Journal of Visualized Experiments

Whole-mount Staining, Visualization, and Analysis of Fungiform, Circumvallate, and Palate Taste Buds

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62126R1
Full Title:	Whole-mount Staining, Visualization, and Analysis of Fungiform, Circumvallate, and Palate Taste Buds
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Neuroscience
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

Whole-mount Staining, Visualization, and Analysis of Fungiform, Circumvallate, and Palate Taste Buds

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KEYWORDS:
taste bud, whole mount, innervation, afferent, tongue, taste

SUMMARY:
This paper describes methods for tissue preparation, staining, and analysis of whole fungiform, circumvallate, and palate taste buds that consistently yield whole and intact taste buds (including the nerve fibers that innervate them) and maintain the relationships between structures within taste buds and the surrounding papilla.

ABSTRACT:
Taste buds are collections of taste-transducing cells specialized to detect subsets of chemical stimuli in the oral cavity. These transducing cells communicate with nerve fibers that carry this information to the brain. Because taste-transducing cells continuously die and are replaced throughout adulthood, the taste-bud environment is both complex and dynamic, requiring detailed analyses of its cell types, their locations, and any physical relationships between them. Detailed analyses have been limited by tongue-tissue heterogeneity and density that have significantly reduced antibody permeability. These obstacles require sectioning protocols that result in splitting taste buds across sections so that measurements are only approximated, and cell relationships are lost. To overcome these challenges, the methods described herein involve collecting, imaging, and analyzing whole taste buds and individual terminal arbors from three taste regions: fungiform papillae, circumvallate papillae, and the palate. Collecting whole taste buds reduces bias and technical variability and can be used to report absolute numbers for features including taste-bud volume, total taste-bud innervation, transducing-cell counts, and the morphology of individual terminal arbors. To demonstrate the advantages of this method, this paper provides comparisons of taste bud and innervation volumes between fungiform and circumvallate taste buds using a general taste-bud marker and a label for all taste fibers. A workflow for the use of sparse-cell genetic labeling of taste neurons (with labeled subsets of taste-transducing cells) is also provided. This workflow analyzes the structures of individual taste-nerve arbors, cell type numbers, and the physical relationships between cells using image analysis

software. Together, these workflows provide a novel approach for tissue preparation and analysis of both whole taste buds and the complete morphology of their innervating arbors.

INTRODUCTION:

Taste buds are collections of 50–100 specialized epithelial cells that bind subsets of chemical-taste stimuli present in the oral cavity. Taste-transducing cells are generally thought to exist as types[1-9], initially based on electron microscopy criteria that were later correlated with molecular markers. Type II cells express phospholipase C-beta 2 (PLCb2)[2] and transient receptor potential cation channel, subfamily M member 5[1] and include cells that transduce sweet, bitter, and umami[1,10]. Type III cells express carbonic anhydrase 4 (Car4)[11] and synaptosomal-associated protein 25[8] and denote cells that primarily respond to sour taste[11]. The cells that transduce saltiness have not been as clearly delineated[12-14], but could potentially include Type II and Type III cells[15-19].The taste-bud environment is complex and dynamic, given that taste-transducing cells continuously turn over throughout adulthood and are replaced by basal progenitors[3,20,21]. These taste-transducing cells connect to pseudo-unipolar nerve fibers from the geniculate and petrosal ganglia, which pass taste information to the brainstem. These neurons have primarily been categorized based on the kind of taste information they carry[22,23] because information about their morphology has been elusive until recently[24]. Type II cells communicate with nerve fibers via calcium homeostasis modulator protein 1 ion channels[25], whereas Type III cells communicate via classical synapses[8,26]. Further characterization of taste bud cells—including transducing cell type lineages, factors that influence their differentiation, and the structures of connecting arbors—are all areas of active investigation.

Taste-bud studies have been hindered by several technical challenges. The heterogenous and dense tissues that make up the tongue significantly reduce antibody permeability for immunohistochemistry[27-29]. These obstacles have necessitated sectioning protocols that result in the splitting of taste buds across sections so that measurements are either approximated based on representative sections or summed across sections. Previously, representative thin sections have been used to approximate both volume values and transducing-cell counts[30]. Thicker serial sectioning allows for the imaging of all taste-bud sections and the summing of measurements from each section[31]. Cutting such thick sections and selecting only whole taste buds biases sampling towards smaller taste buds[32-34]. Nerve innervation estimates from sectioned taste buds have been based on analyses of pixel numbers[13,35], if quantified at all[36-38]. These measurements completely ignore the structure and number of individual nerve arbors, because arbors are split (and usually poorly labeled). Lastly, although peeling away the epithelium does permit entire taste buds to be stained[39,40], it also removes taste-bud nerve fibers and could disrupt the normal relationships between cells. Therefore, investigations of the structural relationships within taste buds have been limited because of this disruption caused by staining approaches.

Whole-structure collection eliminates the need for representative sections and allows the determination of absolute-value measurements of volumes, cell counts, and structure morphologies[41]. This approach also increases accuracy, limits bias, and reduces technical variability. This last element is important because taste buds show considerable biological variability both within[34,42] and across regions[43,44], and whole taste-bud analyses allow absolute

cell numbers to be compared between control and experimental conditions. Furthermore, the ability to collect intact taste buds permits the analysis of the physical relationships between different transducing cells and their associated nerve fibers. Because transducing cells may communicate with each other[45] and with nerve fibers[46], these relationships are important for normal function. Thus, loss-of-function conditions may not be due to a loss of cells, but instead to changes in cell relationships. Provided here is a method for collecting whole taste buds to achieve the benefits of absolute measurements for refining volume analyses for both taste buds and their innervations, taste-cell counts and shapes and for facilitating analyses of transducing-cell relationships and nerve-arbor morphologies. Two workflows are also presented downstream of this novel whole-mount method for tissue preparation: 1) for analyzing taste bud volume and total innervation and 2) for sparse-cell genetic labeling of taste neurons (with subsets of taste-transducing cells labeled) and subsequent analyses of taste-nerve arbor morphology, numbers of taste-cell types and their shapes, and the use of image analysis software to analyze the physical relationships between transducing cells and those between transducing cells and their nerve arbors. Together, these workflows provide a novel approach to tissue preparation and for the analyses of whole taste buds and the complete morphology of their innervating arbors.

PROTOCOL:

NOTE: All animals were cared for in accordance with the guidelines set by the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals. Phox2b-Cre mice (MMRRC strain 034613-UCD, NP91Gsat/Mmcd) or TrkB^{CreER} mice (*Ntrk2$^{tm3.1(cre/ERT2)Ddg}$*) were bred with tdTomato reporter mice (Ai14). Advillin^{CreER} [47] were bred with Phox2b-flpo[48] and Ai65. For 5-ethynyl-2'-deoxyuridine (EdU) injections, the EdU was prepared and doses calculated according to Perea-Martinez et al.[49].

1. Preparation of materials

1.1. Preparation of solutions

1.1.1. Dissolve 5.244 g of monobasic sodium phosphate and 23.004 g of dibasic sodium phosphate in double-distilled water (ddH_2O) on a stir plate. Adjust the pH to 7.4, and bring the total volume to obtain 1 L of 0.2 M sodium phosphate buffer (PB).

1.1.2. Dissolve paraformaldehyde in ddH_2O in a fume hood by heating while stirring on a stir plate until the solution reaches 90 °C. Add 4 M NaOH solution dropwise to clear the paraformaldehyde, and filter the solution using a vacuum Erlenmeyer flask and ceramic filter with filter paper. Add an equal volume of 0.2 M PB, and adjust the pH to 7.4 to obtain 4% PFA in 0.1 M PB.

2. Tissue preparation

2.1. Tissue collection

2.1.1. Sacrifice mice using an anesthetic overdose with a working solution containing 10 mL of

sterile saline and 0.25 mL of a stock solution containing 5 g of 2,2,2-tribromoethanol and 5 mL of tert-amyl alcohol. Perfuse transcardially with 4% PFA in 0.1 M PB; remove the tongues and the palate.

2.1.2. Isolate the circumvallate taste buds using a coronal cut separating the posterior tongue, behind the intermolar eminence; cut off the taste buds with a razorblade; and then bisect the anterior tongue at the midline. Post-fix overnight at 4 °C with 4% PFA in 0.1 M PB.

2.1.3. Cryoprotect the tissue in 30% sucrose overnight at 4 °C.

NOTE: The tissue can be frozen in optimal cutting temperature (OCT) compound using 2-methylbutane chilled in a beaker on dry ice and stored at -80 °C if the procedure has to be paused here.

2.2. Fungiform taste buds

2.2.1. Chill 2-methylbutane in a beaker on dry ice in preparation for step 2.2.8.

2.2.2. Thaw and rinse the tongue in 0.1 M PB. Place one half of the anterior tongue containing the fungiform papillae on a glass slide under a dissecting microscope.

2.2.3. Use blunt-ended forceps and dissection scissors to remove the muscle. Use blunt-ended forceps to hold the tissue open as the lingual epithelium is curved, and ensure a flat orientation by keeping the blades of the coarse dissection scissors parallel to the epithelium.

2.2.4. Discard the ventral non-keratinized epithelium of the tongue as it contains no taste buds.

2.2.5. Use fine dissection scissors for closer dissection to the underside of the keratinized epithelium.

NOTE: It is important to dissect close to the epithelium so that the remaining muscle is of uniform thickness, and the surface is smooth to ensure uniform antibody penetration. The consequence of non-uniform thickness of the remaining muscle will be uneven sectioning on the cryostat, with exposure of the epithelium in areas with less muscle and a thicker layer of muscle for other areas, which impedes antibody penetration.

2.2.6. Use the blunt-ended forceps to lay a piece of epithelium into a tissue mold (muscle side down), and ensure that it lays flat. Once the tissue is flat, add a drop of OCT to the tissue.

NOTE: Given that the tip of the tongue is curved, it may be necessary to make a cut in the epithelium where it is curved so that the tissue can be made to lay flat.

2.2.7. Place the tissue mold on a metal base (previously cooled in dry ice) under the dissecting

scope. Continue to tap the tissue lightly with the forceps until the OCT has frozen to ensure the tissue freezes as flat as possible.

