Intravital Microscopic Interrogation of Peripheral Taste Sensation

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Intravital microscopy is a powerful tool in neuroscience but has not been adapted to the taste sensory organ due to anatomical constraint. Here we developed an imaging window to facilitate microscopic access to the murine tongue in vivo. Real-time two-photon microscopy allowed the visualization of three-dimensional microanatomy of the intact tongue mucosa and functional activity of taste cells in response to topically administered tastants in live mice. Video microscopy also showed the calcium activity of taste cells elicited by small-sized tastants in the blood circulation. Molecular kinetic analysis suggested that intravascular taste sensation takes place at the microvilli on the apical side of taste cells after diffusion of the molecules through the pericycicular capillaries and tight junctions in the taste bud. Our results demonstrate the capabilities and utilities of the new tool for taste research in vivo.

Results

Setup for Live Imaging of the Mouse Tongue. We fabricated a miniature suction tip to grab and pull the tongue out of the oral cavity in a mouse under anesthesia (Fig. 1a, b and Supplementary Fig. S1). The suction tube had a rubber tip with an inner diameter of 1.5 mm. A suction pressure of about 25 mmHg (0.6 g-force) was found to be adequate to hold the externalized mouse tongue reliably without inducing tissue damage even for over 30 min of
operation. Postmortem histological analysis showed no sign of cellular damage and macroscopic deformation of the tongue tissue (Supplementary Fig. S2). The tongue could be released reversibly by reducing the suction pressure. The externalized tongue was sandwiched between custom-designed stainless steel metal plates with gentle pressure (Supplementary Fig. S3). This arrangement stabilized tissue motion to the submicron level without compromising microvascular perfusion into the tissue. The top plate has an opening through which imaging was performed. The opening also allowed tasters to be administered topically onto the tongue (Fig. 1c). In the case of acute experiments, cyanoacrylate tissue adhesive can be used to hold the tongue by gluing the ventral surface to the bottom metal plate (Supplementary Fig. S1). For imaging, a water-immersion objective lens was used with artificial saliva as immersion solvent to maintain the physiological aqueous environment.

Visualization of Taste Buds. To understand endogenous multiphoton contrast in the dorsal surface of the tongue, we imaged unstained tongue with 100-fs excitation pulses at a central wavelength of 800 nm. The tongue exhibited bright multiphoton photoluminescence in the visible spectrum, which readily demarcated different types of papillary structures (yellow in Fig. 2a and Supplementary Fig. S4). The filiform papillae, cone-shaped protrusions covering most of the dorsum, were visualized by bright two-photon autofluorescence with broad emission spectra peaked at about 520 nm, mostly generated from keratin18 in the keratinized stratified squamous epithelium (yellow in Figs. 2A). The extracellular collagen in the connective tissue around and underneath the taste buds in fungiform and circumvallate papillae generated second harmonic generation (SHG) signal, revealing a crater shape composed of fibrillar structure (blue in Fig. 2a and Supplementary Fig. S4). The spectrum of the SHG signal is centered at exactly the half of the excitation wavelength (i.e. $\lambda_{em} = 400\ \text{nm}$ for $\lambda_{ex} = 800\ \text{nm}$). The SHG signal appeared only in the base of the taste bud but not under the entire epithelium. To visualize the taste cells in the taste buds, we loaded an anionic calcium indicator (calcium green-1 dextran) across the tongue dorsum. We placed a piece of dye-soaked paper tissue and applied uniform electrical pulses throughout the tongue surface using a tweezer-type electrode20 (Supplementary Fig. S5). Using this method, we typically found nearly all taste buds (98.9 ± 2.0%; 92 taste buds from 3 mice) stained over the entire image area (>7 mm²).

