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Protocols for two- and three-color fluorescent RNA *in situ* hybridization of the main and accessory olfactory epithelia in mouse

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Abstract

The main and accessory olfactory epithelia of the mouse are composed of many cell populations. Each sensory neuron is thought to express one allele of one of the ∼1000 odorant or ∼300 vomeronasal receptor genes. Sensory neurons die and are replaced by new neurons that differentiate from precursor cells throughout the lifetime of the individual. Neuronal replacement is asynchronous, resulting in the co-existence of cells at various stages of differentiation. Receptor gene diversity and ongoing neuronal differentiation produce complex mosaics of gene expression within these epithelia. Accurate description of gene expression patterns will facilitate the understanding of mechanisms of gene choice and differentiation. Here we report a detailed protocol for two- and three-color fluorescent RNA *in situ* hybridization (ISH) and its combination with immunohistochemistry, or detection of bromodeoxyuridine (BrdU)-incorporated DNA after labeling. The protocol is applied to cryosections of the main and accessory olfactory epithelia in mouse.

Introduction

The olfactory system detects and discriminates an enormous array of chemical compounds (Farbman, 1992). This remarkable capacity of detection is based on diverse cell populations in the olfactory epithelia. Most mammals have a main olfactory system and an accessory olfactory (or vomeronasal) system. In mouse, the main olfactory epithelium (MOE) primarily serves the general detection of conventional odorants. The MOE consists of olfactory sensory neurons (OSNs), their precursors (basal cells), and sustentacular cells. OSNs express ∼1000 odorant receptor (OR) genes, which encode seven-transmembrane domain proteins (Buck & Axel, 1991; Mombaerts, 2004a; Zhang *et al.*, 2004). The expression of OR genes is monoallelic (Chess *et al.*, 1994; Ishii *et al.*, 2001; Strotmann *et al.*, 2000), and is thought to be monogenic such that one neuron expresses one type of receptor-but perhaps not in all cases (Mombaerts, 2004b). OSNs thus form at least 1000 cell populations. The vomeronasal organ (VNO) is physically separated from the MOE, and is thought to be specialized in the detection of pheromones (Halpern & Martinez-Marcos, 2003; Keverne, 1999). The vomeronasal epithelium (VNE) contains two types of vomeronasal sensory neurons (VSNs) that can be distinguished by the expression of G protein α subunits, Gαi2 and Gαo (Berghard & Buck, 1996; Jia & Halpern, 1996). Neurons expressing Gαi2 or Gαo also express members of the vomeronasal receptor V1R superfamily (Dulac & Axel, 1995; Zhang *et al.*, 2004) and V2R superfamily (Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997), respectively. The V1R and V2R repertoires each consist of ∼150 genes encoding seven-transmembrane domain proteins that are unrelated in sequence to each other and to ORs. Monoallelic and monogenic expression of V1R (Rodriguez *et al.*, 1999) and V2R (Del Punta *et al.*, 2002) genes gives rise to at least 300 populations of VSNs. In addition, Gαo-positive neurons also express nine members of another multigene family, *H2-Mv* genes, which are non-classical class I genes of the Major Histocompatibility Complex (MHC) (Ishii *et al.*, 2003; Loconto *et al.*, 2003). The variegated expression of *H2-Mv* genes may generate more diversity within VSNs.

OSNs are replaced by cells that differentiate from basal cells, to maintain chemosensory function of this neuroepithelium throughout life (Carr & Farbman,

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1992; Farbman, 1990; Huard *et al.*, 1998; Schwob, 2002). The MOE thus contains many types of cells at various stages of neuronal differentiation that are closely appositioned at multiple levels within the epithelium. Conventional single-color RNA ISH provides useful information about the expression of a gene and its distribution pattern within a tissue, but information about the cell types is usually limited. Multi-color RNA ISH reveals in greater detail the expression pattern of a gene of interest at the cellular level.

The expression of multiple genes has been visualized simultaneously in cultured human cells with high spatial and temporal resolution using multi-color combinatorial codes (Levsky *et al.*, 2002). Multicolor RNA ISH protocols have been reported for *Drosophila* (Hauptmann, 2001; Kosman *et al.*, 2004). In the MOE and VNE, two-color RNA ISH (fluorescent or non-fluorescent) has provided critical information about the expression of OR, V1R and V2R genes, and about the various cell types (Ishii *et al.*, 2003; Kaluza *et al.*, 2004; Li *et al.*, 2004; Pantages & Dulac, 2000; Rawson *et al.*, 2000; Schwarzenbacher *et al.*, 2004; Shykind *et al.*, 2004; Tian & Ma, 2004; Tsuboi *et al.*, 1999). To study cell proliferation and differentiation in the MOE and VNE, injection of BrdU into the living animal is a standard way to label proliferating cells (Martinez-Marcos *et al.*, 2000; Weiler & Farbman, 1997; Weiler *et al.*, 1999). The detection of BrdU-incorporation in DNA combined with non-fluorescent RNA ISH has been reported for the brain of mouse embryos (Ishii *et al.*, 2000).

Here, we provide a detailed, step-by-step protocol with all reagents and supplies required, for two- and three-color RNA ISH; RNA ISH combined with immunohistochemistry; and RNA-ISH combined with the detection of BrdU-DNA after labeling. The protocol is applied to cryosections of the MOE and VNE of mouse.

