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Cell dissociation from the tongue epithelium and mesenchyme/connective tissue of embryonic-day 12.5 and 8-week-old mice

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Corresponding Author:	Hong-Xiang Liu, PhD, MD University of Georgia College of Agricultural and Environmental Sciences Athens, Georgia UNITED STATES
Corresponding Author's Institution:	University of Georgia College of Agricultural and Environmental Sciences
Corresponding Author E-Mail:	lhx@uga.edu
Order of Authors:	Wenxin Yu
	Mohamed Ishan
	Zhonghou Wang
	Hong-Xiang Liu, PhD, MD
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TITLE:

Cell dissociation from the tongue epithelium and mesenchyme/connective tissue of embryonic-day 12.5 and 8-week-old mice

AUTHORS AND AFFILIATIONS:

Wenxin Yu, Mohamed Ishan, Zhonghou Wang, Hong-Xiang Liu*

Regenerative Bioscience Center; Edgar L. Rhodes Center for Animal and Dairy Sciences, College of Agricultural and Environmental Sciences; University of Georgia, Athens, GA, USA.

wy48803@uga.edu
mohamed.ishan25@uga.edu
zhonghou.wang@uga.edu

*Corresponding author:
lhx@uga.edu

KEYWORDS:

cell dissociation; mouse; tongue; epithelium; mesenchyme; connective tissue; embryo; adult.

SUMMARY:

We have developed a generalized protocol to dissociate a large quantity of high-quality single cells from the epithelium and mesenchyme/connective tissue of embryonic and adult mouse tongues.

ABSTRACT:

Cell dissociation has been an essential procedure for studies at the individual-cell level and/or at a cell-population level (e.g., single cell RNA sequencing and primary cell culture). Yielding viable, healthy cells in large quantities is critical, and the optimal conditions to do so are tissue dependent. Cell populations in the tongue epithelium and underlying mesenchyme/connective tissue are heterogeneous and tissue structures vary in different regions and at different developmental stages. We have tested protocols for isolating cells from the mouse tongue epithelium and mesenchyme/connective tissue in the early developmental [embryonic day 12.5 (E12.5)] and young adult (8-week) stages. A clean separation between the epithelium and underlying mesenchyme/connective tissue was easy to accomplish. However, to further process and isolate cells, yielding viable healthy cells in large quantities, and careful selection of enzymatic digestion buffer, incubation time, and centrifugation speed and time are critical. Incubation of separated epithelium or underlying mesenchyme/connective tissue in 0.25% Trypsin-EDTA for 30 min at 37 °C, followed by centrifugation at 200 x g for 8 min resulted in a high yield of cells at a high viability rate (>90%) regardless of the mouse stages and tongue regions. Moreover, we found that both dissociated epithelial and mesenchymal/connective tissue cells from embryonic and adult tongues could survive in the cell culture-based medium for at least 3 h without a significant decrease of cell viability. The protocols will be useful for studies that require the preparation of isolated cells from mouse tongues at early

developmental (E12.5) and young adult (8-week) stages requiring cell dissociation from different tissue compartments.

INTRODUCTION:

The mammalian tongue is a complex organ critical for taste, speaking, and food processing. It is comprised of multiple types of highly organized tissues compartmentalized by mesenchyme/connective tissue and covered by a stratified epithelial sheet containing taste papillae and taste buds. Cell populations in both tongue epithelium and mesenchyme/connective tissue are heterogeneous. To better understand the functions and distribution of a particular type of cells in the tongue, studies using dissociated cells are necessary. For example, single cell RNA sequencing is a powerful and high-throughput method for transcriptomic profiling in individual cells, which is designed to understand the transcriptome of complex tissue at a single-cell resolution[1-4]. Primary cell culture has been proven to be a useful tool to study the function and differentiation of stem/progenitor cells for taste buds[5,6]. These studies require a large quantity of high-quality isolated cell populations (e.g., sufficient total cell number with proper concentration and high viability).

Thus, there is a need to isolate cells from different regions of the lingual tissues and at different developmental stages. Currently, there is not a detailed protocol available for cell dissociation from the tongue epithelium and underlying mesenchyme/connective tissue. Here, we report an optimized cell dissociation method to prepare cells for experiments requiring a high quality of live cells such as for single cell RNA sequencing and primary stem cell cultures. We found that selection of enzymatic digestion buffer, gentle pipetting, selection of resuspension medium, and optimal centrifugation time and speed are crucial to generate these large quantities of high-quality cells.

PROTOCOL:

Animal use (C57BL/6 mice throughout the study) was approved by the University of Georgia Institutional Animal Care and Use Committee and was in accordance with the National Institutes of Health Guidelines for care and use of animals for research.

