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TITLE:

Investigation of activated mouse olfactory sensory neurons via combined immunostaining and *in situ* hybridization

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KEYWORDS:

Neuronal activation, *In situ* hybridization, Olfactory sensory neurons, Vomeronasal receptors, Immunohistochemistry, Immunofluorescence, pS6 staining

SUMMARY:

This protocol combines *in situ* hybridization with immunofluorescence to identify olfactory and vomeronasal receptor genes expressed in olfactory sensory neurons after activation by chemical stimuli in the mouse.

ABSTRACT:

Animals rely on chemical communication to convey and perceive relevant environmental information, ranging from assessment of food quality to detection of available mating partners or threats. In mice, this task is executed primarily by the olfactory system and its underlying subsystems, including the main and accessory olfactory systems. Both have peripheral organs populated by sensory neurons expressing G-protein coupled receptors able to bind chemical cues that reach the nasal cavity. Even though the molecular characteristics of these receptors is well understood, little is known about their cognate specific ligands. The method described here combines *in situ* hybridization detection of olfactory or vomeronasal receptors with immunodetection of phosphorylated ribosomal protein S6 (pS6) – a marker of neuronal activation. This protocol was devised to identify neurons activated after a single event of exposure to purified or complex chemical stimuli detected by the olfactory organs. Importantly, this technique allows the investigation of neurons triggered in biologically relevant contexts. Ideally, this method should be used to probe the molecular biology of the olfactory system and to study olfactory behaviors.

INTRODUCTION:

Chemosignaling is the most widespread form of communication in animals. In mammals, detection of chemical cues is mediated mainly through olfaction and is paramount for finding food, locating possible mating partners, and avoiding potential predators[1–3]. The mouse olfactory system is further divided into different subsystems, each with their own anatomical, physiological and molecular properties[4]. Among these, the main olfactory system (MOS) and accessory olfactory system (AOS) are the most widely studied and better characterized.

The MOS is mostly responsible for the detection of volatile chemical molecules that reach the nasal cavity. This is accomplished by olfactory sensory neurons (OSNs), which populate the system's sensory interface — the main olfactory epithelium (MOE). Each OSN expresses one out of thousands of olfactory receptors (ORs) in a monogenic and monoallelic fashion[5]. Furthermore, ORs tend to be broadly tuned[6], and a single odorant may bind to more than one OR, generating a combinatorial code for odors[7].

In turn, the AOS is mainly responsible for pheromone and kairomone detection. These ligands activate vomeronasal sensory neuros (VSNs) in the vomeronasal organ (VNO). VSNs in the apical zone of the VNO express receptors from the vomeronasal receptor 1 family (V1Rs)[8], while neurons in the basal zone express the vomeronasal receptor 2 family (V2Rs)[9]. Importantly, it has been shown that some VSNs co-express more than one vomeronasal receptor[10–12].

Despite all available information on the molecular characteristics of ORs and VRs, knowledge on their cognate ligands is still very limited. The identification of specific olfactory neurons responsible for the detection of a molecule or complex stimulus is an important task for those investigating olfaction, olfactory-driven behaviors, and physiological responses. Several strategies have been attempted to deorphanize olfactory receptors, including calcium-imaging[7], single-cell RNA sequencing[13, 14], heterologous receptor expression[15], and strategies based on immediate early genes (IEGs)[16]. More recently, some techniques have been employed to assess the modulatory effects of odorants in sensory neurons, using *in vivo* calcium imaging[17, 18] and fluorescent-protein tagged single-cell RNA sequencing strategies[19]. Most of these techniques demand an artificial setup or are performed on dissociated neurons, making it impossible to simultaneously assess behaviors triggered by the odors used.

The protocol described here allows the identification of sensory neurons activated after a single event of odor exposure, by combining riboprobes that detect specific ORs or VRs with the immunodetection of phosphorylated S6 ribosomal protein (pS6), a proxy for neuronal activation. This method is a reliable way to investigate the identity of sensory neurons activated by different chemosignals in a biologically relevant context.

PROTOCOL:

Animal procedures were carried out in accordance with Animal Protocol #1883-1, approved by the University of Campinas (Institute of Biology's Institutional Animal Care and Use Committee -

Committee for Ethics in Animal Use in Research), which follows the guidelines established by the federal National Council for Animal Experimentation Control (CONCEA).

1. Material preparation

1.1. Prepare 1 L of 3% H_2O_2 (v/v) in 1x PBS. Dilute 100 mL 10× PBS and 100 mL of 30% H_2O_2 in 500 mL of ultrapure water. Complete the volume with ultrapure water. Prepare this solution immediately before use.

1.2. Prepare a humidified chamber containing pieces of lint-free laboratory wipes wet with 5× SSC solution.

1.3. Prepare hybridization wash solutions: 5× SSC (at 55 °C), 2× SSC (55 °C), 0.2× SSC (55 °C) and 0.1× SSC (55 °C and room temperature). Dissolve the appropriate volumes of 20× SSC stock solution in RNase-free ultrapure water. Prepare the diluted solutions fresh before the start of each experiment.

1.4. Prepare immunohistochemistry blocking solution: 1× PBS/1% BSA/0.1% Triton X-100. For 100 mL, dilute 10 mL of 10× PBS, 10 mL of 10% BSA and 3.33 mL of 3% Triton X-100 in 70 mL of ultrapure water. Complete the volume with ultrapure water. Prepare the diluted solutions fresh before the start of each experiment.

1.5. Prepare PTw solution: 1× PBS/0.1% Tween-20. For 100 mL, dilute 1 mL of 10% Tween-20 and 10 mL of 10× PBS in 70 mL of ultrapure water. Complete the volume with ultrapure water. Prepare this solution fresh before the start of each experiment.

1.6. Prepare RNase-free 0.1 M triethanolamine solution. For 250 mL, dilute 3.33 mL of reagent-grade triethanolamine in 200 mL of RNase-free ultrapure water under agitation (use RNase-free glassware and magnetic stirrer when preparing this solution). Adjust pH to 8.0 with HCl. Complete the volume with RNase-free ultrapure water and store protected from light until use. Prepare this solution fresh before the start of each experiment.

1.7. Prepare RNase-free 0.1% H_2O_2 (v/v) in 1× PBS. For 1 L, dilute 100 mL of 10× PBS and 3.3 mL of 30% H_2O_2 in 500 mL of RNase-free ultrapure water. Complete the volume with RNase-free ultrapure water. Prepare this solution immediately before use.

1.8. Prepare RNase-free solutions of the following: 0.2 M HCl; 1 M Tris-HCl pH 8.0; 10 mg/mL yeast tRNA; 10% SDS; 10× PBS; 100× Denhardt's solution; 20× SSC; 50% dextran sulfate; 6 M HCl

1.9. Prepare RNase-free 4% paraformaldehyde fixative solution. For 100 mL, dissolve 4 g of paraformaldehyde in 80 mL of 1× PBS. Adjust pH to 7.4 with 10 M NaOH. Complete the volume with 1× PBS. This solution must be prepared fresh before the start of each experiment.

Caution: Paraformaldehyde is a hazardous substance that can potentially cause harm if inhaled. Prepare solution under a fume hood and wear protective gloves, mask, and safety goggles.

1.10. Prepare RNase-free dissection solution: 30% sucrose/0.45 M EDTA pH 8.0/1× PBS. For 100 mL, dissolve 30 g of sucrose in 60 mL of RNase-free 0.5 M EDTA pH 8.0. Add 10 mL of 10× PBS. Complete the volume with RNase-free 0.5 M EDTA pH 8.0.

1.11. Prepare RNase-free glass or plastic graduated cylinders and beakers.

1.12. Prepare RNase-free pre-hybridization and hybridization solutions: 50% deionized formamide (v/v)/600 mM NaCl/200 μg/mL yeast tRNA/0.25% SDS (w/v)/10 mM Tris-HCl pH 8.0/1× Denhardt's solution/1 mM EDTA pH 8.0/10% dextran sulfate (w/v). For 10 mL in a graduated cylinder, add 5 mL of deionized formamide, 1.2 mL of 5 M NaCl, 200 μL of 10 mg/mL yeast tRNA, 250 μL of 10% SDS, 100 μL of 1 M Tris-HCl pH 8.0, 200 μL of 50× Denhardt's solution, 20 μL of 500 mM EDTA pH 8.0, and 2 mL of 50% dextran sulfate (M.W. 500,000). Complete the volume with RNase-free ultrapure water. Prepare this solution fresh before the start of each experiment.

NOTE: Dextran sulfate is very viscous. It is advisable to prepare an extra 20% of the total volume of hybridization/pre-hybridization solutions to account for pipetting errors. Yeast tRNA may be omitted from the pre-hybridization solution, if desired.

