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(54) Title: METHODS FOR DETERMINING CELLULAR RESPONSE TO STIMULI

(57) Abstract: The present invention provides a method for determining cellular response to stimuli. The cells to be tested, for example, may be part of an intact taste bud.

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METHODS FOR DETERMINING CELLULAR RESPONSE TO STIMULI

Related Application

This patent document claims the benefit of priority of U.S. application serial No. 60/639,152, filed December 22, 2004, which application is herein incorporated by reference.

Background of the Invention

Taste transduction is one of the most sophisticated forms of chemotransduction in animals. Gustatory signaling is found throughout the animal kingdom, from simple metazoans to the most complex vertebrates. Its main purpose is to provide a reliable signaling response to non-volatile ligands. Humans typically distinguish several perceptual taste qualities or modalities: sweet, sour, salty, bitter and umami. Each of these modalities is thought to be mediated by distinct signaling pathways mediated by receptors or channels, leading to receptor cell depolarization, generation of a receptor or action potential, and release of neurotransmitter at gustatory afferent neuron synapses.

Taste transduction in animals is mediated by specialized neuroepithelial cells, referred to as taste receptor cells. These cells are organized into groups of about 40 to 100 cells to form taste buds. Taste buds are ovoid structures and are primarily embedded within the epithelium of the tongue. It is believed that taste transduction is initiated at the apical portion of a taste bud at the taste pore, where microvilli of the taste receptor cells make contact with the outside environment. Various taste stimulants cause either depolarization (*i.e.*, a reduction in membrane potential) or hyperpolarization (*i.e.*, an increase in membrane potential) of taste cells and regulate neurotransmitter release from the cells at chemical synapses with afferent nerve fibers. The primary gustatory sensory fibers, which receive the chemical signals, enter the base of each taste bud. Inter-cellular connections between taste cells in the same bud may also modulate the signals transmitted to the afferent nerve fibers.

Each taste modality is associated with particular types of receptor proteins expressed in some of the cells that form each taste bud. Genes encoding taste receptor proteins for sweet, bitter, umami and salty taste substances have

been cloned from a variety of species, including humans. The nature of the coupling of stimulus-receptor interaction to a cellular response in the receptor cells has also been defined for some receptors. Some of these receptors have been used to develop bioassays for use in identifying potential taste enhancers, blockers and modifiers. Although these "chip" based systems have the potential for high throughput screening of large numbers of compounds, they do not incorporate the normal cellular components of the taste signaling pathways that are required for normal receptor-response coupling. Consequently, these assays are best at providing initial information about binding of potential stimuli with a particular receptor. They do not, however, provide information about a subsequent cellular response, if any, to the test substance.

An alternative approach is to express cloned taste receptors in heterologous cells, typically a mammalian cell line such as human embryonic kidney cells (HEK293), and to measure changes in intracellular calcium induced by taste stimuli. This approach requires coupling between stimulus-receptor interaction and a cellular pathway leading to an increase in calcium, and it permits measurements in many cells at once. The normal cellular organization of the taste receptor unit, the taste bud, however, is lost along with any processing of taste information occurring between cells within the taste bud. This limitation is particularly important in light of recent results suggesting that sweet and bitter receptors are localized in taste cells that do not directly communicate with afferent nerve fibers, but rather communicate with adjacent taste bud cells that are innervated.

Summary of the Invention

Over the years substantial efforts have been directed to the development of various agents that interact with taste receptors to mimic or block natural taste stimulants. Examples of agents that have been developed to mimic sweet tastes are saccharin, monellin, and the thaumatins. Many taste-mimicking or taste-blocking agents developed to-date are not suitable as food additives, however, because they are not economical, are high in calories, or are carcinogenic. Development of new agents that mimic or block the basic tastes has been limited by a lack of knowledge of the taste cell biology involved in the transduction of

taste modalities. Thus, there is a continuing need for new products and methods involved in or affect taste transduction.

The present invention provides a method for determining the functional cellular response of tissue to one or more stimuli. In the present invention, one contacts tissue such as taste tissue (taste buds or taste papillae), from an animal, with one or more stimuli, and quantitatively determines the magnitude of least one cellular signaling event initiated by the stimulus/stimuli. Multiple data values may be collected, such as at differing concentrations of stimulus/stimuli and/or at different time points.

The invention provides methods of testing different taste stimuli, *e.g.*, activators, inhibitors, stimulators, enhancers, agonists, and antagonists of taste cells and tissues. As used herein, the term "taste cells" include neuroepithelial cells that are organized into groups to form taste buds of the tongue, *e.g.*, foliate, fungiform, and circumvallate cells (see, *e.g.*, Roper et al., Ann. Rev. Neurosci. 12:329-353 (1989)). Taste cells also include cells of the palate and other tissues that may contain taste cells, such as the esophagus, the stomach, the gastrointestinal tract or other internal organs such as the liver or pancreas. The taste cells may be taken from a biological sample. Such samples include, but are not limited to, tissue isolated from humans, mice, rats, and pigs. In addition to fresh tissue, biological samples may include sections of tissues such as frozen sections taken for histological purposes. A biological sample is typically obtained from a eukaryotic organism, such as an insect, protozoa, bird, fish, reptile, or mammal (*e.g.*, a rat, mouse, cow, dog, pig, rabbit, chimpanzee, or human). Tissues include tongue tissue and isolated taste buds.

A "functional cellular response" in the context of the present invention includes one or more cellular change in response to any parameter that is indirectly or directly under the influence of the test stimulus, *e.g.*, a functional, physical or chemical effect of the stimulus on the taste cell or tissue. A functional cellular response includes ligand binding, changes in ion flux, membrane potential, current flow, transcription, signal transduction, receptor-ligand interactions, messenger concentrations (including, but not limited to, cyclic AMP (cAMP), inositol trisphosphate (IP_3), or intracellular Ca^{++} or other positive and/or negative ions including, but not limited to, chloride, sodium, or

protons), *in vitro*, *in vivo*, and *ex vivo* and also includes other physiologic effects, such as increases or decreases of neurotransmitter or hormone release.

As used herein, "determining a functional cellular response" means assaying for an increase or decrease in a parameter in or on a cell that is indirectly or directly under the influence of the test stimulus, *e.g.*, functional, physical and chemical effects. Such functional effects can be measured by any means known to those skilled in the art, *e.g.*, changes in spectroscopic characteristics (*e.g.*, fluorescence, absorbance, refractive index), hydrodynamic (*e.g.*, shape), chromatographic, or solubility properties, patch clamping, voltage-sensitive dyes, whole cell currents, radioisotope efflux (or influx), inducible markers; tissue culture cell expression; transcriptional activation; ligand binding assays; voltage, membrane potential and conductance changes; ion flux assays; changes in intracellular second messengers such as cAMP and inositol triphosphate (IP3); changes in intracellular calcium levels; neurotransmitter release, and the like.

"Inhibitors," "activators," and "modulators" are used to refer to inhibitory, activating, or modulating molecules identified using *in vitro* and *in vivo* assays for taste transduction, *e.g.*, ligands, agonists, antagonists, and their homologs and mimetics. Inhibitors are compounds that, *e.g.*, bind to a cell or cell component (e.g., receptor), partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate taste transduction, *e.g.*, antagonists. Activators are compounds that, *e.g.*, bind to a cell or cell component (e.g., receptor), stimulate, increase, activate, facilitate, enhance activation, sensitize or up regulate taste transduction, *e.g.*, agonists. Modulators include compounds that alter the interaction of a polypeptide with receptors or extracellular proteins that bind activators or inhibitor, such as kinases. Modulators include genetically modified, naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like.