1. Animal usage

NOTE: Mice were bred and maintained in the animal facility of the Animal and Dairy Science department at the University of Georgia at 22 °C under 12-h day/night cycles.

1.1. Designate noon of the day of vaginal plug detection in mice as embryonic (E) day 0.5. Use embryos at E12.5 and postnatal mice at 8 weeks of age for the following experiments.

2. Preparation before experiment

NOTE: The instruments required for this protocol are listed in the **Table of Materials**.

2.1. Autoclave instruments before the experiment. Sterilize instruments using a bead sterilizer during the experiment.

2.2. Clean the surgical area, the dissecting microscope, and the biosafety cabinet using alcohol wipes. Turn on the UV light of biosafety cabinet and keep it on for 20 min prior to the experimental procedure.

2.3. Prepare an enzyme mixture of 1:1 dispase (5.0 mg/mL) and collagenase (2.0 mg/mL) to a final concentration of 2.5 and 1.0 mg/mL respectively, and filter the solution using 0.22 µm syringe filter[7].

2.3.1. Prepare 1 mL of enzyme mixture for an adult tongue or 0.5 mL for a specific region of tongue (e.g., posterior, or anterior tongue).

2.3.2. Prepare 2 mL of enzyme mixture for embryonic tongues.

2.4. Make 10 mL of 2.5% BSA in 0.1 M PBS and filter the solution using 1 mL syringe and 0.22 µm syringe filter.

2.5. Make 500 µL of 5% FBS in DMEM/F12.

2.6. Make 3 mL of DMEM/F12 containing 10% FBS and 1% BSA and filter the solution using 1 mL syringe and 0.22 µm syringe filter.

3. Separation of the tongue epithelium from the mesenchyme/underlying connective tissue

3.1. Separation of the epithelium from the mesenchyme of a E12.5 mouse tongue

3.1.1. Euthanize timed pregnant female mice carrying E12.5 embryos by placing it in a CO_2 chamber followed by cervical dislocation.

NOTE: Mouse embryos at E12.5 are collected after 12 pm (afternoon) on the 12th day after detection of vaginal plug in the pregnant female mice.

3.1.2. Transfer mice to the surgical area. Wet the mouse abdomen using 70% ethanol to prevent fur from getting into the operating site.

3.1.3. Open the abdomen using dissecting scissors to expose the uterine horns carrying embryos. Dissect the uterine horns using dissecting scissors and transfer it to 15 mL of fresh Tyrode's solution in a 100 mm culture dish.

3.1.4. Dissect the embryos (**Figure 1A$_1$**) out from uterine horns under a dissecting microscope using mini-scissors and fine forceps.

3.1.5. Carefully open the mouth cavity wide by using fine forceps and dissect the tongue off from the mandible using mini-scissors (**Figure 1A$_2$**).

3.1.6. Wash the tongues using 15 mL of fresh sterile Tyrode's solution in a 100 mm culture dish with a spatula and fine forceps in a biosafety cabinet.

3.1.7. Transfer the tissues to 2 mL of enzyme mixture of dispase (2.5 mg/mL) and collagenase (1.0 mg/mL) in a 35 mm culture dish. Incubate for a 20 min at 37 °C.

3.1.8. Transfer tongues to 15 mL of fresh sterile Tyrode's solution in a 100 mm culture dish and gently remove the mesenchyme from the epithelium from the ventral side using fine forceps.

NOTE: Epithelial sheets may be separated without mechanical force during the incubation.

3.1.9. Wash the separated epithelia and mesenchyme twice in 15 mL of fresh sterile Tyrode's solution in a 100 mm culture dish.

NOTE: The activities of dispase and collagenase will be inhibited by EDTA in the cell dissociation procedure (step 4.1).

3.2. Separation of the tongue epithelium from the underlying connective tissue of adult mice

3.2.1. Euthanize the mouse at 8 weeks of age by placing it in a CO_2 chamber. Confirm that the mouse is euthanized with no forepaw-pinch response.

3.2.2. Transfer the mice to the surgical area. Wet the mouse head using 70% ethanol to prevent fur from getting into the oral cavity.

3.2.3. Cut the corners of the mouth along the cheek using dissecting scissors to open the oral cavity. Dissect the tongue with a mandible (**Figure 1B$_1$**) and place it in a plastic dish with a layer of plastic wrap.

3.2.4. Using surgical forceps to hold the tongue under a dissecting microscope, inject the enzyme mixture of dispase (2.5 mg/mL) and collagenase (1.0 mg/mL) in the sub-epithelial space of tongue through the cutting edge (**Figure 1B$_1$**, arrows) of the posterior tongue.

3.2.4.1. Inject 1 mL of enzyme mixture evenly to the whole tongue for tissue collection from both anterior and posterior tongue.