2.2.8. Once the OCT has frozen, quickly add additional OCT, and place the mold in a beaker of 2-methylbutane (cooled in dry ice) until frozen.

2.2.9. Cryostat sectioning

NOTE: The cryostat is used for fine removal of remaining subcutaneous tissue, which may inhibit antibody penetration (**Figure 1**).

2.2.9.1. Mount the OCT molds on the cryostat, and cut 20 µm sections. Collect each section, and view it under the light microscope to assess its proximity to the base of the epithelium (**Figure 1E–H**).

2.2.9.2. After the tissue is shaved from the underside of the epithelium, thaw the epithelium and rinse it twice in 0.1 M PB on a shaker.

2.3. Circumvallate taste buds

2.3.1. Using a coronal cut with a razorblade, separate the circumvallate papilla from the anterior tongue. Use two parasagittal cuts with the same razorblade to remove the tissue lateral to the papilla under a dissecting scope. Place the papilla in a tissue mold using forceps so that one lateral edge of the circumvallate papilla faces the bottom of the tissue mold.

NOTE: The tissue can be frozen in OCT using 2-methylbutane chilled in a beaker on dry ice and stored at -80 °C if the procedure has to be paused here.

2.3.2. Cut the tissue into 90 mm floating sections on the cryostat.

2.4. Taste buds on the palate

2.4.1. Cut the hard palate anterior to the junction of the soft and hard palate (**Figure 2**). Use scissors to separate the soft palate from the underlying tissue, making sure any remaining bone fragments are cut away. Remove additional muscle and connective tissue.

NOTE: Once removed, all tissue that remains will consist of glands and loose connective tissue, which are lightly adhered to the underside of the palate.

2.4.2. Hold the palate with blunt-ended forceps, and remove the remaining glands and loose connective tissue by gently scraping them with a razor blade.

NOTE: The tissue can be frozen in OCT using 2-methylbutane chilled in a beaker on dry ice and stored at -80 °C if the procedure has to be paused here.

3. **Immunohistochemistry staining**

3.1. Wash the tissues with 0.1 M PB, 3 x 15 min. Place the tissues in 1 mL tubes with blocking solution (3% donkey serum, 0.5% non-ionic surfactant [see the **Table of Materials**], 0.1 M PB) at 4 °C overnight.

3.2. Remove the blocking solution, and incubate the tissue in primary antibody (rabbit anti-PCLβ2) in antibody solution (0.1 M PB, 0.5% non-ionic surfactant) for 5-days at 4 °C.

3.3. Wash with 0.1 M PB, 4 x 15 min each wash, and incubate in secondary donkey anti-rabbit 488 antibody (1:500) in antibody solution for 2 days at 4 °C.

3.4. Wash with 0.1 M PB, 4 x 15 min each wash, and block with 5% normal rabbit serum in antibody solution.

3.5. Wash with 0.1 M PB, 4 x 15 min. Incubate with donkey anti-rabbit blocking antibody (20 µg/mL) in antibody solution for 2 days at 4 °C.

3.6. Wash with 0.1 M PB, 4 x 15 min each wash, and then incubate with primary antibody dsRed (rabbit) conjugated to a fluorescent label (according to the manufacturer's instructions) in an antibody solution for 5 days at 4 °C.

3.7. Wash with 0.1 M PB, 4 x 15 min each wash, and then incubate with primary antibody (goat anti-Car4 (1:500)) in antibody solution for 5 days at 4 °C.

3.8. Wash with 0.1 M PB, 4 x 15 min each wash. Incubate with secondary donkey anti-goat 647 antibody (1:500) in antibody solution for 2 days at 4 °C.

3.9. Wash with 0.1 M PB, 4 x 15 min each wash, and mount (epithelial side up) in aqueous mounting media, and place a coverslip over the tissue section.

NOTE: If using antibodies from different species, as in the case with keratin-8 and dsRed only, add all primary antibodies to the antibody solution in step 3.2 and all secondary antibodies in step 3.3 before proceeding to step 3.9.

4. **Confocal imaging and deconvolution**

4.1. Capture confocal images using a confocal microscope with a 60x objective (Numerical Aperture= 1.40), 4 ms/pixel, zoom of 3, Kalman of 2, and size of 1024 x 1024. Select a step size of 0.47 mm along the z-axis. For capturing innervation to the papilla, use a zoom of 2.5 if the field of view with a zoom of 3 is too narrow to capture all the innervation to the papilla.

4.2. To deconvolute the images, note that some settings will automatically import with the

image; therefore, fill in the remaining details for modality, objective lens, numerical aperture, immersion medium, sample medium, and fluorophores captured in the image. Then, select **3D Deconvolution**.

5. Image analysis

5.1. Taste bud and innervation volume

5.1.1. Import deconvoluted image stacks to a pixel-based image analysis software (see the **Table of Materials**) to determine the taste bud volume and volume of total innervation within the taste bud.

5.1.1.1. Uncheck **Volume** in the main **Object** menu.

5.1.1.2. Select **Add new surfaces** from the **Object** menu. Select **Skip automatic creation, edit manually**, and then select **Contour**.

5.1.1.3. Click on **Select** and observe the arrow appearing as a **+** to help trace the border of the taste bud. Move the slicer and outline the taste bud in each optical section. Once the contours are complete, click on **Create Surface**.

5.1.1.4. Observe the taste-bud volume object now appearing in the main **Object** menu. Locate the volume of the taste bud under **Tools**.

5.2. Volume of innervation within the taste bud

5.2.1. Select the **pencil** icon under the taste bud object, then select **Mask All**.

5.2.2. In the dropdown menu, select the **fluorescent channel** that corresponds with the nerve fiber label. Check **Create Duplicate Channel**.

5.2.3. Check **Set voxels outside surface to:**, and type **0** in the box.

5.2.4. Observe the new channel appearing in the **Display Adjustment** window, which is an unaltered duplicate of the fluorescent channel selected within the taste bud.

5.2.5. In the main **Object** menu, select **Create New Surface**. Uncheck **Skip automatic creation, edit manually**.

5.2.6. Click twice on the **blue arrow** to proceed to the next step.

5.2.7. Click on **Delete**, then click on the **green double arrow** to complete the surface, which represents the volume of the nerve fibers present within the taste bud. To find the value for the

volume, select **Tools** under the **nerve fiber Object** menu, and select **Volume** from the dropdown menu.

5.3. Volume of innervation to the papilla

5.3.1. Create a volume of the taste bud as described in section 5.1.

5.3.2. Select the **pencil** icon under the taste-bud object, then select **Mask All**.

5.3.3. In the dropdown menu, select the **fluorescent channel** that corresponds with the nerve fiber label. Check **Create Duplicate Channel**.

5.3.4. Check **Set voxels inside surface to:** and type **0** in the box. Click **OK**.

5.3.5. Generate a surface by clicking on **Add New Surface**. Select **Segment only a Region of Interest**.

5.3.6. Click on the **blue arrow**, and increase the Z-value so that the region of interest begins at the base of the taste bud.

5.3.7. Click twice on the **blue arrow** to proceed to the next step.

5.3.8. Click on **Delete**, then click on the **green double arrow** to complete the surface, which represents the volume of the innervation to the papilla. To find the value for the volume, select **Tools** under the nerve fiber **Object** menu, and select **Volume** from the dropdown menu.

5.4. Terminal arbor contact analysis

5.4.1. Image preparation

5.4.1.1. Go to the **Edit** menu and select **Crop 3D**. Crop the image on all sides, removing space outside of the taste bud.

NOTE: Crop as close to the taste bud without removing any relevant structure—any excess image will lengthen processing time.

5.4.1.2. Select **Edit** from the main menu, click **Change data type.** Select **To: 32 bit float** from the dropdown menu.

5.4.1.3. Select **Add new surfaces** from the **Object** menu. Click on **Skip automatic creation, edit manually**, select **Contour**, and then drag the slice position to the right to find the total number of optical slices.

5.4.1.4. Generate isometric voxels, and make sure that instead of the voxels being

rectangles (0.0691 x 0.0691 x 0.474) as XxYxZ, respectively, the voxels are 0.0691 x 0.0691 x 0.0691 by dividing the rectangles into cubes with identical values for fluorescence intensity as the original, rectangular voxel. Select **Image Properties**. Then, divide the Z value for Voxel Size by the X or Y value (= 0.474/0.0691), and multiply that value by the number of slices (optical sections) found in the previous step.

5.4.1.5. Go back to **Edit** on the main menu, and select **Resample 3D**.

5.4.1.6. Replace the Z value (number of slices) with the newly calculated value.

5.4.2. Creating automatic surfaces based on fluorescence

5.4.2.1. Click on **Add New Surface** again to add a new surface, deselect **Segment only a region of interest**, and click on **Next**.

5.4.2.2. From the **Source channel** dropdown menu, select the **channel** for one of the receptor cell types. Unselect **Smooth** and continue to the next step.

5.4.2.3. Do not alter anything on the next screen that shows the range of fluorescent intensities present in the image. Click on the **blue arrow** at the bottom again to move to the next step.

5.4.2.4. Click on **Delete**, then click on the **green double arrow** to complete the surface.

5.4.2.5. Go to the **Object** menu on the left hand of the screen where the completed cell surface will appear and will be called something generic such as **Surface 2**. Double click on the **surface name** to name it according to what the label represents.

NOTE: In this case, the surface generated is based on the PLCb2 receptor cell marker.

5.4.2.6. If there are dots instead of a surface, under the menu in the lower left-hand corner, click on **Surface** instead of **Center point**. Then, click **OK** when prompted.

5.4.2.7. Repeat steps 5.3.2.1–5.3.2.5 for the other receptor cell markers and the nerve fiber marker.

5.4.2.8. Save (export) the progress.

5.4.2.9. Click on a cell type **Surface** in the main menu. From that object's menu, click on **Tools**, then click on **Distance Transformation**.

5.4.2.10. Wait for a pop-up box entitled **XTDistanceTransformation** to appear. Select **Outside Surface Object**. Make note of the new channel that appears in the **Display Adjustment** menu called **Distance to *surface name***.