Samples treated with an inhibitor, activator, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition, activation or modulation. Control samples (untreated with an inhibitor, activator, or modulator) are assigned a relative activity value of 100%. Inhibition is achieved when the activity value relative to the control is about 80%, optionally 50%,or 25, or even 0%. Activation is achieved when the

activity value relative to the control is 110%, optionally 150%, optionally 200-500%, or 1000-3000%, or higher.

The terms "isolated," "purified" or "biologically pure" refer to material that is substantially or essentially free from components that normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography.

A "label" or a "detectable moiety" is a composition detectable by, *e.g.*, spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include fluorescent dyes, electron-dense reagents, enzymes, biotin, digoxigenin, or haptens and proteins which can be made detectable, *e.g.*, by incorporating a radiolabel into the peptide.

As used herein, the terms "a" and "an" can mean either single or plural.

Brief Description of the Figures

Figure 1 is a schematic drawing of a taste bud.

Detailed Description of the Invention

The present invention provides a method for determining the functional cellular response of taste tissue to one or more stimuli. The tissue to be tested includes live, intact taste cells. The taste cells may be a part of an intact taste bud. The tissue may be obtained from an animal in the form of a biopsy, or maybe obtained immediately after sacrifice of an animal (*e.g.*, pig). The tissue is prepared for further study. In one embodiment, the harvested tissue is prepared into slices. In another embodiment, individual papillae are isolated. In another embodiment, individual taste buds are isolated. The isolated tissue is then placed either in or on a solid substrate, such as a microscope cover slip. The solid substrate may be coated with a tissue-adhering coating. The tissue may then be placed in a tissue chamber that allows for perfusion of oxygenated cell media and stimuli.

The tissue to be tested may be contacted with a label or a detectable moiety. For example, the tissue may be contacted with a fluorescent dye. In certain embodiments, a dye may be loaded into a tissue or cell.

The tissue to be tested is contacted with one or more test taste stimuli, such as activators, inhibitors, stimulators, enhancers, agonists, and antagonists of taste cells and tissues. The contacting may be achieved by point delivery by proximate delivery of stimulus via micropipette to the taste pore region, or by bulk delivery via perfusion of a known stimulant concentration (*i.e.*, bathing the tissue in stimulant). The stimulus may be in contact with the tissue briefly (*e.g.*, for a few seconds), or for an extended period of time (*e.g.*, for a few minutes). Further, the stimulus may be in contact with the tissue for a period of time, discontinued for a period of time, and then reapplied. This cycling can be repeated numerous times. Alternatively, the stimulus may be applied at one concentration, and then at a later time period, the stimulus may be applied at another concentration, either higher or lower.

One then detects and/or quantitatively determines the magnitude of at least one cellular signaling event initiated by the stimulus/stimuli. A wide variety of techniques and technologies may be used. Examples include wide field fluorescence imaging using native fluorescence or fluorophores with specific binding properties, confocal laser scanning microscopy (*e.g.*, multiphoton confocal laser scanning microscopy). Multiple data values may be collected, such as at differing concentrations of stimulus/stimuli and/or at different time points.

In the method of the present invention, one quantitatively determines the cellular signaling in response to the one or more stimuli. For example, one could determine the cellular signaling response in the absence of a stimulus, determine the response in the presence of the stimulus, and even then determine the response after the stimulus has been removed. In other words, different temporal measurements can be determined and different concentration measurements can be determined.

Live, intact taste cells/buds/tissue

The approach of the present invention permits direct documentation of lateral interactions among cells within taste buds. Several approaches are presented that allow for response measurement of taste tissue system. The taste bud tissue may be extracted from live test subjects from the species under study or from sacrificed animals. Taste cells may be obtained from appropriately

sectioned taste papillae and used as thick sections (200 micrometers thick) that would contain a majority of the taste cell and bud structure in the section. The slice preparation provides experimental access to nearly intact taste buds, which retain most of the morphological and functional organization of *in situ* taste buds.

A taste bud slice preparation also provides the ability to determine if taste responses are modified by lateral and/or sequential processes in cells within the taste buds. For example, dyes sensitive to membrane potential can be used to measure concurrent electrical responses in taste cells. Membrane dyes can also be used to measure synaptic vesicle recycling to determine which cells release neurotransmitter in response to a taste stimulus. Dyes such as Lucifer yellow can be used to assess the effects of electrical coupling between taste cells on signal processing.

For example, in the case of porcine tissue, an intact tongue is procured from a local slaughterhouse and immediately placed in chilled storage (0°C) for transport to the laboratory. The tongue is then examined and sections of tissue are removed containing fungiform papillae from the lateral portion of the tongue. The tissue sections in this case contain not only epithelial tissue, but taste cells and the connective tissue underneath the papillae. The section, or sections, removed in this manner are placed in an appropriate oxygenated storage solution, as described in the references such as Danilova, V., Roberts, T., and Hellekant, G. *Responses of single taste fibers and whole chorda tympani and glossopharyngeal nerve in the domestic pig, Sus scrofa.* Chemical Senses 1999, 24:301-316. In another embodiment, a biopsy is removed from a live animal (e.g., pig) and immediately prepared for examination.

In one embodiment, the tissue section is prepared (*e.g.*, using a vibrating microtome) into a slice section of thickness between 100 and 200 micrometers. This is accomplished using chilled tissue and storage solution such that the sections are cut with minimal cutting artifacts. The individual tissue sections are then placed onto a microscope cover slip coated with a tissue-adhering coating or protein (collagen is typically used). This cover slip is then attached, via suitable removable adhesive or highly viscous, non-reacting grease, to a tissue chamber that allows for perfusion of oxygenated cell media and stimuli. This presentation is then ready to be loaded with appropriate cell signaling dye and

imaged for response to stimulus as a slice section. Alternately, the slice section may be prepared, in a manner similar to that above, after the papillae tissue is loaded with an appropriate signaling dye.

An alternate approach is to utilize intact preparations of taste tissue excised from the tongue as papilla tissue, with epithelial cells and an intact taste pore located on the top of the tissue. For example, a subject animal (*e.g.*, pig or human) is anaesthetized and biopsied along the lateral portion of the tongue, resulting in removal of a block of tissue one to two millimeters on a side. This tissue is then placed directly in chilled storage solution and prepared, under a dissecting microscope, into a tissue sample containing a single fungiform papilla. This is then transferred to a collagen-coated cover slip such that the apical face of the taste bud is pointing up from the cover slip, exposing the taste pore and surrounding epithelial tissue. This sample is thus prepared for subsequent loading of appropriate signaling dye. Examples of general taste tissue preparation and loading protocols and results are given in the following references: Lindemann, B. *Taste reception,* Physiol. Reviews, 1996, 76:718-766; and Caicedo, A., Jafri,S, and Roper, S. D., *In Situ Ca Imaging Reveals Neurotransmitter Receptors for Glutamate in Taste Receptor Cells*, The Journal of Neuroscience, November 1, 2000, *20*(21):7978–7985.