1.13. Prepare thermoplastic laboratory film coverslips. Cut rectangular thermoplastic laboratory film pieces 1-2 mm shorter than the length and width of a microscope slide.

1.14. Prepare TN buffer: 100 mM Tris-HCl/150 mM NaCl. For 100 mL, dilute 10 mL of 1 M Tris-HCl pH 7.5 and 3 mL of 5 M NaCl in 70 mL of ultrapure water. Complete the volume with ultrapure water. Prepare this solution fresh before the start of each experiment.

1.15. Prepare TNB blocking buffer: 0.5% blocking reagent A (w/v) in TN Buffer. For 200 mL, dissolve 1 g of blocking reagent A in 200 mL of TN Buffer (see **Table of Materials** for commercial source of blocking reagent A).

1.16. Prepare TNT buffer: 0.05% Tween-20 in TN Buffer. For 1 L, dilute 5 mL of 10% Tween-20 in 950 mL of TN buffer. Slowly complete the volume with TN buffer to avoid foaming. Prepare this solution fresh before the start of each experiment.

1.17. Prepare Tyramide-Alexa 488 working solution: 1× amplification buffer A/0.0015% H_2O_2/1:100 tyramide-Alexa 488. For 100 μL, dilute 1 μL of 0.15% H_2O_2 and 1 μL of tyramide-Alexa 488 in 98 μL of amplification buffer A (see **Table of Materials** for commercial sources of tyramide-Alexa 488 and amplification buffer A). Prepare this solution immediately before use.

1.18. Prepare Tyramide-Alexa 555 working solution: 1× amplification buffer A/0.0015% H_2O_2/1:100 tyramide-Alexa 555. For 100 μL, dilute 1 μL of 0.15% H_2O_2 and 1 μL of tyramide-Alexa 555 in 98 μL of amplification buffer A (see **Table of Materials** for commercial sources of tyramide-Alexa 555 and amplification buffer A). Prepare this solution immediately before use.

1.19. Prepare Tyramide-biotin working solution: 1× amplification buffer B/0.0015% H_2O_2/1:50 tyramide-biotin. For 100 μL, dilute 1 μL of 0.15% H_2O_2 and 2 μL of tyramide-biotin in 97 μL of amplification buffer B (see Table of Materials for commercial sources of tyramide-biotin and amplification buffer B). Prepare this solution immediately before use.

1.20. Bake glassware at 200 °C for at least 4 h. Treat non-disposable plasticware (including slide racks, Coplin jars and syringes) with 3% H_2O_2 for 15-30 min, followed by thorough washing under RNase-free water. Treat stainless steel metal instruments, such as dissection tools, forceps, and cryostat specimen holders, with 3% H_2O_2 for 15 min, followed by thorough washing under RNase-free water. Clean bench surfaces, heated plate, and dry blocks with RNase cleaning solution.

2. Riboprobe synthesis

NOTE: The following procedure to synthesize 1 kb digoxigenin-labeled cRNA probes for *in situ* hybridization via *in vitro* transcription is based on previously published protocols[20–23].

2.1. Use appropriate oligonucleotide primer pairs to amplify a 1 kb DNA fragment from the gene of interest (e.g., olfactory receptor gene). Select primer pairs that amplify a region present in the mature mRNA to be detected (for example, the gene's coding sequence). A selection of primers for different olfactory receptors is provided in the **Table of Materials**. For details on probes to detect other vomeronasal olfactory receptors, please check previous publications[16,21].

2.2. Run the PCR products on an 0.8% agarose gel and remove the desired 1 kb band.

2.3. Purify the DNA fragment using a suitable mini column-based gel purification kit according to manufacturer's protocol.

2.4. Quantify the purified DNA fragment and clone it into a suitable PCR cloning vector according to manufacturer's protocol. It is advisable to use cloning vectors containing T7 and SP6 RNA polymerase promoters flanking the cloned amplicon.

2.5. Transform the recombinant plasmid into competent recombination-free bacteria via chemical transformation or electroporation according to manufacturer's protocol.

2.6. Seed isolated transformant bacterial colonies into rich medium and isolate the plasmid via DNA mini-preparation according to manufacturer's protocol.

2.7. Perform restriction digestion analysis with suitable restriction enzymes and/or Sanger sequencing to check the identity and orientation of the inserted DNA fragment according to manufacturer's protocol.

2.8. Perform restriction digestion to linearize 10 μg of the plasmid using an appropriate restriction enzyme. To produce an anti-sense riboprobe after *in vitro* transcription, use a restriction enzyme that digests the plasmid DNA at the extremity of the insert opposite to the RNA polymerase promoter located downstream to the gene's coding sequence. To produce sense riboprobes after *in vitro* transcription, use a restriction enzyme that digests the plasmid DNA at the extremity of the insert opposite to the RNA polymerase promoter located upstream to the gene's coding sequence.

2.9. Run an aliquot of the plasmid linearization reaction product on a 0.8% agarose gel to confirm completeness of digestion. Once complete linearization is confirmed, purify the remainder of the reaction using a column-based PCR cleanup kit according to manufacturer's protocol.

2.10. Synthesize digoxigenin-labeled cRNA probes by *in vitro* transcription using 1-2 μg of the linearized plasmid as a template, the appropriate enzyme (e.g., T7 or SP6 RNA polymerase), and a suitable digoxigenin (DIG) labeling kit.

2.11. Remove the DNA template from the reaction by digesting with DNase I at 37 °C for 30 min.

2.12. Purify the cRNA probes using either a mini column-based RNA purification kit or a gel filtration-based purification kit. Elute the resulting probe in 50 μL of RNase-free water.

2.13. Quantify the resulting riboprobe, preferably using fluorometric methods. Usually, the expected yield is above 100 ng/μL.

2.14. Run the riboprobe on an automated electrophoresis system or on an RNase-free 0.8% agarose gel to check for probe quality and degradation.

2.15. Store the synthesized probe at -80 °C until needed.

3. Exposure of mice to olfactory stimuli

3.1. Individually house each animal in a clean cage for at least 24 h prior to exposure. Remove the food grid at least 2 h before exposure. All animals used in this study were 8-12 weeks old male C57BL/6 mice (average weight: 20 g).

3.2. Prepare the appropriate olfactory stimulus. Different stimuli can be used to activate olfactory neurons in the MOE (main olfactory epithelium) or VNO (vomeronasal organ). For a comprehensive list of olfactory stimuli, please consult other publications[3, 16, 22–24]. Liquid stimuli,

such as chemical solutions, urine, or recombinant protein solution, can be deposited on a medical gauze or cotton ball.

3.3. Insert the olfactory stimulus into the cage and leave the animal undisturbed for 1 h.

3.4. Euthanize the mouse subject and quickly dissect the MOE or VNO using RNase-free dissecting tools.

4. Tissue dissection and freezing

4.1. Dissect the MOE or VNO under a stereomicroscope using dissection solution.

4.1.1. For the VNO, remove the thick septal bones, leaving the softer cartilage shells surrounding the organ.

4.2. Prepare specimen for cryo-sectioning.

4.2.1. Blot the specimen dry on a piece of blotting paper and place it inside a histological plastic mold containing embedding medium.

4.2.2. For VNO, arrange each organ vertically and pull it to the bottom of the plastic mold with the help of a 1 mL syringe (**Figure 1A**).

4.2.3. For MOE, place it at the bottom of the plastic mold with the cribriform plate facing down.

4.2.4. Remove the excess of bubbles around the specimen since these can interfere with sectioning.

4.2.5. Freeze specimens on dry ice.

4.2.6. Store the specimen block at -80 °C.

NOTE: Frozen specimens may be stored for several months before sectioning.

4.3. Perform cryo-sectioning.

4.3.1. Turn the cryostat on and set the temperature to -23 °C at least 4 h prior to use.

4.3.2. Remove the frozen block from the histological plastic mold and trim it with a razor blade.

4.3.3. Leave 5 mm around the specimen, as this makes section handling easier (**Figure 1B**).

4.3.4. Use embedding medium to attach the block to the cryostat's specimen holder.

4.3.5. Collect 16 μm sections onto positively charged microscope slides.

4.3.6. Store the slides at -80 °C.

NOTE: Slides may be stored at -80 °C for several months before *in situ* hybridization.

5. Step-by-step *in situ* hybridization protocol

5.1. Dry the slides for 10 min using a hair dryer. Use the warm jet to defrost the slides, then switch to room-temperature jet until the embedding medium is opaque.

5.2. Add 500 μL per slide of RNase-free 4% paraformaldehyde fixative solution and incubate at 23-26 °C for 15 min to fix the histological sections.

