

Live Cell Calcium Imaging of Dissociated Vomeronasal Neurons

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Abstract

Sensory neurons in the vomeronasal organ (VNO) are thought to mediate a specialized olfactory response. Currently, very little is known about the identity of stimulating ligands or their cognate receptors that initiate neural activation. Each sensory neuron is thought to express 1 of approximately 250 variants of Vmn1Rs, Vmn2Rs (A, B, or D), or FPRs which enables it to be tuned to a subset of ligands (Touhara and Vosshall, *Annu Rev Physiol* 71:307–332, 2009). The logic of how different sources of native odors or purified ligands are detected by this complex sensory repertoire remains mostly unknown. Here, we describe a method to compare and analyze the response of VNO sensory neurons to multiple stimuli using conventional calcium imaging. This method differs from other olfactory imaging approaches in that we dissociate the tightly packed sensory epithelium into individual single cells. The advantages of this approach include (1) the use of a relatively simple approach and inexpensive microscopy, (2) comparative analysis of several hundreds of neurons to multiple stimuli with single-cell resolution, and (3) the possibility of isolating single cells of interest to further analyze by molecular biology techniques including in situ RNA hybridization, immunofluorescence, or creating single-cell cDNA libraries (Malnic et al., *Cell* 96:713–723, 1999).

Key words Vomeronasal, Calcium imaging, Neurons, Dissociated

1 Introduction

In mice, the vomeronasal organ (VNO) has been implicated in mediating reception of a variety of ligands informing both interspecies and intraspecies interactions [1]. Sensory neurons within the VNO can each express 1 of approximately 250 receptors. With such a complex receptor family of interest, ligand identification and receptor deorphanization have become important in order to study the mechanisms of sensory detection and function. Calcium imaging of dissociated sensory neurons is a relatively simple technique to identify and analyze ligands detected by the VNO [2]. Moreover, because the closely packed neurons are dissociated into individual sensory neurons, single cells can be easily isolated following calcium imaging for analysis by conventional molecular biology [3].

Activation of the vomeronasal sensory neurons (VSNs) occurs upon binding of ligands by the cognate receptors, followed by a signaling cascade that leads to opening of a Trpc2-associated calcium channel [4]. This in turn results in a high influx of extracellular calcium ions [5], a property that has been exploited to identify activated cells using chemical dyes or genetically encoded marker proteins that detect changes in intracellular calcium (reviewed by Grienberger, 2012 [6]). This principle can be combined with simple microscopy to study the changes in calcium concentration within individual VSNs. The densely packed VSNs must first be separated by physical dissociation followed by mild protease digestion. The separated neurons are plated on coverslips at a density that enables analysis of individual neurons. This process is referred to as a “dissociated preparation” of VSNs. The attached VSNs are incubated in a solution containing a calcium-sensing chemical dye to “load” the neurons with the fluorescent indicator. A dissociated preparation allows each neuron in the field of view to be independently selected as “regions of interest” or areas on the coverslip for commercially available software to monitor changes in calcium concentration. The recorded change in fluorescence of the dye reflects the changes in intracellular calcium within the selected VSNs. Rapid changes in calcium can be recorded and the timing of each rise in calcium can be correlated to specific ligands being introduced in the cell environment. The collected data can be studied for an in-depth comparative analysis of ligands that may broadly or specifically activate populations of VSNs.

Dissociation of the VSNs allows for a relatively rapid and even uptake of chemical dyes within each cell. Consequently, this preparation results in a highly reliable resolution of ligand responses by sensory neurons normally present in both superficial and deep layers. The preparation is highly efficient; from three mice, up to four coverslips can be prepared, each containing between 150 and 500 cells that can be imaged. Since the timing of each stimulant delivery is known, the window of time during which a neural response may occur can be precisely defined. This allows us to parse out spontaneous changes in intracellular calcium from real responses to presented ligands. Additionally, the preparation is easy to learn and does not require highly specialized equipment for imaging or analysis of gathered data. Following imaging of a dissociated preparation, it is possible to physically isolate single cells of interest from the coverslip for molecular analysis, for instance RNA in situ hybridizations, immunofluorescence, and creation of single-cell cDNA libraries. The ability to do subsequent analysis on individual activated cells is a distinct advantage of imaging dissociated neurons over whole epithelium or slice preparations.