3.2.4.2. Inject 0.5 mL of enzyme mixture locally to the anterior tongue for tissue collection from tongue tip or to the posterior tongue for circumvallate papilla tissue collection.

NOTE: The tongue will swell as the enzyme accumulates (**Figure 1B$_2$**). Gentle injection of the enzyme can prevent pressure from damaging the epithelium and keep as much enzyme as possible in the tongue.

3.2.5. Wrap the tongue with plastic wrap and incubate the tongue for 30 min at 37 °C.

3.2.6. Use mini-scissors to dissect the tongue tip and/or circumvallate papilla, and use spatula and fine forceps to transfer tissue to 15 mL of fresh sterile Tyrode's solution in a 100 mm culture dish.

3.2.7. Separate the epithelium from the underlying connective tissue in the enzyme-digested sub-epithelial space using mini-scissors. Trim the tissues to a proper size according to the requirement of downstream experiments.

3.2.8. Wash the separated epithelium and underlying connective tissue twice in 15 mL of fresh sterile Tyrode's solution in a 100 mm culture dish.

NOTE: The activities of dispase and collagenase will be inhibited by EDTA in the cell dissociation procedure (step 4.1).

4. Cell dissociation

NOTE: The cell dissociation protocol described here can be applied to the tongue epithelium and mesenchyme/connective tissue in both E12.5 embryonic and 8-week-old mice. To reduce the cell loss during agitation and transfer of cell suspension, use commercial low retention pipette tips or pre-coated pipette tips with 2.5% BSA in 0.1 M PBS at pH 7.4[8].

4.1. Transfer the tissues using spatula and fine forceps to 3 mL of 0.25% trypsin-EDTA in a new 35 mm culture dish for 30 min at 37 °C. Gently agitate tissues every 5 min with 1 mL pipette tips.

NOTE: Do not cut the pipette tip, as the cutting edge can physically damage the dissociated cells.

4.2. Add 500 µL of 5% FBS in DMEM/F12 to stop the reaction and transfer the medium to a 5 mL low binding centrifuge tube.

4.3. Centrifuge cell suspension at 200 x *g* for 8 min at room temperature and remove the supernatant.

4.4. Gently re-suspend cells in 3 mL of DMEM/F12 containing 10% FBS and 1% BSA using 1 mL pipette tips and filter the cells using a 70 µm cell strainer, followed by a 35 µm cell strainer.

4.5. Centrifuge cell suspension at 200 x *g* for 8 min at room temperature. Remove most of the medium and leave 50-300 µL as the final volume to re-suspend cells.

NOTE: Adjust the concentration of cells according to requirements of downstream experiments by changing the final volume of single cell suspension.

5. Cell counting and viability test using hemocytometer

NOTE: To improve measurement accuracy, 3 technical replicates are recommended for each sample.

5.1. Gently mix 5-10 µL of the single cell suspension with an equal volume of Trypan blue and add onto hemocytometer.

NOTE: Clean the hemocytometer thoroughly using 70% ethanol before use. Dusts particles on the hemocytometer will be stained in dark blue and affect the accuracy of viability test. Check the hemocytometer under a microscope.

5.2. Count the total cell number, the number of live cells (white), and the number of dead cells (dark blue) stained by Trypan blue (**Figure 2**, arrows) respectively in 4 squares with 16 grids (**Figure 2**) using inverted microscope with imaging system.

5.3. Calculate the cell concentration:

$$\text{Cell concentration} = \frac{\text{Total cell number of all 4 squares}}{4} \times 2\ (\text{diluted factor}) \times 10^4\ \text{cells/mL}$$

5.3.1. Calculate the viability:

$$\text{Viability} = \frac{\text{Number of live cells of 4 squares}}{\text{Total cell number of all 4 squares}} \times 100\%$$

REPRESENTATIVE RESULTS:

Separation of the tongue epithelium from the underlying mesenchyme/connective tissue

In the embryonic mouse tongue, a gap in the sub-epithelial space is visible after proper enzyme digestion. Epithelial sheets of some tongues are separated without mechanical force during the incubation.

In the adult mouse tongue, a successful enzyme injection is indicated by the swelling in the injected areas (**Figure 1A$_2$**), which suggests that the enzyme can be held by the tongue. Insufficient enzyme and/or deep needle insertion to the mesenchyme and/or tongue epithelial penetration by needle will induce a partial swelling of the injection area or no swelling at all. After enzyme digestion, the underlying connective tissues with proper enzyme digestion become loose and sticky. A gap in the sub-epithelial space is visible when gently lifting the edge of the epithelial sheet.