Interestingly, even though the calcium dye and electrical field were applied to the tongue surface homogeneously, the taste cells in taste buds were predominantly stained (Fig. 2b). We also observed the dye adsorbed onto the surface of the lingual epithelium, but the nonspecific dye was mostly washed away after rinsing with artificial saliva and almost completely removed by the time of imaging at 1–2 days after the staining. This selective intracellular loading is attributed to the high electrical conductivity of taste buds, compared to the surrounding, which results in high electrophoretic force into the taste cells. Blood vessels were stained by intravenously administered rhodamine-B dextran. In a 3D reconstructed view, individual taste cells have characteristic spindle shapes with their basolateral sides surrounded by the collagenous connective tissue (Fig. 2c and Supplementary Video S1). Projection of the microvilli towards the taste pores at the apical side was apparent21. A schematic of microanatomy of a taste bud is depicted in Fig. 2d (Supplementary Fig. S6).

Functional Calcium Imaging of the Taste Cells. Following the bulk loading of the calcium dye, we studied functional activity of taste cells in the taste buds (Fig. 3a). To quantify the tasteant-evoked change in cellular calcium level, we took volumetric z-stacks of calcium

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**Figure 1** Setup for Intravital Tongue Imaging. (a) Schematic. (b) Photograph. (c) Schematic of the setup for topical administration of aqueous tastant solution.

**Figure 2** Microanatomy of the Tongue Mucosa. (a) Comprehensive view of the tongue mucosa taken with two-photon excitation. Yellow, autofluorescence from keratinized lingual epithelial cells of filiform papillae. Blue, second-harmonic signal from collagen around individual taste buds. This label-free image comprises a mosaic of $15 \times 19$ images, each acquired with an integration time of $2\ \text{s}$ and an axial resolution of about $10\ \text{μm}$ using a $10 \times 0.25\ \text{NA}$ objective lens. (b) Image after electrophoretic bulk loading of calcium-green dextran. The image was taken using a $10 \times 0.25\ \text{NA}$ objective lens. Arrows indicate taste buds (green). (c) A 3D rendered view of a taste bud. The receptor cells (green) show typical spindle shape with their villi projecting towards the apical side. Red, blood vessels visualized by intravenous rhodamine-B dextran (70 kDa). The taste bud is surrounded by collagenous structure (blue). Scale bar, $20 \times 20 \times 20\ \text{μm}^3$. (d) Schematic of a tongue mucosa in cross-sectional view.
fluorescence in a taste bud before and after tastant application. We noticed subtle motion artifact at the moment of applying tastant solution. To compensate this, we aligned two z-stack images by using intensity-based registration algorithm and computed the relative calcium-fluorescence change ($\Delta F/F$) measured by fluorescence intensity. (c) Response to repeated stimuli of a sweet tastant: Na-saccharine, 40 mM. Artificial saliva (AS) and saccharin (Sac) were applied alternatively with a period of ~10 min. The calcium response map are shown.

**Vascular Physiology of the Tongue.** Intact vascular physiology is a hallmark of in vivo system. To study vascular perfusion near taste buds, we injected rhodamine-B dextran (70 kDa) intravenously as a blood tracer. Non-labeled red blood cells (RBCs) were identified as non-fluorescent dark objects flowing in blood vessels. The blood vessels were located about 80 μm deep from the tissue surface, beneath the layer of filiform papillae (Fig. 2d). The imaging depth of two-photon microscopy was greater than 240 μm, sufficient to visualize the epithelial vasculature comprehensively (Fig. 4a and Supplementary Fig. S8; Supplementary Video S2). Beneath each filiform papilla, there was a curly capillary without branching (Fig. 4a, marked by arrows). By contrast, each taste bud had complex vascular architecture composed of richly branched capillaries forming at least one closed loop around the taste cells (Fig. 4b). To understand microvascular perfusion in the taste bud, we quantified the flow velocity in each vascular segment by tracking RBC movement (Fig. 4c). Although the specific vascular architecture varied among the taste buds, several common features were identified as follows. A feeding arteriole bifurcates and anastomoses to form a closed loop. The multiple branches eventually drain into one or two venules with larger diameters (Fig. 4d, e). The capillaries in the taste buds are highly tortuous, much more than those elsewhere including the brain cortex (Fig. 4f). We presume that the curly shape of the vessels in the tongue renders mechanical flexibility essential for dynamic tongue motion.