5.4.2.11. Select the **Nerve Fiber surface** from the **Object** menu. Click on the **pencil** icon and then **Mask All**.

5.4.2.12. From the dropdown menu that appears, select the new channel **Distance to *surface name***. Make note of the new channel that appears in the **Display Adjustment** window called **Masked Distance to *surface name***.

5.4.2.13. Create a **new object** using the main **Object** menu. Unselect **Segment only a region of interest** and click on the **blue arrow**. Select the **Masked Distance to PLCb2** channel and uncheck **Smooth**.

5.4.2.14. On the next screen, check if there are any regions where the taste receptor cells are within the smallest distance from the nerve fibers discernable by this software. To do this, set a limit of 0.01–0.011 µm to check for any receptor cell fluorescence this close to the nerve fibers.

5.4.2.15. Type **0.01** in the green box on the left side to set the lower threshold, press **Tab**. Then click on the **red button** and type **0.11** to set the upper threshold. Press **Tab** and then click on the **blue arrow at the bottom** to move on to the next step.

5.4.2.16. Click on **Delete** and then the **green double arrow** to finish.

5.4.2.17. Rename this surface **Within 0.01-0.11 of PLCb2** in the **Object** menu. Select **Within 0.01–0.11 of PLCb2 surface** in the **Object** menu.

5.4.2.18. Select the **pencil** then click on **Mask All**. Select the **red (nerve fiber) channel** from the dropdown menu and click **OK**. Make note of the new channel that appears in the **Display Adjustment** window called **Masked CHS2**.

5.4.2.19. Click on the **name of the channel**; rename the channel **Within 0.01–0.11 of PLCb2**.

NOTE: This is a fluorescent channel that represents a duplicate of the red fluorescent channel present within the surface created.

5.4.2.20. Click on **white** in the middle of the **color selector**. Select a color that contrasts with the colors of the structures.

5.4.2.21. Export (i.e., save) the file at this stage.

5.4.2.22. Repeat steps 5.4.2.9–5.4.2.21 for other taste receptor cell markers.

5.4.2.23. Export (i.e., save) the file at this stage.

NOTE: To analyze the proximity of one labeled cell type to another, simply replace the Nerve Fiber surface in step 5.4.2.11 (and the following steps) with the object of interest and the equivalent components that pertain to each subsequent steps.

6. Neuron arbor reconstruction and absolute cell number quantification

6.1. Terminal arbor tracing and analysis

6.1.1. Open the deconvoluted image file in a 3D vector-based image analysis software (see the **Table of Materials**), select **Trace**, click on **Neuron**, and then click on **Dendrite**.

6.1.2. Scroll to the base of the taste bud in the image stack. Trace each fiber to the end while scrolling through the image stack.

6.1.3. When at the branch end, right click on the end and select **Ending**. At branch points, right click and select **Bifurcating Node**.

NOTE: This enables tracing one branch to the end and then returning to the bifurcating point and tracing the other branch with the program recognizing that this tracing is still of the same neuron.

6.1.4. Save the data file as a .DAT file, which can then be opened for analysis in the 3D vector-based image analysis software.

7. Cell number quantification

7.1. Quantify the labeled taste bud cells in any image analysis software package as long as distinct markers for receptor cell types anchored to the z-position can be placed at the nuclear level.

REPRESENTATIVE RESULTS:

Staining of the lingual epithelium with antibodies to dsRed and keratin-8 (a general taste-bud marker) labeled both whole taste buds and all taste-bud innervation in Phox2b-Cre:tdTomato mice[50,51] (**Figure 3A)**. Imaging these taste buds from their pores to their bases gave the highest resolution x-y plane images (**Figure 3A,B**). The contour function of the pixel-based imaging program was used to outline the periphery of the taste bud in each section (**Figure 3B**), and then generate a surface (**Figure 3C**) that represented taste bud volume. Masking (or duplicating) the fluorescence associated with the taste-bud label only within the surface created a new channel that contained only this fluorescence and eliminated any papilla staining obscuring the taste bud (**Figure 3D**). The nerve fiber fluorescence within the taste bud was masked (**Figure 3E**) and used to automatically create a surface representing the volume of innervation within it (**Figure 3F**). A similar approach was also used to measure taste-bud volume and that of its associated innervation in circumvallate taste buds (**Figure 3G**). Representative measurement data revealed no correlations between taste-bud volumes and innervation volumes in either the fungiform (p = 0.115) or the circumvallate (p = 0.090) measurement regions (**Figure 3H**).

The administration of a low dose of tamoxifen in *TrkB*^{CreER}:tdTomato mice causes gene recombination and the labeling of a small number of neurons so that taste buds are innervated by zero to a few labeled terminal arbors (the neuronal portion within the taste bud). The lingual epithelium was stained using an anti-dsRed antibody for the terminal arbors and anti-Car4 (sour) and anti-PLCβ2 (sweet, bitter, and umami) antibodies for the taste-transducing cells (**Figure 4A**). A vector-based image analysis program was used to trace the labeled terminal arbors (**Figure 4B**). The orthogonal heights of the arbors associated with the blue and green tracings were 33.4 μm (**Figure 4C**) and 32.4 μm (**Figure 4D**), respectively. The 3D Convex Hull measurements (i.e., the extent of the terminal arbor within the taste bud) for the blue terminal arbor was 644.0 μm^3 and 3647.0 μm^3 for the green arbor. The dendrogram for the green tracing is shown in **Figure 4E** with branch lengths measured in microns. The green arbor had seven branch ends and a total length of 183.4 μm. Quantification of the absolute numbers of PLCβ2+ and Car4+ cells revealed that this taste bud had 17 PLCβ2+ cells and two Car4+ cells. Using cell pixel-based imaging software to determine the closest proximity between nerve fibers and taste-transducing cells revealed that out of a total of 19 taste-transducing cells in the taste bud, the blue terminal arbor (shown in red in **Figure 4F,G**) was within 200 nm (the resolution of the light microscope) of the light blue Car4+ cell (white areas indicated by arrows in **Figure 4G**). The terminal arbor associated with the green tracing is shown in magenta (**Figure 4F** and **Figure 4H**) and is within 200 nm of both the light and dark blue Car4+ cells (white areas in **Figure 4H**). As the next closest cell to these arbors was more than 200 nm away, there was an unlabeled voxel separating the two structures.

Dividing progenitor cells were labeled using injections of EdU on Days 0, 1, and 3, and tissues were collected on Day 4. Whole-mount keratin-8 and EdU staining of fungiform taste buds revealed that EdU-labeled cells were present both within and outside of the taste buds (**Figure 5A–C**). Individual EdU+/keratin-8+ cells (teal and yellow) and EdU+/keratin-8– nuclei (purple and magenta) were segmented (**Figure 5B,C**). The dark blue cell shown was keratin-8+ and had an elongated shape consistent with mature taste-transducing cells. These surfaces are shown with the taste bud oriented from pore-to-base (**Figure 5B**) and along the long axis of the taste bud (**Figure 5C**). Each structure could be viewed in individual optical slices by masking the fluorescence within each structure (**Figure 5D–F**). The magenta and purple nuclei are outside of the keratin-8+ border of the taste bud indicated by the white-dotted outline (**Figure 5D,E**). The yellow, teal, and blue cells were within the taste bud (**Figure 5D–F**). Individual taste-transducing cells could be reconstructed using pixel-based imaging software of either Car4 labeling (**Figure 6A–C**) or PLCβ2 labeling (**Figure 6D–F**). A pixel-based imaging software was be used to measure the closest proximity between cells revealed that a Car4+ cell (same cell as shown in **Figure 6B**) was within 200 nm of a single PLCβ2+ cell (**Figure 6G**, green). The area where the cells were within 200 nm of each other is shown in white (**Figure 6G**) and indicated by white arrows. The next closest cell was more than 200 nm away and is shown in yellow in **Figure 6H,I** in two different orientations. **Figure 7** demonstrates the isolation and analysis of the innervation terminating within the papilla (but outside the taste bud) and includes its distribution around the taste bud and its distance from the epithelium.

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of lingual epithelium for fungiform taste-bud staining. (**A**) View of the cut tongue with epithelium and muscle labeled prior to any dissection. (**B**) Once enough muscle has been removed, there is only a small amount of remaining muscle on the underside of the epithelium. In addition to evaluating the progress of the dissection by viewing the cut side of the epithelium, (**C**) laying the epithelium flat on a glass slide under the dissecting scope reveals that some portions of the tissue are evenly translucent (purple rectangle); enough muscle has been removed from this area. In contrast, the blue arrows indicate regions on the left where there is more muscle that needs to be removed. Once the entire underside of the epithelium is similar to the area in the purple rectangle, proceed to the next step. (**D**) After portions of the epithelium have been frozen with the muscle side down, additional muscle and lamina propria are removed as thin sections using the cryostat. When sectioning is complete, the remaining epithelium is thin and translucent. (**E–F**) Serial sections (20 µm) were collected on a glass slide, and each section was viewed under a fluorescent microscope before cutting the next section. Well below the epithelium, muscle fibers are oriented in multiple directions so that muscle fibers are present both in cross section and along the muscle fiber (**E**, red rectangle). The serial sections in **E–F** demonstrate the transition from muscle fibers oriented in multiple directions (**E**, red rectangle) to muscle fibers being oriented mostly in one direction (**F**, red rectangle), which is indicative of the muscle-lamina propria border. Another region of the same piece of tissue (yellow rectangles) demonstrates that when the muscle fibers are oriented in one direction, the next section will likely yield connective tissue because all muscle has been removed from that region. The blue rectangles both represent the underside of the epithelium. If taste buds are present on the section (**G**, red arrows), too much tissue has been removed. Ideally, sectioning is complete when the underside of the epithelium (but no taste buds) is visible in the removed sections (**F**, yellow rectangle). Although areas with muscle fibers oriented in the same direction (**E**, yellow rectangle and **F**, red rectangle) are suitable for sectioning, areas where the muscle fibers are oriented in multiple directions (**E**, red rectangle) should be avoided. (**G**) Once sections include the underside of the epithelium/lamina propria, it is only possible to cut a few additional sections before too much of the epithelium has been removed and sections include taste buds. (**H**) The most common mistake is revealed by cryostat sections where epithelium is seen at the edge of the tissue, muscle is seen inside of the epithelium, and OTC/sparse muscle is present in the middle. This is most often due to not laying the tissue flat on the bottom of the tissue mold before freezing or insufficient flattening with blunt-ended forceps. Scale bars in **A–C** = 1 mm; scale bars in **E, F, H** = 100 µm; scale bar in **G** = 50 µm.