Taste tissue (taste buds or taste papillae) can be isolated from a wide variety of animals. For instance, rodents are subjects of studies involving stimulus of taste sensory systems in the work presented in Caicedo, A., Kim, K.N. and S.D. Roper. 2002, *Individual mouse taste cells respond to multiple chemical stimuli.* J. Physiol. 544.2:501-509. In addition, feline specimens have been used for taste response studies, according to the following references: Boudreau, J.C. and N. Alev. 1973. *Classification of chemoresponsive tongue units of the cat geniculate ganglion.* Brain Res. 54:157-175; Boudreau, J.C. and T.E. Nelson. 1977. *Chemical stimulus determinants of cat geniculate ganglion chemo-responsive group I discharge.* Chem. Senses Flavour. 2:353-374; Boudreau, J.C., Bradley, B., Bierer, P., Kruger, S. and C. Tsuchitani. 1971. *Single unit recordings from the geniculate ganglion of the facial nerve of the cat.* Exp. Brain Res. 13:461-488; Krimm, R.F. and D.L. Hill. 1998. *Innervation of single fungiform taste buds during development in rat* J. Comp. Neurol. 398:13-24; and Boudreau, J.C., Sivakumar, L., Do, L.T., White, T.D., Oravec, J. and

N.K. Hoang. 1985. *Neurophysiology of geniculate ganglion (facial nerve) taste systems: species comparisons.* Chem. Senses. 10:89-127.

Additionally, canines are subjects of studies involving stimulus of taste sensory systems in the work presented in the following references: Kumazawa T. and K. Kurihara. 1990. *Large synergism between monosodium glutamate and 5'-nucleotides in canine taste nerve responses.* Am. J. Physiol. 259:R420-R426; Kumazawa T., Nakamura M., Kurihara K. 1991. *Canine taste nerve responses to umami substances.* Physiol. Behav. 49:875-881; Kumazawa T., Kurihara K. 1990. *Large enhancement of canine taste responses to sugars by salts,* J. Gen. Physiol. 95:1007-1018; Nakamura M., Kurihara K. 1990. *Non-specific inhibition by amiloride of canine chorda tympani nerve responses to various salts: do Na^+-specific channels exist in canine taste receptor membranes*?. Brain Res. 524:42-48; and Nakamura M., Kurihara K. 1991. *Canine taste nerve responses to monosodium glutamate and disodium guanylate: differentiation between umami and salt components with amiloride.* Brain Res. 541:21-28.

The isolation of taste sensory systems (taste buds or taste papillae) from taste tissue is not limited to mammalian subjects. For instance, species of fish have been the subject of taste system study, according to the following representative references: Caprio, J. 1975. *High sensitivity of catfish taste receptors to amino acids.* Comp. Biochem. Physiol. A. 52:247-251; Brand, J.H., Teeter, J.H., Kumazawa, T., Huque, T., and Bayley, D.L. 1991. *Transduction mechanism for the taste of amino acids.* Physiol. Behav. 49: 899-904; Zviman, M.M., D. Restrepo, and J.H. Teeter. 1996. *Single taste stimuli elicit either increases or decreases in intracellular calcium in isolated catfish taste cells.* Journal of Membrane Biology. 149: 81-88; Hayashi, Y., M.M. Zviman, J.G. Brand, J.H. Teeter and D. Restrepo. 1996. *Measurement of membrane potential and $[Ca^{2+}]_I$ in cell ensembles: Application to the study of glutamate taste in mice.* Biophysical Journal. 71: 1057-1070; Finger, T.E., Bryant, B.B., Kalinoski, D.L., Teeter, J.H., Böttger, B., Grosvenor, W., Cagan, R.H. and J.G. Brand. 1996. *Differential localization of putative amino acid receptors in taste buds of the channel catfish, Ictalurus punctatus.* Journal of Comparative Neurology. 373: 129-138; and Ashworth, R. *Approaches to measuring calcium in zebrafish: focus on neuronal development,* Cell Calcium 35 (2004) 393–402.

Imaging system consisting of fluorescence imaging of cellular signaling events

The imaging of cellular signaling events is performed using a variety of techniques and technologies. The prevailing tool in this area of endeavor is optical microscopy of fluorescent structures and features in cells, which encompasses a broad spectrum of specific equipment and approaches. First, wide field fluorescence imaging using both native fluorescence and addition of fluorophores with specific binding properties to cell species is a widely used tool. In addition, confocal laser scanning microscopy (CLSM) of fluorescently labeled cells with ultraviolet or visible light excitation is a tool gaining increased use in cell studies. Alternatively, confocal laser scanning microscopy of fluorescently labeled cells with multiphoton excitation using a near-infrared laser is a methodology that allows for deeper tissue sampling and reduced tissue damage due to the optical effects exploited in multiphoton excitation of fluorescent molecules.

Fluorescence is the result of a three-stage process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific stimulus.

In stage one, a photon of energy $h\nu_{EX}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

In stage two, the excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (stage one) return to the ground state (S_0) by fluorescence emission. Other processes such as collisional quenching, fluorescence resonance energy transfer (FRET) and intersystem crossing (see below) may also depopulate S_1. The fluorescence quantum yield, which is the

ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these processes occur.

During stage three of the fluorescence excitation/emission process, a photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon $h\nu_{EX}$. The difference in energy or wavelength represented by ($h\nu_{EX} - h\nu_{EM}$) is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

The entire fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching, see below), the same fluorophore can be repeatedly excited and detected. The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques. For polyatomic molecules in solution, the discrete electronic transitions represented by $h\nu_{EX}$ and $h\nu_{EM}$ are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected (see below). With few exceptions, the fluorescence excitation spectrum of a single fluorophore species in dilute solution is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength. Additional information regarding optical processes involved in fluorescence can be found in Lakowizc, J.R., *Principles of Fluorescence Spectroscopy, 2nd Edition*, 1999, Kluwer Academic/Plenum Publishers.

Fluorescence microscopy is very useful due to the inherent specificity afforded by the application of fluorescent labels to specific cell targets. As an example, Molecular Probes Corporation markets and sells a complete library of standardized fluorescent tags functionalized to allow for specific assays and tagging experiments. For instance, calcium sensitive dyes respond to changes in intracellular calcium ion concentration by changing the excitation or emission properties of the dye. For the calcium ion indicators fura-2 and indo-1 the free and ion-bound forms of fluorescent ion indicators have different emission or excitation spectra. With this type of indicator, the ratio of the optical signals can be used to monitor the association equilibrium and to calculate ion concentrations. Ratiometric measurements eliminate distortions of data caused by photobleaching and variations in probe loading and retention, as well as by instrumental factors such as illumination stability. Lifetime measurements of fluorophores in different environments allow for quantitative measurement of the environment perturbation, regardless of differences in dye distribution and optical scattering, making this an attractive mode of measurement in living tissue.

Wide-field fluorescence microscopy is very widely used to obtain both topographical and dynamic information. It relies on the simultaneous illumination of the whole sample. The source of light is usually a mercury lamp, giving out pure white light. Optical filters are then used in order to select the wavelength of excitation light (the excitation filter). Excitation light is directed to the sample via a dichroic mirror (*i.e.*, a mirror that reflects some wavelengths but is transparent to others) and fluorescent light detected by a camera (usually a CCD camera). Thus both the illumination and detection of light covering the whole visual field of the chosen microscope objective is achieved simultaneously.