Methods and materials

GENERAL CONSIDERATIONS

- RNA probe preparation and hybridization steps are carried out in a ribonuclease (RNase)-free location, ideally in a dedicated room.
- RNase-free solutions are used from the time of animal dissection to hybridization. There is no need to use RNase-free solutions during the subsequent wash and detection steps.
- Solutions are treated with diethyl pyrocarbonate (DEPC) at 1 ml/L to inactivate RNase, except for Tris-Cl (pH 8.0), sodium dodecyl sulfate (SDS) and sucrose solutions which are dissolved in DEPC-treated water.
- Staining jars, glassware and slide racks are baked at 200° C for 4 h.
- Useful references are Hirota *et al.* (1992), Ishii *et al.* (2003), Kosman *et al.* (2004), Montmayeur *et al.* (2001), and Tsuboi *et al.* (1999).
- "x" indicates the number of times to repeat.

PROBE PREPARATION

(1) A specific probe sequence for the target gene can be determined by comparing probe sequences to the mouse genomic DNA sequence database (http://genome.ucsc.edu/). Probe specificity is further confirmed by Southern blotting, if necessary. In our experience, cross-hybridization does not occur if sequence identity is below 80% and hybridization is carried out at 65° C.

PCR products or DNA restriction fragments (200–1500 bp) are subcloned into a plasmid vector such as pGEM T-easy vector (Promega) or pBluescript II (Stratagene), which contain promoter sites for *in vitro* RNA transcription.

For this paper, RNA probes were prepared for odorant receptors *M*71 (Rothman *et al.*, 2005; nucleotide 64-930 from GenBank accession no. AF281061), *P*2 (nucleotide 54-948 from AF 247657), *MOR28* (Tsuboi *et al.*, 1999), *MOR23* (nucleotide 8052-8945 from X92969), and *MOR32-4* (nucleotide 1-939 from AY073335); for markers *CBR2* (carbonyl reductase 2; nucleotide 5-466 from BC010758), *GAP43* (growth associated protein 43; nucleotide 147-860 from NM_008083), and *OMP* (olfactory marker protein; nucleotide 820-2891 from U012139); for vomeronasal receptors *V2ra*, *V2rb*, *V2rf* (Ishii *et al.*, 2003); for MHC genes *M*11 (nucleotide 2-596 from AF539596), *M*10.4 (nucleotide 62-951 from AF539600), and *M*9 (nucleotide 2-596 from AF539595).

(2) Linearize 10 μg plasmid with an appropriate restriction enzyme.

(3) Extract twice with phenol/chloroform (1:1) followed by a single chloroform extraction.

(4) Precipitate plasmid DNA by adding 0.1x volume of 3 M sodium acetate pH 5.2, and 2.5 volume of ethanol, place at -20° C for 30 min, then centrifuge for 10 min at 20,000 g.

(5) Wash the pellet with 70% ethanol, centrifuge at 20,000 g, remove supernatant completely and air dry the pellet.

(6) Dissolve air-dried pellet in 10 μl TE (10 mM TrisCl pH 8.0, 1 mM EDTA) and determine the concentration using 1 μl of the resuspended DNA solution in 100 μl TE by measuring the absorbance of 260 nm. Only 0.5 μg of the linearized plasmid is required in the subsequent transcription reaction. Linearized plasmids can be stored at -20° C.

(7) Set up the transcription reaction listed below on ice in a tube, and incubate at 37° C for 2 h.

Template DNA	0.5 μg
RNA labeling mixture (DIG, FLU, DNP or BIO)	1 μl
10x transcription buffer (provided with enzyme)	1 μl
RNasin, an RNase inhibitor (40 U/μl)	0.25 μl
RNA polymerase (T7, SP6 or T3; 20 U/μl)	1 μl
Adjust with H_2O (Sigma) to total	10 μl

Note: RNA labeling mixtures for DIG (digoxigenin), FLU (fluorescein), and BIO (biotin) are available from Roche. For DNP (dinitrophenyl) labeling, use DNP-11UTP and NTPs in the transcription reaction as listed below, for a total of 10 μl.

100 mM GTP	1 μl (final 10 mM)
100 mM ATP	1 μl (final 10 mM)
100 mM CTP	1 μl (final 10 mM)
100 mM UTP	0.65 μl (final 6.5 mM)
10 mM DNP-UTP	3.5 μl (final 3.5 mM)
H_2O (Sigma)	2.85 μl

(8) Add 1 μl of DNase I (2 U/μl) and incubate at 37°C for 15 min.
(9) Stop the reaction by adding 1 μl of 200 mM ethylenediamine tetraacetic acid (EDTA) pH 8.0.
(10) Precipitate RNA probe with 1.25 μl of 4 M LiCl and 37.5 μl of ethanol.
(11) Place at -20°C for 2 h, or at -80°C for 30 min, then centrifuge at 20,000 g for 15 min at 4°C.
(12) Wash with ice-cold 70% ethanol, discard supernatant completely and air-dry.
(13) Dissolve the pellet in 25 μl of H_2O. Verify the presence of reaction products by running an aliquot on a 1.5% agarose gel.

BrdU ADMINISTRATION

BrdU (Sigma) was injected intraperitoneally (50 mg/kg of body weight) into 2 wk old mice. Mice were dissected 24 h later.

MOUSE DISSECTION

(1) Anesthetize the mouse with intraperitoneal injection of sodium pentobarbital (60 mg/kg).
(2) Perfuse the mouse with ice-cold 4% paraformaldehyde (PFA) in Phosphate-buffered saline (PBS) pH 7.4.
(3) Dissect the olfactory organs.
(4) Incubate the tissue in 4% PFA/PBS overnight at 4°C.
(5) Decalcify specimen by incubation in 500 mM EDTA pH 8.0/PBS overnight (for young mice less than 3 wk) or two nights (for older mice) at 4°C.
(6) Incubate in 10% Sucrose/PBS for 2 h at 4°C.
(7) Incubate in 20% Sucrose/PBS for 2 h at 4°C.
(8) Incubate in 30% Sucrose/PBS for 3 h to overnight at 4°C.
(9) Embed the tissue in OCT compound and freeze in a mixture of dry ice and ethanol. Embedded samples can be stored at -80°C for up to one year.