The most prominent advantage of using chemical dyes over genetic markers is their high sensitivity in that they undergo a large change in fluorescence in response to single-action potentials [7].

Without this added sensitivity, a subset of relevant and functional neuronal activities would go undocumented, further impairing the ability to fully understand vomeronasal function. We use UV excitable ratiometric dye Fura-2-AM. Once bound to Ca^{2+} , the absorption of Fura-2-AM shifts, which can be observed by scanning the excitation spectrum from 300 to 400 nm, whereas the emission spectrum stays at ~ 510 nm (product information—Invitrogen). In the calcium-free state, fluorescence at 380 nm is higher than at 340 nm whereas in the bound state, the fluorescence at 380 nm decreases and that at 340 nm increases. The simultaneous change in absorption spectra results in a more reliable measurement of calcium concentration. By measuring fluorescence at both 340 and 380 nm a relative quantification of calcium concentrations can be made based on the 340/380 fluorescence values (product information—Invitrogen). The use of the ratio also aids in reducing artifactual information by accounting for differences in cell thickness, uneven dye loading, loss of dye, and photobleaching [8]. Several other commercially available fluorescent dyes may also be used; however an advantage of Fura-2-AM over other similar dyes is that it can be used in combination with and does not interfere with the fluorescence spectrum of GFP. Addition of chemical modifications, such as the acetoxymethyl (AM) ester [9, 10], to calcium indicators enhances their permeability and facilitates more uniform uptake by cells. Greater uptake of the dye reduces the number of dye micelles observed, which can produce a background signal as flecks of fluorescence during imaging. Noise can be further reduced by using pluronic acid, which aids in dispersion of Fura-2-AM, increasing dye uptake efficiency and breaking up fluorescent aggregates.

Despite the simplicity and sensitivity of using a dissociated preparation and calcium-sensing dye-based fluorescence imaging, the technique is not without disadvantages. Protease digestion, albeit mild, followed by dissociation of the closely packed neurons reduces overall cell health compared to vibratome slices or whole-epithelium preparations where cell connections are not entirely disrupted. A limited number of sequential ligand pulses can be perfused over the neurons before their reduced cell health affects reliable cell responses. Therefore, each step of the protocol must be carried out within a minimal amount of time so as to preserve cell health. UV phototoxicity may also reduce the longevity of the neurons during imaging; thus designing shorter experiments yields more reliable results. For each experiment, the time of stimulant or buffer delivery must be precisely noted so that data analysis can be carried out with reliable knowledge of which stimuli are responsible for cell depolarization events. Most calcium dye indicators when used in large concentrations have a calcium buffering effect whereby the peak amplitude of calcium transients is significantly reduced [11], so indicator loading must be carried out carefully to

maintain a good signal-to-noise ratio. Lastly, since the topological structure of the VNO is lost during dissociation, all information on the spatial arrangement of the neurons is irretrievable, though molecular characterization can inform on whether imaged cells are from the apical or the basal layer of the VNO. By designing experiments while keeping these caveats in mind, a vast amount of information can be gathered about the neurons of interest.

The combined use of chemical dye Fura-2-AM and a dissociated cell preparation enables characterization of the response profile of a single neuron when presented with particular ligands. With these tools, stimuli relevant to the VNO can be identified and the specificity and tuning of vomeronasal neurons can be more fully understood.

2 Materials

2.1 Dissection of VNO

1. 1× Phosphate buffer saline (PBS): pH 7.4 (*see Note 1*).
2. Forceps and surgical scissors.

2.2 Dissociation of Neurons

1. DL-Cysteine-HCl (1 M): Dissolve 15.7 mg DL-cysteine-HCl in 100 μ L distilled water (*see Note 2*).
2. Ethylene diamine tetra acetate (EDTA, 100 mM).
3. Papain: Dissolve papain in water to 0.22 U/ μ L concentration, sterile filter, and divide into 11 μ L aliquots for single use (*see Note 3*).
4. Dissociation buffer (in 1× PBS): 1.1 mM EDTA; 5.5 mM DL-cysteine-HCl; papain 2.2 U/mL. Mix 5.5 μ L DL-cysteine-HCl solution (1 M), 11 μ L EDTA solution (100 mM), and 10 μ L papain (0.22 U/ μ L) in 1 mL 1× PBS (*see Note 4*).
5. DNase solution: 10× DNase buffer; DNase I; 1× PBS. Mix 200 μ L of 10× DNase buffer and 800 μ L 1× PBS, add 50 U DNase I, and mix well (*see Note 5*).