Alternatively, laser scanning confocal microscopy (LSCM or CLSM, abbreviated CLSM in this document) may be used to excite and collect fluorescence from a system under study. Measuring fluorescence by CLSM differs from wide-field fluorescence microscopy in a number of ways. First, the light source is one or more laser(s). This has two consequences. First, the excitation light bandwidth is determined by the source, not the excitation filter and thus is much narrower than in wide field fluorescence microscopy (2-3 nm

rather than 20 - 30 nm). Second, in order to illuminate the whole visual field, the laser beam has to be rapidly scanned across the area in a series of lines, much like a TV image is generated. The fluorescence detected at each point is measured in a photomultiplier tube (PMT), and an image is built up. This method of illumination has enormous advantages in that it is possible to illuminate selected regions of the visual field allowing complex photobleaching protocols to be carried out to investigate the rates of lateral travel of fluorophores and for the excitation of different fluorophores in different regions of the same cell. The major difference between fluorescence microscopy and LSCM is the presence of a pinhole in the optical path. This is a device that removes unwanted, out-of-focus fluorescence, giving an optical slice of a 3-dimensional image. In one embodiment of the imaging protocol, excitation light incident on the sample excites the object of interest, and gives high-resolution fluorescent image with a minimum amount of haze or out-of-focus light reaching the detector. In order to obtain such an image, the pinhole is placed in front of the detector photomultiplier tube and blocks the passage of this out-of-focus light into the PMT. This means that the only light to enter the PMT, and thus be detected, comes from near the focal plane of the objective lens of the microscope. As this is taken across the area of the sample, it produces an image that is a slice through the object and surrounding material. This is known as "optical slicing" and allows the observer to see inside the object of interest. This gives clear images, with fine detail observable.

In addition to the above advantages, by altering the focus of the microscope, images can be obtained at different depths. Each image is called a z-section, and can be used to reconstruct an image of the 3-dimensional object. As an analogy, this technique is like cutting an object into slices, and then stacking the slices back on top of each other to reconstruct the shape of the object. This principle can similarly be achieved using multiple z-sections. If images are "stacked" on top of one another in the correct order, a single image of the object can be generated.

In an alternative embodiment, multiphoton CLSM may be used, where multiphoton excitation of the fluorophores of interest is achieved. As stated earlier, the energy of emitted fluorescent light is less than that of the incident light. Also, fluorophores that absorb red light do not emit green fluorescence.

This holds true for almost all fluorescent applications. Under appropriate conditions, however, the generation of high energy fluorescence using low energy incident light is achieved by delivering multiple photons of excitation light to the same point in space in a sufficiently short time that the energy effectively is summed and so acts as a higher energy single photon. The arrival of the first photon causes the electron to become excited, but not sufficiently to reach a more stable state. This excess energy is lost very quickly, but if a second photon is delivered rapidly enough, the electron acts as if a high-energy single photon has been delivered, resulting in fluorescence emission from the focal point fluorophores.

The timescale of electronic excitation is incredibly short. The second photon must arrive within <0.1 femtoseconds, so photons must be delivered in rapid succession. The power required to deliver such a rapid continuous stream of photons into a particular position in space, however, is enormous, up to about 1-Terawatt/cm^2. In order to prevent damage to biological samples, pulsed lasers are typically used. A typical multiphoton excitation laser is the Titanium:Sapphire laser, which delivers pulses of photons of about 100 femtoseconds duration separated by about 12-40 nanoseconds, at wavelengths ranging from ~700 to 1000 nanometers. Thus, although the pulses of light are of extremely high intensity, the average power delivered to the sample is relatively low.

Several advantages exist with regard to employing multiphoton excitation in fluorescence imaging. First, light from the high intensity red to near infrared laser scatters less efficiently than lower intensity blue light, so objects of interest can be imaged in thicker sections of tissue than in conventional CLSM. Thicker tissue slices are likely to be healthier, and the cells being observed are less likely to have been damaged in the preparation of the sample. Second, the lower overall energy of the excitation light means that less phototoxic damage is caused during viewing and less photobleaching is seen, extending the time that cells can be observed. Third, multiphoton CLSM is innately confocal, *i.e.*, no pinhole is required. Excitation of the fluorophore can only occur where the two photons can interact. Given the quadratic nature of the probability of two photons interacting with the fluorophore in the necessary timescale, excitation only occurs in the focal plane of the objective lens. This

provides cleaner images. Finally, the high repetition rate pulsed laser used in multiphoton CLSM is uniquely suited to performing rapid fluorescent lifetime measurements. Thus, a cell with an appropriate calcium sensitive dye, like calcium green dextran, is imaged using multiphoton fluorescence lifetime imaging with a properly configured CLSM to yield deep tissue measurement of intracellular calcium release in a receptor cell, such as a taste receptor cell.

Additional information regarding biological applications of fluorescence microscopy in cell signaling studies may be found in the following references: Gratton, E., Breusegem, S., Sutin, J., Ruan, Q. and N. Barry. 2003. *Fluorescence lifetime imaging for the two-photon microscope: time-domain and frequency-domain methods*. J. Biomed. Optics 8:381-390; Koester, H.J., Baur, D., Uhl, R. And S.W. Hell. 1999. *Ca^{2+} fluorescence imaging with pico- and femtosecond two-photon excitation: signal and photodamage*. Biophys. J. 77:2226-2236; Konig, K. et al. 1997. *Cell damage in UVA and CW/femtosecond NIR microscopes*. SPIE vol. 2983:37-44; Liu, D. and E.R. Liman. 2003. *Intracellular Ca^{2+} and the phospholipids PIP_2 regulate the taste transduction ion channel TRPM5,* 2003. PNAS 100:15160-15165.

Quantitative stimulus delivery system applied to live tissue

The ability to provide known concentrations of taste stimuli to the preparation under precise temporal and spatial control is critical to quantitative determination of cellular signaling in response to stimulus. The delivery of stimuli may be by any one or several methods. In one embodiment of the present invention, for example, the delivery of stimuli is achieved by point delivery by proximate delivery of stimulus via micropipette to the taste pore region. In another exemplary embodiment, the delivery of stimuli is achieved by bulk delivery via perfusion of known stimulant concentration, effectively bathing the tissue in stimulant.

In one embodiment of a proximate delivery approach, slices of taste papillae containing taste buds are mounted on glass cover slips coated with adhesive protein. The cover slips form the bottom of the recording/perfusion chamber, which is attached to the stage of the microscope. A continuous background flow of oxygenated, physiological saline (*e.g.*, Tyrode solution) is supplied to the chamber from a temperature-controlled reservoir via an

independently heated perfusion line. The direction of flow across the slice is oriented from the base to the apical region of the taste bud. Taste stimuli or mixtures of stimuli are applied from an independent, 8-line, temperature-controlled perfusion pencil. Each of seven lines are attached to pressurized solution reservoirs via miniature (normally-closed) solenoid valves. The 8th line is attached via a normally-open solenoid valve to a regulated vacuum line and vacuum trap. The solenoids are under computer control so that a stimulus valve opens while the vacuum line is simultaneously closed for a predetermined time. This device is positioned with the delivery tip close to and slightly distal to the apical end of the taste bud. This allows small puffs of up to seven different stimuli to be applied just to the apical, receptive ends of the taste cells. By adjusting the pressure in the stimulus reservoirs, the position of the stimulus delivery tube, and the rate of background perfusion, stimuli can be confined to just the apical surfaces of the taste bud cells. It is desirable to prevent activation of cellular pathways in the basolateral membranes of the taste bud cells that normally do not interact with taste stimuli on the surface of the tongue, which could lead to erroneous conclusions about taste response properties. The vacuum line in the stimulus delivery manifold is typically closed slightly after activation of the chosen stimulus valve and acts to prevent mixing of stimuli by evacuating solution leaking from closed lines and for priming the selected delivery tube. Solutions are continuously removed from the chamber by a vacuum line set to provide a constant fluid level and to pull solution without "jitter" or "slurping" at the solution surface.