5.3. Wash the slides twice in RNase-free 1× PBS for 5 min each.

5.4. Add 500 μL per slide of RNase-free 0.2 M HCl and incubate at 23-26 °C for 10 min.

5.5. Wash the slides twice in RNase-free 1× PBS for 5 min each.

5.6. Add 500 μL per slide of RNase-free 0.1% H_2O_2/1× PBS and incubate for 30 min at 23-26 °C. Perform this step in the dark to prevent light-induced degradation of hydrogen peroxide.

5.7. Wash slides twice in RNase-free 1× PBS for 5 min each.

5.8. Perform acetylation by transferring slides to a jar containing 250 mL of RNase-free triethanolamine 0.1 M pH 8.0 and a magnetic stir bar, in a fume hood. Add 625 μL of acetic anhydride under constant stirring. Turn stirring speed down, add another 625 μL drop by drop onto the slides, then turn stirring off and incubate for 10 min.

5.9. Wash slides twice in RNase-free 1× PBS for 5 min each.

5.10. Wash slides once in RNase-free 1× PBS at 60 °C for 5 min.

5.11. Perform pre-hybridization by removing slides from 1× PBS one at a time and blotting each of them dry using lint-free laboratory tissue paper. Pipet 400 μL of pre-hybridization solution onto each slide and place it inside a humidified chamber pre-warmed at 60 °C in a water bath or heated oven. Incubate at 60 °C for 1 h.

5.12. Heat up enough hybridization solution to 60 °C, aliquot 200 μL per slide in microfuge tubes, and heat them up to 85 °C for 10 min.

5.13. Add the appropriate cRNA probe(s) to the hybridization solution tubes (1 μg/mL each probe) and denature at 85 °C for 5 min.

5.14. For hybridization, take one slide at a time from the humidified chamber, blot it dry and place it onto a 60 °C heated plate. Add 200 μL of riboprobe-containing hybridization solution and cover the slide with a glass coverslip. Incubate for 12-16 h in the humidified chamber at 60 °C.

NOTE: Do not allow slides to cool down after pre-hybridization. Hybridization temperature must be empirically determined for each probe. Sixty degrees Celsius is suggested as a starting point, since it generally works well for 1 kb probes.

5.15. Heat SSC wash solutions to 55 °C in separate jars and place them in a water bath at the appropriate temperature.

5.16. After hybridization is finished, remove the coverslip from each slide using a bath containing 5× SSC solution at 55 °C. Hold each slide horizontally inside the warm SSC solution. When the coverslip has detached, tilt the slide and let the coverslip fall down to the bottom.

5.17. Wash with 2× SSC at 55 °C for 30 min in a slide jar.

5.18. Wash with 0.2× SSC at 55 °C for 20 min in a slide jar.

5.19. Wash with 0.1× SSC at 55 °C for 20 min in a slide jar.

5.20. Transfer to 0.1× SSC at 23-26 °C and incubate for 3 min in a slide jar.

5.21. Wash in PTw for 10 min in a slide jar.

5.22. Wash twice in TN Buffer, for 5 min each, in a slide jar.

5.23. Pipet 600 μL of TNB blocking buffer onto each slide and incubate for 3 h at 23-26 °C.

5.24. Pipet 600 μL of primary antibody solution (peroxidase-conjugated anti-DIG antibody diluted 1:400 in TNB solution) onto each slide. Carefully overlay a piece of thermoplastic laboratory film onto each slide and incubate for 12-16 h.

5.25. Wash slides 6 times with TNT Buffer for 5 min each, under mild stirring in a slide jar.

5.26. Pipet 100 μL of tyramide-biotin working solution onto each slide. Cover with thermoplastic laboratory film coverslips and incubate for 12 min at 23-26 °C.

NOTE: Perform this incubation step in a staggered fashion, with 2 or 3 slides per batch, to allow good control over incubation times. Duration of tyramide incubation may need to be optimized, depending on cRNA probe sensitivity and concentration.

5.27. Wash slides 6 times with TNT Buffer for 5 min each, under mild stirring in a slide jar.

5.28. Pipet 200 µL of solution containing peroxidase-conjugated streptavidin (SA-HRP, diluted 1:100 in TNB buffer) onto each slide and cover with a thermoplastic laboratory film coverslip. Incubate at 23-26 °C for 1 h.

5.29. Wash slides 6 times with TNT Buffer for 5 min each, under mild stirring in a slide jar.

5.30. Pipet 100 µL of tyramide-Alexa 488 working solution onto each slide, cover with thermoplastic laboratory film coverslip and incubate at 23-26 °C for 12 min in the dark.

5.31. Wash slides 6 times with TNT Buffer for 5 min each, under mild stirring in a slide jar.

5.32. Incubate in 3% H_2O_2/1× PBS in a slide jar for 1 h, to inactivate peroxidases from the previous steps.

5.33. Wash slides 6 times with TNT Buffer for 5 min each, under mild stirring in a slide jar.

6. pS6 immunostaining

6.1. Pipet 500 µL of 4% paraformaldehyde/1× PBS solution onto each slide and incubate at 23-26 °C for 15 min to fix sections.

6.2. Pipet 500 µL of 1× PBS/0.1% Triton X-100 onto each slide and incubate for 5 min to permeabilize sections.

6.3. Wash twice with 1× PBS in a slide jar.

6.4. Pipet 2-3 drops per section of commercially available tyramide signal amplification blocking solution B (**Table of Materials**) and incubate at 23-26 °C for 1 h.

6.5. Pipet 400 µL of immunohistochemistry blocking solution onto each slide and incubate at 23-26 °C for 30 min to block sections.

6.6. Pipet 200 µL of rabbit anti-pS6 primary antibody solution (diluted 1:200 in immunohistochemistry blocking solution; final concentration: 1 µg/mL) onto each slide, cover with thermoplastic laboratory film coverslip, and incubate at 4 °C for 12-16 h.

6.7. Wash 3 times with 1× PBS/0.1% Triton X-100, for 5 min each in a slide jar.

6.8. Pipet 2-3 drops per slide of commercially available peroxidase-conjugated anti-rabbit secondary antibody solution (**Table of Materials**) and incubate at 23-26 °C for 1.5 h.

6.9. Wash 3 times with 1× PBS/0.1% Triton X-100, for 5 min each in a slide jar.

6.10. Blot slides dry and pipet 100 to 200 µL of tyramide-Alexa 555 working solution onto each slide, overlay with a piece of thermoplastic laboratory film coverslip, and incubate for 7 min in the dark.

6.11. Wash twice with 1× PBS for 5 min each in a slide jar.

6.12. Pipet 500 µL of diluted nuclear stain (in 1× PBS) onto each slide and incubate at 23-26 °C for 30 min.

6.13. Wash twice with 1× PBS at 23-26 °C for 5 min each in a slide jar.

6.14. Mount slides with anti-fading mounting medium and let the mounting medium cure for 24h in the dark.

6.15. Store slides at the appropriate temperature until imaging, to avoid fluorescence fading.

7. Microscopy imaging

7.1. Image slides using a epifluorescence or confocal microscope equipped with appropriate filters for the fluorophores used.

REPRESENTATIVE RESULTS:

The current protocol aims at obtaining microscopy images in which the experimenter's gene of interest and the neuronal activity marker pS6 are fluorescently labeled. The described method involves Tyramide Signal Amplification, which produces clear and strong labeling with little to no background. pS6 immunostaining appears as cytoplasmic fluorescent signal that usually fills the entire neuronal cell body, whereas *in situ* hybridization signal for olfactory receptor neurons shows as cytoplasmic staining. pS6 is a transient marker of olfactory neuron activation and therefore the associated immunostaining signal will appear stronger in cells that have been activated in the 1 h period preceding sensory organ fixation. Fluorescence crosstalk between staining for pS6 and receptors is not expected, provided the appropriate bandpass microscopy filters are used.

Labeled sections can be used to evaluate co-localization between the two types of staining, allowing for the identification of genes expressed by the population of activated neurons under study. **Figure 2A,B** shows VNO images from a mouse exposed to cat odor, with co-localization of signals for V2R receptors in clade A4 and for pS6, indicating that neurons expressing those olfactory receptors are activated by the stimulus used. Conversely, when activated neurons do not express the olfactory receptors under scrutiny, no co-localization between signals is expected (**Figure 2C,D**).

Challenges inherent to this protocol include maintaining the integrity of cryostat sections, particularly because the olfactory organs are dissected without fixation. This may result in torn sections (**Figure 3A**) or partially deformed or curled sections during subsequent incubations (**Figure 3B**). Another potential problem is high fluorescence background staining, possibly derived from insufficient blocking or excessive signal development in amplification steps, in both pS6 immunohistochemistry (**Figure 3C**) or *in situ* hybridization steps (**Figure 3D**).