Figure 2: Dissection of palate for staining. (**A**) The palate was dissected first using thin blade scissors to cut the hard palate, (**B**) then using the same scissors to separate the soft palate from the underlying connective tissue. After removing the tissue from the oral cavity, any remaining tissue was removed with the scissors. At this point, all that may remain are glands on the back of the soft palate. A razorblade was used to gently scrape away these glands. The (**C**) back and (**D**) epithelial surface of the completed dissection of the palate are shown.

Figure 3: Measuring volume in whole-mount taste buds. (**A**) Whole-mount taste buds were imaged from the taste pore to the base of the taste bud so that the plane of highest resolution is

the x-y plane. Each optical slice was viewed in vector-based image analysis software, and the contour function was used to manually outline the periphery of the taste bud stained with keratin-8. (**B**) An example of one optical slice is provided. (**C**) The position of this representative section along the long axis of the taste bud is shown by the yellow line. After each optical section was outlined, a surface was created that represents the volume of the taste bud (white). Masking or duplicating the fluorescent channel corresponding to the taste bud (keratin-8 in **D**) or the tdTomato-labeled innervation (pseudo-colored blue in **E**) within the volume representing the taste bud. The fluorescence within the taste bud in (**E**) was used to generate a surface representing the volume of innervation within the taste bud (**F**, blue). (**G**) A similar approach was applied to whole-mount circumvallate taste buds imaged in the same orientation as the fungiform taste bud in **A**. (**H**) Measuring the volume of fungiform and circumvallate taste buds and their respective volume of innervation revealed that there is no correlation between the taste bud and innervation volume for taste buds sampled for either region. Scale bars in **A–D, F** = 4 µm; scale bar in G = 5 µm. This figure has been modified from Ohman-Gault et al.[50]. Abbreviations: FF = fungiform; CV = circumvallate.

Figure 4: Representative terminal arbors in fungiform taste buds using sparse cell genetic labeling. (**A**) Whole-mount taste bud stained with taste-receptor-cell markers Car4 (white) and PLCb2 (green). (**B**) This taste bud has two labeled terminal arbors, which are shown with the taste bud removed after reconstructing the fibers. (**C**) The blue arbor has 6 branch ends and an orthogonal height in the taste bud of 33.4 µm and (**D**) the green arbor has 7 branch ends. (**E**) The dendrogram corresponding to the green arbor is provided with each segment length in micrometers. (**F–H**) The distance between structures was measured. (**F–G**) The blue tracing in **C** was segmented and is shown in red. (**G**) The areas where this terminal arbor is within 200 nm of the light blue Car4+ cell are indicated by white arrows. (**F, H**) The terminal arbor represented by the green reconstruction is shown in magenta. (**H**) The magenta arbor (associated with the green tracing in 4B,D) is within 200 nm of both the dark and light blue Car4+ cells. Scale bar in **A, B** = 4 µm; scale bars in **F–H** = 5 µm.

Figure 5: Whole-mounts can be used to track incorporation of new taste bud cells. Mice were injected with EdU to label dividing progenitors on Days 0, 1, and 3 and sacrificed on Day 4. (**A, B**) Cells labeled with EdU (green) can be identified both around and within the taste bud, which is labeled with keratin-8 (A, white, B, gray). (**B, C**) Individual EdU-labeled, keratin-8+ cells inside the taste bud and keratin-8–, EdU-labeled nuclei are segmented outside the taste bud. (**D–F**) The fluorescence within each structure segmented in **A–B** was masked and can be seen in cross-section. The perimeter of the taste bud is outlined with a white dotted line (**D–F**). (**D**) The yellow cell is within the taste bud and is both EdU-labeled and keratin-8+. The magenta nucleus is outside the taste bud and is keratin-8–. (**E**) The teal cell is inside the taste bud and both EdU-labeled and keratin-8+. The purple EdU-labeled nucleus is keratin-8– and outside of the taste bud (white arrow). (**F**) The blue cell is keratin-8+ and elongated, consistent with mature taste-transducing cells. Scale bars in **A–C** =3 µm; scale bars in D = 2 µm; scale bars in **E, F** = 4 µm. Abbreviation: EdU = 5-ethynyl-2'-deoxyuridine.

Figure 6: Shapes of whole taste bud cells can be analyzed along with their relationships with

other taste bud cells. (**A–F**). Segmenting individual taste bud cells to create surfaces isolates individual taste bud cells, facilitating clear visualization. Individual (**A–C**) Car4+ and (**D–F**) PLCβ2+ cells show the variation in individual cell shapes. (**G**) The closest PLCβ2+ cell to the Car4+ cell in **B** was determined to be within 200 nm (at a single small 0.5 μm^2 location indicated by arrow). The next closest cell was greater than 300 nm away and distinguishable as a separate structure from the segmented Car4+ cell. (**H, I**) The next closest cell was segmented; and the masked fluorescence is shown in yellow. The three closest points for the next closest cell (yellow) are indicated by arrowheads in **H, I.** Scale bars in **A–C** = 3 μm; scale bars in **D, E** = 4 μm; scale bar in **F** = 2 μm; scale bars in **G–I** = 3 μm.

Figure 7: Quantifying innervation to the papilla. (**A**) Some labels for taste neurons also label innervation to the papilla. (**B**) The innervation within the taste bud is separated from the innervation outside the taste bud by segmenting the taste bud (as described for **Figure 3**), (**C**) masking the innervation inside the taste bud (red), and then masking the innervation outside of the taste bud only (dark blue). The volume of innervation to the taste bud (red) was 1649.6 mm^3. The innervation outside the taste bud will include taste fibers underneath the papilla that should not be included in the quantification of the innervation to the papilla. (**D**) The fluorescence of the innervation to the papilla was masked (light blue). The volume of innervation to the papilla was 121.8 mm^3. Scale bars in A–D = 4 mm.

DISCUSSION:

The development of an approach to consistently collect and stain whole taste buds from three oral cavity taste regions (fungiform, circumvallate, and the palate) provides significant improvements for analyzing taste-transducing cells, tracking newly incorporated cells, innervation, and relationships between these structures. In addition, it facilitates the localization of a potential secondary neuron marker both within or outside of a labeled population[50]. This is particularly relevant given that gustatory papillae also receive robust somatosensory innervation[52,53], which may also label some taste neurons. The papillae housing taste buds can also be imaged using a lower magnification. This permits visualization of the innervation to the entire papilla, as well as to the taste buds, and enables independent analyses of the innervation that penetrates the taste bud and the surrounding nerve fibers.

Somatosensory nerve endings in the skin can be distinguished based on their organization around hair follicles and their relationships to other components of the epithelium; parallel analyses in gustatory papillae may yield similar characterizations[54,55]. Establishing a normal foundation for the relationships within, and the composition of, taste buds and papillae will serve as a baseline for determining the mechanisms underlying deficits in peripheral taste functions[56,57]. The taste bud is a dynamic sensory end-organ where cell turnover and terminal arbor remodeling are coordinated by a variety of factors[58]. Investigations into the potential circuitry within the taste bud[59], disease processes[57], and chemotherapies that disrupt normal taste function[58] could be enhanced by this method, which maintains whole taste buds and nerve fibers intact. The whole-mount method described here both expands the possibilities for analysis and refines the measurements that are possible.

Given that the tongue is a dense and heterogenous tissue, and that the taste bud itself contains many cell-to-cell junctions that limit permeability[27], developing an approach to accomplish whole-mount staining of taste buds presented a significant challenge. Previous methods involved taking representative sections[60] or cutting thicker sections, which then limited antibody penetration[32-34]. In addition, the selection of whole taste buds from these thicker sections biased the data toward smaller taste buds. Alternatively, peeling the epithelium is likely to disrupt taste bud nerve fibers; these are not specifically labeled when this approach is used[39,40]. Nerve arbors form a large plexus within a taste bud[26,50,61], so it is unclear whether arbor removal disrupts the normal relationships between other cells in the taste bud. In contrast, the present whole-taste-bud method permits absolute numbers and measurements to be quantified. This staining permits many receptor-cell features (type, shape, and location) and the terminal arbors (as well as relationships between them) to be preserved and analyzed.

There are several limitations to this approach. In particular, some antibodies that have been used in thin sections[62] do not work in whole-mounts, which will limit the types of structures that can be examined. In addition, as confocal microscopy resolution is limited, the structural detail analyzed from individual cells, and from relationships between cells will also be limited[24]. For example, cells can be determined to be within 200 nm of each other, but specialized structures between cells (e.g., synapses)[63] cannot be examined. Lastly, not all cell types can be labeled using this approach. For example, it has proven to be difficult to specifically label cells that transduce salt in this preparation. These cells could be a subset of a combination of Type 1, Type II, and Type III cells[14-19,64]. Type I cells, which are primarily supporting cells, cannot be examined in whole-mounts because they appear to wrap around other cells, making them difficult to distinguish as separate entities[65]. Having a reliable marker for salt-transducing cells would allow for more comprehensive analyses[14,66]. Likewise, as PLCβ2 staining represents taste cells capable of transducing multiple types of stimuli, a label that permitted further separation of this cell type would also be an improvement.

The following are important preparatory steps that require care. First, ensure that the muscle layer that remains after dissection is even and as thin as possible. If this layer is not thin, antibody penetration will not be uniform. Second, it is crucial that the pieces of epithelium lay flat in the bottom of the tissue mold before freezing, and that blunt-ended forceps be used to lightly press on the tissue until it is frozen. When the minimal amount of muscle (in an even layer) remains on the underside of the epithelium, as few as three cryostat sections will reach the underside of the epithelium. Positioning of the tissue in a cryostat, so that sections are taken across the whole tissue face, sometimes results in portions of the tissue being removed unevenly. For these reasons, it is strongly recommended to avoid additional thawing, further dissection, and refreezing the tissue. Instead, care should be taken to evaluate tissue dissection before freezing the tissue.