2.3 Plating of Neurons

1. Plating media: 10 % supplemented Dulbecco's modified Eagle medium (D-MEM). D-MEM High Glucose (1×)+ GlutaMAX+Sod.Pyr, heat-inactivated fetal bovine serum (FBS). Mix 90 mL D-MEM with 10 mL heat-inactivated FBS and divide into 12 mL single-use aliquots. Pre-warm aliquots to 37 °C before use (*see Note 6*).
2. Concanavalin A (0.5 μ g/ μ L)-coated coverslips: Solid Concanavalin A (type V), distilled water. Weigh out 10 mg Concanavalin A and dissolve in 20 mL distilled water to obtain a 0.5 μ g/ μ L solution. Divide into 1–2 mL single-use aliquots. Incubate coverslips overnight in Concanavalin A solution at

4 °C, with continuous shaking. Place them in a coverslip rack to dry in a laminar flow chamber for 2–7 h and store at 4 °C (*see Note 7*).

3. Stacks (optional): Prepare stacks by cutting off a center portion of P-200 tips (Fig. 2). Autoclave the stacks before use.

2.4 Calcium Imaging

1. Hank's Balanced Salt Solution (HBSS) 10×, anhydrous calcium chloride 1,400 mg/L, magnesium chloride-6H₂O 1,000 mg/L, magnesium sulfate-7H₂O 1,000 mg/L, potassium chloride 4,000 mg/L, potassium phosphate monobasic 600 mg/L, sodium chloride 80,000 mg/L, sodium phosphate dibasic-7H₂O 900 mg/L, dextrose 10,000 mg/L (*see Note 8*).
2. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1 M (*see Note 9*).
3. Imaging buffer: Mix 50 mL HBSS (10×), 5 mL HEPES (1 M), and 445 mL of distilled water for a final volume of 500 mL (*see Note 10*).
4. Pluronic acid 20 %: Add 20 mg pluronic acid to 100 μL DMSO in an Eppendorf tube, warm in 37 °C water bath for 10–15 min with intermittent vortexing to dissolve (*see Note 11*).
5. Fura-2-AM dye (1 μg/μL): Dissolve 50 μg Fura-2-AM in 50 μL DMSO by pipetting up and down several times (*see Note 12*).
6. Loading solution: Mix 5 μL Fura-2-AM solution with 2.5 μL pluronic acid solution by pipetting up and down several times. Add 1,000 μL imaging buffer (*see Note 13*).

2.5 Equipment

1. 37 °C cell culture incubator with 5 % carbon dioxide.
2. Certified class II biological safety cabinet with laminar flow.
3. Centrifuge machine with swinging bucket rotor for 15 mL conical tubes.
4. Dissection microscope.
5. Phase contrast microscope with 10× and 20× objective lenses.
6. Inverted deconvolution microscope with a 20× fluar 0.75 objective or an upright microscope with water immersion objective.

3 Methods

All reagents and chemicals should be of cell culture grade. For best results, all solutions should be prepared using sterile technique and stored at the appropriate temperature for future use. Each step must be carried out within minimal time to ensure optimal health of the neurons.

3.1 VNO Dissection

1. Transfer 1 mL 1× PBS to a well in a 4-well plate and set on ice. Transfer 20 mL 1× PBS each into two petri dishes and chill on ice.
2. Prepare papain-containing dissociation buffer immediately prior to starting dissections. Incubate on ice.
3. Isolate both VNO lobes from a total of three mice transferring each upon removal to a petri dish containing chilled PBS on ice from **step 1**. For best results, this step should be completed within 10–15 min.
4. Move VNOs to the second petri dish containing chilled PBS one at a time. Remove cartilage from each VNO lobe and transfer tissue to chilled PBS in the 4-well plate prepared in **step 1**.

3.2 Dissociation of Vomeronasal Neurons

1. Once all VNOs have been removed from their cartilage, carefully aspirate PBS in the well and replace with 1 mL ice-cold dissociation solution prepared in Subheading 3.1, **step 2**.
2. Dissociate each lobe of the VNO by tearing with fine forceps into minute pieces (Fig. 1). For best results, complete within 10 min.
3. Transfer solution containing dissociated VNO tissue to a 15 mL Falcon tube and incubate at 37 °C for 15–20 min with continuous shaking (at approximately 225 rpm).
4. Prepare the DNase solution.
5. Add DNase solution to dissociated VNO tissue and gently triturate until all aggregates are dispersed (*see Note 14*).