Next, we studied microvascular permeability in taste buds. When administered intravenously, small-molecular fluorescein (330 Da) rapidly permeated out of the luminal compartment and began to fill in the taste bud through paracellular diffusion (Fig. 4g). There was no noticeable diffusion of the dye to the apical side (Fig. 4h), indicating that the transport of the molecules is blocked by the tight junctions that separate the basolateral and the apical side. In the basolateral side, the extravasated dye reached the 25%, 50%, 75% and 90% levels of the maximum equilibrium concentration in 2.2, 3.6, 6.3 and 11 s, respectively, from the onset of the fluorescence signal (Fig. 4i). To verify this time delay sis indeed due to molecular diffusion, we also tested dextran-conjugated fluorophores with different molecular weights of 4 kDa, 10 kDa, and 70 kDa, respectively. The diffusion time increased linearly with the hydrodynamic molecular size, in agreement with the Stokes-Einstein diffusion theory (Fig. 4j). No diffusion of 70 kDa molecules was observed. These results clearly show that small-sized molecules up to 10 kDa in blood circulation can reach the basolateral side of taste cells through diffusion within one minute. We note that all the molecules including fluorescein did not reach the apical taste pore where intraoral taste sensing takes place, conceivably due to tight junctions between taste cells.

**Intravascular Taste Sensation.** Having confirmed the molecular diffusion from blood circulation, we investigated the possibility of taste sensation from the pericellular blood vessels, a phenomenon called intravascular taste. Although phenomenological intravascular taste has long been known and was even used in the clinic to measure an arm-to-tongue circulation time, its mechanism has been only speculated, and it remains controversial whether taste cells mediate intravascular taste sensation. To address this, we imaged the taste bud in real time while administrating a tastant with an intravenous catheter via the retro-orbital route. An artificial sweetener, saccharin, was chosen because it is known to generate intravascular taste sensation in humans and rodents. Rhodamine-B dextran (183 Da) was added to the tantant solution to visualize the arrival of the injected tantant to the pericellular capillary. Upon a bolus intravenous injection of the mixture of saccharin and dye, real-time fluorescence imaging indicated the arrival of these molecules to the vessel (Fig. 5a and Supplementary Fig. S9). The arrival time from the injection point was 4.8 ± 0.5 s (Fig. 5b). The arrival of the molecules was followed by rapid calcium response in a subset of taste cells, consistent with apical taste stimulation. The intravascular taste sensation by taste cells is not limited to saccharin. Intravenous administration of a physiologic tastant, NaCl (500 mM) also elicited robust calcium response of subpopulation of taste cells (data not shown).

The latent time from the arrival of the molecule to the onset of the calcium response was 1.4 ± 0.9 s, and the peak calcium response appeared 16.0 ± 1.4 s after the onset (Fig. 5b). Our previous measurement of extravasation kinetics predicted that it would take about 10 s, or less, for the tantant to reach 90% concentration at the basolateral side of taste cells (Fig. 4j). We attributed the extra >6 s of delay to the additional diffusion time taken for the molecules to diffuse through the tight junction and reach the apical sensing site—i.e. microvilli—of the taste cells. To verify this conjecture, we tested another sweet tantant molecule, sucrose, with a larger molecular size of 342 Da. Sucrose is small enough to extravasate and permeate through the basolateral space but supposed to be too large to cross the tight junction in the taste bud. As expected, sucrose did not elicit any response from the taste cells even at a near-maximal physiologic concentration of 500 mM in the blood circulation (Fig. 5c). This discrete size discrepancy further suggests that intravascular sensing occurs at the microvilli of the receptor cells.
**Figure 4 | Vascular physiology of the tongue mucosa.** (a) Images at various depths from the surface. The right-most image is a projection of 40 z-stacks with an axial spacing of 2 μm. Arrows indicate the capillaries beneath filiform papillae (FP). (b) A 3D rendered view of the vasculature in the taste bud (TB) demarcated by the dotted rectangle in (a). Arrows indicate the direction of blood flow. (c) Line-scan images and measured RBC velocity for the vessel segments, i–v, indicated in (b). (d) Measured lumen diameters in the capillaries (C), arteriole (Ar), and venule (Ve) in FP and TB. ***, P < 0.01. (e) Histogram of the number of capillary branches in each FP and TB in a fungiform papilla. (f) Measured vascular tortuosity in the tongue vs. brain cortex. Tortuosity is defined as the arc-chord ratio of vessel segments. *, P < 0.05. (g) Fluorescence images of a blood vessel in a TB. Following administration of Rhodamine-B dextran (red, 70 kDa), images were taken before (left) and 1 min after (right) injection of fluorescein (green, 330 Da) via an intravascular catheter. (h) Image of a TB at the depth of the taste pore 5 min after the injection of fluorescein (green). The dark spot at the center corresponds to the location of taste pore. Bottom, intensity profile across a central axis (x, dotted line). (i) Transport kinetics of various dyes at the basolateral side of taste cells. t = 0 corresponds to the arrival of dye at the pericellular blood vessel. (j) Relationship between the hydrodynamic radii of the injected molecules and the measured diffusion times (symbols) to reach specific concentration % levels relative to the steady-state maximum. Dashed lines, linear fit at various concentration levels. For molecules smaller than fluorescein, the diffusion time is expected to be shorter than 10 s (dotted lines). Scale bars, 50 μm in (a), (b), and (g).