Overall, the method for whole-mount tissue preparation presented here can be used for collecting whole taste buds as well as the surrounding papilla from three taste-bud regions: fungiform, circumvallate, and the palate. Although a variety of disease conditions[56,57] and chemotherapies[56] are known to disrupt taste function, the mechanisms underlying these changes

remain unknown. Using the whole-mount staining approach for taste buds presented here represents a robust experimental design where both taste-transducing cells and their nerve fibers could be labeled to determine whether a deficit is due to loss of a specific cell type, compromised terminal arbor morphologies, disrupted relationships between taste-transducing cells, or to disrupted relationships between transducing cells and their nerve fibers. Additionally, it would be possible not only to quantify the absolute number of labeled new cells in taste buds, but also to quantify the number of new taste-transducing cells (EdU-labeled) of a defined type (i.e., PLCβ2+ or Car4+). Whether these new cells develop normal shapes and incorporate normally into the taste bud (i.e., move into the taste bud following treatment) could also be examined. Many of these measures, along with taste bud number, can all be made from the same tissue, limiting the number of different animals needed for an experiment. These possibilities could facilitate the streamlining of experimental methods to provide clinical interventions for taste deficits, as well as provide insight into the normal mechanisms underlying taste function.

ACKNOWLEDGMENTS:

We thank Kavisca Kuruparanantha for her contributions to tissue staining and the imaging of circumvallate taste buds, Jennifer Xu for staining and imaging of innervation to the papilla, Kaytee Horn for animal care and genotyping, and Liqun Ma for her tissue staining of the soft-palate taste buds. This project was supported by R21 DC014857 and R01 DC007176 to R.F.K and F31 DC017660 to L.O.

DISCLOSURES:

The authors have nothing to disclose.

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881

Figure 1

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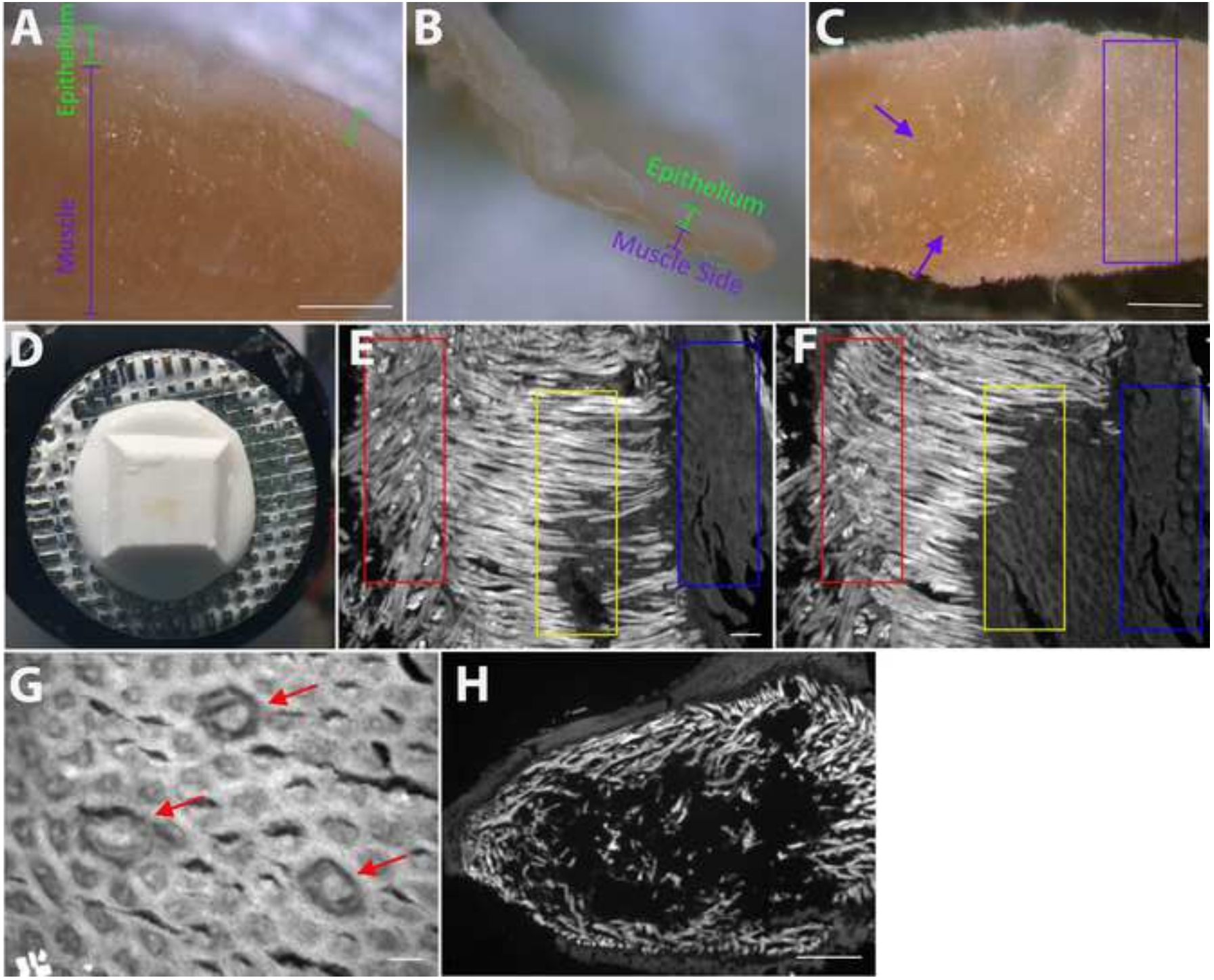
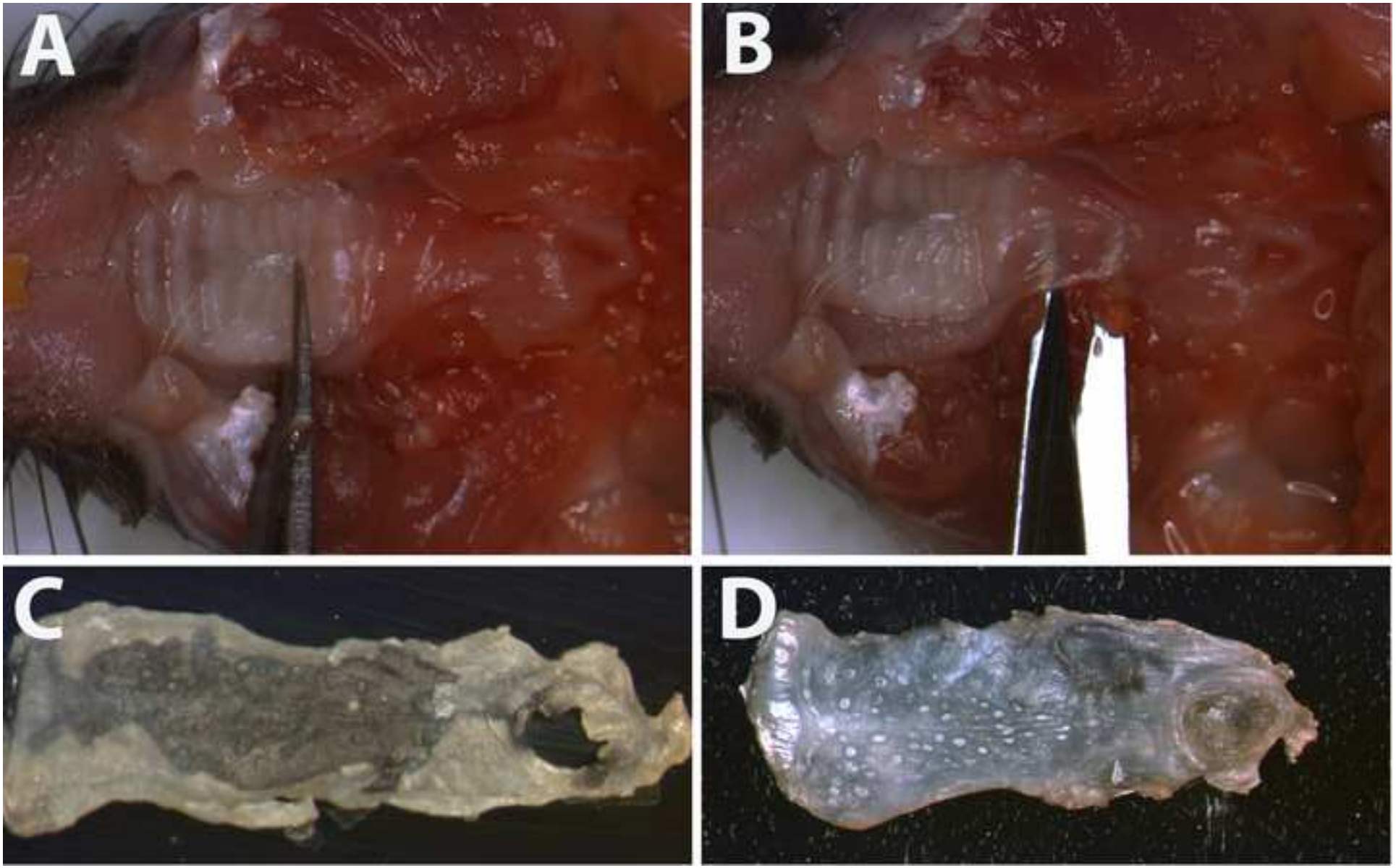