SECTIONING

Cut 10 μm sections using a cryostat, and collect sections on slides taking straight from the box. Sections can be stored at -20°C or -80°C for several months. Sample boxes containing the slides are stored in a zipper bag to avoid accumulation of frost on the sections.

HYBRIDIZATION

Steps 3–15 except 12 and 13 are performed in a staining jar containing 200 ml solution. Steps 12 and 13 are performed in a 2 L-glass beaker containing 600 ml solution. Slides are in a staining rack at steps 3–15.

(1) Take a sample box from the -20° or -80°C freezer. Open the sample box at -20°C to prevent samples from accumulating frost. Remove slides and quickly dry sections with a hair dryer. First use hot air for a few seconds, then use cold air for 30 sec.
(2) Seal the edge of a slide with a Super Pap pen liquid blocker.
(3) Incubate the samples in 4% PFA/PBS at room temperature (RT) for 15 min.
(4) Wash in PBS at RT for 3 min.
(5) Incubate the slides in 0.1% H_2O_2/PBS at RT for 30 min (666 μl of 30% H_2O_2 in 200 ml PBS) to block endogenous peroxidases.
(6) Wash in PBS at RT for 3 min ($\times 2$).
(7) Incubate the slides in 10 μg/ml Proteinase K solution in TE at 37°C for 5–12 min (no more than 5 min for two-color ISH combined with immunohistochemistry). Solution contains 128 μl of 15.6 mg/ml Proteinase K, 2 ml of 1 M Tris-Cl at pH 8.0, 0.4 ml of 0.5 M EDTA in 200 ml H_2O.
(8) Incubate the sections in 4% PFA/PBS at RT for 10 min.
(9) Wash in PBS at RT for 3 min.
(10) Incubate the slides in 0.2 M HCl (3.44 ml of HCl in 200 ml H_2O) at RT for 10 min
(11) Wash in PBS at RT for 3 min.
(12) Pre-incubate the slides in 0.1 M Triethanol amine-HCl, pH 8.0, at RT for 1 min. Solution is Triethanol amine 7.95 ml, 12 N HCl 1.35 ml in 600 ml H_2O.
(13) Slowly add 1.5 ml of Acetic anhydrate to the solution made in step 12, and incubate at RT for 10 min while gently stirring. Acetic anhydrate will dissolve slowly during this period.
(14) Wash in PBS at RT for 3 min.
(15) Dehydrate the samples in a series of 60%, 80%, 95%, 100%, and 100% ethanol at RT for 90 sec each.
(16) Air-dry the samples.
(17) Prepare fresh hybridization solution by mixing the reagents listed below. Solution can be prepared during the pretreatment steps. 200 μl is required for hybridization of a single slide.

Components of hybridization solution	Stock solution	4 ml scale (for 20 slides)
50% Formamide (deionized)	100%	2 ml
10 mM Tris-Cl, pH8.0	1 M	40 μl
200 μg/ml yeast tRNA	8 mg/ml	100 μl
10% dextran sulfate	50%	0.8 ml
1x Denhardt's solution	50x	80 μl
600 mM NaCl	5 M	480 μl
0.25% SDS	10%	100 μl
1 mM EDTA, pH8.0	0.5 M	8 μl
H_2O		392 μl

(18) Dispense 200 μl aliquots into a single 1.5 ml tube for each slide, and incubate at 85°C in a heating block for 10 min.
(19) Add DIG-, FLU-, BIO- and/or DNP-labeled RNA probes to each tube containing hybridization solution: final concentration 0.1–0.2 μg/ml or 0.1–0.2 μl of stock probes/slide, 0.5–1.0 μl of stock probes/slide for DNP . Denature the probe at 85°C for 3 min.
(20) Apply 200 μl of the heated hybridization solution containing probes onto the samples on each slide.
(21) Cover samples with parafilm cut to the same size of the slide to prevent evaporation of the hybridization

solution. Be careful to avoid bubbles between the parafilm and the slide.

(22) Incubate sample slides in a tightly sealed moisture chamber (50% formamide) at 65°C overnight (>12 h) in the dark.

WASH

Solutions that are used at 37° or 65°C should be pre-warmed. From this step reagents are not required to be RNase-free.

- 300 ml of 5× standard saline citrate (SSC) (225 ml of H_2O, 75 ml of 20× SSC)
- 200 ml of 2× SSC/50% formamide (80 ml of H_2O, 20 ml of 20× SSC, 100 ml of formamide)
- 600 ml of TNE (10 mM Tris-Cl pH 7.5, 500 mM NaCl, 1 mM EDTA: 533 ml of H_2O, 6 ml of 1M Tris-Cl, pH 7.5, 60 ml of 5 M NaCl, 1.2 ml of 0.5 M EDTA)
- 200 ml of 2× SSC (180 ml of H_2O, 20 ml of 20× SSC)
- 200 ml of 0.2× SSC (200 ml of H_2O, 2 ml of 20× SSC)
- 200 ml of 0.1× SSC (200 ml of H_2O, 1 ml of 20× SSC)

(1) Carefully remove the parafilm from the slide without causing damage to the sections by incubating each slide in 5× SSC at 65°C.
(2) Wash the slides in 2× SSC, 50% formamide at 65°C for 30 min with gentle agitation.
(3) Wash the slides in TNE at 37°C for 10 min with gentle agitation.
(4) Incubate in 20 μg/ml RNase A in TNE at 37°C for 30 min.
(5) Wash the slides in TNE at 37°C for 10 min with gentle agitation.
(6) Wash in 2× SSC at 65°C for 20 min with gentle agitation.
(7) Wash in 0.2× SSC at 65°C for 20 min with gentle agitation.
(8) Wash in 0.1× SSC at 65°C for 20 min with gentle agitation.

