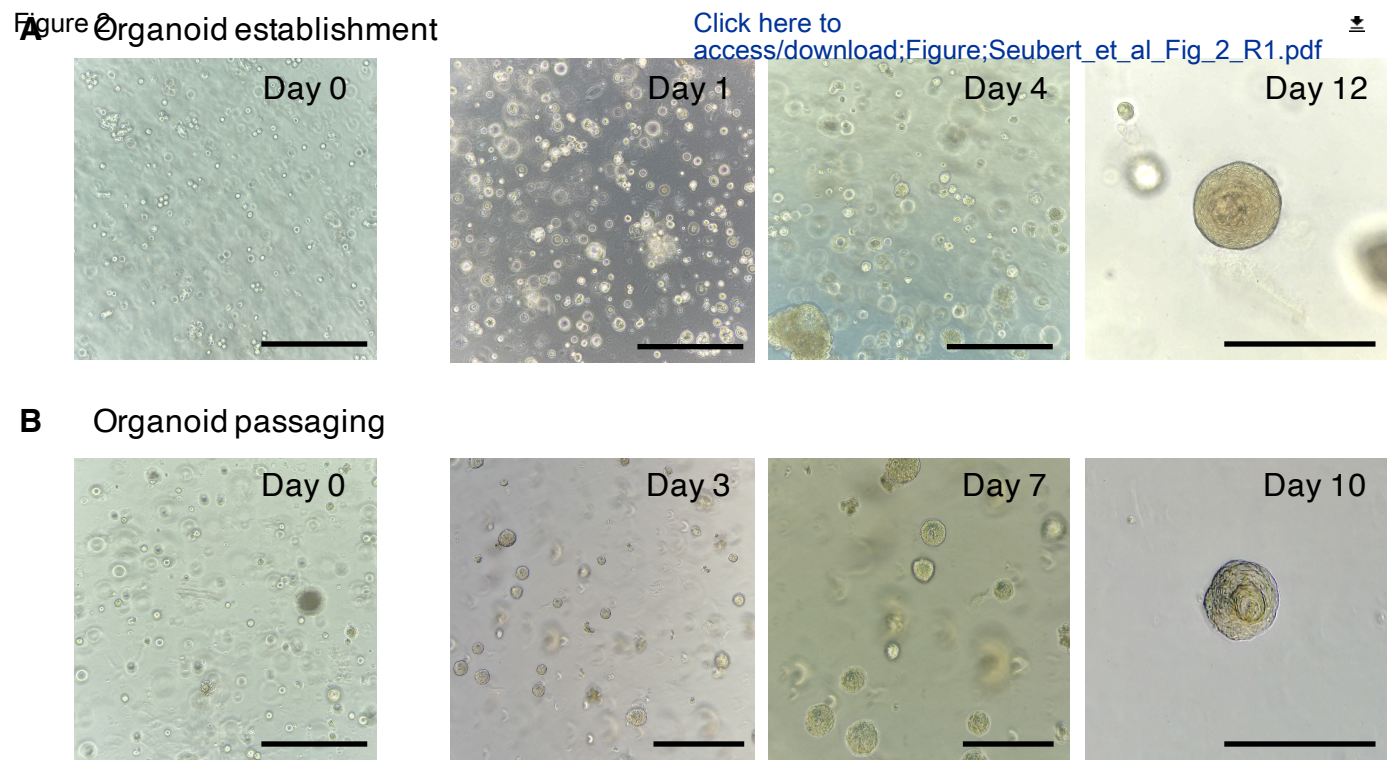
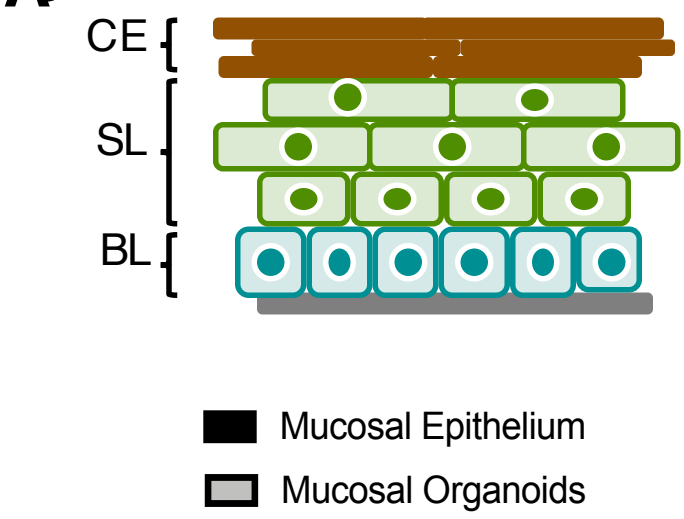
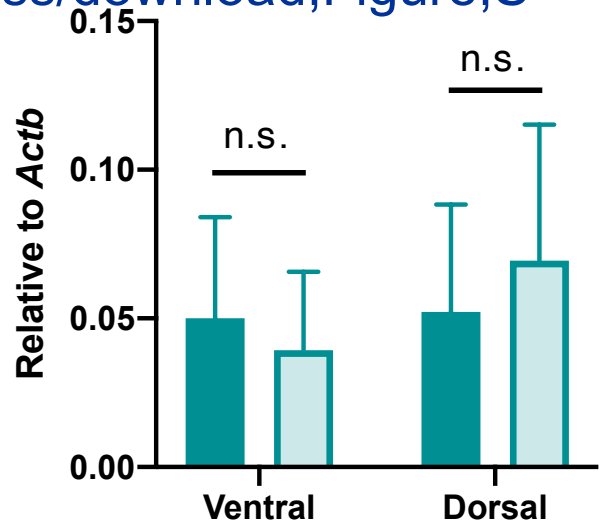