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation showing vertical placement of the VNOs inside the embedding medium to obtain transversal cryostat sections. (A) Use of a syringe to place the VNOs inside the histology mold, with the bone shell facing sideways. **(B)** Instructions for trimming the specimen block and attaching it to the cryostat holder (grey).

Figure 2. Representative images of dual *in situ* hybridization/pS6 immunohistochemistry staining of VNOs after mouse exposure to cat odors. (A) Microscopy image after *in situ* hybridization using riboprobes for V2R receptors in clade A4 (green fluorescence) and pS6 immunostaining (red). Middle panel: overlay of dual staining and nuclear counterstaining (blue). Arrowheads indicate co-localization of *in situ* hybridization and pS6 immunohistochemistry signals. **(B)** High magnification images from different VNO sections subjected to dual staining in **A**. **(C)** Microscopy image after *in situ* hybridization using probes for V2R receptors in clade A8 (green fluorescence) and pS6 immunohistochemistry (red). Middle panel: overlay of dual staining and nuclear counterstaining (blue). **(D)** High magnification images from different VNO sections subjected to the same dual staining as in **C**. Scale bars are 100 μm.

Figure 3. Representative suboptimal results. (A) Damaged, teared VNO (arrow). **(B)** Curled VNO section (arrow). **(C)** High background in pS6 immunostaining signal. Arrow shows an example of border effect. **(D)** High background staining after riboprobe *in situ* detection. Arrowheads indicate true staining. Scale bars are 100 μm.

Table 1. Troubleshooting table with the most frequent problems and proposed solutions. This table presents steps to troubleshoot the protocol, in cases where tears are found in sections or when there is difficulty sectioning. Other problems are also addressed, such as loss of sections during staining, low *in situ* hybridization signal, and high staining background.

DISCUSSION:

The protocol described here reliably identifies sensory neurons activated by chemical cues in the olfactory system through a combination of *in situ* detection of OR or VR receptors with immunodetection of pS6, an indirect marker of neuronal activity. The experimenter must take extra caution to maintain histology integrity and perform all steps before hybridization under RNase-free conditions. Failure to do so may cause mRNA degradation and compromise riboprobe labeling. Care must be exercised during dissection of the olfactory organs, as unfixed tissue is particularly fragile and may be easily damaged.

In situ hybridization in this protocol involves two rounds of signal amplification, and it must be

noted that fluorescence intensity may be affected by factors related to hybridization or signal development. For example, incubation temperatures and periods must be empirically optimized. Weak labeling may be due to low probe concentration, high hybridization temperature, or short tyramide labeling. Conversely, high background may be due to low hybridization temperature or over-development in tyramide incubation steps. These parameters must be adjusted to attain optimal signal-to-background ratio (see Table 1 for a thorough troubleshooting discussion).

Similarly, pS6 immunodetection may lead to low fluorescence intensity or high background staining. These problems generally arise from inadequate duration of tyramide incubation steps, insufficient blocking, or inadequate quenching of endogenous peroxidases (**Table 1**).

Despite such methodological concerns, the procedure described here has important advantages. pS6 immunodetection is simpler and faster than double *in situ* hybridization with riboprobes designed to target popular IEGs, such as *c-fos*, *Arc*, and *Egr-1*. The method described here generally produces very clear labeling in olfactory sensory neurons[23, 25, 26]. Furthermore, the technique allows the identification of activated neurons in biologically relevant contexts (i.e., upon the detection of odors from a potential mating partner or from a predator), in experimental paradigms where behaviors can be simultaneously assessed and recorded. Other types of investigation aiming to detect olfactory neuron activation do not share this advantage, as they employ artificial settings in which the animal is head-fixed or euthanized prior to neural recordings or calcium imaging. The ability to identify neurons activated by specific chemical cues in freely moving mice is a powerful asset in several research areas, ranging from those studying the molecular biology of the olfactory system and chemical ecology to those interested in behavioral neuroscience and ethology. This protocol provides a reliable method for the investigation of chemodetection in these studies.

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We thank GAG Pereira and JA Yunes for resources, APF Ferreira and WO Bragança for administrative and technical help, and the Life Sciences Core Facility (LaCTAD-UNICAMP) staff for help with confocal microscopy. This work was supported by the Sao Paulo Research Foundation (FAPESP; grant numbers 2009/00473-0 and 2015/50371-0 to F.P.), by PRP/UNICAMP (grant numbers 2969/16, 725/15, 348/14, and 315/12 to F.P.), by FAPESP fellowships to V.M.A.C. (2014/25594-3, 2012/21786-0, 2012/01689-0), T.S.N. (2012/04026-1), and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) fellowship to T.S.N.

DISCLOSURES:

The authors declare they have no competing interests of any kind.

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619

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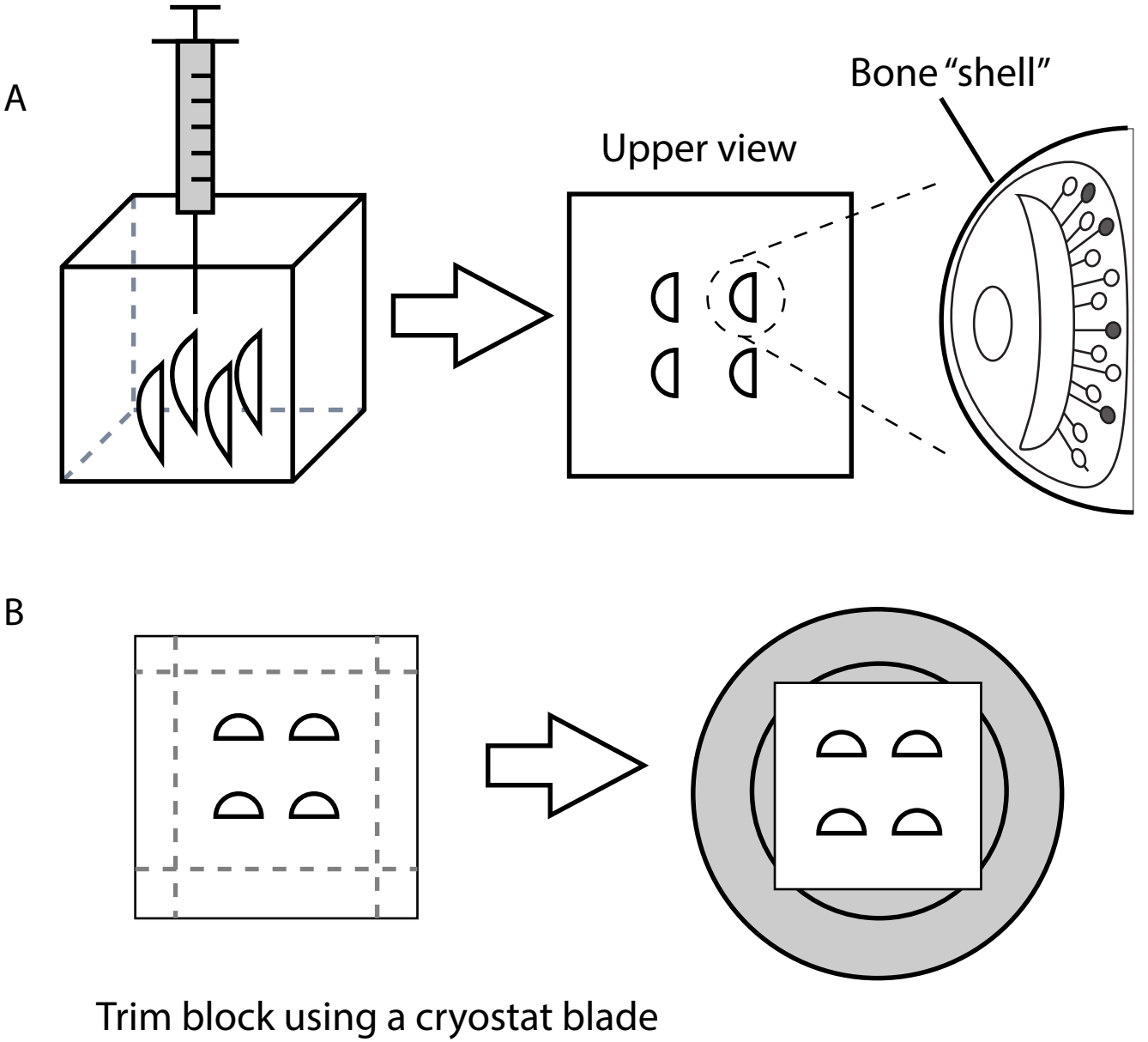