DETECTION

- 200 ml of TN buffer (100 mM Tris-Cl pH 7.5, 150 mM NaCl: 174 ml of H_2O, 6 ml of 5 M NaCl, 20 ml of 1 M Tris-Cl pH 7.5)
- 3 × 1 L of TNT (TN buffer + 0.05% Tween 20: TN buffer [870 ml of H_2O, 30 ml of 5 M NaCl, 100 ml of 1M Tris-Cl pH 7.5] + 500 μl of Tween20)
- TNB buffer (0.5% NEN Blocking reagent in TN buffer). Keep at −20°C for long-term storage.
- Tyramide-biotin (NEL700A) is dissolved in 300 μl dimethylsulfoxide (DMSO) to prepare a stock solution. Aliquots can be stored at 4°C.
- Streptavidin (SA)-Alexa488 or 633 is dissolved in 1 ml PBS (with sodium azide 2 mM). Aliquots can be stored at −20°C.
- 300 ml of detection buffer (100 mM Tris-Cl pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$: 30 ml of 1 M Tris-Cl pH 8.0, 6 ml of 5 M NaCl, 3 ml of 1 M $MgCl_2$, 261 ml of H_2O)
- Fast Red TR solution 25 mg/ml in H_2O (5 mg of Fast Red TR is dissolved in 200 μl H_2O). Can be stored for 4 wks at 4°C.
- Anti-FLU-POD (horse-radish peroxidase) is dissolved in 1 ml redistilled H_2O (final concentration, 150 U/ml). Reconstituted antibody is dispensed into aliquots, and can be stored at 4°C, or at −80°C for long term.
- Anti-BrdU-POD is dissolved in 1 ml redistilled H_2O (final concentration, 15 U/ml). Reconstituted antibody is dispensed into aliquots, and can be stored at 4°C, or at −80°C for long term.

Notes:

- The amounts of reagent are for one slide.
- Steps 11–13 are omitted for two-color RNA ISH with DIG- and FLU-labeled probes.
- When anti-BIO is used, steps 12-13 have to be performed between steps 4 and 5.
- The concentration of primary antibodies must be optimized because antigenicity may be altered due to the proteinase K treatment. A higher concentration of antibodies is necessary in this protocol, compared with conventional immunohistochemistry.

(1) Incubate in TN buffer at RT for 5 min with gentle agitation.
(2) Apply 600 μl TNB buffer to each slide and incubate in a moisture chamber at RT for 30 min.
(3) Drain off the TNB buffer and apply 200 μl of TNB buffer containing the following combinations of antibodies and incubate in a moisture chamber at 4°C overnight:

Experiment	Antibody A	Antibody B	Antibody C
Two-color ISH	anti-DIG-AP	anti-FLU-POD	
Three-color ISH	anti-DIG-AP	anti-FLU-POD	anti-DNP or anti-BIO
Two-color ISH with IHC	anti-DIG-AP	anti-FLU-POD	antibody for antigen
Two-color ISH with BrdU	anti-DIG-AP	anti-BrdU-POD	anti-DNP or anti-BIO

The working concentration of each antibody is:

Anti-DIG-AP: 1/500–1/1000
Anti-FLU-POD: 1/100–1/200
Anti-DNP: 1/400–1/800
Anti-BIO: 1/100–1/500
Anti-BrdU-POD: 1/30

In this paper, anti-GAP43 (1/200) or anti-laminin (1/200) were used for two-color RNA ISH combined with immunohistchemistry.

(4) Drain off the antibody solution and wash in TNT buffer at RT for 5 min with agitation (×3).
(5) Apply 150 μl of tyramide-biotin (1/50) diluted with 1× amplification diluent and incubate in a moisture chamber at RT for 10 min. Do not incubate samples for longer than 10 min.
(6) Wash in TNT buffer at RT for 5 min with gentle agitation (×3).
(7) Apply 200 μl of SA-Alexa 488 or 633 (1/300) diluted in TNB buffer and incubate in a moisture chamber in the dark for 30 min.
(8) Drain off the SA solution and wash in TNT buffer at RT for 5 min in the dark with agitation (×3).
(9) Incubate in detection buffer at RT for 10 min in the dark with gentle agitation.
(10) Apply 600 μl of HNPP/Fast Red solution (10 μl of HNPP, 10 μl of Fast Red TR solution in 1 ml of detection

