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).