Figure 2

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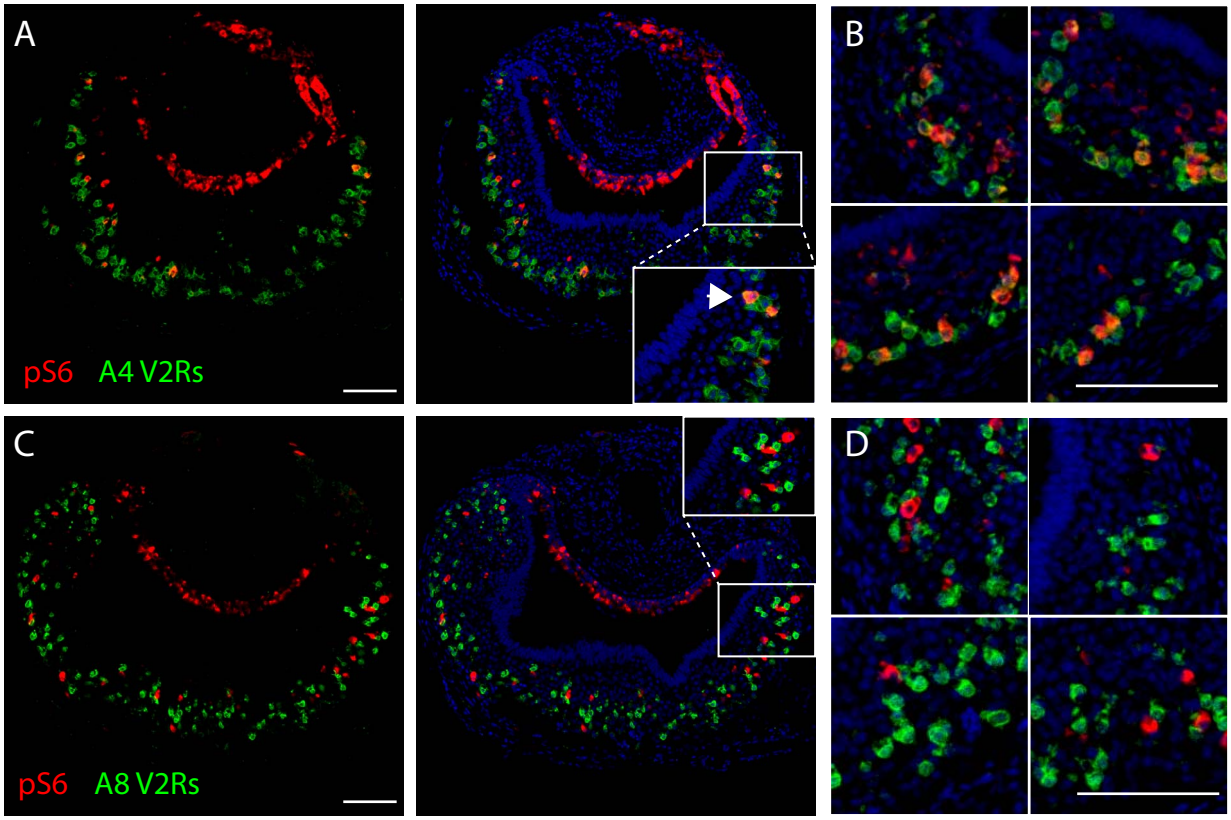

Figure 3

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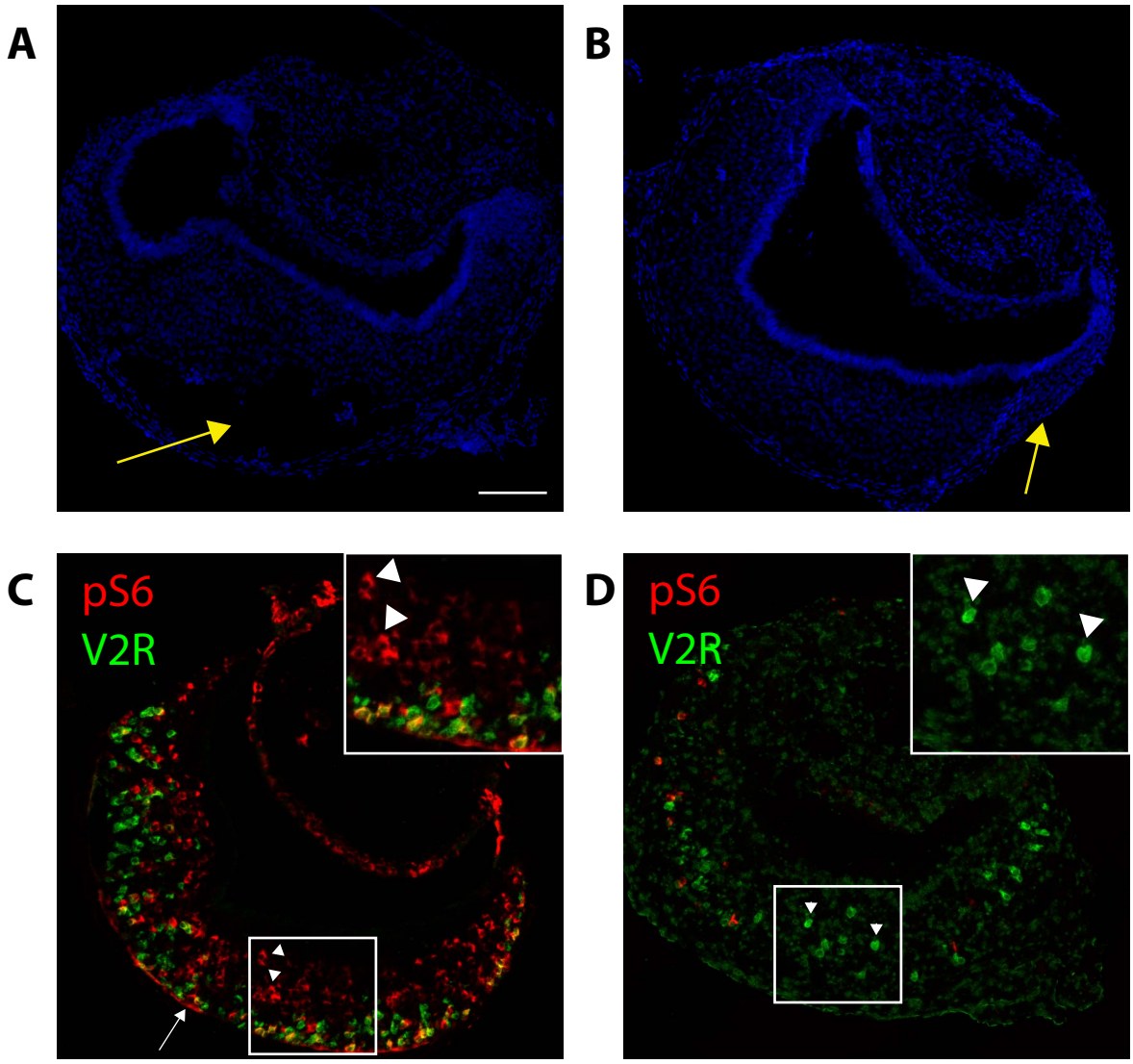

Table 1

Click here to access/download;Table;Table 1.xlsx

Problem	Possible causes	Proposed solution
Tears in sections or difficulty when sectioning	Bones are impairing sectioning process	Replace cryostat blade or change cryostat block orientation until section quality improves. Usually, the remaining bones, if present, are positioned opposite to the side where the cryostat blade first hits the specimen.
Loss of sections during staining	Use of non-adhesive microscope slides	Use silanized or other type of positively-charged slides. Consider changing brands.
	Harsh conditions *in situ* hyb steps	Handle slides carefully during staining washes, especially when inserting and removing from wash solutions and during coverslip displacement.
Low *in situ* hybridization signal	Insufficient probe hybridization	Increase probe concentration. Reduce hybridization or probe wash temperature.
	Poor tyramide signal ampification	Extend duration of tyramide amplification steps.
High staining background	Insufficient blocking	Check blocking solution concentration or extend blocking duration.
	Excessive tyramide signal ampification	Reduce duration of tyramide amplification steps.
	Sections drying up during staining	Maintain sections covered in solution at all times. Minimize time between washes, during which slides are outside the staining jars and may dry up.

