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METHODS ARTICLE

Taste Bud Labeling in Whole Tongue Epithelial Sheet in Adult Mice

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Molecular labeling in whole-mount tissues provides an efficient way to obtain general information about the formation, maintenance, degeneration, and regeneration of many organs and tissues. However, labeling of lingual taste buds in whole tongue tissues in adult mice has been problematic because of the strong permeability barrier of the tongue epithelium. In this study, we present a simple method for labeling taste buds in the intact tongue epithelial sheet of an adult mouse. Following intralingual protease injection and incubation, immediate fixation of the tongue on mandible in 4% paraformaldehyde enabled the *in situ* shape of the tongue epithelium to be well maintained after peeling. The peeled epithelium was accessible to taste bud labeling with a pan-taste cell marker, keratin 8, and a type II taste cell marker, α-gustducin, in all three types of taste papillae, that is, fungiform, foliate, and circumvallate. Overnight incubation of tongue epithelial sheets with primary and secondary antibodies was sufficient for intense labeling of taste buds with both fluorescent and DAB visualizations. Labeled individual taste buds were easy to identify and quantify. This protocol provides an efficient way for phenotypic analyses of taste buds, especially regarding distribution pattern and number.

Introduction

Taste buds, each of which consists of about 80 specialized epithelial cells, are sensory organs for taste that transduce gustatory stimuli into neural signals conveyed to the central nervous system.[1,2] In mammals, taste buds are primarily located in the tongue. The lingual taste buds reside in taste papillae, that is, fungiform, foliate, and circumvallate, each with a unique topographic distribution pattern on the tongue. Additional features of taste buds, for example, short life span and continuous turnover,[3] nerve dependence for the maintenance, enable the system to serve as an ideal model for studying pattern formation during development, degeneration and regeneration, tissue engineering for nerve repair, and drug screening in regenerative medicine.

The mouse genome is well characterized and amendable to manipulations. Mice with designed genetic backgrounds provide opportunity to characterize gene functions in the development and maintenance of taste organs. Structural and molecular analyses, for example, scanning electron microscopy (SEM), *in situ* hybridization, and immunohistochemistry in whole-mount mouse tissues, provide an efficient way for phenotypical evaluation of many organs. However, due to the strong permeability barrier of the epithelium in late embryos and postnatal animals,[4–8] molecular labeling in whole tongue tissues has been limited to embryonic tissues when the tongue epithelium is more permeable.[4]

In mice, early lingual taste buds are not histologically observed until birth, and taste bud development is essentially a postnatal process[1,4,9] when the strong permeability barrier has already developed.[4–8] The penetration of antibodies and molecules becomes poor in postnatal tongues.[4–8] To evaluate the maintenance, degeneration, and regeneration of mature taste buds, conventional sectioning procedures have been adopted, which are very useful for detailed analyses at the cellular level, but less efficient at obtaining overall information about the distribution pattern and number of taste buds.

To evaluate the phenotypic alterations of mature taste buds in an efficient manner, labeling with pan- and/or type-specific markers of taste bud cells in whole tongue tissues needs to be established. In the present study, we report a simple protocol to label lingual taste buds in an intact tongue epithelial sheet that has the *in situ* shape. This is a useful method for a quick evaluation of the distribution pattern and quantification of taste buds in adult mice.

Materials and Methods

Animals

Adult (2–6 months) wild-type C57BL/6 male and female mice were used throughout the study. Animal use was in

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compliance with the institutional animal care protocols (The University of Georgia Institutional Animal Care and Use Committee, AUP No. A2013 07-013) and the National Institutes of Health Guidelines for care and use of animals in research.

Preparation of whole tongue epithelial sheet

The procedure for preparing tongue epithelial sheets is summarized and illustrated in Fig. 1. Mice were euthanized with CO_2, and the tongue with mandible was dissected (Fig. 1A). A mixture of collagenase A (1 mg/mL, Cat No. 10103578001; Roche Diagnostics) and dispase II (2.5 mg/mL; Cat No. 10374300; Roche Diagnostics) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) (Cat No. CP4390-48; Denville Scientific, Inc.) was injected into the tongue from the posterior cutting end of the tongue using a 30G needle. The tip of the needle was placed in the subepithelial space during the injection to enable the enzymes to efficiently separate the epithelium from the underlying connective tissue. Approximately 1.5 mL of the enzyme solution was injected into the entire tongue. After the injection, the tongue and mandible were incubated at 37°C for 30 min with the tongue surface wetted by the same enzyme mixture, and then immediately fixed in 4% paraformaldehyde (PFA) (Cat No. 15710; Electron Microscopy Sciences) in 0.1 M PBS for 1 h at room temperature. The intact whole tongue epithelial sheet was peeled off from the lamina propria using forceps in 0.1 M PBS and rinsed thrice in 0.1 M PBS for 30 min each (30 min ×3) at room temperature under constant shaking. For DAB reactions, the PFA-fixed tongue and mandible were rinsed with 0.1 M PBS and bleached with 6% hydrogen peroxide (Cat No. 216763; Sigma Aldrich) in methanol at room temperature for 1 h under constant shaking before peeling the tongue epithelial sheet. The peeled tongue epithelial sheet was then rinsed in 0.1 M PBS and further processed for immunoreactions with DAB or fluorescence visualizations, as described below.