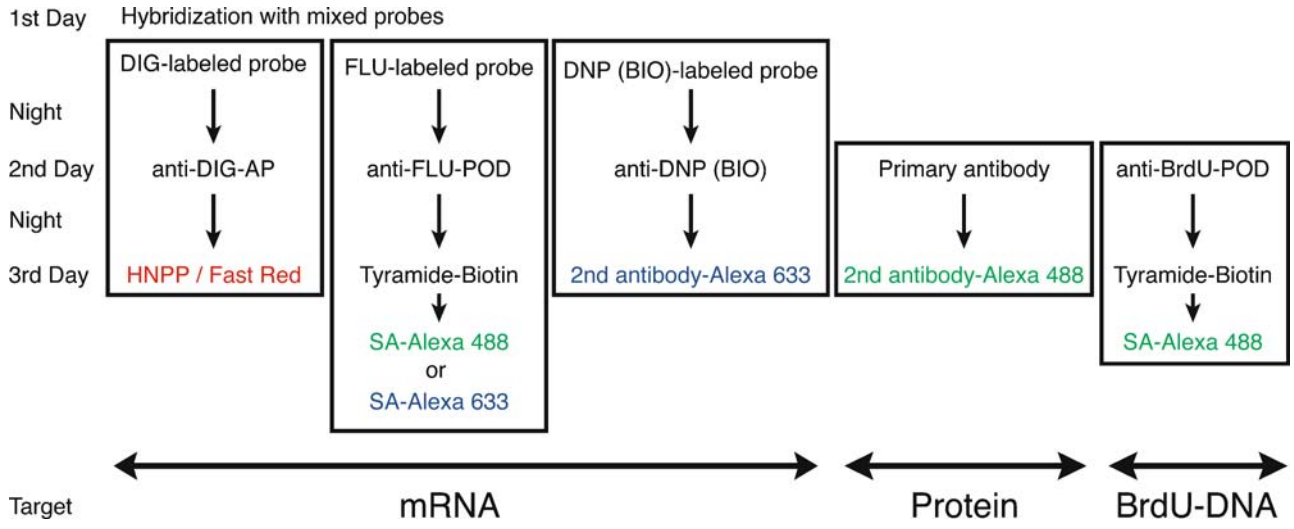

Fig. 1. Overview of the protocol. The strategy to detect three mRNAs, a protein and a BrdU-DNA is shown. Three target mRNAs are hybridized with DIG-, FLU- and DNP (BIO)-labeled probes followed by reaction with appropriate antibodies. The alkaline phosphatase-based detection for DIG, the horse-radish peroxidase-based detection for FLU and the non-enzyme-based primary/secondary antibodies detection for DNP (BIO) are used. A protein is detected by the combination of primary and secondary antibodies. BrdU-incorporated DNA is reacted with horse-radish peroxidase-conjugated anti-BrdU antibody followed by the detection with combination of tyramide-biotin and fluorescent dye-conjugated streptavidin.

buffer: filtrate this mixture with a 0.2 μm nylon syringe filter) and incubate in a moisture chamber at RT for 30 min in the dark.

(11) Drain off the HNPP/Fast Red solution, wash in TNT buffer at RT for 5 min in the dark with gentle agitation ($\times 3$).

(12) Apply 200 μl of Alexa 633 or 488-conjugated anti-rabbit IgG (1/500) diluted in TNB buffer and incubate in a moisture chamber for 2 hr at RT in the dark.

(13) Drain off the antibody solution, wash in TNT buffer at RT for 5 min in the dark with gentle agitation ($\times 3$).

(14) Wash in PBS for 5 min and observe samples directly in PBS or mounted with 100 ul of Vectashield mounting medium.

Dephosphorylated-HNPP/Fast Red TR, Alexa Fluor 488, and Alexa Fluor 633 can be exited by the 543 nm line of the HeNe1 laser, the 488 nm line of the argon-ion laser, and the 633 nm of the HeNe2 laser, respectively. Filter sets of FITC/Rhodamine/Cy5 in a Zeiss LSM 510 confocal microscope are suitable.

List of reagents is shown in Table 1.

Results

OVERVIEW OF METHODS

Figure 1 shows an overview of the strategy to detect two or three mRNAs, a protein and BrdU-DNA. For RNA ISH we used four types of hapten-labeled nucleotides incorporated in riboprobes: digoxigenin (DIG), fluorescein (FLU), dinitrophenyl (DNP) and biotin (BIO). Simultaneous detection of four different RNAs is theoretically feasible based on these four haptens, with consideration given to the species of origin for the antibodies. The riboprobes are visualized simultaneously in three ways. First, anti-hapten antibody conjugated with alkaline phosphatase is combined with HNPP/Fast Red alkaline phosphatase substrate. Second, anti-hapten antibody conjugated to a different enzyme, horse-radish peroxidase (POD), is combined with tyramide signal amplification (TSA). Third, anti-hapten antibody is combined with fluorescent dye-conjugated secondary antibody. The combination of these three methods enables the simultaneous detection of three different target RNAs. Alternatively, sequential TSA reactions are possible if the first peroxidase activity is inactivated using hydrogen peroxide (Paratore *et al.*, 1999; Zaidi *et al.*, 2000) or hydrochloric acid (Wilkie & Davis, 1998). Multiple combinations of primary anti-hapten antibodies and fluorescent dye-conjugated secondary antibodies can be applied by choosing primary antibodies from different host species and by using appropriate secondary antibodies. If two-color ISH is performed, the combination of an alkaline phosphatase-based and a POD-based detection method (for DIG and FLU, respectively) is recommended because of the higher sensitivity as compared to direct dye-coupled secondary antibody detection or using BIO and DNP as probe labels.

To combine RNA ISH with immunohistochemistry, incubation with the primary antibody reaction is performed along with application of anti-hapten antibodies for RNA ISH. Signals are then detected with fluorescent dye-conjugated secondary antibody. For BrdU-DNA detection, anti-BrdU-POD is applied with other anti-hapten antibodies, and signals are visualized using the TSA method.

Table 1. Reagent list for multi-color ISH.