Another interesting kinetic feature we observed was that the calcium response attenuated rapidly within 1 min from its onset. This is more than one order of magnitude shorter than the plasma half-life of saccharine in circulation, which is ~10 min in rodents. We reasoned that the taste cells have sensory adaptation to intravascular saccharin as well as to intraoral tastant. To test this hypothesis, we monitored the calcium response of taste cells as saccharin was administered repeatedly at 10 min interval (Fig. 5d). Indeed our measurement showed that the peak response decreased drastically in the second injection of saccharine (Fig. 5e). At the third injection, the calcium response decreased down to the noise level (Fig. 5f). This result is in agreement with the previous observations of rapid attenuation in responsiveness with exposure to prolonged sweet taste.

**Discussion**

**Current Capabilities and Future Outlook.** We have described microscopic interrogation of the tongue in live animals for the first time to our knowledge. Using a video-rate two-photon microscopy, we demonstrated that our approach is well suited for studying physiologic dynamics, such vascular perfusion, molecular diffusion, and functional mapping of taste cells, in the tongue. We further applied the technique to study intravascular taste sensation and revealed that small-molecular tastants (saccharine and sodium chloride) injected into the blood circulation elicits calcium activity of taste cells. Intravital microscopy of the tongue is expected to enable numerous studies previously difficult or not possible with ex vivo preparations. We envision an optogenetic approach to control taste cells by introducing light-sensitive proteins into the cells. Then, the imaging setup demonstrated here can be used to not only read their responses but also to activate or de-activate individual taste cells to study intercellular circuitry. Furthermore, the penetration depth of multiphoton microscopy is sufficient to view the entire taste buds in mice and may be extended to measure the activities of the underlying gustatory nerves to study functional connectome and information processing between taste cells to the nerve fibers. This capability may help to address the grand debate in the field of taste: whether taste cell processing between taste cells to the nerve fibers. This capability may help to address the grand debate in the field of taste: whether taste cell processing is a labeled-line system or combinatory one. It should be possible to perform tongue imaging in conjunction with conventional neurotechniques such as nerve electrophysiology and neuronal calcium imaging for comprehensive understanding of the taste transduction pathway (Supplementary Fig. S10). Microfluidic channels may be integrated into the tongue window to allow controlled delivery of multiple tastants at steady-state flow and enable studies with delicate combinations of different taste qualities. Furthermore, endoscopic probes can be employed to gain access to other regions in the oral cavity, such as posterior part of the tongue, buccal mucosa, and palates.