Click here to access/download;Table of Materials;JoVE_Materials_revised.xlsx

Name of Material/ Equipment	Company	Catalog Number
10x phosphate-buffered saline (PBS)	Thermo Fisher Scientific	AM9625
20x amplification diluent (reaction buffer from Alexa Fluor 555 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40923 or B40922
20x SSC	Merck (Calbiochem)	8310-OP
30% Bovine Serum Albumin (BSA)	Merck (Sigma-Aldrich)	A9576
30% hydrogen peroxyde (H_2O_2)	Merck (Sigma-Aldrich)	H1009
Acetic anhydride	Merck (Sigma-Aldrich)	320102
Agarose	Merck (Sigma-Aldrich)	A9539
Amplification diluent (from TSA Biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
Anti-pS6 (Ser 244/247) rabbit polyclonal antibody	Thermo Fisher Scientific	Cat# 44-923G, RRID:AB_2533798
Blocking buffer 1x (from Alexa Fluor 488 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40923 or B40922
Blocking reagent (from TSA Biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
DAPI nuclear stain	Thermo Fisher Scientific	D1306
Denhardt's solution (50x)	Merck (Sigma-Aldrich)	D9905
Deoinized formamide	Merck (Sigma-Aldrich)	F9037
Dextran sulfate solution (50%)	Merck (Chemicon)	S4030

Ethylene-diamine-tetraacetic acid (EDTA)	Merck (Sigma-Aldrich)	E9884
Hoechst 33342 nuclear stain	Thermo Fisher Scientific	H1399
Hydrochloric acid (HCl)	Merck (Sigma-Aldrich)	320331
Olfactory stimuli	n/a	Papes et al. (2010), Carvalho et al. (2015), Nakahara er al. (2016), Carvalho et al. (2020)
Paraformaldehyde	Merck (Sigma-Aldrich)	P6148
Peroxidase-conjugated anti-digoxigenin antibody (Fab fragments)	Merck (Roche)	11207733910; RRID:AB_514500
Peroxidase-conjugated anti-rabbit secondary antibody (polyHRP-conjugated goat anti-rabbit reagent from Alexa Fluor 488 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40922
Peroxidase-conjugated streptavidin (from TSA biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
ProLong Gold antifade mountant	Thermo Fisher Scientific	P36934
RNase-free ultrapure water	Thermo Fisher Scientific	10977015
Sodium chloride (NaCl)	Merck (Sigma-Aldrich)	S9888
Sodium dodecyl sulfate (SDS)	Merck (Sigma-Aldrich)	436143
Sodium hydroxide (NaOH)	Merck (Sigma-Aldrich)	S8045
Sucrose	Merck (Sigma-Aldrich)	S0389
Triethanolamine	Merck (Sigma-Aldrich)	T58300

Triton X-100	Merck (Sigma-Aldrich)	X100
Trizma hydrochloride (Tris-Cl)	Merck (Sigma-Aldrich)	T5941
Tween-20	Merck (Sigma-Aldrich)	822184
Tyramide-Alexa 488 conjugate (from Alexa Fluor 488 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40922
Tyramide-Alexa 555 conjugate (from Alexa Fluor 555 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40923
Tyramide-Biotin conjugate (from TSA Biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
Yeast tRNA	Merck (Roche)	10109495001
Critical Commercial Assays and Animals		
Alexa Fluor 488 Tyramide SuperBoost kit	Thermo Fisher Scientific	B40922
Alexa Fluor 555 Tyramide SuperBoost kit	Thermo Fisher Scientific	B40923
DIG RNA Labeling Kit (SP6/T7)	Merck (Roche)	11175025910
High Sensitivity RNA ScreenTape Analysis reagents (buffer, ladder, and tape)	Agilent	5067-5580, 5067-5581, and 5067-5579
Mouse: C57BL/6J inbred strain	Jackson Laboratories	Stock No: 000664; RRID:IMSR_JAX:000664)
ProbeQuant G-50 Micro Columns	Cytiva Biosciences	28903408
QIAquick gel extraction kit	Qiagen	28506
RNeasy MinElute cleanup kit	Qiagen	74204
TSA Biotin kit	Akoya Biosciences (Perkin Elmer)	SAT700001EA
Oligonucleotides		
5’ – AAACTTCATCCTTACAGAATGGCAG – 3’	Integrated DNA Technologies	n/a

5’ – ACTGGCTTTGGGACAGTGTGAC – 3’	Integrated DNA Technologies	n/a
5’- GGTAATATCTCCATTATCCTAGTTTCCC – 3’	Integrated DNA Technologies	n/a
5’ – TTGACCCAAAACTCCTTTGTTAGTG – 3’	Integrated DNA Technologies	n/a
5’ – ATGGGAGCTCTAAATCAAACAAGAG – 3’	Integrated DNA Technologies	n/a
5’ – TAGAAAACCGATACCACCTTGTCG – 3’	Integrated DNA Technologies	n/a
5’ – TACATCCTGACTCAGCTGGGGAACG – 3’	Integrated DNA Technologies	n/a
5’ – GGGCACATAGTACACAGTAACAATAGTC – 3’	Integrated DNA Technologies	n/a
5’ – GAGGAAGCTCACTTTTGGTTTGG – 3’	Integrated DNA Technologies	n/a
5’ – CAGCTTCAATGTCCTTGTCACAG – 3’	Integrated DNA Technologies	n/a
5’ – TGGGTTGGAGGCTTATCATACCTG – 3’	Integrated DNA Technologies	n/a
5’ – AAGAACAACACAGAGTCTTGATGTC – 3’	Integrated DNA Technologies	n/a
5’ – AGAAGTAACTAACACCACTCATGGC – 3’	Integrated DNA Technologies	n/a
5’ – TTAGTGCACCTTTCTTTGCAAC – 3’	Integrated DNA Technologies	n/a
5’ – TAACAGCTCTTCCCATCCCCTGTTC – 3’	Integrated DNA Technologies	n/a
5’ – TAGGGTTGAGCATGGGAGGAACAAGC – 3’	Integrated DNA Technologies	n/a
5’ – CACTGGATCAACTCTAGCAGCACTG – 3’	Integrated DNA Technologies	n/a

5’ – CTGCCCTTCTTGACATCTGCTGAG – 3’	Integrated DNA Technologies	n/a
5’ – ATCGGATCCACTGCTTTAGCATTTCTTACAGGACAG – 3’	Integrated DNA Technologies	n/a
5’ – ATCCTCGAGTCATGCCTCTCCATAAGCAAGGAATTCCAC – 3'	Integrated DNA Technologies	n/a
5’ – TAGGAAGCTATTTGCCTTGTTTCCAC – 3’	Integrated DNA Technologies	n/a
5’ – AGGAGATTTTACCAACCAGATTCCAG – 3’	Integrated DNA Technologies	n/a
5’ – CTCTAAGAACAGCAGTAAAATGGATCT – 3’	Integrated DNA Technologies	n/a
5’ – ATGGGAATGACCAACTTAGGTGCA – 3’	Integrated DNA Technologies	n/a
5’ – ATCCCATGGCTGAGAACATGTGCTTCTGGAG – 3’	Integrated DNA Technologies	n/a
5’ – ATCCTCGAGTCAGTCTGCATAAGCCAGATATGTCAC – 3’	Integrated DNA Technologies	n/a
5’ – ATCGGATCCGCTGATTTTATTTCTCCCAGATGCTTTTGG – 3’	Integrated DNA Technologies	n/a
5’ – ATCCTCGAGTCATGGTTCTTCATAGCTGAGAAATACAAC – 3’	Integrated DNA Technologies	n/a
5’ – TGGGTGTCTTCTTTCTCCTCAAGA – 3’	Integrated DNA Technologies	n/a
5’ – GGTGACCCATATTCTCTGTATAACTGT – 3’	Integrated DNA Technologies	n/a
5’ – GATGTTCATTTTCATGAGAGTCTTCC – 3’	Integrated DNA Technologies	n/a
5’ – CATTTGTGGATGACATCACAATTTGG – 3’	Integrated DNA Technologies	n/a
5’ – TTTATGGCAAATTTCACTGATCCCG – 3’	Integrated DNA Technologies	n/a

5’ – AGTGGGTCTTTCTTAGAAAGGAGTG – 3’	Integrated DNA Technologies	n/a
5’ – ACATGAACCAGAATTTGAAGCAGGC – 3’	Integrated DNA Technologies	n/a
5’ – GCCAAGAAAGCTACAGTGAAACC – 3’	Integrated DNA Technologies	n/a
5’ – AGGTGAAGAAATGGTATTCTTCCAG – 3’	Integrated DNA Technologies	n/a
ACTGTGGCCTTGAATGCAATAACT – 3’	Integrated DNA Technologies	n/a
5’ – TTCCTAAAGAACACCCTACTGAAGCATCG – 3’	Integrated DNA Technologies	n/a
5’ – CATATTCCACAGAAGAGAAGTTGGAC – 3’	Integrated DNA Technologies	n/a
5’ – TTGAGGTGAGAGTCAACAGTTTAGAC – 3’	Integrated DNA Technologies	n/a
5’ – CCCTTGTTGCACAAAATGATGATGTGA – 3’	Integrated DNA Technologies	n/a
5’ – ATCCCATGGAGTCAGAGTATCTACTACACCATGATGG – 3’	Integrated DNA Technologies	n/a
5’ – ATCCTCGAGTCAATCATTATAGTCCAGAAAGGTGACAG – 3’	Integrated DNA Technologies	n/a
Recombinant DNA		
pGEM-T-Easy vector	Promega	A1360
Other materials		
1mL syringes	Fisher Scientific	14-829-10F
Conical tubes (15 mL and 50 mL)	Fisher Scientific	14-432-22, 14-959-49B
Coplin jars	Fisher Scientific	12-567-099, 07-200-81
Cryostat	Leica Biosystems	CMS 1850
Dissecting tools and forceps	Roboz	RS-6802, RS-8124, RS-7110, RS-5111
Dry bath	n/a	n/a