Effect of cell dissociation on total cell number and viability

With step 4, E12.5 tongues, the epithelial sheets, and thin layers of mesenchyme immediately under the epithelium of tongues were pooled, respectively. Manual cell counting using a hemocytometer (**Figure 2**) demonstrated that the protocol yielded 63,917 cells in total with a viability of 95.2% from the epithelial sheets (around 0.3 mm^2 in size per tongue) (**Figure 1A$_3$**), and 294,333 cells in total with a viability of 96.3% from the mesenchyme (around 0.3 mm^2 in size per tongue) (**Figure 1A$_4$**).

Using 10 adult tongues at 8 weeks of age, the pieces of the epithelial sheets of the tongue tip (where taste buds are densely distributed), epithelial sheets of circumvallate papillae, and thin layers of connective tissue immediately under the epithelium of circumvallate papillae were pooled, respectively. A manual cell count using the hemocytometer (**Figure 2**) demonstrated that the protocol yielded 187,333 cells in total with a viability of 95.4% from epithelial sheets of tongue tip (around 0.075 mm^2 in size per tongue) (**Figure 1B$_3$**), 544,000 cells in total with a viability of 96.3% from epithelial sheets of circumvallate papillae (around 0.1 mm^2 in size per tongue) (**Figure 1B$_5$**), and 150,500 cells in total with a viability of 93% from connective tissues (around 0.1 mm^2 in size per tongue) (**Figure 1B$_6$**).

FIGURE AND TABLE LEGENDS:

Figure 1. Tissue preparation for cell dissociation. A) Representative images of an E12.5 whole embryo (A_1), dorsal view of the dissected tongue (A_2), and epithelial sheets (A_3) and mesenchyme (A_4) separated from tongues. **B**) Representative images of an adult tongue before (B_1) and after enzyme injection (B_2). Dorsal view of an epithelial sheet (B_3) and mesenchyme (B_4) of tongue tip, and an epithelial sheet (B_5) and underlying connective tissue (B_6) of circumvallate papilla. Scale Bbars: 1 mm in A_1, A_3, A_4, B_1, B_2; 200 µm in A_2; 100 µm in B_3, B_4, B_5, and B_6.

Figure 2. Representative images of isolated cells visualized in hemocytometer. A) Isolated cells from epithelial sheets (A_1) and mesenchyme (A_2) of embryos at E12.5. **B**) Isolated cells from epithelial sheets (B_1) and underlying connective tissue cores (B_2) of circumvallate papillae at 8 weeks of age. Dash lines encircle the grids amplified in top right corner. Arrows point to dead cells stained by Trypan blue. Scale bars: 100 µm for B_1, B_2, B_3, and B_4; 25 µm for high power images in top right corner.

DISCUSSION:

To date, there has not been a detailed protocol available for cell dissociation from the tongue epithelium and underlying mesenchyme/connective tissue. This current cell dissociation protocol provides a reproducible procedure to generate a single cell suspension with a high cell viability (>90%) from mouse tongue tissues, including epithelial sheets and mesenchyme/connective tissues at both embryonic and postnatal stages even though isolated cells from E12.5 and adult mice are different in size. For example, isolated cells from the epithelium and mesenchyme of E12.5 mouse tongues are consistent and small in contrast to a large variability (from small to large) of cells from 8-week-old mouse tongue. With the advantage of the high viability (>90%) of isolated cells, the yielded single cell suspension can facilitate the downstream experiments requiring high quality of viable cells (e.g., single cell RNA

sequencing and primary cell cultures).

To guarantee the high viability of cells, careful attention must be paid to several important factors. A proper digestion time with dispase and collagenase mixture can effectively separate the epithelium from mesenchyme/underlying connective tissue while preserving the viable cells. A 20 min incubation for the embryonic tongue and a 30 min incubation for the adult tongue are recommended. Even though epithelial sheets can be easily peeled after enzyme digestion, cutting with scissors for the separation is recommended, which may preserve the stem cell population in the basal area of the tongue epithelium[9]. The choice of 0.25% trypsin-EDTA over trypsin alone is highly recommended for dissociating cells as 0.25% trypsin-EDTA can dissociate cells more efficiently and keep cells in separation compared to the trypsin alone. The use of cell culture based medium (DMEM/F12 containing 10% FBS and 1% BSA) to re-suspend cells after enzymatic digestion is crucial and contributes to the higher viability of isolated cells compared to cell suspension medium (e.g., 1% BSA in 0.1 M PBS). In addition, isolated cells have a higher survival rate in this medium over time: at least 3 h without significant decrease of cell viability. The pipetting technique is another critical factor to ensure a high cell viability. Gently aspirating the supernatant and re-suspending cells using a pipette can reduce extra cell loss and physical damage to cells.