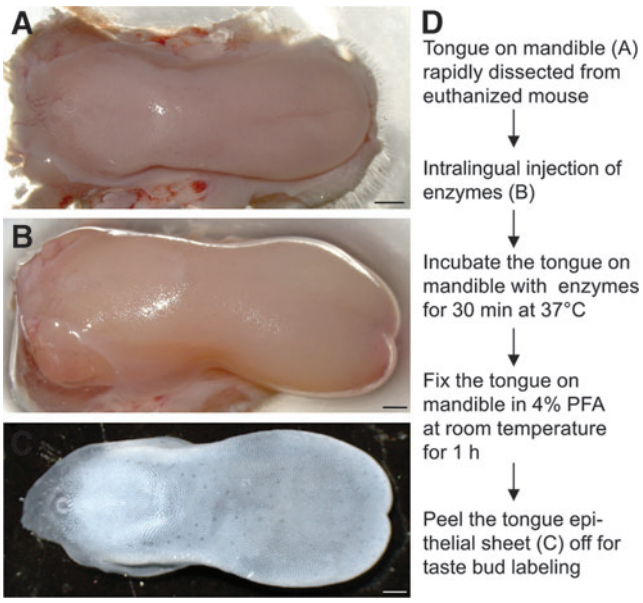

FIG. 1. Illustration of the procedures for tongue epithelial sheet preparation. **(A)** Tongue on mandible dissected from the mice; **(B)** Tongue on mandible after the injection of collagenase A and dispase II; **(C)** Peeled epithelium sheet; **(D)** Flow of the major steps. Scale bars: 1 mm. Color images available online at www.liebertpub.com/tec

Taste bud labeling in tongue epithelial sheets

The whole epithelium was incubated with 10% normal donkey serum (NDS) (Cat No. S30—100 mL; Millipore) in 0.1 M PBS containing 0.3% Triton-X100 (Cat No. X100—100 mL; Sigma) (PBS-X) for 1 h at room temperature to block nonspecific staining. Epithelial sheets were then incubated overnight at 4°C with primary antibody against keratin 8 (Krt8) (1:500, TROMA-I; Developmental Studies Hybrydoma Bank) or α-gustducin (1:250, sc-395; Santa Cruz Biotechnology) in 1% NDS in PBS-X. An epithelial sheet processed without the addition of Krt8 primary antibody was used as a negative control. The epithelium was then washed for 30 min ×3 with 0.1 M PBS on a shaker.

Fluorescence labeling. The epithelium was incubated with Alexa Fluor 488-conjugated donkey anti-rat secondary antibody (1:500, Cat No. A-21208; Life Technologies) or Alexa Fluor 647-conjugated donkey anti-rabbit secondary antibody (1:500, Cat No. A-31573; Life Technologies) in 1% NDS in PBS-X overnight at 4°C. The epithelium was rinsed for 30 min ×3 with 0.1 M PBS and photographed under an SZX2-ILLT Olympus stereomicroscope.

Diaminobenzamide reaction. The rinsed epithelial sheet was incubated with biotin-conjugated goat anti-rat secondary antibody (1:250, Cat No. BA-9401; Vector Laboratories, Inc.) in 1% NDS in PBS-X overnight at 4°C. Following PBS rinses for 30 min ×3 at 4°C on a shaker, the epithelium was further incubated with the ABC reagents (Cat No. PK-6200; Vector Laboratories, Inc.) in PBS-X overnight at 4°C. The rinsed epithelium (PBS for 30 min ×3) was preincubated with nickel-intensified DAB reaction solution (Cat No. SK-4100; Vector Laboratories, Inc.) without H_2O_2 at room temperature for 30 min, followed by visualization with DAB reaction solution with 0.0003% H_2O_2 in 0.2% bovine serum albumin in PBS-X until the signal development was satisfactory. The epithelium was rinsed in 0.1 M PBS and photographed under an SZX2-ILLT Olympus stereomicroscope.