In an alternative embodiment, pieces of tongue epithelium containing taste buds are removed from the underlying tissue using gentle enzymatic treatment. The epithelium is mounted in a trans-epithelial chamber that allows independent perfusion of the basolateral (serosal) and apical (mucosal) surfaces. This permits the normal polarity of the epithelium and the taste buds in it to be maintained. The basolateral chamber is perfused with oxygenated Tyrode solution that has been pre-heated to minimize out-gassing and formation of bubbles. Manipulations of basolateral ion channels and transports are manipulated by application of Tyrode solution containing pharmacological agents by switching to other perfusate reservoirs through a bank of valves. The apical chamber is used either in an open configuration with a dipping objective

or closed, with a cover glass attached to the top of the chamber. In either case, stimuli is applied from temperature-controlled reservoirs attached to the apical chamber via computer-controlled solenoid valves attached to a manifold attached to the chamber with a short length of tubing. This system is either gravity fed via constant flow syringes or through pressurized reservoirs. In either case, up to 24 independent stimuli can be applied to the apical chamber and then rinsed out under computer control with the present system.

Collection of stimulus/response data structure from imaging/stimulus system

A stimulus/response data structure may be collected from a live cell preparation in the following general manner. Cell and/or tissue sample is obtained and mounted in a suitable oxygenated bath to allow for live cell imaging. The cell/tissue is exposed to signaling-sensitive fluorescent dye such that the intracellular matrix (or membrane, in the case of membrane potential-sensitive dyes, and recycling synaptic vesicles, in the case of synaptic activity markers) is loaded with dye. The cell and/or tissue sample is transferred to the imaging platform that contains the stimulus system and a perfusion chamber, and supporting apparatus, to allow for constant perfusion of the sample during the experimental data collection. Image data is collected from the cell and/or tissue sample, using appropriate image modality and acquisition parameters to provide a baseline or standard resting response image data matrix prior to quantitative stimulus.

Stimulus or series of stimuli are applied in the form of one or more solutions of known concentrations of stimulus prepared in surrogate or synthetic saliva matrices. The application of stimulus is proximate to the taste pore and is administered via a micropipette system having control of stimulus flow and pipette position. Alternatively, the application of stimulus is administered via the perfusion medium, thus bathing the cell and/or tissue sample in a known concentration of stimulus. Before, during, and after the application of known stimulus to the cell and/or tissue sample, image data are collected in such a manner that a number, if not all, of the cells comprising the taste bud are represented in the field of view captured by the imaging system. The image data are collected in such a manner that data corresponding to multiple time points for each phase (before, during, and after stimulus) are collected. Image data

collected at each time point may be comprised of two or three spatial dimensions.

By way of example, an intact pig tongue is procured from a local slaughterhouse and immediately placed in chilled storage (4°C) for transport to the laboratory. The tongue is then examined and sections of tissue are removed containing fungiform papillae from the lateral portion of the tongue. The tissue sections in this case contain not only epithelial tissue but taste cells and the connective tissue underneath the papilla. The section, or sections, removed in this manner are placed in an appropriate oxygenated Tyrode storage solution.

The tissue section is prepared, using a vibrating microtome, into a slice section of thickness between 100 and 200 micrometers. This is performed using chilled tissue and storage solution such that the sections are cut with minimal cutting artifacts. The individual tissue sections are then placed onto a microscope cover slip coated with a tissue-adhering coating or protein (collagen is typically used). This cover slip is then attached, via suitable removable adhesive or highly viscous, non-reacting grease, to a tissue chamber that allows for perfusion of oxygenated cell media and stimuli. This presentation is then iontophoretically loaded with a dye, such as 5 mg/mL calcium green dextran (3000 MW), and imaged for response to glucose stimulus as a slice section. The loading of calcium sensitive dye is verified via standard wide field epifluorescence microscopy. The tissue slice preparations are then transferred to the measurement chamber on the recording microscope, such as a multiphoton confocal laser scanning microscope with epifluorescence optical filters designed to allow for multiphoton excitation of the dye and recording of emission above 530 nm. A continuous background flow of oxygenated, physiological saline (Tyrode solution) is supplied to the chamber from a temperature-controlled reservoir via an independently heated perfusion line. The direction of flow across the slice is oriented from the base to the apical region of the taste bud. Taste stimuli or mixtures of stimuli are applied from an independent, 8-line, temperature-controlled perfusion pencil. Each of seven lines is attached to pressurized solution reservoirs via miniature (normally-closed) solenoid valves. The 8th line is attached via a normally open solenoid valve to a regulated vacuum line and vacuum trap. The solenoids are under computer control so that a stimulus valve opens while the vacuum line is simultaneously closed for a

predetermined time. This device is positioned with the delivery tip close to and slightly distal to the apical end of the taste bud.

For stimulus one, at time = zero, the perfusion and stimulus chambers allow flow with no stimulus, and one or more image frames are acquired whereby the intensity of the generated fluorescence is acquired on a per pixel basis along one plane through the tissue, capturing several, if not all, of the cells in the tissue in the field of view. These frames are referred to as the baseline frames, prior to stimulus. After 2-5 minutes of perfusion with no stimulus, a low concentration stimulus of glutamate (0.05 mmol/L) in artificial saliva perfusion medium is applied via a single pipette channel proximate to the taste pore for a predefined length of time, such as 120 seconds. An initial response to the stimulus is recorded via the fluorescence imaging system upon activation of the stimulus channel. Subsequently, two or more additional image frames are recorded during the stimulus time period, generating a series of frames collected during stimulus. The stimulus is then discontinued and the perfusion continues with no stimulus present, effectively rinsing stimulus from the region of interest around the taste pore. After the stimulus is removed, and at time intervals corresponding to 60 seconds, additional frames are recorded using the fluorescence imaging system in order to develop three frames of image data corresponding to the resting, or post stimulus, response of the taste system under study after stimulus one.

For stimulus two, the perfusion and stimulus chambers allow flow with no stimulus, and single image frame is acquired whereby the intensity of the generated fluorescence is acquired on a per pixel basis along one plane through the tissue, capturing several, if not all, of the cells in the tissue in the field of view. This frame is referred to as the baseline frame, prior to stimulus for stimulus two and may be different in intensity from the baseline stimulus one frame. After 2-5 minutes of perfusion with no stimulus, a low concentration stimulus of glutamate (0.25 mmol/L) in artificial saliva perfusion medium is applied via a single pipette channel proximate to the taste pore for a predefined length of time, such as 120 seconds. An initial response to the stimulus is recorded via the fluorescence imaging system upon activation of the stimulus channel. Subsequently, two additional image frames are recorded during the stimulus time period, generating a series of frames collected during stimulus.