Electrophoresis equipment	Fisher Scientific	09-528-110B
Fine point paintbrushes	Winsor & Newton	10269097
Fluorescence or confocal microscope	Leica Microsystems	TCS SP5II
Heated plate	Fisher Scientific	HP88850200
Humidified chamber (if used at higher temperatures, it will need to be sealed inside a plastic Tupperware container)	Thermo Fisher Scientific	22-045-034
Lint-free laboratory Kimwipes	Kimberly-Clark	34120
Microcentrifuge	Eppendorf	5401000013
Mouse cages	InnoVive	M-BTM, MVX1
PCR Thermocycler	Thermo Fisher Scientific	4375786
Pipette p1000, p200, and p20 disposable tips	Fisher Scientific	02-707-408, 02-707-411, 02-707-438
Plastic histology embedding mold	Thermo Fisher Scientific	22-19
Qubit 4 Fluorometer	Thermo Fisher Scientific	Q33238
Razor blades or scalpels	Fisher Scientific	12-640
RNA tapestation or BioAnalyzer	Agilent	4200 TapeStation System or 2100 Bioanalyzer Instrument
RNase-free glass or plastic graduated cylinders and beakers	Fisher Scientific	10-462-833, 02-555-25B, 02-555-25D
Stereomicroscope	Leica Microsystems	M80
SuperFrost Plus microscope slides	Thermo Fisher Scientific	12-550-15
Parafilm	Bemis Company	PM999
Water bath	Thermo Fisher Scientific	TSCIR19

Comments/Description
n/a
refered to as Amplification Buffer A in working solutions for tyramide-Alexa 488 or tyramide-Alexa 555 signal amplification
n/a
n/a
n/a
n/a
n/a
refered to as Amplification Buffer B in working solution for tyramide-biotin signal amplification
n/a
refered to as blocking solution B in the tyramide signal development step
refered to as blocking reagent A in the formulation for TNB buffer
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refered to as peroxidase-conjugated anti-DIG antibody
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refered to as anti-fading mounting medium
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refered to as automated electrophoresis system
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refered to as gel filtration-based purification kit
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Olfr692

Olfr692
Olfr124
Olfr1509
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Olfr691
Olfr638
Olfr569
Vmn2r1

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Vmn2r2
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Vmn2r41
Vmn2r46

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Vmn2r90
Vmn2r107
Vmn2r83
reccommended PCR cloning vector
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refered to as lint-free laboratory tissue paper
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refered to as highly sensitive fluorometric method
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referred to as positively charged microscope slides
refered to as thermoplastic laboratory film
n/a

Rebuttal Letter & Cover Letter
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From:
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Visiting Professor
Department of Pediatrics, School of Medicine
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February 3rd, 2021

Dear Dr. Vineeta Bajaj,
Review Editor
&
Dr. Benjamin Werth,
Senior Science Editor - Medicine|Chemistry|Biochemistry
Journal of Visualized Experiments (JoVE)

Please, find enclosed our revised manuscript entitled **"Investigation of activated mouse olfactory sensory neurons via combined immunostaining and *in situ* hybridization",** which we would like to be considered for publication in *JoVE*, as part of the article collection '*Chemical Communication in Living Systems'*, organized by guest editor Dr. Alex Artyukhin.

In this protocol, histological sections of the nervous system are labeled to detect activated neurons expressing marker pS6 and cells expressing a gene of interest via a combination of immunostaining and *in situ* hybridization with cRNA probes. The protocol was devised to identify neurons activated after a single event of exposure to purified or complex olfactory stimuli, in biologically relevant contexts, allowing histological investigation and assessment of behavioral responses in the same subjects. This method can be used to deorphanize olfactory receptors, the vast majority of which do not have known ligands.

In the journal's decision letter, it was indicated that we should upload the video for review, but video production is virtually impossible for us at the moment, due to the COVID-19 pandemic. With cases going up in the second wave of COVID in Brazil, our university has been completely shut down for non-essential activities until further notice. In an e-mail communication with Dr. Benjamin Werth dated September 2020 (which we reproduce at the end of this cover letter), it was stated that the video material could be sent later, when possible, and that the manuscript would be reviewed/published in full text format in the meantime. Can you please confirm this possibility, as it is currently impossible for us to produce and send the video component? In the revised manuscript, we highlighted the steps in the protocol which will appear in the video.

We are also sending a response to the editor's comments below. A point-by-point rebuttal letter to address the reviewers' comments is attached. Once again, thank you for considering our manuscript. Please, do not hesitate to contact me should you have any further questions.

Sincerely,

Prof. Fabio Papes

Response to editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully revised the manuscript to correct any grammar and spelling issues.

2. Please provide an email address for each author. Also indicate the corresponding author correctly.

E-mail addresses are now provided for each author in the title page. The corresponding author is Dr. Fabio Papes, who is now correctly indicated in the title page.

3. Please include a statement about the confirmation to the institutional animal ethics guidelines for this study at the start of the protocol.

We have included a statement on the use of animal subjects at the beginning of the protocol (starting in line 80).

4. Please ensure all units are correctly written. Please use SI units, and include a single space between the quantity and the unit. E.g. Lines 438, 443: "µm.." instead of "µM..", Line 268: " mL" instead of "cc", Line 305: "625 µL" instead of "625µl", etc.

We have carefully revised all units in the main manuscript text and supplementary tables to conform with SI nomenclature. Some µm units had been mistakenly written as µM, but this has now been fixed in lines 486 and 491.

5. All the necessary reagents/solutions must be uploaded to the table of materials including company name and catalog number. The protocol should contain only action items that direct the reader to do something.

We have listed all the necessary reagents in lines 86-199 (in alphabetical order) and included information on commercially available sources (company name and catalog number) in the accompanying Table of Materials spreadsheet. We ensured that all items in the step-by-step protocol beginning in line 257 direct the reader to execute some action in the protocol.

6. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

In the originally submitted manuscript, Table 1 was inadvertently inserted into the final pdf by the journal's editorial manager. In the revised version, we ensured that all tables were removed from the main manuscript text file. Rather than placing the components needed for preparing the necessary solutions in tables, we now describe each formulation and preparation steps using complete sentences (example in lines 96 and 97). Legends and descriptions for Table 1 and for the Table of Materials are given in the text, in lines 470-501.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of

phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We carefully revised the entire manuscript to direct the reader to perform the steps in the imperative tense.

8. Please ensure you answer the "how" question, i.e., how is the step performed?

The revised version now includes a complete description of how to perform each step (add these volumes, pipet those solutions, etc.).

9. Lines 246-47: Please provide appropriate details about the animals used, their strain, sex, age, weight etc..

This information has now been added to the text (lines 259-261).

10. Please define all abbreviations before use. E.g. Line 249: MOE.

Abbreviations are defined the first time they are used (example in line 264).

11. Lines 192-236: If this part of the protocol has been previously published, it could be referenced here.

This has now been properly referenced in line 203.

12. Line 277, 289: Please include this as a "Note".

This problem has been fixed in lines 290 and 299.

13. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Kimwipes, RNEasy MinElute Cleanup kit, etc.

We have removed all mention to commercial sources from the main manuscript file, including brand names and commercial reagent names. A description of preferred or critical commercial sources (kits, for example) is now given in the Table of Materials spreadsheet.

14. Please include a single line spacing between successive protocol steps, and avoid the use of personal pronouns in the protocol section. E.g. "we", "our", etc.

The revised manuscript is now written in the imperative tense or using the third person. Single line spacing has been added between each step in the protocol, starting in line 201.

15. Please sort the Materials Table alphabetically by the name of the material. (E.g. Lines 91-189. The chemicals and instruments used should be listed in the Table of Materials).

Both the list of materials in lines 88-192 and the Table of Materials spreadsheet have been ordered alphabetically to the best extent possible.

