**Response to Reviewers**  
  
**Reviewer #1:**  
Manuscript Summary:  
This is a wonderful and interesting work. The idea is smart and the discussion is reasonable. The following weakness needs to be improved to enrich its value.

We appreciate the reviewer’s positive feedback and recognition of the impact of this work.   
  
Minor Concerns:  
**1. As being mentioned that the size of tumors localized deep in the submucosa of buccal mucosa is measured by caliber, how authors can assure that the growth curve in Fig. 1B is accurate. This picture might need to be replaced by showing only the final volume or weight of the tumors yielded at the endpoints.**

This is an excellent point. While what the reviewer suggested is reasonable, we decided to take a more thorough and detailed approach at addressing this highly pertinent question. We conducted serial radiographic imaging with CT simultaneously with manual caliper measurement. The CT-based gross tumor volume was generated by contouring each and every section and volumetric measurements were assessed using ITK-SNAP Software .Tumor volume measurements were then correlated with caliper measurement and a correlation coefficient was obtained. These data are now shown in Figure 2C, which show excellent correlation R2 = 0.8493. We further performed histological confirmation of the exophytic pattern of tumor growth and lack of tumor invasion into major neighboring structures such as the tongue, esophagus or bronchus. We added a sentence to clarify this point in the results section (shown below).

“Tumors did not invade into the tongue or other nearby organs (esophagus, bronchus, thymus) as assessed histologically.”

**2. It is not sure whether the findings in Fig. 1 are owing to the discrepancy of in vitro or in vivo growth. Authors should provide the in vitro growth curve of both cell lines to signify their growth potential during cultivation.**

We thank the reviewer for highlighting this point. We have added the following sentence to our results section:

“In vitro assessment of LY2 and B4B8 cell proliferation showed that both cell lines have similar doubling times (21 hours for LY2 and 23 hours for B4B8).”

**3. Scale bar of Fig. 2A should be provided as a reference for tumor size evaluation.**

A scale has been added.

**4. The description of (A) and (B) content in Fig. 3 should be detailed.**

A description has been added.

**5. To reorganize right panel of Fig. 3B is required. Myeloid cells and lymphoid cells should be shown in distinctive clusters.**

We have addressed this comment by splitting the graph into 2 graphs with myeloid and lymphoid cells presented separately (Figure 3C and Figure 3D).

**6. If possible, immune profile of B4B8 xenograft should be integrated in paper to compare with LY2 cells. This reviewer would think that the higher survival of B4B8 recipients may be associated with the stronger immunity. The comparison suggested would signify more the power of this study.**

We agree with the reviewer that adding flow cytometry data on B4B8 tumors may shed light on a different immune landscape in HNSCC. However, this is beyond the scope of this “methods” paper as dictated by the journal instructions. Immune profile data with these tumors has been published elsewhere (PMID: 30042205; PMID: 29123967). Our goal from this paper was to provide the scientific community detailed technique for inducing orthotopic HNSCC tumors. We hope that this has been achieved, especially after integrating reviewers’ feedback.

**Reviewer #2:**  
Manuscript Summary:  
This manuscript describes the development of a murine model system that is clinically- and physiologically-relevant to explore human head and neck squamous cell carcinoma. The authors use syngeneic and orthotopic murine cell injection to the buccal mucosa which makes it relatively simple and easy to measure the tumor site, closely mimics human HNSCC, and has the advantage of an immunocompetent mice model system. The symptoms the mice demonstrated such as jaw displacement, and the histological and tumor microenvironment features show relevance to human head and neck squamous cell carcinoma.

Many thanks for the kind feedback.   
  
Minor Concerns:  
**1. The approved AICUC protocol details and number should be reported.**

We have included this information in the methods section of the manuscript:

“All animal procedures were performed in accordance with an approved institutional animal care and use committee (IACUC) protocol of the University of Colorado Denver (approval # 00250).”

**2. In page 3, section 15.1, RBC lysis buffer - the composition of the buffer should be described in detail.**

We added a description of the RBC lysis buffer in the appropriate section of the protocol:

“RBC lysis buffer is comprised of ammonium chloride, sodium bicarbonate and disodium.”

In addition, the specific product used is listed in the Jove excel file which is part of the manuscript.

**3. In page 3, section 15.4: HBSS the composition of buffer should be described in detail.**

We added a description of the RBC lysis buffer in the appropriate section of the protocol:

“HBSS is comprised of HBSS is comprised of potassium chloride, sodium chloride, sodium bicarbonate, sodium phosphate dibasic, sodium phosphate monobasic and glucose.”

In addition, the specific product used is listed in the Jove excel file which is part of the manuscript.

**4. Page 4 line 194: "tumor size" should be tumor volume.**

We thank the reviewer for highlighting this inadvertent mistake. We have corrected this.

**5. In the entire text, there should be a space left between the values and units, for example in section 14.1, "1-2mm" should be 1-2 mm, or in section 14.2, "50mL" should be 50 mL, etc.**  
Our apologies. This too has been corrected.

*In compliance with data protection regulations, please contact the publication office if you would like to have your personal information removed from the database.*

**All editorial comments below have been addressed in the manuscript.**

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

Done  
2. Please provide an email address for each author.

Done  
3. Keywords: Please provide at least 6 keywords or phrases.

Done  
4. Please add a Long Abstract (150-300 words) before Introduction section. It should include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.

Done  
5. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

Done  
6. Please define all abbreviations before use.

Done

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Done  
8. Please add more details 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. 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. See examples below.

Done  
9. 2.1: How long does it take to reach 70% confluence?  
10. 3.2: Please specify the reaction conditions (temperature and time).  
11. 3.4: Resuspend cell in what media? Please specify. How is the cell number counted?  
12. 6.1: Please specify the age, gender and strain of mice as well as the concentration of isoflurane.  
13. 12: Please specify all surgical instruments used throughout the protocol.  
14. 13.1: Please specify the concentration of formalin and the temperature.  
15. 15.3: Please specify incubation temperature.  
16. 17.1: What volume is considered to be appropriate?  
17. 18: Please describe how to perform staining and analyze data.  
Done

18. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.  
Done

19. Please include single-line spaces between all paragraphs, headings, steps, etc.  
Done

20. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less 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.  
Done

21. P

lease highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Done

22. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Done

23. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

Done  
  
24. Figures 1 and 2: Please include a space between the number and the units of the scale bar.

Done

25. Figure 3: Please explain both panels (A and B) in the figure legend. Please include a space between all numbers and their corresponding units (i.e., 5 mL, 50 mL, 37 °C, 70 µm). Please use the micro symbol µ instead of u. Please define error bars in the figure legend.

Done

26. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We have added the following sentences in the appropriate parts of the discussion:

“Although, injection of cells into the buccal mucosa is straightforward, positioning of the needle and depth of penetration are critical to prevent puncture through the skin and ensure cells are not injected subcutaneously. We recommend inserting the needle intra-orally while it is perfectly parallel to the buccal and tilting by no greater than 10 degrees when ready to inject.”

“To ensure maximum retrieval of viable single cells from tumors, use of digestion enzymes is necessary. Collagenase-based digestion enzymes can be harsh on cells and optimization of the concentration and type of collagenase maybe necessary for different tumor types. We compared 5 digestion enzymes before determining that Collagenase III which was used in this study is the optimal method for digestion of squamous cell tumors.”

27. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

Done

28. Please 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. See the example below:  
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).  
Done

29. References: Please do not abbreviate journal titles.

The Jove Endnote reference style was downloaded and applied and yet journal titles were abbreviated in this file. If you have an endnote compatible file, we can use that instead.

30. Table of Equipment and Materials: Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Done