3.3 Plating Vomeronasal Neurons

1. Add 10 mL of pre-warmed D-MEM–FBS media. Tap several times to mix and spin at 1,000 rpm (200g) for 5 min, at room temperature.

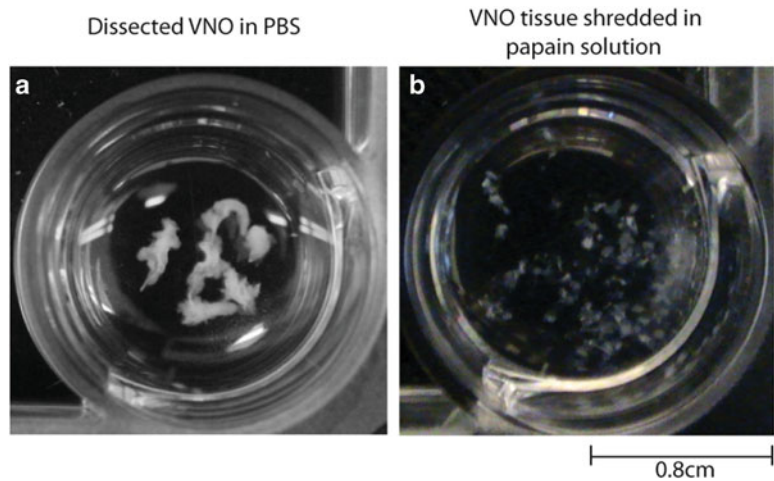


Fig. 1 Dissociating VNO tissue in protease solution (a) before and (b) after. Three whole VNOs are depicted before and after

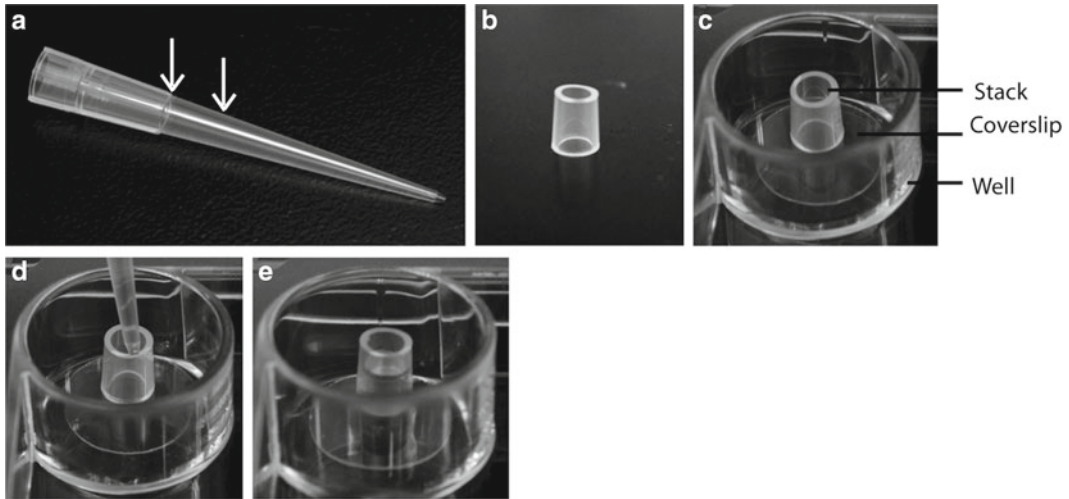


Fig. 2 (a and b) Preparing a stack. (a) Cut a 200 μL pipette tip at the positions indicated by *white arrows*. (b) A stack obtained from the pipette tip. (c) Stack placed on a Concanavalin A-coated glass coverslip in a well. (d) Pipetting cell suspension in media inside the stack. (e) Media containing dissociated neurons pipetted in stacks, ready to be incubated at 37 $^{\circ}\text{C}$

2. Place one Concanavalin A-coated coverslip per well in a new 4-well plate. Place a stack in the center of each coverslip to concentrate application of cells (Fig. 2).
3. Aspirate supernatant without disturbing the pellet. Gently resuspend in 100 μL of D-MEM-FBS media.
4. Transfer 25 μL of resuspended VSNs onto each coverslip inside the stack.
5. Incubate at 37 $^{\circ}\text{C}$ for 45–60 min. Check to see if cells are attached to the coverslip using a phase contrast microscope (density similar to Fig. 3).