Enzymes
- RNase A, 50 mg, Sigma (St. Louis, MO) #R4642
- Proteinase K, 5 ml, Roche (Indianapolis, IN) #03115828001
- DNase I, 2000U (2 U/ul), Ambion (Austin, TX) #2222
- T7 RNA polymerase, Roche #10881767001, 1000 unit
- SP6 RNA polymerase, Roche #10810274001, 1000 unit
- T3 RNA polymerase, Roche #11031163001, 1000 unit

Hapten-labeled nucleotides
- DIG RNA labeling mix, 10× conc., Roche #11277073910
- Fluorescein RNA labeling mix, 10× conc., Roche #11685619910
- DNP-11-UTP, Perkin Elmer (Boston, MA) #NEL555
- Ribonucleoside triphosphate set, Roche #11277057001
- BIO RNA labeling mix, 10× conc., Roche #11685597001

Detection reagents and antibodies
- HNPP fluorescent detection set, Roche #11758888001
- TSA biotin system for 100–150 slides, Perkin Elmer #NEL700A
- Streptavidin Alexa Fluor 488 conjugate, 1 mg, Molecular Probes (Eugene, OR) #S11223
- Streptavidin Alexa Fluor 633 conjugate, 1 mg, Molecular Probes #S21375
- Anti-fluorescein-POD, Fab fragments, Roche #11426346001
- Anti-digoxigenin-AP, Fab fragments, Roche #11093274910
- Rabbit anti-biotin, Enzo Life Science (Farmingdale, NY) #43861
- Anti-dinitrophenyl-KLH, rabbit IgG fraction, Molecular Probes #A6430
- Anti-bromodeoxyuridine-POD, Fab fragments, Roche #11585860001
- Rabbit anti-laminin, Sigma #L9393
- Rabbit anti-GAP43, Chemicon (Pittsburgh, PA) #AB5220
- Alexa Fluor 488 goat anti-rabbit IgG (H + L), Molecular Probes #A11008
- Alexa Fluor 633 goat anti-rabbit IgG (H + L), Molecular Probes #A21071

Other reagents
- Diethyl pyrocarbonate, Sigma #D5758-25ML
- Bromodeoxyuridine, Sigma #B5002
- Paraformaldehyde, 500 g, Fisher Scientific (Pittsburgh, PA) # 04042-500
- Phosphate Buffered Saline tablets, Sigma #P4417
- Sucrose, 1 kg, Sigma #S0389-1KG
- Tissue-Tek O.C.T compound, Sakura (Torrance, CA) #4583
- EDTA (Ethylenediaminetetraacetic acid) disodium salt, dihydrate, Sigma #E5134
- Lithium chloride anhydrous, Sigma #L4408
- TRIZMA base (Tris[hyroxymethyl]aminomethane) for the hybridization step, Sigma #T6066
- Tris for wash and detection steps, 1 kg, Fisher Scientific #BP152-1
- Sodium acetate, 500 g, Fisher Scientific #S210-500
- Sodium chloride for the wash and detection steps, 3 kg, Fisher Scientific #S271-3
- Sodium chloride for the hybridization step, Sigma #S7653
- Acetic anhydride, 500 ml, Fisher Scientific #A10-500
- Triethanolamine, 500 ml, Fisher Scientific #T350-500
- Hydrochloric acid, 500 ml, Fisher Scientific #A144S-500
- Chloroform, 500 ml, Fisher Scientific #C298-500
- Phenol:chloroform:isoamyl alcohol, UltraPure, Invitrogen (San Diego, CA) #15593-031
- Ethyl alcohol, AAPER Alcohol and Chemical Co (Shelbyville, KY)
- Magnesium chloride hexahydrate, 500 g, Sigma #M2670-500G
- Sodium citrate, 3 kg, Fisher Scientific #S279-3
- Formamide for the wash step, 500 ml, Roche #11814320001
- Formamide (deionized) for the hybridization step 100 ml, Sigma #F9037
- Yeast tRNA, Sigma #R6875
- Denhardt's solution, Sigma #D2532
- Dextran sulphate, 50 g, Amersham Biosciences (Piscataway, NJ), #US70796-50 g
- Hydrogen peroxide (30% solution), 100 ml, Fisher Scientific # H325-100
- RNasin ribonuclease inhibitor, 2500 U, Promega (Madison, WI) #N211A
- Vectashield mounting medium, Vector laboratories (Burlingame, CA) #H-1000
- Tween 20, 500 ml, Fisher Scientific #BP337-500
- Dimethyl sulfoxide, Sigma #D2650
- Sodium azide, Sigma #S8032
- Water, Sigma #W4502

Miscellaneous
- RNase-free microtube 1.5 ml, Kimble/Contes (Vineland, NJ) #749510-1590,
- Fisherbrand Superfrost/Plus microscope slides precleaned, Fisher Scientific #12-550-15
- Super Pap pen, Ted Pella (Redding, CA) #22311
- Parafilm, Pechiney Plastics Packaging (Menasha, WI) #PM-996
- Ziploc storage gallon (26.8 cm × 27.9 cm), Johnson (Racine, WI)
- Millex GP syringe driven filter unit, Millipore (Billerica, MA) #SLGP033RS
- Staining rack, stainless steel, Shandon (Waltham, MA) #109
- Staining dish/cover 20-slide unit, Wheaton Science Products (Milville, NJ) #900203
- Dry bath incubator (heat block), Fisher Scientific #11-718
- Laboratory stirrer/ hotplate, Corning (Acton, MA) #PC-220
- Mini hybridization oven, Bellco Glass (Vineland, NJ) #7930-10110
- Hot shaker for the wash steps at 37°C and 65°C, Bellco Glass #7746-22110
- Gyrotary shaker for the wash and detection steps, New Brunswick Scientific (Edison, NJ) #G-2
- Cryostat, Leica (Bannockburn, IL) #CM305
- Centrifuge, Eppendorf (Hamburg, Germany) #5417R

There are many possible combinations of hapten-labeled nucleotides for RNA ISH, immunohistochemistry, and BrdU-DNA detection. Among these possibilities, Figure 1 shows the methods that we have tested and found to work well for the main olfactory and vomeronasal epithelia in mouse.