Figure 2



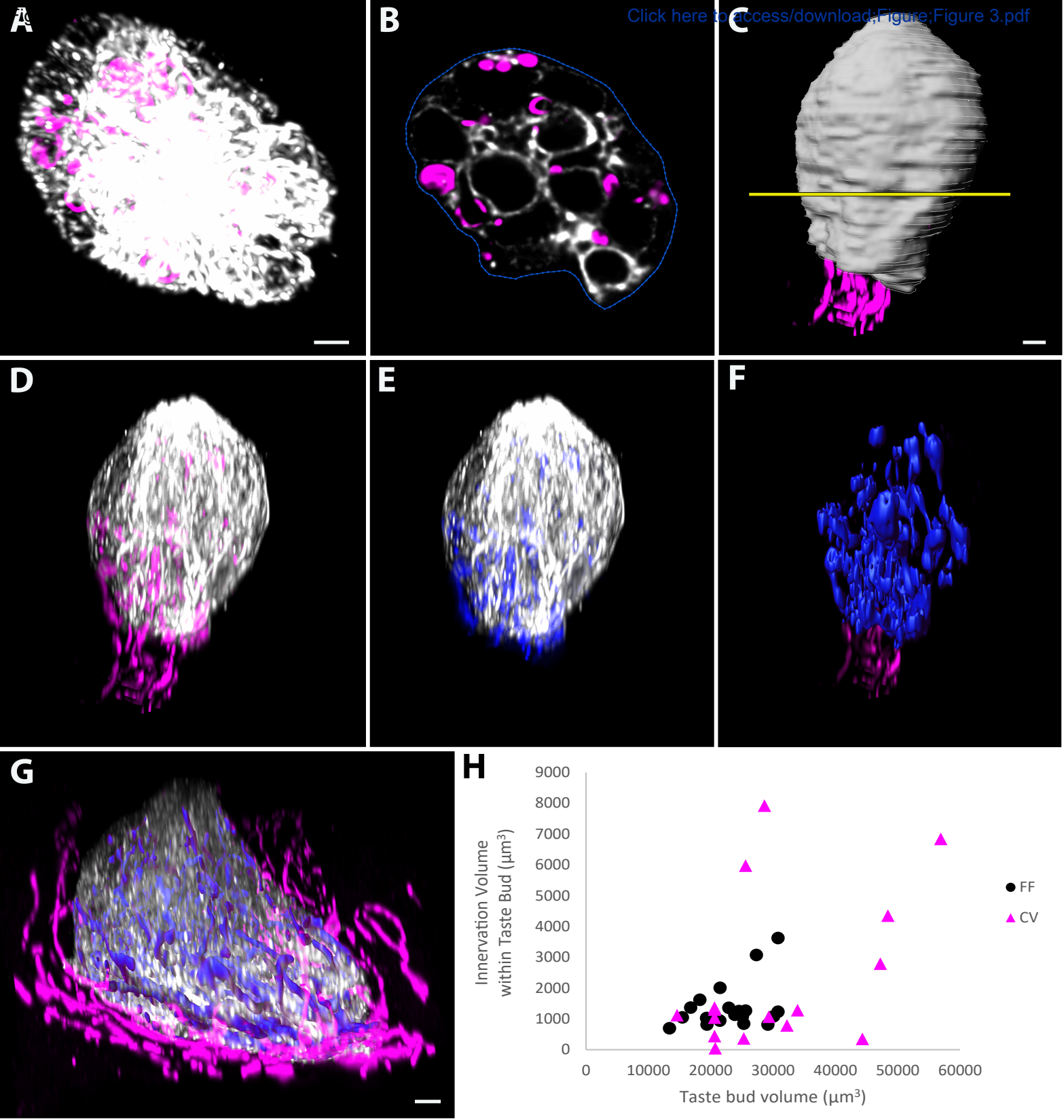


Figure 4

Click here to access/download;Figure;Figure 4 Revised 2.tif

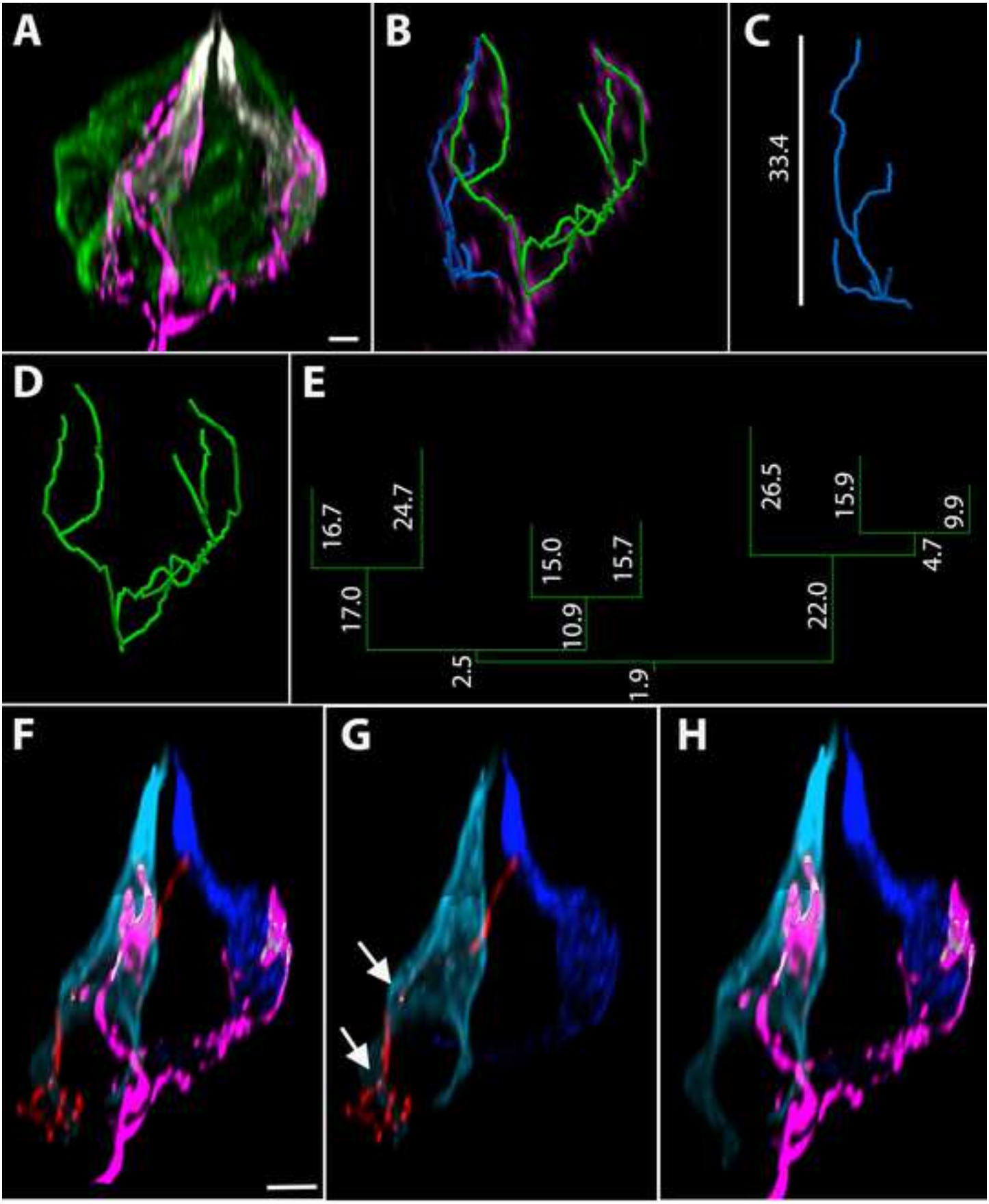


Figure 5

Click here to access/download;Figure;Figure 5 (1).tif

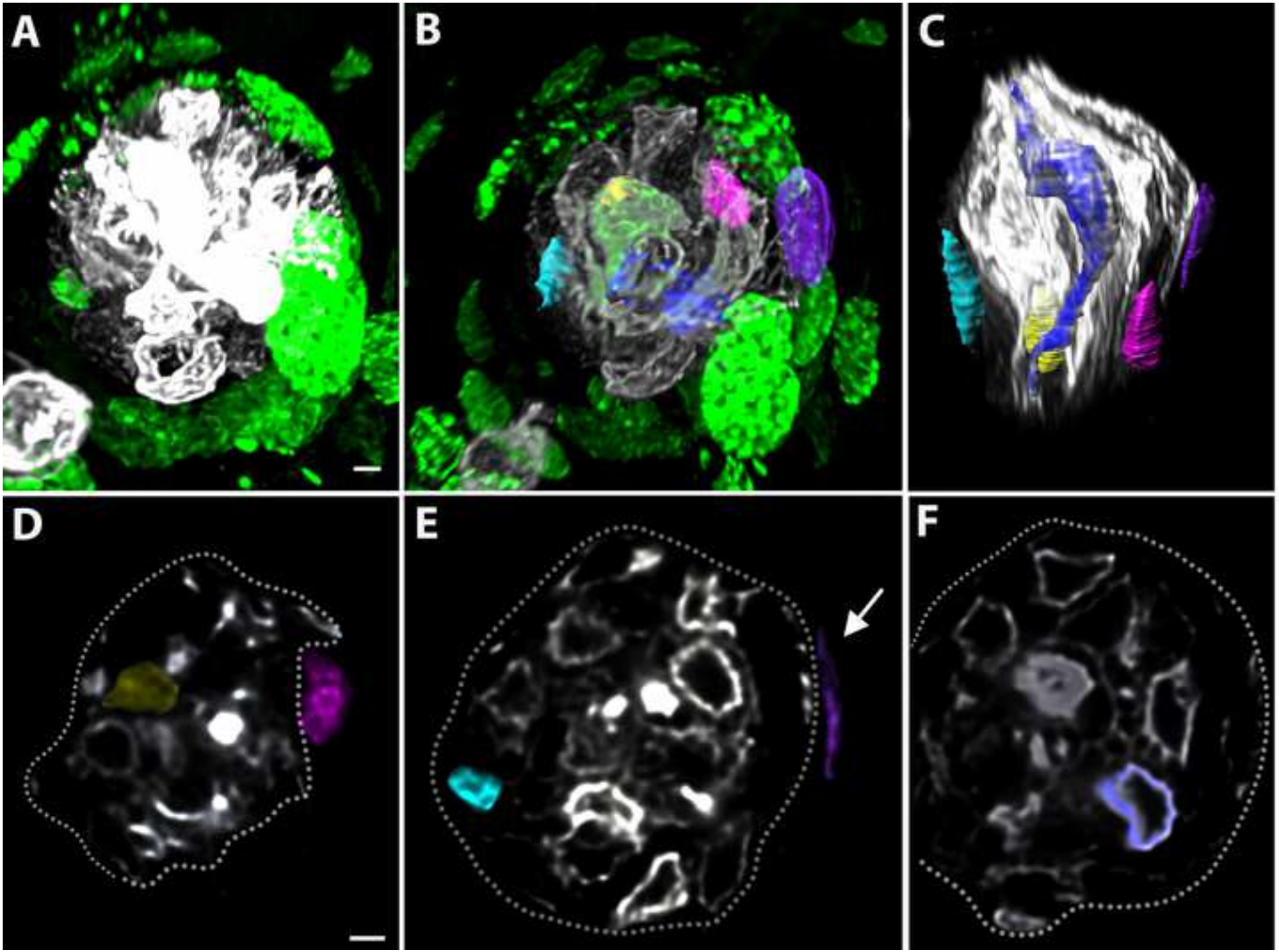


Figure 6

Click here to access/download;Figure;Figure 6 Revised 2.tif

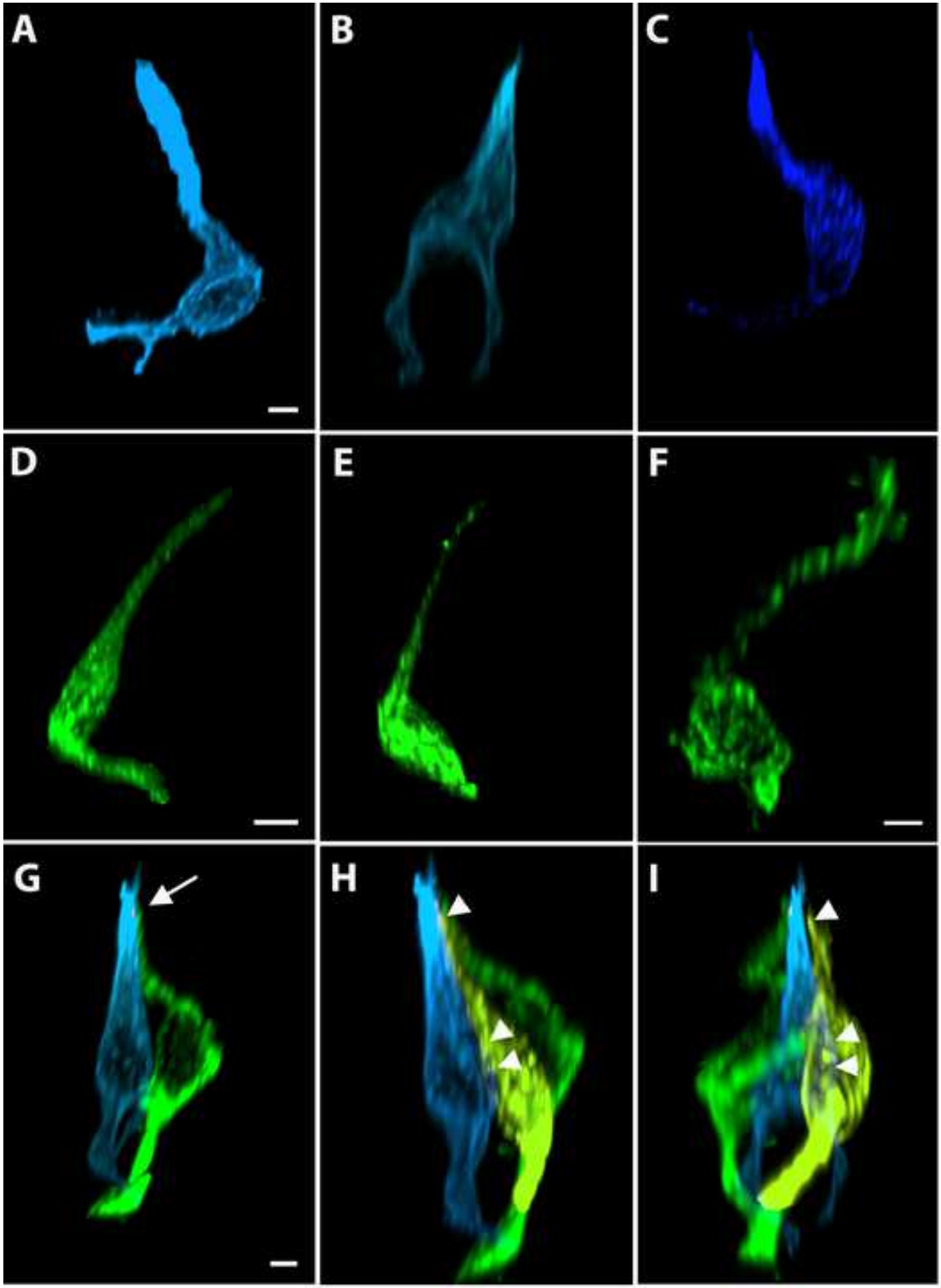
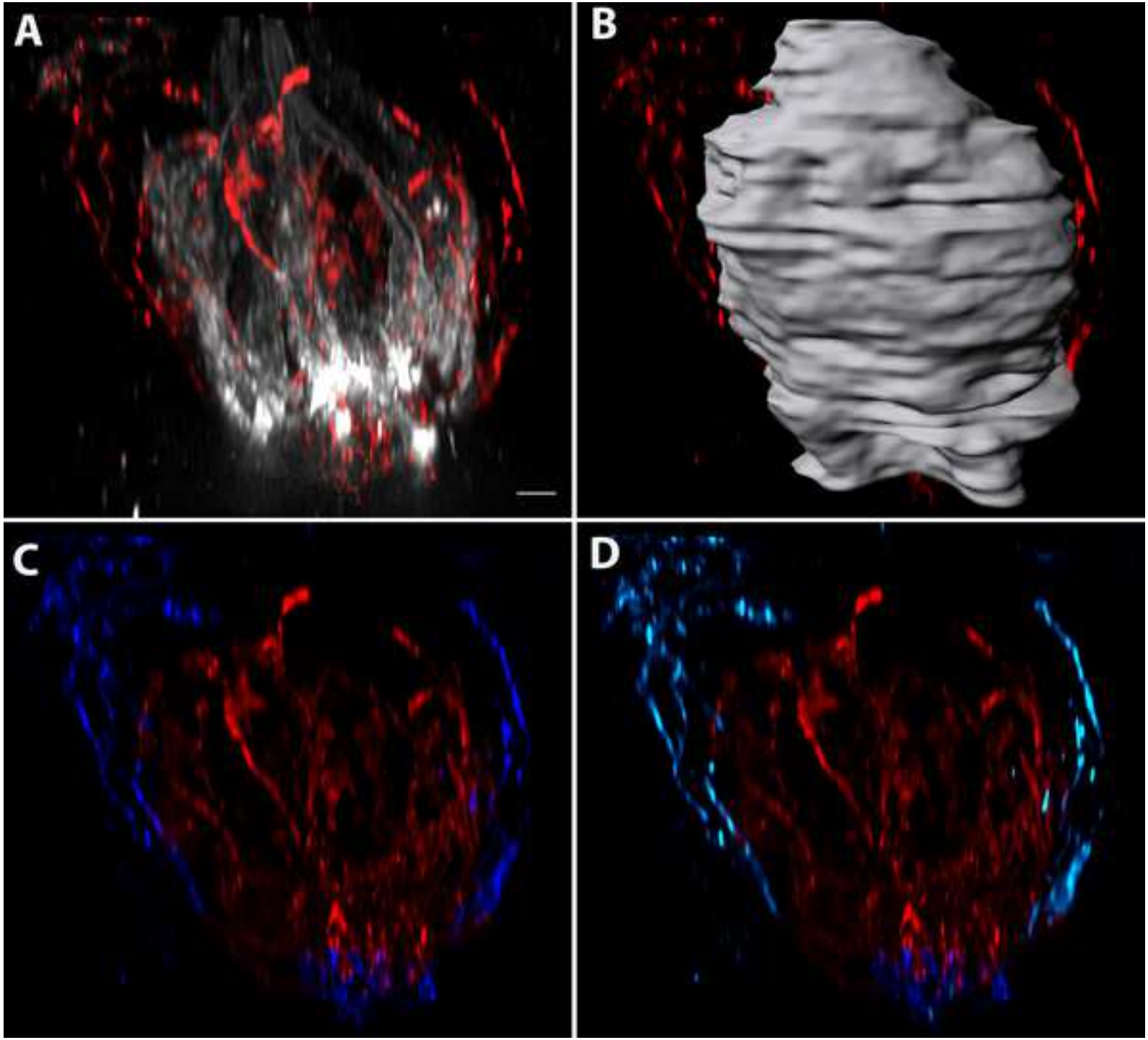


Figure 7



Supplies
2,2,2-Tribromoethanol
2-Methylbutane
5-ethynyl-2'-deoxyuridine (EdU)
Advillin^{CreER}
Ai65
AffiniPure Fab Fragment Donkey Anti-Rabbit IgG
Alexa Fluor 647 AffiniPure Donkey Anti-Rat IgG
AutoQuant X3 software
Blunt-ended Forceps
Click-iT Plus EdU Cell Proliferation Kit
Coverglass
Cytokeratin-8
Dissection Scissors (coarse)
Dissection Scissors (fine)
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488
Donkey anti-Rabbit, Alexa Fluor 555
DyLight 405 AffiniPure Fab Fragment Bovine Anti-Goat IgG
Fluoromount G
Glass slides
Goat anti-Car4
Imaris
Neurolucida 360 + Explorer
Normal Donkey Serum
Normal Rabbit Serum
Olympus FV1000
Paraformaldehyde
Phox2b-flpo
Rabbit anti-dsRed
Rabbit anti-PLCβ2
Sodium Phosphate Dibasic Anhydrous
Sodium Phosphate Monobasic
tert-Amyl alcohol
tdTomato reporter mice
Tissue Molds
Tissue-Tek O.C.T. Compound
Triton X-100
TrkBCreER mice (Ntrk2tm3.1(cre/ERT2)Ddg