3.4 Loading Vomeronasal Sensory Neurons with Calcium- Sensing Dye

1. Thaw Fura-2-AM at room temperature.
2. Prepare dye loading solution, cover in aluminum foil, and store in the dark through experiment.
3. Remove stack from the coverslip, transfer the coverslip to a well, and gently layer with 250 μL dye loading solution. Incubate the coverslip for 15–30 min at room temperature, covered in aluminum foil.
4. Remove coverslip from dye solution and assemble it on the stage. Gently cover the cells with 250 μL of imaging buffer to prevent drying (*see Note 15*). Coverslip is now ready for imaging.

3.5 Calcium Imaging

1. Prior to imaging, wash stimulant/buffer delivery system and accessory tubes thoroughly with distilled water. Other wash solutions may also be used based on the solubility of stimulants used.

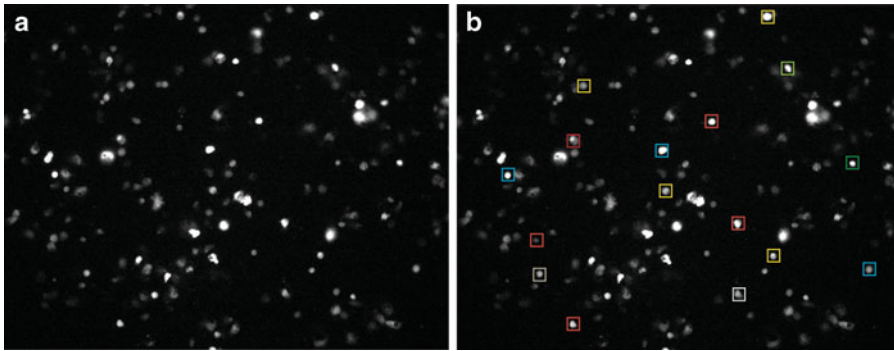


Fig. 3 (a) A field of view of dissociated cells under 380 nm. (b) Individual cells selected, shown in *colored squares* as “regions of interest”

2. Dilute stimulants in imaging buffer to desired strength.
3. Set up dye-loaded coverslip on microscope and set perfusion system up to wash cells with imaging solution.
4. Using a data collection software such as MetaFluor, select each dissociated cell as an individual “region of interest” (Fig. 3). Select an empty region of the coverslip for background subtraction during data collection (*see Note 16*).
5. Apply stimulant(s) for desired duration followed by imaging buffer for an intervening duration to wash cells before perfusing the next stimulant (*see Note 17*). Following the last test stimulant, perfuse a pulse of a known activator of the cells of interest (for a positive control). If such a positive control ligand is not defined for cells of interest, pool individual stimulants to pulse at the end (*see Notes 18–20*).

3.6 Data Analysis

1. Identify the cells that show an increase of the 340/380 ratio measuring more than 1.5 times the baseline signal during the time window(s) of positive control or pooled stimulant application. Graphing software such as Microsoft Excel may be used to do this (*see Note 21*). Plot the 340/380 fluorescence ratio for each region of interest that shows the increase in calcium coinciding with the delivery of the positive control or pooled stimulant.
2. Precisely define the time during the experiment when stimulus was perfused over the cells. In case the stimulants are delivered through a tube, determine the length of time required for the stimulant front to reach the coverslip so that the time of delivery of stimuli is defined precisely (*see Note 22*). If analyzing by a trace plot, annotate the plot from the time the stimulus reaches the coverslip to the time the subsequent wash reaches the coverslip.