THREE COLOR *in situ* HYBRIDIZATION

Following hybridization with DIG-, FLU- and DNP (BIO)-labeled probes, samples were incubated with anti-FLU-POD, anti-DIG-AP and rabbit anti-DNP simultaneously. After incubation with tyramide-biotin followed by streptavidin (SA)-Alexa 488, the FLU-labeled probe was visualized. Then using HNPP/Fast Red alkaline phosphatase substrate, the DIG-labeled probe was visualized in a different color. For detection of the DNP-labeled probe, a secondary antibody, Alexa 633-conjugated anti-rabbit IgG, was used. Thus the three types of hybridization signals can be visualized by their green, red and far-red fluorescence, respectively. When a BIO-labeled probe is used instead of a DNP-labeled probe, BIO should be visualized with the secondary antibody before the detection with tyramide-biotin/SA-Alexa 488 is applied, to avoid cross-reaction of SA-Alexa 488 to the BIO-labeled probe. Thus we prefer DNP to BIO.

The three-color ISH method was used to detect the expression of three OR genes, *M*71, *P*2 and *MOR28* in the MOE (Fig. 2A). As expected, the expression

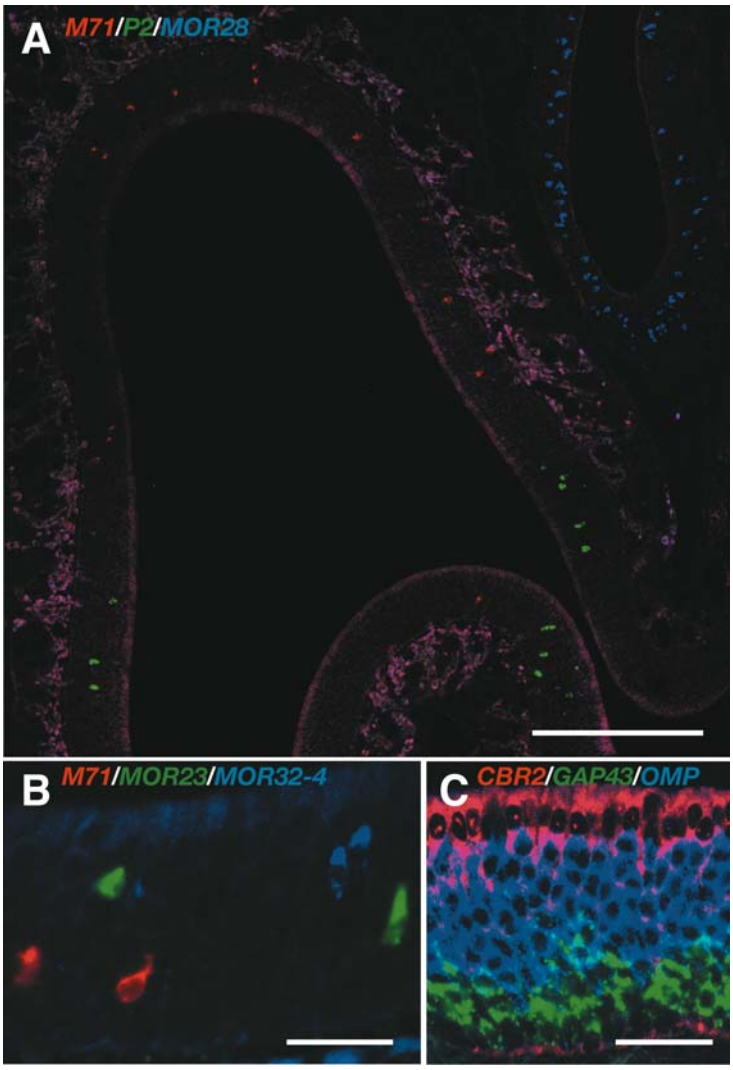

Fig. 2. Three-color RNA ISH in the MOE. Expression of three *OR* genes, *M*71 (DIG, red), *P*2 (FLU, green) and *MOR28* (DNP, blue) is detected, and the distribution of OSNs in the MOE is shown in (A). Expression of *M*71 (DIG, red), *MOR23* (FLU, green) and *MOR32-4* (BIO, blue) is detected in (B); there is a high background signal in the upper epithelium, where the sustentacular cells reside. Their region of expression overlaps in the MOE but they are expressed in distinct cells. Expression of three markers, *CBR2* (DIG, red) for sustentacular cells, *GAP43* (FLU, green) for immature OSNs, and *OMP* (DNP, blue) for mature OSNs is visualized in (C). Bars = 300 μm in (A), and 30 μm in (B) and (C).