The stimulus is then discontinued and the perfusion continues with no stimulus present, effectively rinsing stimulus from the region of interest around the taste pore. After the stimulus is removed, and at time intervals corresponding to 60 seconds, additional frames are recorded using the fluorescence imaging system in order to develop three frames of image data corresponding to the resting, or post stimulus, response of the taste system under study after stimulus two.

For stimulus three, the perfusion and stimulus chambers allow flow with no stimulus, and single image frame is acquired whereby the intensity of the generated fluorescence is acquired on a per pixel basis along one plane through the tissue, capturing several, if not all, of the cells in the tissue in the field of view. This frame is referred to as the baseline frame, prior to stimulus for stimulus three and may be different in intensity from the baseline stimulus two frame. After 2-5 minutes of perfusion with no stimulus, a low concentration stimulus of glutamate (0.75 mmol/L) in artificial saliva perfusion medium is applied via a single pipette channel proximate to the taste pore for a predefined length of time, in this case 120 seconds. An initial response to the stimulus is recorded via the fluorescence imaging system upon activation of the stimulus channel. Subsequently, two additional image frames are recorded during the stimulus time period, generating a series of frames collected during stimulus. The stimulus is then discontinued and the perfusion continues with no stimulus present, effectively rinsing stimulus from the region of interest around the taste pore. After the stimulus is removed, and at time intervals corresponding to 60 seconds, additional frames are recorded using the fluorescence imaging system in order to develop three frames of image data corresponding to the resting, or post stimulus, response of the taste system under study after stimulus three. The collection of these three time-dependent stimulus/response image data sets results in single image data structure that is then used to generate a quantitative relationship between stimulus and taste tissue response as determined by intensity-based calcium green dextran fluorescence measurements of changes in intracellular calcium.

An alternative embodiment of this experiment is as follows. A subject pig is anaesthetized and biopsied along the lateral portion of the tongue, resulting in removal of a block of tissue five millimeters on a side. This tissue is then placed directly in chilled storage solution and prepared, under a dissecting

microscope, into a tissue sample containing a single fungiform papilla. This is then transferred to a collagen-coated cover slip such that the apical face of the taste bud is pointing up from the cover slip, exposing the taste pore and surrounding epithelial tissue. This cover slip is then attached, via suitable removable adhesive or highly viscous, non-reacting grease, to a tissue chamber that allows for perfusion of oxygenated cell media and stimuli. This presentation is then iontophoretically loaded with 5 mg/mL calcium green dextran (3000 MW) and imaged for response to glucose stimulus as a slice section. The loading of calcium sensitive dye is verified via standard wide field epifluorescence microscopy by application of dye to the apical end of the taste bud through the taste pore.

The intact tissue preparations are then transferred to the measurement chamber on the recording microscope, in this case a multiphoton confocal laser scanning microscope with epifluorescence optical filters designed to allow for multiphoton excitation of the dye and recording of time correlated picosecond fluorescence lifetime emissions above 530 nm in such a manner as to allow for fluorescence lifetime images to be generated. This is accomplished via a time correlated single photon counting detector attached to the output port of the confocal laser-scanning microscope (such as a Leica MP-FLIM system).

The epithelium is mounted in a trans-epithelial chamber that allows independent perfusion of the basolateral (serosal) and apical (mucosal) surfaces. This permits the normal polarity of the epithelium and the taste buds in it to be maintained. The basolateral chamber is perfused with oxygenated Tyrode solution that has been pre-heated to minimize out-gassing and formation of bubbles. Manipulations of basolateral ion channels and transports can be manipulated by application of Tyrode solution containing pharmacological agents by switching to other perfusate reservoirs through a bank of valves. Stimuli are applied from temperature-controlled reservoirs attached to the apical chamber via computer-controlled solenoid valves attached to a manifold attached to the chamber with a short length of tubing.

For stimulus one, at time = zero, the perfusion and stimulus chambers allow flow with no stimulus, and single image frame is acquired whereby the intensity of the generated fluorescence is acquired on a per pixel basis along one plane through the tissue, capturing several if not all of the cells in the tissue in

the field of view. This frame is referred to as the baseline frame, prior to stimulus. After 5-7 minutes of perfusion with no stimulus, a low concentration stimulus of glutamate (0.05 mmol/L) in artificial saliva perfusion medium is applied via a single pipette channel proximate to the taste pore for a predefined length of time, in this case 240 seconds. An initial response to the stimulus is recorded via the fluorescence imaging system upon activation of the stimulus channel. Subsequently, two additional image frames are recorded during the stimulus time period, generating a series of frames collected during stimulus. The stimulus is then discontinued and the perfusion continues with no stimulus present, effectively rinsing stimulus from the region of interest around the taste pore. After the stimulus is removed, and at time intervals corresponding to 120 seconds, additional frames are recorded using the fluorescence imaging system in order to develop three frames of image data corresponding to the resting, or post stimulus, response of the taste system under study after stimulus one.

For stimulus two, the perfusion and stimulus chambers allow flow with no stimulus, and single image frame is acquired whereby the intensity of the generated fluorescence is acquired on a per pixel basis along one plane through the tissue, capturing several if not all of the cells in the tissue in the field of view. This frame is referred to as the baseline frame, prior to stimulus for stimulus two and may be different in intensity from the baseline stimulus one frame. After 5-7 minutes of perfusion with no stimulus, a low concentration stimulus of glutamate (0.25 mmol/L) in artificial saliva perfusion medium is applied via a single pipette channel proximate to the taste pore for a predefined length of time, such as 240 seconds. An initial response to the stimulus is recorded via the fluorescence imaging system upon activation of the stimulus channel. Subsequently, two additional image frames are recorded during the stimulus time period, generating a series of frames collected during stimulus. The stimulus is then discontinued and the perfusion continues with no stimulus present, effectively rinsing stimulus from the region of interest around the taste pore. After the stimulus is removed, and at time intervals corresponding to 120 seconds, additional frames are recorded using the fluorescence imaging system in order to develop three frames of image data corresponding to the resting, or post stimulus, response of the taste system under study after stimulus two.

For stimulus three, the perfusion and stimulus chambers allow flow with no stimulus, and single image frame is acquired whereby the intensity of the generated fluorescence is acquired on a per pixel basis along one plane through the tissue, capturing several if not all of the cells in the tissue in the field of view. This frame is referred to as the baseline frame, prior to stimulus for stimulus three and may be different in intensity from the baseline stimulus two frame. After 5-7 minutes of perfusion with no stimulus, a low concentration stimulus of glutamate (0.75 mmol/L) in artificial saliva perfusion medium is applied via a single pipette channel proximate to the taste pore for a predefined length of time, in this case 240 seconds. An initial response to the stimulus is recorded via the fluorescence imaging system upon activation of the stimulus channel. Subsequently, two additional image frames are recorded during the stimulus time period, generating a series of frames collected during stimulus. The stimulus is then discontinued and the perfusion continues with no stimulus present, effectively rinsing stimulus from the region of interest around the taste pore. After the stimulus is removed, and at time intervals corresponding to 120 seconds, additional frames are recorded using the fluorescence imaging system in order to develop three frames of image data corresponding to the resting, or post stimulus, response of the taste system under study after stimulus three. The collection of these three time-dependent stimulus/response image data sets results in single image data structure that is then used to generate quantitative relationship between stimulus and taste tissue response as determined by emission lifetime-based calcium green dextran fluorescence measurements of changes in intracellular calcium.