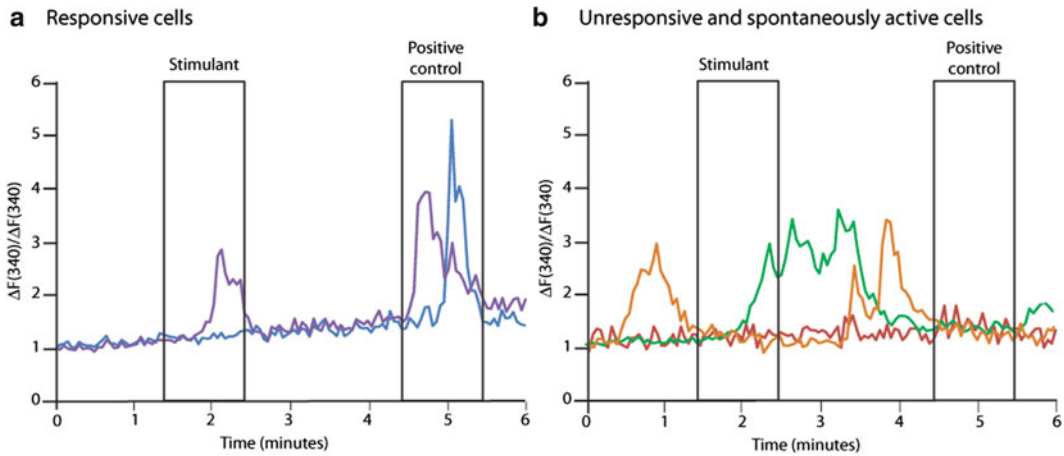


Fig. 4 Example of output data. Each *colored line* represents the calcium trace of a single neuron, the *black rectangles* represent time windows for application of stimulus. **(a)** Responsive cells. *Purple*: Cell showing rise in intracellular calcium on application of test stimulus and positive control. *Blue*: Cell showing rise in intracellular calcium on application of positive control only. **(b)** Unresponsive and noisy cells. *Red*: Unresponsive cell. *Orange*: Cell showing intracellular calcium increase before application of test stimulus and positive control. *Green*: Cell showing intracellular calcium increase randomly on application of test stimulus but not to positive control. (X-axis: Ratio of fluorescence change measured at 340 nm and that measured at 380 nm, Y-axis: Time (in min))

3. To define a cell of interest as “responsive,” we use the following criteria (Fig. 4):

- Cells must respond to the positive control stimulus pulse at the end.
- An increase in fluorescence ratio measuring at least 1.5 times the baseline during the time of interest is counted as a response.
- Cells must only respond during the time that stimuli are present on the coverslip (*see Note 23*).
- Cells must not show an increase in calcium greater than 1.5 times the baseline signal outside of the stimulus windows.

4 Notes

1. PBS used for dissection may be kept chilled on ice as VNOs are dissected. Chilled PBS is helpful in keeping the neurons alive if dissection takes longer than 15 min.
2. Prepare fresh each day.
3. Papain aliquots dissolved in distilled water may be stored at $-20\text{ }^{\circ}\text{C}$ up to several months. To avoid repeated freeze–thaw

- cycles, they are best divided into single-use aliquots. Thaw before preparing dissociation buffer.
4. Prepare fresh each day. Once prepared, keep dissociation buffer on ice until use to avoid losing enzyme activity.
 5. Prepare DNase solution right before use while neurons are incubating in 37 °C shaker. If DNase buffer is not available, this may be substituted by other buffers in which DNase is fully active, for instance 10× PCR buffer or 10× endonuclease buffer.
 6. Prepare 10 % (by volume) FBS-supplemented DMEM in the laminar flow chamber to keep it contamination free and store aliquots at 4 °C. Pre-warm each aliquot at 37 °C at the start of the experiment to avoid adding cold media to cells.
 7. Of the available reagents, we have found that the use of Concanavalin A to coat coverslips allows for the maximum number of cells to adhere after plating. Aliquots of 0.5 µg/µL Concanavalin A may be stored at -20 °C and thawed before use.
 8. Store 10× HBSS at 4 °C after opening.
 9. Store 1 M HEPES at 4 °C.
 10. Make fresh each day. Keep at room temperature after preparation.
 11. Make fresh every 7–10 days. Store at room temperature.
 12. Fura-2-AM is light sensitive. Once dissolved, keep Fura-2-AM solution wrapped in aluminum foil at -20 °C. Thaw before use each day.
 13. Prepare loading solution fresh each day. Wrap loading dye in aluminum foil and store at room temperature while experiment is in progress.
 14. At the end of protease digestion, the pieces of tissue appear to aggregate together. After addition of DNase, the pieces of tissue appear to be more disintegrated: fewer and smaller pieces of tissue should be seen in suspension. The solution should appear more turbid than that in **step 2** (Subheading 3.2).
 15. Do not pipet the solution directly on the coverslip. Instead pipet the solution on the side of the stage and tip the stage to cover the cells. This will prevent cells from being forcibly dislodged from the coverslip.
 16. For urinary proteins and total urine, we have empirically determined that setting the 340–380 gain controls at a ratio of 3:1 obtains the optimal signals to differentiate neuronal responses from noise in our setup. This may have to be adjusted for different ligands and instrumental setups.
 17. We apply stimuli such as mouse urinary proteins or total urine for 1 min alternating with buffer for 2 min. These durations of

stimulus and buffer pulses may have to be varied according to the nature and concentrations of different ligands.