The concentration of isolated cells in a single cell suspension is another important consideration for downstream experiments. In a total given cell number, cell concentration can be adjusted based on the final volume of resuspended solution. For the first trial when total cell number is unknown, resuspension of isolated cells should start at a low volume. Then, isolated cells can be diluted based on the requirements of downstream experiments. Of note, in our culture medium based solution, cellular aggregates were found when the concentration was higher than 4000 cells/µL (around 200 cells per square in hemocytometer). The optimal concentrations range from 500 to 2500 cells/µL.

ACKNOWLEDGMENTS:
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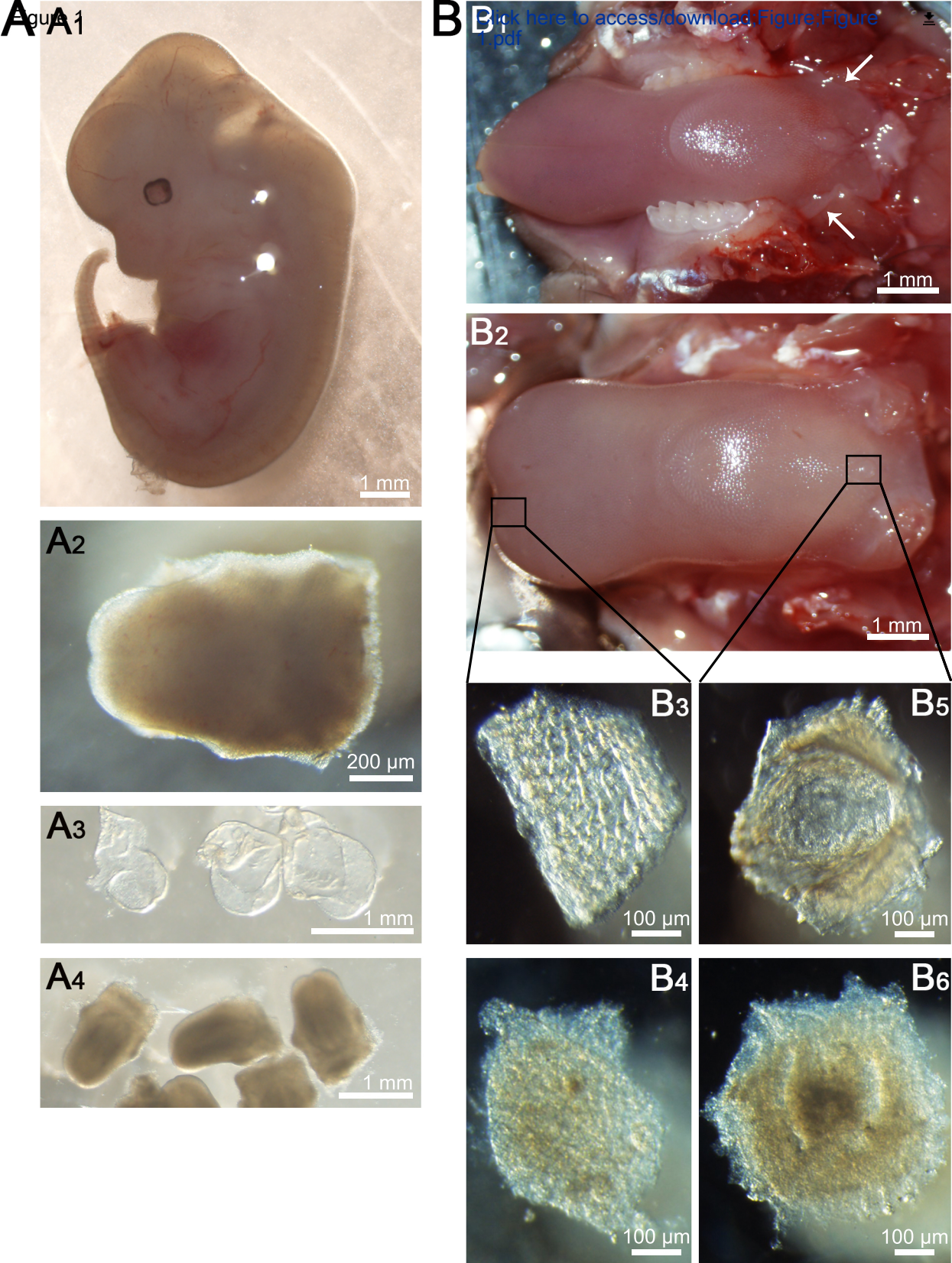
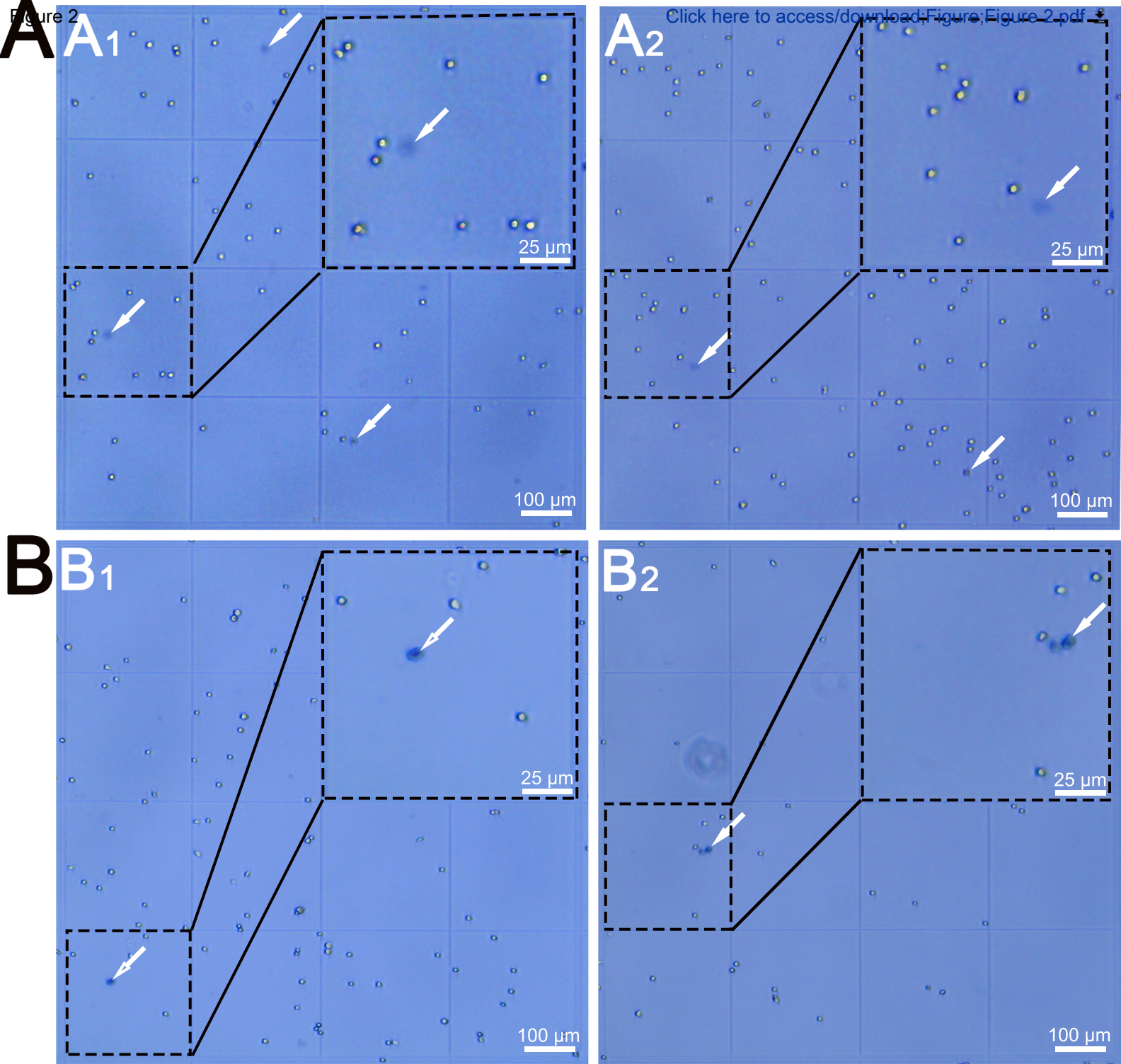
DISCLOSURES:
No conflicts of interest declared.