Quantitative relationship development between image data and stimulus

Once the stimulus-dependent image data are collected, a quantitative relationship between the stimulus applied and the response of the cellular system is developed. This may be achieved by the following stepwise general approach. First, the image data is standardized with respect to feature position (x,y axis), feature intensity (z axis), and feature time (t axis). This includes, *e.g.*, defining onset of stimulus application (initial time or Ti) and the disappearance of stimulus response (final time or Tf) for each series of images associated with a particular stimulus application; registering each image within such a series such

that spatial information in each image in the series may be correlated to subsequent images (Ti+1 to Tf) within that series (and collection of series for a sequence of stimulus applications); and normalizing the baseline and dynamic ranges for the responses between each image within such a series.

Next, a Region or Regions of Interest (ROIs) are defined within each of the standardized images that contain phenomena related to the magnitude of the applied stimulus. The information within such ROIs is used to extract vector or scalar quantities that represent the independent variables in the comparison step.

Independent variables from the standardized data ROIs are then extracted. For magnitude or concentration measurements, this includes reducing the collection of pixel values for a given image ROI to a scalar quantity. For temporal measurements, this includes reducing time-dependent intensity changes in lifetime or intensity ratio within a series of image ROIs to vector quantities. For spatial measurements, this includes reducing, *e.g.*, diffusion events across ROIs in the (x,y) space to vector quantities. This may include multiple scalar/vector extractions for more than one ROI within each image. After extraction, the independent variables are then grouped into a single vector to represent a particular stimulus application.

Next, the known stimulus applications (dependent variables) are compared against their corresponding independent variable vectors. The comparison may include elements of classification, regression, or qualitative procedures that are used to rank the data according to the applied stimuli. Such procedures define a model which, when applied to an independent variable vector collected on a stimulated sample in the future, yield a classification estimate or predicted stimulus magnitude. In addition, this procedure yields estimates of statistical quantities such as correlation coefficients for the independent vector components and confidence limits for the classification estimate or stimulus magnitude prediction.

By way of example, image data standardization is begun by applying an intensity correction to each image within the set so that each image's response to a pre-defined internal standard condition is the same. For example, an inert fluorescent dye of known concentration is injected into the field of view along with the applied stimulus, and the response of this dye is defined to have a certain magnitude. Alternatively, the image data set is normalized to maximum

and minimum values of intensity and this normalization is applied to subsequent images for comparison.

Following this step, the set of images are corrected for random movement of objects within the field of view during data acquisition. The mathematical procedure of correcting arbitrary differences between a set of multidimensional collections of values is called Procrustes Rotation. Such procedures determine a linear transformation (translation, reflection, orthogonal rotation, and scaling) of the points in a matrix Y (or in this case a digital image) to best conform them to the points in another matrix X. Different methods, such as promax, orthomax, or varimax rotations can be applied, which differ mainly in the criterion they use to estimate differences in the alignment fit. Standardizing the images with respect to time includes defining the onset of stimulus application (initial time or Ti) and the disappearance of stimulus response (final time or Tf) for every collected set of images. Ti could be defined as the first image in the series to show any data point response above a pre-defined baseline or un-stimulated condition, and Tf could be defined as the first image after Ti to register no data point responses above the baseline condition (*i.e.*, the imaged field of view has returned to an "un-stimulated" state).

Having corrected the raw image data, the informative ROIs of the images is then isolated. This can be done manually by having a trained operator input image coordinates or interactively draw perimeters around ROIs in a reference image. A more rigorous way is to calculate the magnitude of response for each data point in the set of images between Ti and Tf. Those values with maximum responses below a certain threshold are discarded as "noise" or "background." Further, the remaining data points are grouped into similar types according to the pattern of their response to the applied stimuli as a function of time. For example, certain regions of the field of view might record transitory "spikes" in response along the time axis, while others demonstrate a steady-state increase in response throughout the entire period from Ti to Tf. This pattern recognition can also be done manually. Alternatively, quick mathematical routines such as Principal Components Analysis (PCA) or Cosine Correlation Analysis (CCA) can be used to define the different types of informative data points in the image. Likewise, if temporal information is not important for quantification, the average or summed response of each data point could be calculated across the time axis

of a given set of images. Groups of interconnected data points with similar response levels are then defined as individual ROIs. Regardless of the procedure used, the ROI definitions only need to be done once, since all of the images in a set (and across sets collected for the same field of view) have been aligned with respect to each other, so that an ROI in any given image is in the exact same place on all other images within a set, and among different image sets.

Depending upon how the ROIs were defined, the information within them may have to be transformed into scalar or vector quantities to make it appropriate for comparison to the magnitude of the applied stimulus. For temporal measurements, this includes reducing time-dependent intensity changes in lifetime or intensity ratio within a series of image ROIs to vector quantities. The most straightforward way to do this is to average together all of the time profiles within a given ROI to form one vector of time responses. For ROIs that include spatial gradients, such as a cell membrane that demonstrates diffusion of a stimulus from its outer to inner boundaries over the time-span of the stimulus application, diffusion gradients could be calculated mathematically by measuring the differences between nearest-neighborhood data points from image to image over the time-span. This is similar to calculating a derivative via finite-differences. The diffusion pathway is identified as the chain of data points that exhibits the largest negative residual differences among subsequent image subtractions. For example, one subtracts the cell membrane ROI of image Ti+1 from image Ti. The data point with the largest negative magnitude in this residual image (which should be on the outer boundary of the membrane) will be the start of the diffusion gradient. Then one subtracts image Ti+2 from image Ti+1, and finds the data point with the largest negative residual that is close in space to the previous maximum residual points. This step is repeated for all of the remaining images up to Tf (or until the maximum negative residual "wave" has traversed the entire width of the membrane). Then, one calculates the rate of residual intensity transfer in units of distance/time across the width of the membrane. This procedure thereby results in a scalar quantity (diffusion rate) that is compared to, for example, stimulus magnitude or stimulus type. Furthermore, several such waves from different parts of the membrane can be calculated in order to determine an average diffusion rate. Note that several other quantities can be calculated for a set of images from different ROIs that

exhibited different types of information (*i.e.*, magnitude/static, spatial, and/or temporal). After calculating these values, they are then grouped into a single set of numbers (a vector) to represent the independent variables that are to be compared to a particular stimulus application.