UNICAMP

Fabio Papes <papesf@unicamp.br>

Re: Abstract submission for Methods Collection

Benjamin Werth <benjamin.werth@jove.com> 3 de setembro de 2020 16:36
Para: Fabio Papes <papesf@unicamp.br>

Hi Fabio,

Yes, due to COVID we now allow authors to submit the text first, without the video. We'll still ask you to send the video component as soon as possible, but in the meantime we can review/publish the full text article.

Best,

Benjamin

On Thu, Sep 3, 2020 at 2:35 PM Fabio Papes <papesf@unicamp.br> wrote:

> Dear Dr. Werth,
>
> How are you? We are in the process of writing the manuscript and will be ready to submit it in time. However, we are planning to produce the videos at a later date, when the pandemics situation allows. According to the JoVE website, this is allowed, is that correct?
>
> Thank you! Best regards,
>
> Fabio Papes
>
> Em qui., 3 de set. de 2020 às 09:01, Benjamin Werth <benjamin.werth@jove.com> escreveu:
>
> > Dear Dr. Papes,
> >
> > I'm just writing to check in. How is your manuscript/video coming along? Please let me know if you have any questions regarding the manuscript or the publication process.
> >
> > I look forward to receiving your video article on **October 5.**
> >
> > Best regards,
> >
> > Benjamin
> >
> > --
> > Benjamin Werth
> > Senior Science Editor - Medicine|Chemistry|Biochemistry
> > 1 Alewife Center Suite 200 Cambridge MA 02140
> >
> > ---
> >
> > JoVE is the world's first and only scientific video journal, with the mission of transforming scientific research through visualization of scientists' work. As a multi-disciplinary peer-reviewed methods journal, JoVE publishes over 1,000 video articles yearly, filmed at the leading academic institutions around the world. With over 7,000,000 users worldwide, JoVE provides a unique opportunity to showcase research expertise and promote efficient knowledge transfer in the scientific community and beyond. All JoVE articles are peer-reviewed and indexed in PubMed, Web of Science, and other relevant databases. Authors may produce their own video or use JoVE video production services.
> >
> > On Wednesday, August 5, 2020 at 9:18 AM, Benjamin Werth <benjamin.werth@jove.com> wrote:

Dear Dr. Papes,

I'm pleased to inform you that your abstract has been accepted by the Guest Editors - we are looking forward to including your video article in the Methods Collection on chemical communication in living systems!

The next step is to submit a video article in JoVE's format - you can prepare the manuscript using this Template and Instructions for Authors document, and follow these video format guidelines.

Additional information on the video production process, including publication fees, can be found in the authors section of the JoVE website.

You will be receiving an official invitation to publish with JoVE today. In the email there is a link to accept this invitation and directions on how to login/submit your manuscript. I will be in touch again closer to the tentative **October 5th** submission date. In the meantime, if you have any questions please do not hesitate to email or call me.

Best regards,

Benjamin

--
Benjamin Werth
Senior Science Editor - Medicine|Chemistry|Biochemistry
1 Alewife Center Suite 200 Cambridge MA 02140

JoVE is the world's first and only scientific video journal, with the mission of transforming scientific research through visualization of scientists' work. As a multi-disciplinary peer-reviewed methods journal, JoVE publishes over 1,000 video articles yearly, filmed at the leading academic institutions around the world. With over 7,000,000 users worldwide, JoVE provides a unique opportunity to showcase research expertise and promote efficient knowledge transfer in the scientific community and beyond. All JoVE articles are peer-reviewed and indexed in PubMed, Web of Science, and other relevant databases. Authors may produce their own video or use JoVE video production services.

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Benjamin Werth
Senior Science Editor - Medicine|Chemistry|Biochemistry
1 Alewife Center Suite 200 Cambridge MA 02140

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Response to reviewer comments

Please, find below a point-by-point response to the comments sent by the reviewers on our manuscript entitled "Investigation of activated mouse olfactory sensory neurons via combined immunostaining and *in situ* hybridization", by Carvalho et al..

Reviewer #1:
Manuscript Summary:
This protocol combines in situ hybridization with immunodetection of a marker of neuronal activity in the vomeronasal organ of the mouse, aimed to identify receptors activated by more or less specific olfactory ligands. Steps are presented in a clear way with numerous useful details.

Major Concerns:
My only comment is about novelty, since similar protocols have been already published also by the same authors addressing analogous questions. From my understanding of the editorial policy and because of the singular format of this journal this is not a relevant concern. In that case, the manuscript is acceptable for publication.

We thank the reviewer for carefully evaluating our manuscript and for acknowledging its importance to those studying activity and function in the nervous system. Our previous publication (Nakahara et al. 2020, *Star Protocols* 1: 100153) focused on a technique that combined immunostaining and *in situ* hybridization to detect activation of neurons in the brain with temporal resolution. The present manuscript focuses on a technique to molecularly characterize activated neurons. Considering that the two protocols have substantially different details, we anticipate the present piece will be valuable to the scientific community, especially when accompanied by the explanatory video component.

Minor Concerns:
typos: Page 24: "10x phosphate-bufered saline (PBS)"
Page 24: "30% Hydrogen Proxyde Solution"

We have now carefully revised the manuscript to correct for typos and inconsistencies.

Reviewer #2:
Manuscript Summary:
The authors present a detailed protocol for the deorphanization of ORs and/or VRs by combining receptor-specific in situ hybridization with immunohistochemistry for the activity marker pS6 following activation of sensory neurons by chemical cues. This is a valuable method for scientists in the field and I can recommend publication in JoVE.
For minor comments and questions, see below.

We thank the reviewer for carefully evaluating our manuscript and revised it to fix the problems indicated.

Major Concerns:
none

Minor Concerns:
General comments:

Check consistency of upper and lower case usage, as well as subscript and superscript. (see for example 1.29. - 1.32. and H2O2 in associated table).

We have now carefully revised the manuscript to correct for typos and inconsistencies.

Notes and Questions:
Line 122: RNAse-free Paraformaldehyde fixative solution
NaOH 10 M, n/a (to adjust pH) - Please name the specific pH.

We have now included information on the pH adjustment of paraformaldehyde fixative in line 139.

Line 124: Prepare under fume hood would be an asset.

We updated the hazardous information on paraformaldehyde fixative in line 143 to indicate that this solution should be prepared under a fume hood.

Table 1.3.4. and 1.36. Tyramide Alexa final concentration is 1/50 instead of 1/100 (2 µl in 100 µl)!

We apologize for the inadvertent mistake. The tyramide-biotin signal amplification kit recommends a 1:100 dilution for this reagent, but we know from experience that 1:50 works best. This has now been fixed in line 188.

Line 140 1.25. PTw Solution - Explain abbreviation PTw.

The formulation for PTw is now given in line 117. It is important to mention that PTw is not a commercial brand name. This abbreviation stems from the solution components, which are PBS and Tween-20.

Line 300: Why "in the dark"?

We perform this step in the dark to prevent light-induced degradation of hydrogen peroxide. This is now indicated in the protocol (line 316).

Lines 366-367: What is the difference between blocking solution (4.1.4.) and immunohistochemistry blocking solution (4.1.5.)?

The blocking solution used in step 4.1.4 (step 6.4 in the revised manuscript) is the commercial solution from the SuperBoost signal amplification kit (Thermo Fisher Scientific), and this is now indicated in the text (in generic terms), in lines 409-410. A reference to the complete commercial source is given in the Table of Materials spreadsheet. The immunohistochemistry blocking solution in step 4.1.5 (step 6.5 in the revised manuscript) is described in the list of reagents (lines 111-114). The difference between the two solutions in consecutive steps of the protocol has now been clarified in the text. The reason why we use two steps of blocking is that this was empirically determined to be more effective in reducing background staining in this type of combined immunostaining / *in situ* hybridization protocol.

Line 369: Give the specific final concentration (µg/ml) of the pS6 antibody as it may vary with supplier and/or production date.

The pS6 antibody we list in the Table of Materials has no concentration provided by the vendor. However, we experimentally determined the affinity purified antibody to be at an approximate concentration of 200 μg/mL. Information on the final concentration of the diluted antibody is now included in the revised manuscript (line 416), to allow reproducibility.

Line 390: As the mounting medium is water-based, storage at -20°C is questionable as it may screw morphology.

The commercial mounting medium we used in this paper (ProLong Gold; Thermo Fisher Scientific) recommends curing the histological slides at room temperature and then storing it at -20°C, even though it is water-based. Because we cannot add commercial sources to the text, as per the journal's policies, we decided to generically instruct the reader to store the slides at the appropriate temperature (line 440), which will vary depending on the type of mounting medium used. We thank the reviewer for carefully pointing out the need for revision here.