REFERENCES:
1 Grada, A., Weinbrecht, K. Next-generation sequencing: methodology and application. *The Journal of investigative dermatology.* **133** (8), e11 (2013).
2 Whitley, S. K., Horne, W. T., Kolls, J. K. Research techniques made simple: methodology and clinical applications of RNA sequencing. *Journal of Investigative Dermatology.* **136** (8), e77-e82 (2016).

3 Schaum, N. et al. Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris: The Tabula Muris Consortium. *Nature.* **562** (7727), 367 (2018).
4 Sukumaran, S. K. et al. Whole transcriptome profiling of taste bud cells. *Scientific reports.* **7** (1), 1-15 (2017).
5 Ren, W. et al. Transcriptome analyses of taste organoids reveal multiple pathways involved in taste cell generation. *Scientific Reports.* **7** (1), 1-13 (2017).
6 Ren, W. et al. Single Lgr5-or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo. *Proceedings of the National Academy of Sciences.* **111** (46), 16401-16406 (2014).
7 Venkatesan, N., Boggs, K., Liu, H.-X. Taste bud labeling in whole tongue epithelial sheet in adult mice. *Tissue Engineering Part C: Methods.* **22** (4), 332-337 (2016).
8 Nguyen-Ngoc, K.-V. et al. in *Tissue Morphogenesis*. 135-162 (Springer, 2015).
9 Okubo, T., Clark, C., Hogan, B. L. Cell lineage mapping of taste bud cells and keratinocytes in the mouse tongue and soft palate. *Stem Cells.* **27** (2), 442-450 (2009).