**Apical Sensing of Intravascular Taste.** Our study on intravascular taste provides direct evidence that it is the taste cells that mediate intravascular taste. Our data also suggest that the microvilli in the apical side of taste buds are the common active sensing site for...
intravascular and intraoral taste. It has previously been questioned whether intravascular sensing should occur at the basolateral side of the taste cells because of the presence of the tight junctions that separate the apical and basolateral spaces. This was supported by experimental observations that amiloride (230 Da) administered topically did not affect the basolateral side and vice versa. However, another study with electron micrographs suggested that tight junctions in taste buds are relatively leaky by showing diffusion of lanthanum (139 Da) across the tight junction. In the present study, we provided quantitative evidence favoring apical sensing hypothesis by measuring the kinetics of cellular calcium activity in response to intravenously administered saccharin (183 Da) at real-time. The response of taste cells was significantly delayed compared to the estimated diffusion kinetics of saccharin to basolateral space, which can be reconciled by additional diffusion length to reach the apical site. We further verified that intravenous sucrose (342 Da), which is impermeable to the tight junction, failed to elicit activation of taste cells. Taken together, our results suggest that intravascular taste elicited by saccharine is sensed by microvilli at the apical side. It is known that some chemicals (e.g., salt, alcohol) from ingested foods are transported into blood circulation. Our result indicates that intravascular sensing of these chemicals may contribute to the taste sensing of certain foods and drinks.

Other Applications in Peripheral Taste Research. Beyond taste sensation, intravital tongue imaging is expected to provide a wide range of applications, particularly for pathogenesis and homeostatic maintenance, by allowing longitudinal observation of cellular dynamics over prolonged period of time. The lingual keratinized epithelial cells constituting the filiform papillae are one of the most rapidly regenerating cells in the body, with a typical turn over time of 10 days in human. Their rapid proliferation is closely associated with the genesis of squamous cell carcinoma and oral mucositis after cancer therapy. Observing cellular dynamics during the disease progression and therapeutic interventions would facilitate deeper understanding on cellular mechanisms. Moreover, dynamic repopulation of the taste cells, and their renewed connectivity to theafferent nerve fibers should offer an exciting model to study highly orchestrated cellular maintenance and plasticity. Structural and functional mapping of vascular network in the taste bud may also be useful to elucidate the functional role of vascular perfusion in peripheral taste sensation and to measure the potential spatiotemporal correlation (i.e. neurovascular coupling) between neuronal activity and vascular perfusion in the tongue.

**Methods**

**Mouse preparation.** All experiments were conducted with 7- to 10-week-old male BALB/c mice (Jackson Laboratory). The mice were anesthetized by intraperitoneal injection of ketamine (90 mg/kg; Pfizer) and xylazine (9 mg/kg; Bayer Healthcare) in phosphate buffered saline (PBS; Life Technologies). The tongue of an anesthetized mouse was gently pulled out with a rubber-tip forceps and stabilized either by holding with a suction grabber with a pressure of 25 mmHg or by gluing onto a bottom metal plate with cyanoacrylate tissue adhesive (~2 μl) (see Supplementary Fig. S1). A top metal plate was then placed onto the tongue with gentle pressure, monitoring the apparent color of the tongue by naked eyes to ensure normal perfusion. Under intravital microscopy, normal blood perfusion was readily confirmed by observing the apparent color of the tongue by naked eyes to ensure normal perfusion. Under intravital microscopy, normal blood perfusion was readily confirmed by observing the apparent color of the tongue by naked eyes to ensure normal perfusion. Under intravital microscopy, normal blood perfusion was readily confirmed by observing the apparent color of the tongue by naked eyes to ensure normal perfusion. Under intravital microscopy, normal blood perfusion was readily confirmed by observing the apparent color of the tongue by naked eyes to ensure normal perfusion. Under intravital microscopy, normal blood perfusion was readily confirmed by observing the apparent color of the tongue by naked eyes to ensure normal perfusion.
Apical taste stimulation. Imaging experiments were conducted 1 to 2 days after the calcium loading procedure. To remove nonspecifically bound dyes in the epithelium, artificial saliva was applied several times to the epithelium. Electrical stimulation was applied through a tweezer-type electrode (Tweezertrode, Harvard Apparatus) mounted on an A3 microscope slide (2 slides) to maximise the stimulation area. Stimuli were composed of 10 s bursts consisting of 200 ms duration square wave pulses at 10 Hz. The laser was set to a power of 300 mW for red fluorescence. The microscope was set to a gain of 2.5% w/v.