The comparison may include elements of classification, regression, or qualitative procedures that are used to rank the data according to the applied stimuli. Such procedures define a model which, when applied to an independent variable vector collected on a sample stimulated in a similar manner in the future, yields a classification estimate or predicted stimulus magnitude. In addition, this procedure yields estimates of statistical quantities such as correlation coefficients for the independent vector components and confidence limits of, for example, a stimulus type classification estimate or stimulus magnitude estimate. An example of a classification type of comparison is to perform PCA on a collection of vectors calculated from several image sets that were exposed to different types of stimuli (e.g., sweet, bitter, salt). The PCA scores of this reduced data set clusters in the principal components space according to stimulus type. As an example of a quantitative regression application, the level of applied sweet stimulus in an unknown sample could be predicted using a multivariate correlation algorithm such as non-iterative Partial Least Squares (PLS). PLS is applied to a set of response vectors calculated from several applications of known levels of sweetness in order to construct a pattern of correlations between the calculated response vectors and their corresponding level of sweet stimulus. This pattern of correlations constitutes a mathematical model that can be applied to future response vectors in order to yield a prediction of applied sweetness.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. A method for determining a functional cellular response of taste tissue from an animal to one or more test stimuli comprising contacting the taste tissue with one or more test stimuli, and quantitatively determining a magnitude of at least one functional cellular response initiated by the one or more test stimuli.

2. The method of claim 1, wherein the taste tissue comprises a detectable moiety.

3. A method for determining a functional cellular response of taste tissue that comprises a detectable moiety to one or more test stimuli, comprising contacting the taste tissue with one or more test stimuli, and detecting a functional cellular response of the taste tissue to the one or more stimuli by detecting a change in state of the detectable moiety.

4. A method for identifying a stimuli that affects taste, comprising contacting taste tissue that comprises a detectable moiety with one or more test stimuli and detecting a functional cellular response of the taste tissue to the one or more stimuli by detecting a change in state of the detectable moiety.

5. The method of claim 4 further comprising comparing the response of the taste tissue to the one or more test stimuli to the response of the taste tissue to one or more control stimuli.

6. The method of any one of claims 2-5, wherein the detectable moiety is detectable by spectroscopic, photochemical, biochemical, immunochemical, physical, or chemical means.

7. The method of any one of claims 2-6, wherein the detectable moiety is a fluorescent dye, an electron-dense reagent, an enzyme, biotin, digoxigenin, or a hapten or a protein that has been made detectable.

8. The method of any one of claims 2-7, wherein the detectable moiety is a calcium-sensitive dye.

9. The method of claim 8, wherein the calcium-sensitive dye is a calcium-sensitive dye.

10. The method of claim 9, wherein the calcium-sensitive dye is Calcium Green.

11. The method of any one of claims 2-7, wherein the detectable moiety is a voltage-sensitive dye.

12. The method of any one of claims 1-11, wherein the taste tissue is obtained from a eukaryotic organism.

13. The method of claim 12, wherein the eukaryotic organism is a mammal.

14. The method of claim 13, wherein the mammal is a rat, mouse, cat, cow, dog, pig, rabbit, chimpanzee, or human.

15. The method of claim 14, wherein the mammal is a pig.

16. The method of any one of claims 1-15, wherein the taste tissue comprises an intact taste papilla.

17. The method of any one of claims 1-16, wherein the taste tissue comprises an intact taste bud.

18. The method of any one of claims 1-17, wherein the detecting or determining is by means of optical microscopy.

19. The method of claim 18, wherein the optical microscopy is wide field fluorescence imaging microscopy, laser scanning confocal microscopy, or multiphoton fluorescence lifetime imaging microscopy.

20. The method of any one of claims 1-19, wherein the one or more test and/or one or more control stimuli are sweet, sour, salty, bitter and/or umami.

21. The method of any one of claims 1-19, wherein the one or more test and/or one or more control stimuli is an agonist or antagonist.

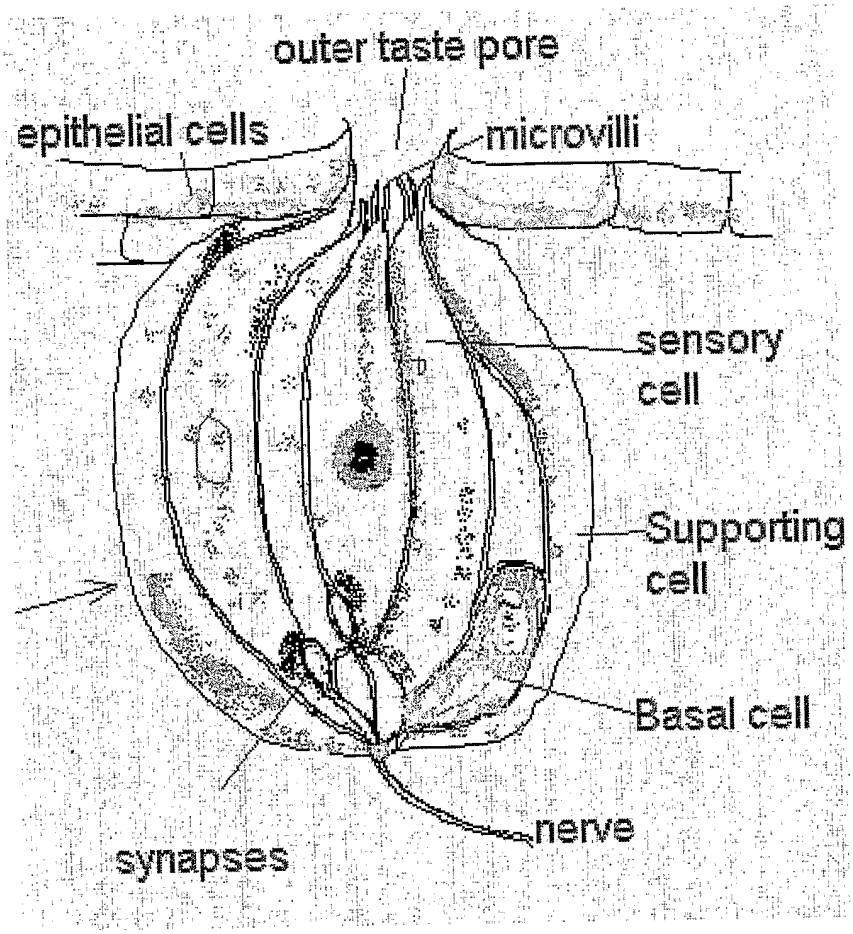
22. One or more stimuli identified according to the method of any one of claims 1-21.

23. The use of the one or more stimuli of claim 22 to prepare an ingestible substance.

24. An ingestible substance comprising the one or more stimuli of claim 22.

25. A method to produce a perceptual taste quality comprising administering to a mammal an effective amount of the one or more stimuli of claim 22 so as to produce a perceptual taste quality.

Figure 1


outer taste pore
epithelial cells
microvilli
sensory cell
Supporting cell
Basal cell
nerve
synapses

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/41946

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **G01N 33/53**(2006.01)

USPC: 435/7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Caicedo et al. Taste receptor cells that discriminate between bitter stimuli Science 2001, Vol. 291, page 1557-1560. Especially Figure 1 and Figure 4	1-4
X	Richter et al. Sour taste stimuli evoke Ca+2 and pH responses in mouse taste cells Journal of Physiology 2003 Vol. 547, page 475-483, Abstract and Figure 1 to Figure 2.	1-6
A	He et al. Partial rescue of taste responses of alpha-gustducin in null mice by transgenic expression of alpha-transducin Chem. Senses 2002 Vol. 27, page 719-727. whole document	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
26 April 2006 (26.04.2006)

Date of mailing of the international search report
07 JUN 2006

Name and mailing address of the ISA/US
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Telephone No. 571-272-0814

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/41946

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 7-25 are improper multiple dependent claims.

3. ☒ Claims Nos.: 7-25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.