Photography and data analysis

Taste bud-labeled tongue epithelial sheets were transferred to 0.1 M PBS in a 100-mm culture dish precoated with 3% agarose gel at the bottom. The epithelial sheets were immobilized by pinning the edges onto the gel using fine pins (Cat No. 10131-05; Fine Scientific Tools) and photographed under an SZX2-ILLT Olympus stereomicroscope. CellSens Dimension software was used for analysis.

To further observe labeled taste buds at the cellular level, laser scanning confocal microscopy was conducted. The tongue epithelial sheets (sock shaped) with fluorescence labeling were cut into three pieces (ventral, anterior 2/3, and posterior 1/3 of dorsal tongue epithelium), spread onto a glass slide, and cover-slipped with ProLong® Gold antifade mounting medium (Cat No. P36934; Life Technologies). Z-stack confocal images of taste buds in fungiform, foliate,

and circumvallate papillae were taken under an LSM710 laser confocal microscope using ZEN 2012 software.

Quantification of taste buds

The tongue epithelial sheets were cut into three pieces, that is, the anterior tongue region with all the fungiform taste buds, two foliate, and the single circumvallate papilla regions. The anterior tongue epithelium was spread onto a glass slide and cover-slipped. The ducts of von Ebner's gland attached to the foliate and circumvallate papillae were removed, and the trenches of the papilla folds were cut, separated, and spread to visualize all the labeled taste buds. Images of fungiform, foliate, and circumvallate papilla regions were taken, and the number of Krt8-positive taste buds was counted for each region of the oral tongue. The data are presented as mean ± SD ($n = 5$).

Results

Fixation before peeling maintained the in situ *shape of the tongue epithelial sheet*

Without PFA fixation, intact tongue epithelial sheets were easy to peel off after intralingual injection and incubation with the mixture of collagenase A and dispase II. However, the separated epithelial sheets were soft and sticky, and the shape could not be maintained. To solve this issue, we fixed the tongue on mandible with 4% PFA at room temperature for 1 h following the protease incubation. Peeling of the intact tongue epithelial sheet was easy after the fixation. The whole tongue epithelial sheet was intact, and the *in situ* shape was perfectly maintained during and after the immunohistochemistry processes (Figs. 1C; 2A$_1$, B$_1$, C$_1$; 3A).