Vascular imaging. After anesthesia, the mouse was intravenously injected with rhodamine-B dextran (70 kDa) or TRITC-dextran (2 mDa, TdB Consultancy AB) dissolved in phosphate-buffered saline (100 µl, 2.5% w/v).

Electrophoretic staining of taste cells. After anesthesia, the mouse was placed in the supine position, and the tongue was gently externalized using the rubber-tipped forceps. A piece of paper tissue (~4 by 4 mm) soaked with calcium green dextran (5% w/v) was placed on the anterior dorsal surface of the tongue and then electric pulses (5 Vpp, 2 Hz, pulse width: 100 ms, duration: 10 s) from a function generator were applied through a tweezer-type electrode (Tweezertrode, Harvard Apparatus) for electrophoresis (Supplementary Fig. S5). The stained tongue was rinsed with artificial saliva several times to remove nonspecifically bound dyes in the epithelium. Imaging experiments were conducted 1 to 2 days after the calcium loading procedure.

Apical taste stimulation. For apical taste stimulation, fresh saliva was obtained by externalizing the tongue and placing it in a tube that contained 4% formalin for 48 hours or longer. The tongue was frozen-sectioned with 5 µm-thin sections and stained with hematoxylin and eosin (H&E). Optical images of the slides were obtained by using a bright-field microscope (IX71, Olympus) with a 4 × objective lens.

Data analysis. Image processing plugins for Fiji (ImageJ, NIH) and Matlab (Mathworks) were used for image registration, stitching, arithmetic operation, filtering and quantification. The calcium activity from taste cells was monitored by using time-lapse z-stack images acquired, typically, over the middle portion of the taste bud where cell bodies are clearly distinguished. We confirmed if the adjacent planes (2–4 µm above or below) exhibited consistent activity patterns to validate the measurement. GraphPad Prism software was used for statistical analysis. Data are expressed as mean ± standard error. Statistical differences were analyzed by t-test or ANOVA where indicated and p-value lower than 0.05 was considered to be statistically significant.

Two-photon microscopy and Spectroscopy. A home-built two-photon microscope with a Ti:Sapphire laser (MaTl DeepSee, Newport) was used for imaging. A laser scanner comprised a rotating polygon mirror (x-axis) and a galvano-scanner mirror (y-axis), generating a frame rate of 30 Hz. The laser power was adjusted by using a rotatable half-wave plate coupled with a polarizing beam splitter. A 1.0 NA water-immersion objective lens (Olympus) was used for all experiments, if not stated otherwise.

The system had three detection channels, each consisting of a photomultiplier tube and dichroic filters: 405 ± 10 nm for blue second harmonic generation signal, 525 ± 25 nm for green autofluorescence, and 600 ± 50 nm for red fluorescence. For the spectral analysis of two-photon luminescence, the light emitted from the tissue was coupled to a multimode optical fiber (FT1500EMT, Thorlabs) and analyzed by a spectroscope (Dongwoo Optron) with an electron-multiplying CCD (Newport).

Intravascular taste. For measuring the response of taste cells to intravascular tastant (saccharine), a movie of fluorescence images of taste cells loaded with calcium green dextran was acquired at 30 frames per second, during and after the retro-ocular injection of the tastant (30 µl in PBS) into the bloodstream. For adaptation experiment (Fig. Sd–i), the same amount of tastant was injected multiple times with a 10 min interval.

Histology. After euthanasia, the tongue was excised from the mouse and fixed in 4% formalin for 48 hours or longer. The tongue was frozen-sectioned with 5 µm-thin sections and stained with hematoxylin and eosin (H&E). Optical images of the slides were obtained by using a bright-field microscope (IX71, Olympus) with a 4 × objective lens.

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Author contributions
M.C. and S.H.Y. designed the research. M.C. and W.M.L. developed the imaging system. M.C. performed experiments and analyzed data. M.C., W.M.L. and S.H.Y. discussed the results. M.C. and S.H.Y. wrote the manuscript.

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