18. A positive control pulsed at the end of the experiment helps to identify cells that survived through the entire experiment. Only these cells can be investigated for their response to all the ligands presented during the course of the experiment. Another possible positive control for neurons is potassium chloride [12].
19. In our setup, a perfusion rate of approximately 5 mL per minute is optimal to obtain laminar flow of buffer over the neurons. This may have to be optimized differently for each setup.
20. To obtain the most accurate results, it is necessary to image each coverslip quickly. Depending on the quality of the preparation, and the nature and concentration of ligands, up to five total pulses may be applied, spanning approximately 15 min of imaging time. To maximize speed, subsequent coverslips should be incubated in dye solution while the previous is being imaged such that one can complete imaging four coverslips in 90 min or less. If the cells are imaged for longer than 15 min, the constant exposure to pulses of ligands and UV light may adversely affect cell viability, leading to less accurate output. As previously noted, one preparation yields four coverslips. If each coverslip is imaged within 15–20 min, there is no substantial loss of cell health between neurons from the first and last coverslip. Cell health may not be optimal if neurons are not imaged within 3 h of plating.
21. We have empirically determined, for mouse urine and urinary proteins, that a 1.5-fold increase in the 340–380 nm ratio is a good indicator of neuronal response. This may have to be optimized for various ligands and instrument setup. We use this cutoff to sort our data, separating cells that show an increase in calcium during presentation of the positive control from the rest of the imaged cells. This way the analysis can be focused on responsive cells instead of every single imaged cell, many of which show no changes in calcium during the experiment.
22. Delivery time may be determined by introducing a bubble or a colored dye in the delivery tube and then monitoring the time taken by the bubble or the dye front to reach the end of the tubing with a stopwatch or a timer.
23. There are spontaneous changes in calcium in cells, a natural phenomenon noticed in many types of preparations [12]. Being able to differentiate spontaneous activity and ligand-based activity is important in order to use this technique to follow ligand activity. A precise notation of the window of stimulus perfusion and repetitive pulses of the same stimulus enables correlation between stimulus perfusion and calcium changes within the cell.

References

1. Touhara K, Vosshall LB (2009) Sensing odorants and pheromones with chemosensory receptors. *Annu Rev Physiol* 71:307–332
2. Malnic B, Hirono J, Sato T, Buck LB (1999) Combinatorial receptor codes for odors. *Cell* 96(5):713–723
3. Munger SD, Leinders-Zufall T, Zufall F (2009) Subsystem organization of the mammalian sense of smell. *Annu Rev Physiol* 71:115–140
4. Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A et al (2007) Identification of protein pheromones that promote aggressive behaviour. *Nature* 450(7171):899–902
5. Kaupp UB (2010) Olfactory signalling in vertebrates and insects: differences and commonalities. *Nat Rev Neurosci* 11(3):188–200
6. Grienberger C, Konnerth A (2012) Imaging calcium in neurons. *Neuron* 73(5):862–885
7. Smetters D, Majewska A, Yuste R (1999) Detecting action potentials in neuronal populations with calcium imaging. *Methods* 18(2):215–221
8. Becker PL, Fay FS (1987) Photobleaching of fura-2 and its effect on determination of calcium concentrations. *Am J Physiol* 253(4 Pt 1):C613–C618
9. Tsien RY (1988) Fluorescence measurement and photochemical manipulation of cytosolic free calcium. *Trends Neurosci* 11(10):419–424
10. Tsien RY (1981) A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* 290(5806):527–528
11. Saftienku EE, Teslenko VI (ed) (1995) Effect of fura-2 on calcium transients and its dependence on kinetics and location of endogenous buffers (a model study). Kluwer Academic-Plenum, New York
12. Holy TE, Dulac C, Meister M (2000) Responses of vomeronasal neurons to natural stimuli. *Science* 289(5484):1569–1572