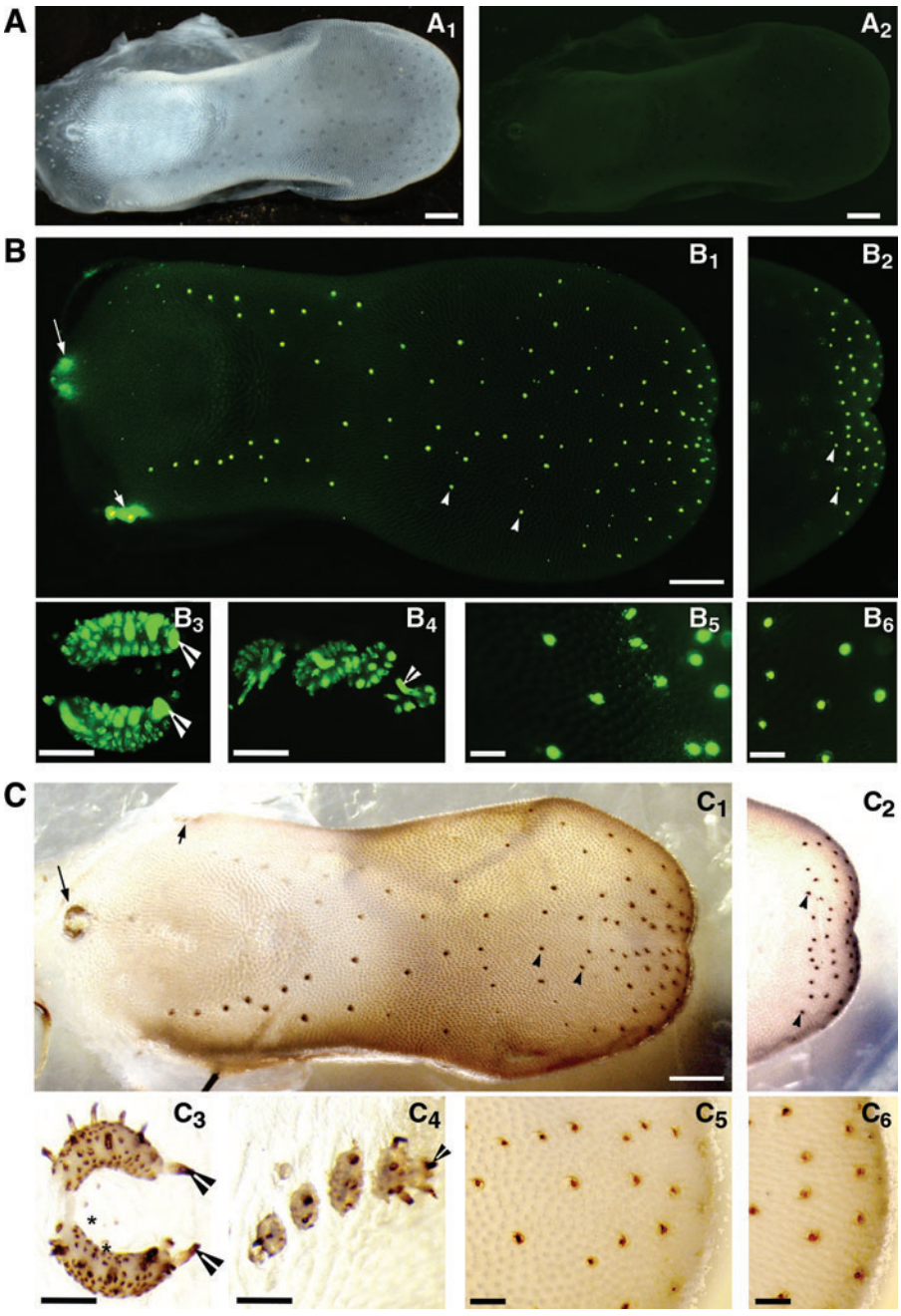

FIG. 2. **(A)** Negative control without addition of primary antibody against Krt8. No labeling was found in the taste buds (A$_1$, bright field; A$_2$, fluorescent image). **(B, C)** Taste bud labeling with a pan-taste cell marker, keratin 8, in the whole tongue epithelium sheet, with fluorescence **(B)** and DAB **(C)** visualizations. B$_1$ and C$_1$: dorsal view of the entire tongue epithelium. B$_2$ and C$_2$: ventral view of the anterior tongue epithelium showing fungiform taste bud labeling (*arrowheads*). The *in situ* shape of the tongue epithelium was well maintained. Krt8-labeled taste buds were observed in fungiform (*arrowheads*), foliate (*short arrows*), and circumvallate (*long arrows*) papillae. At a higher magnification, individual taste buds were easy to identify in circumvallate (B$_3$, C$_3$), foliate (B$_4$, C$_4$), dorsal side fungiform (B$_5$, C$_5$), and ventral side fungiform (B$_6$, C$_6$) papillae. *Asterisks* are placed beside the taste buds in the apical region of circumvallate papilla (B$_3$, C$_3$). *Open arrowheads* in B$_{3\text{-}4}$, C$_{3–4}$ point to von Ebner's glands that were also labeled with Krt8 in addition to taste buds. Scale bars: 1 mm in A$_{1,2}$, B$_{1,2}$, and C$_{1,2}$, 200 μm in B$_{3–6}$, C$_{3–6}$. Color images available online at www.liebertpub.com/tec