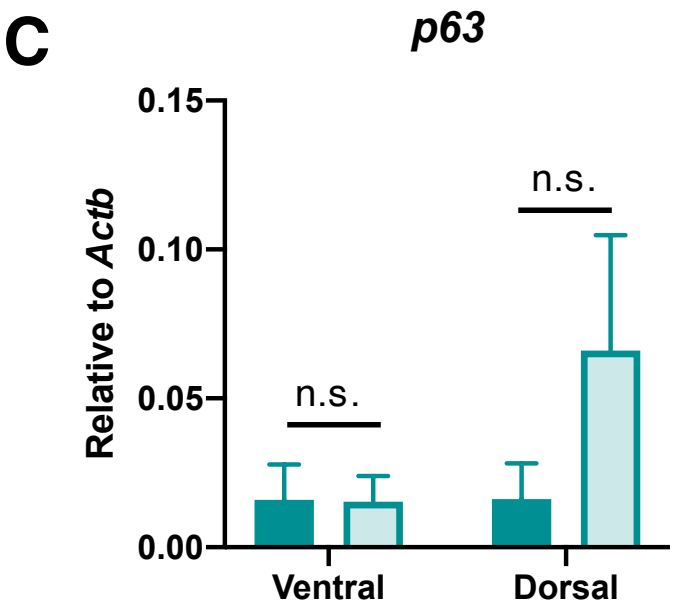
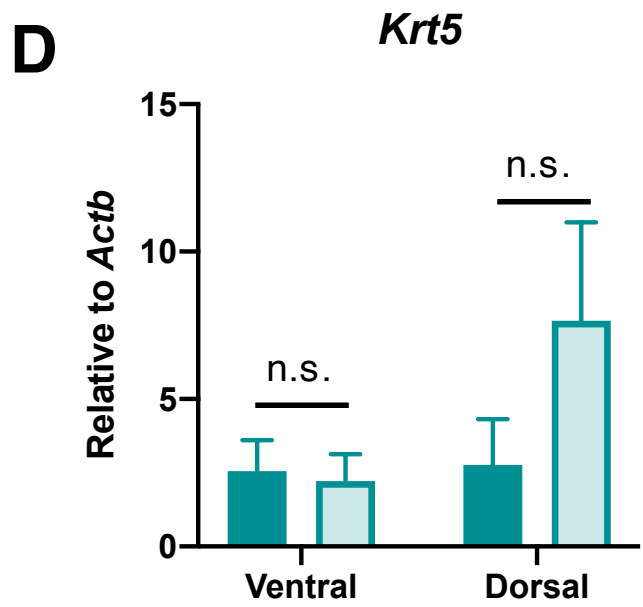