Name of Material/ Equipment	Company	Catalog Number	Comments/Description
bovine serum albumin (BSA)	Gold Biotechnology	A-420-100	
C57BL/6 mouse (C57BL/6J)	The Jackson Laboratory	000664	
collagenase (Collagenase A)	Sigma-Aldrich	10103586001	
culture dish (35 mm in diameter)	Genesee Scientific	32-103G	
culture dish (100 mm in diameter)	Genesee Scientific	32-107G	
dispase (Dispase II)	Sigma-Aldrich	04942078001	
dissecting scissors (Student Fine Scissors)	Find Science Tool	91460-11	
DMEM/F12	Gibco	11320033	
fetal bovine serum (FBS)	Hyclone	C838U82	
fine forceps (Dumount #3 Forceps)	Find Science Tool	11293-00	
hemocytometer	Hausser Scientific	3520	
inverted microscope with imaging system (EVOS XL Core Cell Imaging System)	Life Technologies	AMEX1000	
low retention pipette tips	METTLER TOLEDO	17014342	
mini-scissors (Evo Spring Scissors)	Fine Science Tool	15800-01	
plastic warp	VWR	46610-056	
spatula (Moria Spoon)	Fine Science Tool	10321-08	
surgical forceps (Dumount #2 Laminectomy Forceps)	Fine Science Tool	11223-20	
Trypan blue	Gibco	15250061	
Tyrode’s solution	Sigma-Aldrich	T2145-10L	made from Tyrode's salts
0.25% typsin-EDTA	Gibco	25200056	
0.1 M Phosphate-Buffered Saline (PBS)	Hoefer	33946	made from 1 M PBS
0.22-µm syringe filter	Genesee Scientific	25-243	
70% ethanol	Koptec	233919	made from 100% ethanol
1-mL syringe	BD	8194938	
5-mL low binding microcentrifuge tube	Eppendorf	30122348	
30-G needle	BD	9193532	
35-µm cell strainer	Falcon	64750	
70-µm cell strainer	Falcon	64752	

Our responses are in red under each comment. The changes indicated here are tracked within the manuscript for to identify all of the edits.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thanks for the reminder. We have done thorough proofreading by all authors.

2. Please avoid abbreviations (e.g., wk) in the title.

We have modified based on your comment (line 2-3).

3. 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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The texts have been revised accordingly.

4. Please move all information on materials used (reagents, equipment) to the Table of Materials.

All information on materials has been removed from the test and a table has been generated and included.

5. 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: Dispase II etc

We have revised the names accordingly, e.g., changed Dispase II and Collagenase A to dispase and collagenase.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

More details have been added.

7. Please highlight up to 3 pages of the Protocol (including headings and spacing) that

identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

In the revised version, we identified and highlighted in yellow the essential steps; see the content between section 3 and section 4 (line 120 to 234, 1/2 page 3 – 1/2 page 6).

8. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in Figure Legend 1.

We have added scale bars in Figure 1 and scale values in legend of Figure 1 (line 299-300)

9. As we are a methods journal, please add limitations and significance with respect to existing methods to the Discussion.

Currently there is not a detailed protocol available for cell dissociation from tongue epithelium and underlying mesenchyme/connective tissue. We have optimized the cell dissociation method in our lab. In the revised version, we have added a sentence of significance with respect to existing methods to the Introduction (line 65-69) and Discussion (line 311-312).

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

The names of the material have been sorted alphabetically.

Reviewers' comments:

Reviewer #1:

The manuscript described a cell dissociation method for embryonic and adult mouse tongues. I only have a few minor concerns about the manuscript.

Decimals should be consistent. For example, both 5.0 mg/ml or 5 mg/ml were used in the text.

We have modified for consistency throughout the manuscript.

Protocol 2.2: "Switch on the UV" is misleading. Did the authors mean to leave the UV on for some time before the procedure and switch it off during the procedure?

In the revised version, we have clarified the use of UV light (line 100-101). Turn on the UV light of biosafety cabinet and keep it on for 20 min prior experimental procedure.

Protocol 2.5 should be 2.6 (line 111)

It has been corrected.

Protocol 3.1: I am not sure about using the word "dam" in 3.1.1 (lines 118 and 122). Should it be pregnant female mice or female mice?

We have made it clear in the revised manuscript, i.e., pregnant female mice (line 124 and 128).

Protocol 3.1.6: It is better to define the size of the culture dish rather than use the word small.