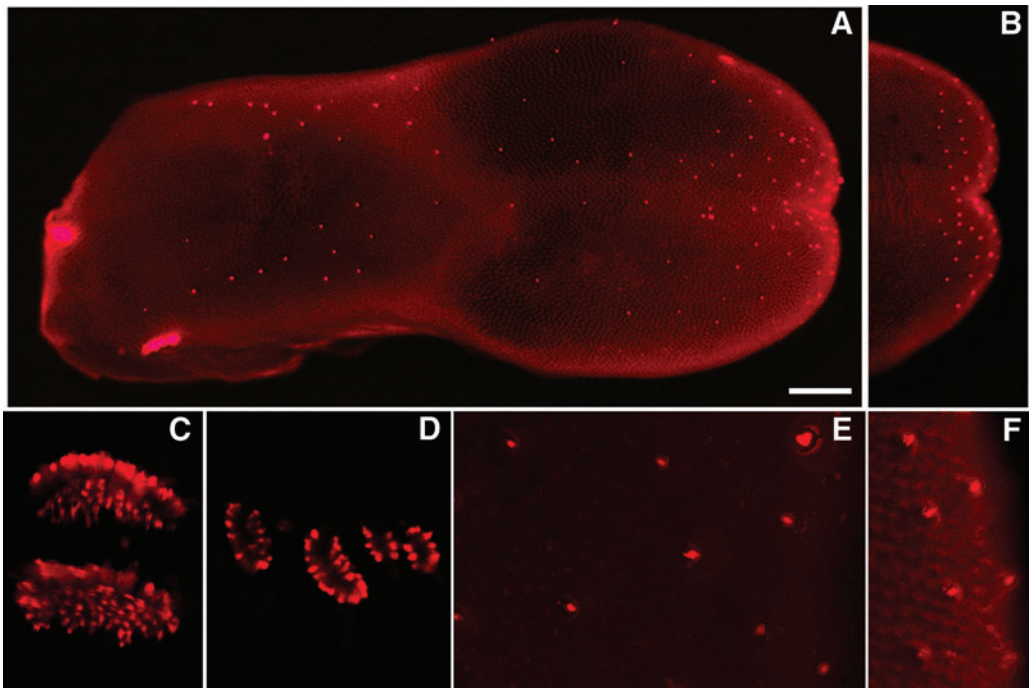

FIG. 3. Type II taste bud cell labeling with α-gustducin in the whole tongue epithelium. **(A)** Dorsal view of the whole tongue epithelium with taste buds in fungiform, foliate, and circumvallate papillae. **(B)** Ventral view of the anterior tongue epithelium with fungiform taste bud labeling. **(C–F)** Higher magnification of the taste papillae showing individual α-gustducin-labeled taste buds in circumvallate **(C)**, foliate **(D)**, and fungiform papillae **(E)**, and also the ventral view of the anterior tongue epithelium **(F)**. Scale bar: 1 mm in **(A)** also applies to **(B)**, and 250 μm in **(C)** also applies to **(D–F)**. Color images available online at www.liebertpub.com/tec

Taste buds were reliably labeled in an adult mouse tongue epithelial sheet

Importantly, without the addition of the primary antibody against Krt8, no signals were observed in the entire epithelial sheet (Fig. $2A_2$). With the monoclonal antibody against Krt8, taste buds were reliably and reproducibly labeled in intact tongue epithelial sheets (Fig. 2B, C). $Krt8^+$ taste buds were observed within all three types of taste papillae, that is, fungiform (arrowheads), foliate (short arrows), and circumvallate (long arrows).

Each fungiform papilla on the anterior 2/3 of the oral tongue contained a single taste bud. Occasionally, fungiform papillae without a $Krt8^+$ taste bud were observed. Fungiform taste buds distributed in the posterior region proximate to the foliate papillae were also seen. Multiple taste buds were labeled in foliate and circumvallate papillae, and each individual taste bud was easily identifiable (Fig. $2B_3$, B_4, C_3, C_4). The circumvallate taste buds were mostly located in the trenches of the papilla wall with a few in the apical surface of the papilla (Fig. $2B_3$, C_3, asterisks). $Krt8^+$ signals were also seen in the ducts of von Ebner's glands adjacent to the foliate and circumvallate papillae (Fig. $2B_3$, B_4, C_3, C_4, open arrowheads). The numbers of fungiform, foliate, and circumvallate taste buds are listed in Table 1.

Table 1. Number and Distribution of $Krt8^+$-Labeled Taste Bud Cells in the Oral Tongue

	Dorsal	*Ventral*
Fungiform	83 ± 10	42 ± 6
	Right	*Left*
Foliate	116 ± 24	110 ± 44
	Apex	*Trenches*
Circumvallate	8 ± 2	192 ± 35

Type II taste cell marker, α-gustducin, was used to test whether specific taste cell types may be labeled in the whole tongue epithelial sheet (Fig. 3). Taste buds were labeled distinctly with α-gustducin in all three types of papillae (Fig. 3A–F).

Further observations of keratin 8-labeled taste buds at the cellular level

Z-stack images of fungiform, foliate, and circumvallate taste buds were taken from the tongue epithelium with Krt8 immunofluorescence labeling under a laser scanning confocal microscope (Fig. 4). Individual $Krt8^+$ taste buds were easy to identify from the apical to basal regions. The signals were more intense in the basal half of the taste bud cells (Fig. 4).