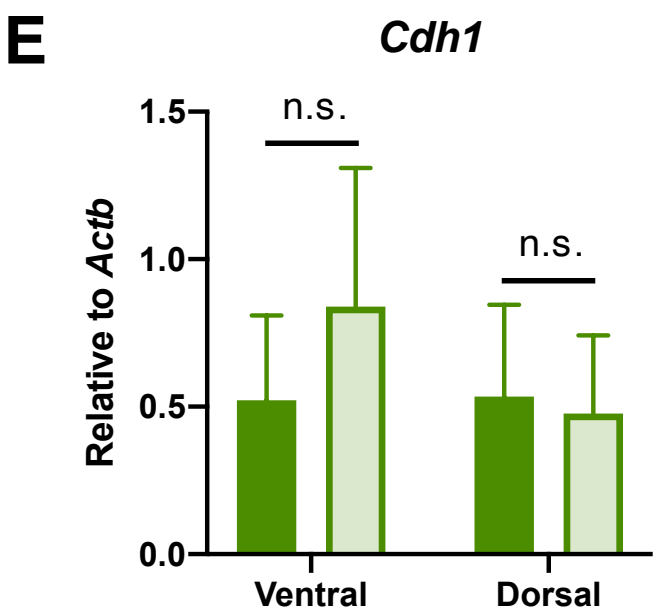
Figure 3











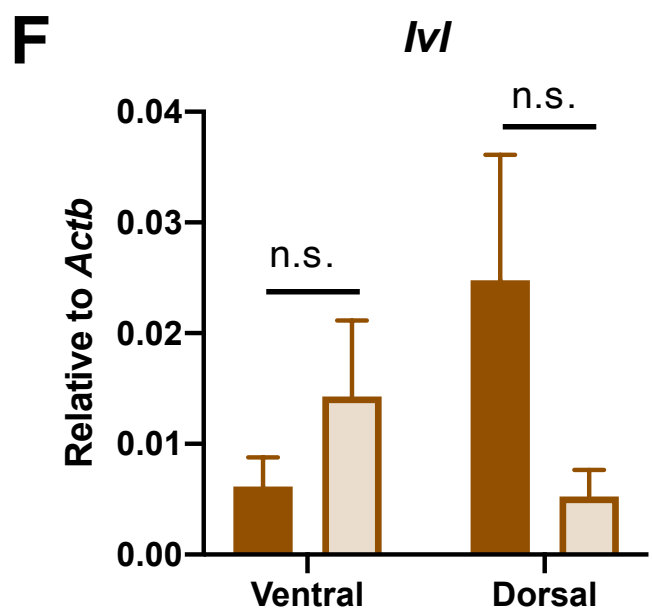


Figure 4

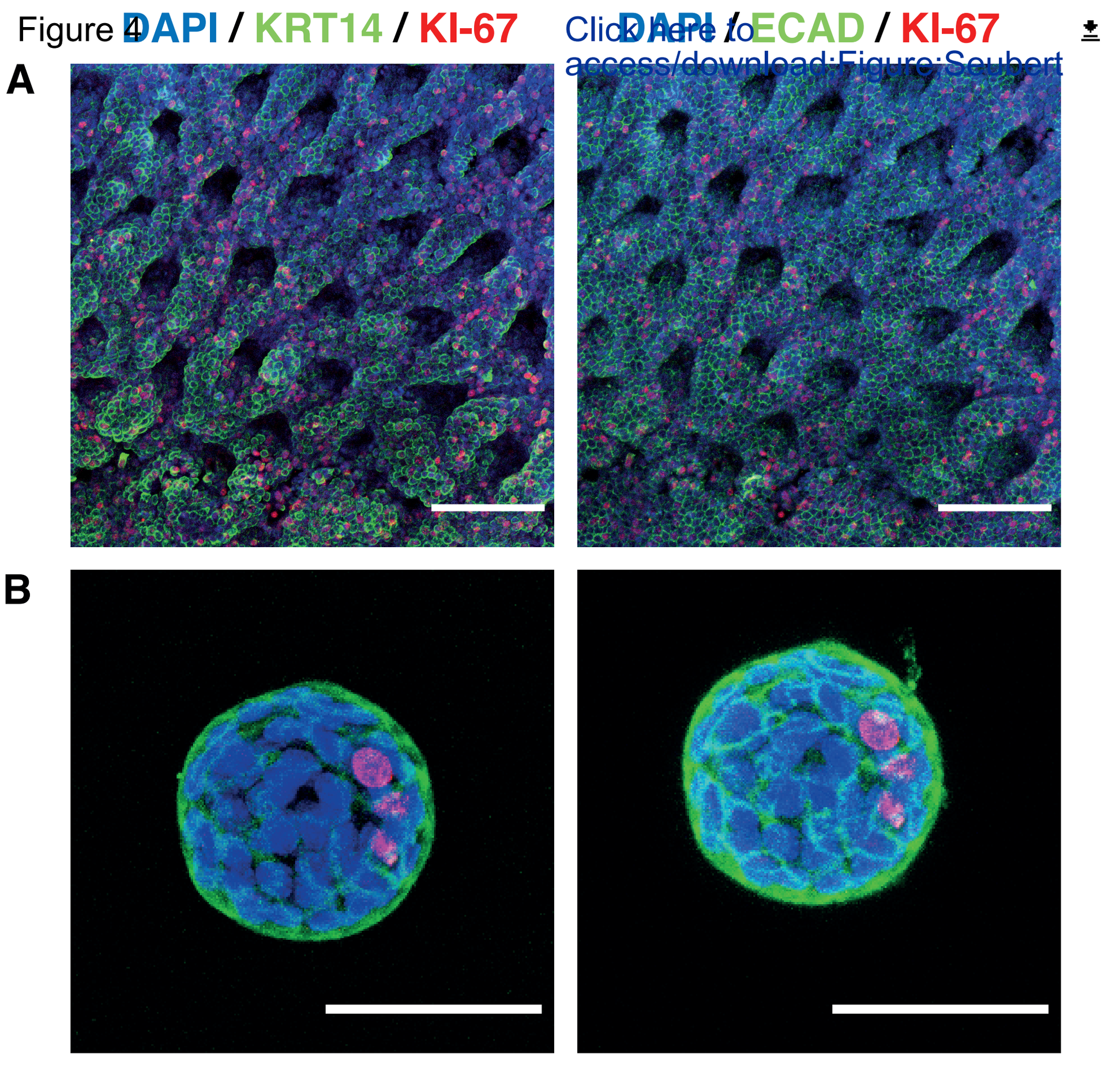


Table 1

Click here to access/download

Table of Materials

Seubert_et_al_Table_2_Medium_R1.xlsx

JoVE62529R1 – Rebuttal Letter

All textual changes have been underlined in the revised manuscript. Point-by-point responses to the editorial and production comments as well as the reviewers' comments are provided below.

RESPONSE TO EDITORIAL AND PRODUCTION COMMENTS

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

Response: This has been done.

2. Please provide an email address for each author.

Response: The email addresses are as follows:
Anna C. Seubert, anna.seubert1@uni-wuerzburg.de;
Marion Krafft, marion.krafft@uni-wuerzburg.de;
Kai Kretzschmar, kai.kretzschmar@uni-wuerzburg.de.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Response: This has been done.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Response: This has been done.

5. Please add more details to your protocol steps. For each step, please ensure you answer the "how" question, i.e., how is the step performed?

Response: This has been done.

6. 1: How is the euthanasia performed in your experiment? How do you perform the cuts, how long is the incision?

Response: The requested information has been added (lines 132/133 and 139-141).

7. 1.8: How do you identify the soft, rough, dorsal, and ventral mucosa? How big is each part?

Response: The requested information has been added (lines 155-159).