We have clarified the size as “35-mm” of culture dish.

Protocol 3.2.4.1 should be 3.2.4.2 (line 162)

It has been corrected.

Reviewer #2:
Manuscript Summary:
Yu et al have developed a nice protocol to isolate lingual epithelium and mesenchyme from tongues of mid-gestation embryos and 8-week-old adult mice. The protocol is quite straightforward and should be of major interest to the field. There are some portions that are not clear however, and these are indicated below.

Major Concerns:
None

Minor Concerns:
Points of clarification
1. In the introduction, the authors use the phrase "strict cell quality". Please

For better clarity, we have changed the phrase to “requiring high quality of live cells” (line 68).

2. Preparation Note before starting. Use of syringe filters should include pore size. Also spell out EVOS - is this microscope essential or can another type be used?

We have clarified the pore size of syringe filter (0.22-μm). The EVOS microscope is not essential, so we changed our description to “an inverted microscope”.

3. Make sure to use filter (verb) and filtrate (noun) appropriately in methods.

Both words (filter and filtrate) can be verb and noun. Nevertheless, we have modified the text based on your suggestions.

4. Please add plastic wrap to the table of materials.

We have added the “plastic wrap” to the table of materials.

5. In Fig 1, please provide images of separated mesenchyme from E12.5 embryos and 8-week-old mice.

We have added the images of separated mesenchyme from E12.5 embryos and 8-week-old mice in Figure 1.

6. In the cell dissociation section 4, the first note indicates that spin velocity and time

impact cell viability and yield and the authors provide a large range of g and time. Please provide the optimal (x g + time) for E12.5 epithelia and for adult epithelia here.

In the revised version in 4.3 (line 212), we have specified the spin velocity, i.e., 200 g for 8 min for both E12.5 and adult epithelia. We have added “Note 1” before the procedure to clarify that the protocol in the cell dissociation section is good for tongue epithelium and mesenchyme/connective tissue of E12.5 embryos and 8-week-old mice (line 203-204).

7. In representative results, in the first section, replace "swell" with "swelling"

We have replaced “swell” with “swelling”. (line 266 and 269)

8. For Fig 2, which 4 squares of the grid are typically selected? Is this done randomly - as cells are not evenly distributed in the hematocytometer in the images shown.

We counted all 4 squares within the hematocytometer. The images shown here are the representative images. A description has been provided in section 5 (line 249).

9. "Line 246 With 4 E12.5 tongues, the epithelial sheets and thin layers of mesenchyme immediately under the epithelium of tongues were pooled, respectively"

a. It is unclear above in the protocol if these tissues are handled differently? Or is the protocol identical for isolated epithelia and mesenchyme, isolated from embryos and adults? Please clarify.

We used the same protocol for cell dissociation of tongue epithelium and mesenchyme/connective tissue of E12.5 embryos and 8-week-old mice. We have added “Note 1” before the procedure in cell dissociation section to address this issue. (line 203-204)

b. "around 0.3 mm2 in size per tongue" in line 250. "(around 0.1 mm2 in size per tongue)" in line 261. These isolated mesenchyme pieces should be depicted in additional micrograph panels in Fig 1 as mentioned above.

We have added the images of separated mesenchyme from E12.5 embryos and 8-week-old mice in Figure 1.

10. It would be helpful if the authors would discuss any differences noted in isolated epithelial v mesenchyme populations and/or if these cell populations differed between embryonic and adult stages.

Isolated cells from epithelium and mesenchyme of E12.5 tongue are consistent and small in contrast to a large variability (from small to large) of cells from 8-week-old tongue. We have added a description in discussion section (line 315-318).

Reviewer #3:
Manuscript Summary:
This protocol was developed well for the cell dissociation from tongue. As recent research methods such as single cell RNA sequencing, and primary culture have been widely used, a protocol for separating cells from tissues seems to be very useful. However, there are some minor concerns with the manuscript.

Major Concerns:
(1) Please write the mouse strain.

We have added the mouse strain used (C57BL/6 wild type mice) in this protocol. (line 77)

(2) Usually after incubation of the tissue with enzyme for the separation, the samples should put in the media and FBS solution on ice for the stop enzyme activity

Unlike trypsin, dispase and collagenase activities are not inhibited by serum. Instead, their activities are inhibited by EDTA and EGTA. We washed the separated tissues and later transfer them to trypsin-EDTA for cell dissociation. In the revised manuscript, we have added a note for clarity (line 156-157 and 198-199).

(3) Figure2 is not clear.

We have inserted high-power images in the top right corner to improve clarity.

Minor Concerns:
(1) Authors are necessary to describe in more detail the items required for this protocol. Such as the pore size of the filter, tubes…

We have added the detailed information for the materials used.