Discussion

In the present study, we report a simple protocol to label mature taste buds in the whole tongue epithelium in adult mice while maintaining the *in situ* shape of the epithelial

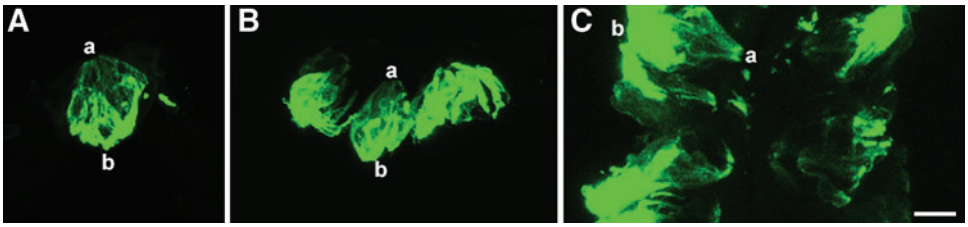

FIG. 4. Laser scanning confocal photomicrographs of individual taste buds in fungiform **(A)**, foliate **(B)**, and circumvallate **(C)** papillae. Keratin 8-positive (*green*) taste bud cells were more intensely labeled in the basal regions (b) than the apical (a) areas. Scale bar: 20 μm in C applies to all images. Color images available online at www.liebertpub.com/tec

sheet. This protocol provides a valuable tool and an efficient way for phenotypical analysis of lingual taste buds, for example, distribution pattern, number, and size. The method may be applicable to labeling taste buds with other molecular markers, molecular labeling in other specialized epithelial appendages, and molecular labeling in other species that include humans.

Scanning electron microscopy is a useful tool to evaluate the structure and pattern formation of lingual papillae, including the taste papillae. However, under certain conditions, taste papillae do not reliably contain taste buds[10–12]; thus, phenotypical alterations of taste papillae cannot be applied to the phenotype of taste buds. Tissue sections, followed by taste bud cell labeling, are very useful for detailed analysis, but it is time-consuming to obtain general information for overall changes of taste buds, for example, distribution pattern and number. In this study, we report that peeling of the epithelial sheet, following incubation of the tongue with intralingually injected proteases, and immediate 4% PFA fixation of the tongue and mandible enable us to maintain the *in situ* shape of the tongue epithelium and to reliably label the taste buds in all three types of taste papillae.

The protocol is simple and efficient. Overnight incubation with primary and secondary antibodies was sufficient for obtaining intense signals of Krt8-positive taste buds with both DAB and fluorescence visualizations. Information about number, size, and distribution pattern of taste buds is easy to collect and analyze from the immunoreacted tongue epithelial sheets. Thus, this technique provides a unique way to efficiently evaluate the mature taste bud phenotype in postnatal animals under light microscopy in addition to other techniques such as SEM and tissue sectioning. Moreover, laser scanning confocal imaging of the immunoreacted epithelium allows us to examine taste buds at the cellular level.

Recently, Dando *et al.* (2015) demonstrated the existence of a highly impermeable barrier surrounding taste buds.[8] The penetration of small dye molecules is limited by both the apical tight junctions and the strong barrier around the taste bud cells, which can be compromised by 75% dimethyl sulfoxide (DMSO).[8] We tested the incubation of adult mouse tongues with 75% DMSO for different time periods (1–30 min), followed by immunoreactions, and found that the tongues were dramatically misshapen and the penetration of antibodies was not sufficient to generate detectable signals after overnight incubation (data not shown).

As reported by Dando *et al.* (2015), the taste bud barrier was partially disrupted in a peeled lingual epithelium with subepithelial injection of an enzyme cocktail of collagenase and dispase. Redigestion of the peeled epithelium with proteases could make the taste buds more accessible to small molecules of fluorescent dyes. In the present study, we found that digestion with a single dose of collagenase and dispase was enough to compromise the permeability barrier of the peeled epithelium for antibody penetration. Our observation that the basal half of the taste bud cells is more intensely labeled supports the idea that protease-induced disruption of the epithelial permeability barrier predominantly occurs in the basal region of the taste bud cells.

Taken together, we report that taste bud labeling in the intact tongue epithelial sheet, with a well-maintained *in situ* shape, is an efficient method for phenotypical analysis of mature taste buds in adult mice.

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Author Contributions

N.V. participated in designing and conducting experiments, data collection and analysis, and manuscript drafting. K.B. participated in designing and conducting experiments, and manuscript editing. H.-X.L. participated in designing and conducting experiments, data analysis, and writing the manuscript.

Disclosure Statement

No competing financial interests exist.

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