8. 2: How do you ensure that the injection is at lamina propria

Response: The requested information has been added (lines 181-183).

9. 3.1: How small the pieces are?

Response: The requested information has been added (line 204).

10. 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. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: RNeasy Micro Kit, iScript cDNA Synthesis Kit, StepOne Plus Thermocycler, SsoAdvanced Universal SYBR Green Supermix, ProLong Gold 287 Antifade Mountant, etc.

Response: This has been corrected.

11. Please include a single line space between each step, substep and note in the protocol section.

Response: This has been done.

12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: This has been done.

13. Please do not abbreviate the journal titles in the reference section.

Response: This has been done.

14. Please sort the materials table in alphabetical order.

Response: This has been done.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1:
Manuscript Summary:
This work is interesting. The development of standard protocol for establishing oral mucosa organoid will certainly bestow advantages in understanding the biological process and the drug effects. There are concerns to be addressed:

Major Concerns:
Nil

Minor Concerns:
1. The information about BME is empty. There so many different types of BME. A table to give the details of BME being tested or validated would be necessary.

Response: We thank the referee for pointing this out. We have amended table 1 accordingly.

2. In line 164, digest the tissue with 0.125 % trypsin in complete medium could be wrong. In such condition, the activity of trypsin is largely attenuated.

Response: The stated trypsin concentration of 0.125% is indeed correct, which is in line with previous publications (Driehuis et al., 2019; Driehuis, Kretzschmar and Clevers, 2020).

3. In line 179, 10 µl droplet could be erroneous. Usually, a droplet is about 40 - 50 µl. As a result, 10 µl in here is nearly impossible, and it could be the typo of 40 µl.

Response: The recommended droplet volume is indeed 10 µl. This is in line with several previously published protocols on organoid cultures (e.g. Driehuis, Kretzschmar & Clevers, 2020).

4. In line 175, it is questionable how the accurate cell number (10,000/40 µl) can be obtained as plenty of ingredients are mixed with cells in solution.

Response: This concentration of cells has been described in several other published papers on organoid cultures (e.g. Driehuis, Kretzschmar & Clevers, 2020; Boonekamp et al., 2019). Usually, organoids are taken up in Advanced DMEM/F12+++ medium and then mixed with BME before plating, as described in lines 231-235.

5. Line 178, the writing of "dilution of BME >70 % might lead to insufficient solidification" could cause misunderstanding. The correct meaning is the concentration of BME < 30% would be difficult to get solidification.

Response: We apologize for this ambiguous phrasing. We have clarified the manuscript text accordingly (lines 234/235).

6. Fig. 3 "Mucosal epithelium" being labelled should be Mucosal tissue. No epithelium stripping has been done for sample preparation in this experiment.

Response: "Mucosal epithelium" is correctly used here. In this experiment, the input RNA was isolated from the stripped epithelium (separated from the underlying connective tissue), as described in step 3.1.4.

7. Fig. 4A, Rt panel, Blue fluorescence in the diagram should be converted to green fluorescence (for E-cad).

Response: This has been done.

Reviewer #2:
Manuscript Summary:
This protcol described an optimised methodology for the establishment and maintenance of oral mucosal organoid cultures from murine tongue epithelium. The experimental steps are clear.

Minor Concerns:
The scale bar should be added in figure 1 and 2.

Response: This has been done.

There is no statistical analysis for the gene expression analysis of tongue epithelium and oral mucosal organoids in figure 3.

Response: Statistical analysis had been performed. However, we did not observe statistically significant differences. For clarification, we have amended the manuscript and figure accordingly.

References:

Boonekamp *et al.* Long-term expansion and differentiation of adult murine epidermal stem cells in 3D organoid cultures. *Proc Natl Acad Sci U S A.* **116** (29), 14630-14638 (2019).

Driehuis, Kretzschmar & Clevers. Establishment of patient-derived cancer organoids for drug-screening applications. *Nat Protoc.* **15** (10), 3380-3409 (2020).

Driehuis *et al.* Oral Mucosal Organoids as a Potential Platform for Personalized Cancer Therapy. *Cancer Discov.* **9** (7), 852-871 (2019).