Zenon Alexa Fluor 555 Rabbit IgG Labeling Kit

Company
ACROS Organics
ACROS
The Jackson Laboratory, Stock No: 032027
The Jackson Laboratory, Stock No: 021875
Jackson ImmunoResearch
Jackson Immuno Research
Media Cybernetics
Fine Science Tools
Molecular Probes
Marienfeld
Developmental Studies Hybridoma Bank (DSHB), (RRID: AB_531826)
Roboz
Moria
ThermoFisher Scientific
ThermoFisher Scientific
Jackson Immuno Research
Southern Biotech
Fisher Scientific (Superfrost Plus Miscroscope Slides)
R&D Systems
Bitplane
MBF Biosciences
Jackson Immuno Research
Equitech-Bio, Inc
EMD
The Jackson Laboratory, Stock No: 022407
ıing Colors DsRed Polyclonal Antibody; Clontech Clontech Laboratories, Inc. (63249
Santa Cruz Biotechnology
Fisher Scientific
Fisher Scientific
Aldrich Chemical Company
Ai14, The Jackson Laboratory, Stock No: 007914
Electron Microscopy Sciences
Sakura
BIO-RAD
https://www.jax.org/ ISMR Cat# JAX:027214, RRID:IMSR_JAX:027214

ThermoFisher Scientific

Catalog #	Comment
AC421430100	
126470025	
711-007-003	15.5 μL/mL
712-605-150	(1:500)
FST 91100-12	
C10637	Follow kit instructions
107242	
Troma1 supernatant	(1:50, store at 4 °C)
RS-5619	
MC19B	
A21206	(1:500)
A31572	(1:500)
805-477-008	(1:500)
0100-01	
12-550-15	
AF2414	(1:500)
pixel-based image analysis software	
3D vector-based image analysis software	
017-000-121	
SR30	
(multi-Argon laser with wavelengths 458, 488, 515 and additional HeNe lasers emitting 543 and 633)	
PX0055-3	4% in 0.1 M PB
632496	(1:500)
Cat# sc-206	(1:500)
BP332-500	
BP330-500	
8.06193	
70180	
4583	
#161-0407	non-ionic surfactant for immunohistochemistry

Z25305	Follow kit instructions

Dear Sir,

We are resubmitting our revised manuscript entitled “Whole Mount Staining, Visualization, and Analysis of Fungiform, Circumvallate, and Palate Taste Buds.” for publication in *JoVE.*

We would like to thank the reviewers for their valuable comments and feel the manuscript has been improved by addressing their concerns/questions. We added images of the tissue dissection process and checkpoints to Figure 1 to address the Reviewer’s question about troubleshooting. We also added Figure 7 to illustrate visualization and quantification of innervation to the papilla. We also submitted the manuscript for professional grammar editing. Due to the extensive suggestions by the grammar editor, it was not possible to track all of those changes. We did track all significant content changes in response to the reviewers’ comments. We respond to each comment below. Below reviewer comments are in plain text and our responses are in ***bold italics***.

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. Please define all abbreviations at first use.

We revised and submitted the manuscript to a professional grammar editor for review.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Imaris; Neurolucida 360; Avertin; FST 91100-12; RS-5619 (Roboz); MCB19B (Moria); Zenon; AutoQuant X3 (Media Cybernetics, Maryland);

Changes made as suggested.

3. Please revise the text to avoid the use of any personal pronouns (e.g., “I” (line 95), "we" (several places), "you", "our" etc.).

Changes made as suggested.

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

Changes made as suggested.

5. Please include a section "Figure and Table Legends" to follow the representative results section (before the discussion section) and include the figure legends in this legends section.

Change made as suggested.

6. As we are a methods journal, please revise the Discussion to also include with citations:
a) Any limitations of the technique
b) The significance with respect to existing methods

Limitation of the technique and elaboration of the significance of this method were added to the discussion section.

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

Change made as suggested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this article, the authors reported their developed methods for collecting, imaging, and analyzing whole
taste buds and individual taste nerve arbors from three taste regions: fungiform papillae, circumvallate papillae, and palate. The manuscript was well justified and written. The advantages of this method over alternative methods with references to relevant studies were clear. The descriptions for procedures were appropriate and in detail.

Minor Concerns:
1) A better explanation and clarity of the procedures about immunohistochemistry is recommended. For examples, EdU and Krt8 labeling was mentioned in the results but not mentioned in the methods. The authors performed immunostaining using primary antibodies derived from the same species of animals, which raises a question of what should be conducted differently when using primary antibodies from different species. In line 242, was the secondary antibody fluorophore-conjugated?

This information was added.

2) It would help readers better if discussions about the troubleshooting and limitations of the method were elaborated.

Figure 1 was expanded to include additional images of the tissue dissection process, checkpoints, and discussion of troubleshooting was expanded in the Discussion section.

3) The use of values and units need to be consistent.

We identified some inconsistencies with our number of significant figures which we have resolved. We are uncertain to which other specific issues the reviewer is referring but we have reviewed the manuscript with this suggestion in mind and hope that we have sufficiently addressed this concern.

4) Very minor issues:
Page 3, line 37: was "to for" meant to be "to"?

Change made as suggested.

Page 4, line 95: was "I" meant to be "we"?

All personal pronouns have been removed as required by the journal.

Reviewer #2:
Manuscript Summary:
This is a thorough description of a procedure that will be of use to the community of scientists studying oral chemoreception and oral irritation. While the MS is reasonably focused on taste buds, it might also be useful for visualizing free nerve endings in the oral epithelium and the authors may wish to add comments or even photos documenting this.

We appreciate this suggestion and have added Figure 7 to illustrate how this method facilitates 3D visualization and analysis of free nerve endings to in the gustatory papilla. We also included suggestions in the Discussion of quantifications and analyses that are possible with this approach.

Major Concerns:
I did not find a table of specification of materials and equipment. Of most importance is sources and RRID numbers for antibodies, Type of confocal microscope utilized including information on light source and wavelengths. Kind of laser –

Information was added.

Minor Concerns:

Introduction: In the description of cell types, the authors omit mention of salty which probably includes some Type II cells and some Type III cells.

Salt cells were added to the introduction and to the limitation section of the discussion.

123 1.1.1 PB: 0.2 M Sodium Phosphate Buffer
124 1.1.1. Dissolve Sodium Phosphate Monobasic and Sodium
125 Phosphate Dibasic in ddH2O on a stir plate. Adjust pH to 7.4.

Give amount of each phosphate salt in g/L to prepare this buffer.

Information was added.

132 Add equal volume of 0.2M PB to bring to 4% PFA. Bring pH to 7.4.

Note that the final solution is 4% PFA in o.1M buffer

Change made as suggested.

149 2.4.1. Thaw and rinse the tongue in 0.1M PB. Place.

Why put the tissue in oct if you are just going to thaw it and rinse it in buffer?

The tissue can be frozen if you need a stopping point in the procedure. However, it is not necessary. This has been clarified in the protocol.

169 2.4.5. Use the blunt ended forceps to lay a piece of epithelium into a tissue mold and
170 ensure it lays flat.

State Which side is up? Presume muscle side down to permit cutting removal?

Change made as suggested.

180 2.4.8. Once the OCT has frozen, quickly add additional OCT and place mold in beaker of
181 Benzyl Alcohol in a container of dry ice until frozen.

Maybe warn people to chill benzyl alcohol. - before step 2.4.1??

Change made as suggested.

185 2.4.9.1. Additional sectioning on the cryostat are used for fine.

"are" should be "is"

Change made as suggested.

Fig. 1 Add labels for muscle, epithelium, etc. - It would be helpful to show aa series of sections from too much muscle to just right to too far. And then use arrows to show where you see the bases of taste buds in the too far section.

We expanded Figure 1 to include images of the dissection process before shaving off muscle on the cryostat. We also added additional dark blue rectangles to Figure 1 E-F to identify the underside of the epithelium. Descriptions of what can be seen within the red, yellow, and blue rectangles in E-F is described in the figure legend. The description of Figure 1G was altered to point out that this is an example of when you have cut too far. Additional description of how to determine when you have cut just enough without going too far has also been added.

193 2.4.9.3. Once the tissue was sectioned it was thawed…

maybe use the phrase shaved off instead of sectioned. Sectioned implies that the tissue is cut through which is not what you are doing here.

Change made as suggested.

198 2.5.1. Using a coronal cut with a razorblade, separate the circumvallate papilla from
199 the anterior tongue. Use two sagittal cuts with.

Strictly speaking these cuts are parasagittal (parallel to the midline), not sagittal (on the midline)

Change made as suggested.

202 bottom of the tissue mold. Covered the tissue with OCT

…. is there a freezing step with benzyl alcohol? Or dry ice? How is this frozen?

Taste buds on the palate. Are they frozen first? Step 1: thaw?

Information was added.

226 3.4. Wash with 0.1 M PB, 4x15 mins each and incubate in secondary Donkey anti-Rabbit
488 antibody (1:500) for 2 days at 4* C.

Is there no triton in 2* ab solution or in any solutions thereafter?

Change made as suggested.

239 3.8. Wash with 0.1 M PB, 4x15 mins each and then incubate in primary antibody: goat anti-Car4 (1:500) for 5.

No triton?

Change made as suggested.

4.1. Confocal images were captured with a 60x objective (Numerical Aperture= 1.40),
251 4ms/pixel, zoom of 3, Kalman of 2, and size of 1024x1024. The step size along the z axis
252 was selected as 0.47μm.

Specify the microscope model? Laser intensity? etc.

Information was added.

471 terminal arbor was 644.02 μm3 and 3647.02 μm3.

Isn't this too many Significant digits?

Change made as suggested.

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Title of new article	Whole Mount Staining, Visualization, and Analysis of Fungiform, Circumvallate, and Palate Taste Buds.
Lead author	Lisa C Ohman
Title of targeted journal	JoVE
Publisher	MyJoVE Corporation
Expected publication date	Oct 2020
Portions	The data in Figure 9g is being combined with similar data from another taste region to be plotted for comparison.
Requestor Location	University of Louisville 511 South Floyd Street Room 111 LOUISVILLE, KY 40202 United States Attn: University of Louisville
Publisher Tax ID	EU826007151

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