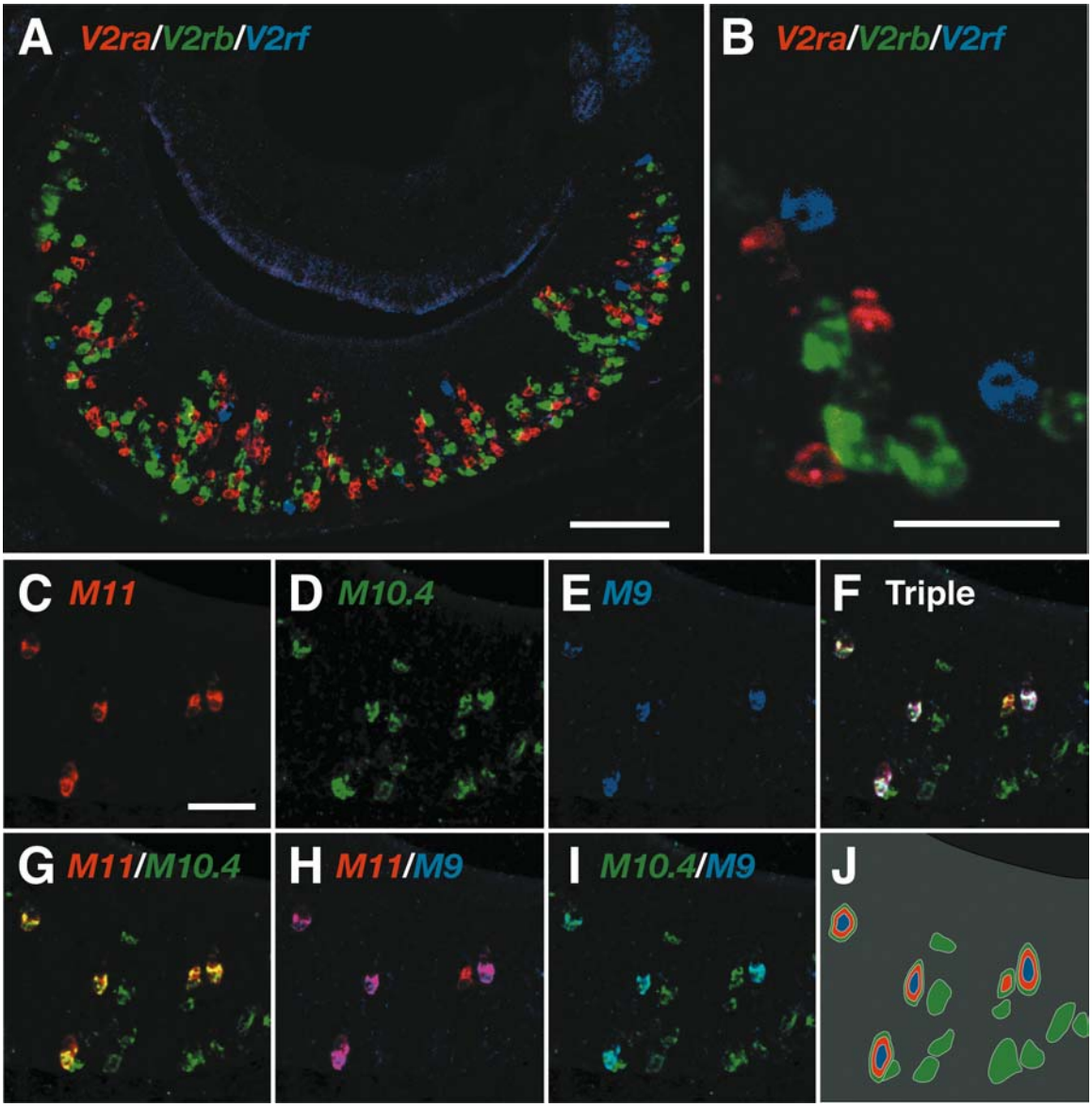

Fig. 3. Three-color RNA ISH in the VNE. Expression of three *V2R* genes, *V2ra* (DIG, red), *V2rb* (FLU, green) and *V2rf* (DNP, blue) is detected in (A), and high magnification is shown in (B). These genes are expressed in distinct cells. Expression of three *H2-Mv* genes, *M*11 (DIG, red, C), *M*10.4 (FLU, green, D) and *M*9 (DNP, blue, E) is shown. All combinations of merged pictures are shown (F–I). Overlaps of green/red, blue/red, green/blue, and green/red/blue, result in yellow, purple, turquoise, and white, respectively. A schematic overview of *H2-Mv* gene expression in the 15 cells is shown as colored profiles (J). Bars = 100 μm in (A), 30 μm in (B) and 50 μm in (C).

pattern of each OR gene is punctate and confined to a particular region of the MOE. Expression of the OR genes *M*71, *P*2 and *MOR28* was observed in different regions in the MOE, and their relative expression patterns could be compared in the same sample by three-color RNA ISH. Similarly, the expression of three OR genes that are expressed in overlapping regions of the MOE, *M*71, *MOR23* and *MOR32-4*, was detected using a BIO-labeled probe for *MOR32-4* (Fig. 2B). The three main cell types in the MOE, sustentacular cells, mature olfactory neurons and immature neurons, were visualized using probes for, respectively, carbonyl reductase 2 (*CBR2*)(Yu *et al.*, 2005), olfactory marker protein (*OMP*) and growth associated protein 43 (*GAP43*), respectively, thus revealing the three basic cell types in the MOE (Fig. 2C).

In the VNE the expression of three VR genes, *V2ra*, *V2rb* and *V2rf*, was investigated (Fig. 3A). Each probe detects several subfamily members. At high magnification (Fig. 3B), these genes are expressed in distinct, non-overlapping populations of cells. By contrast, *H2-Mvs*, a family of nine class Ib genes of the MHC, show variegated expression in overlapping populations of cells in the basal VNE (Ishii *et al.*, 2003).

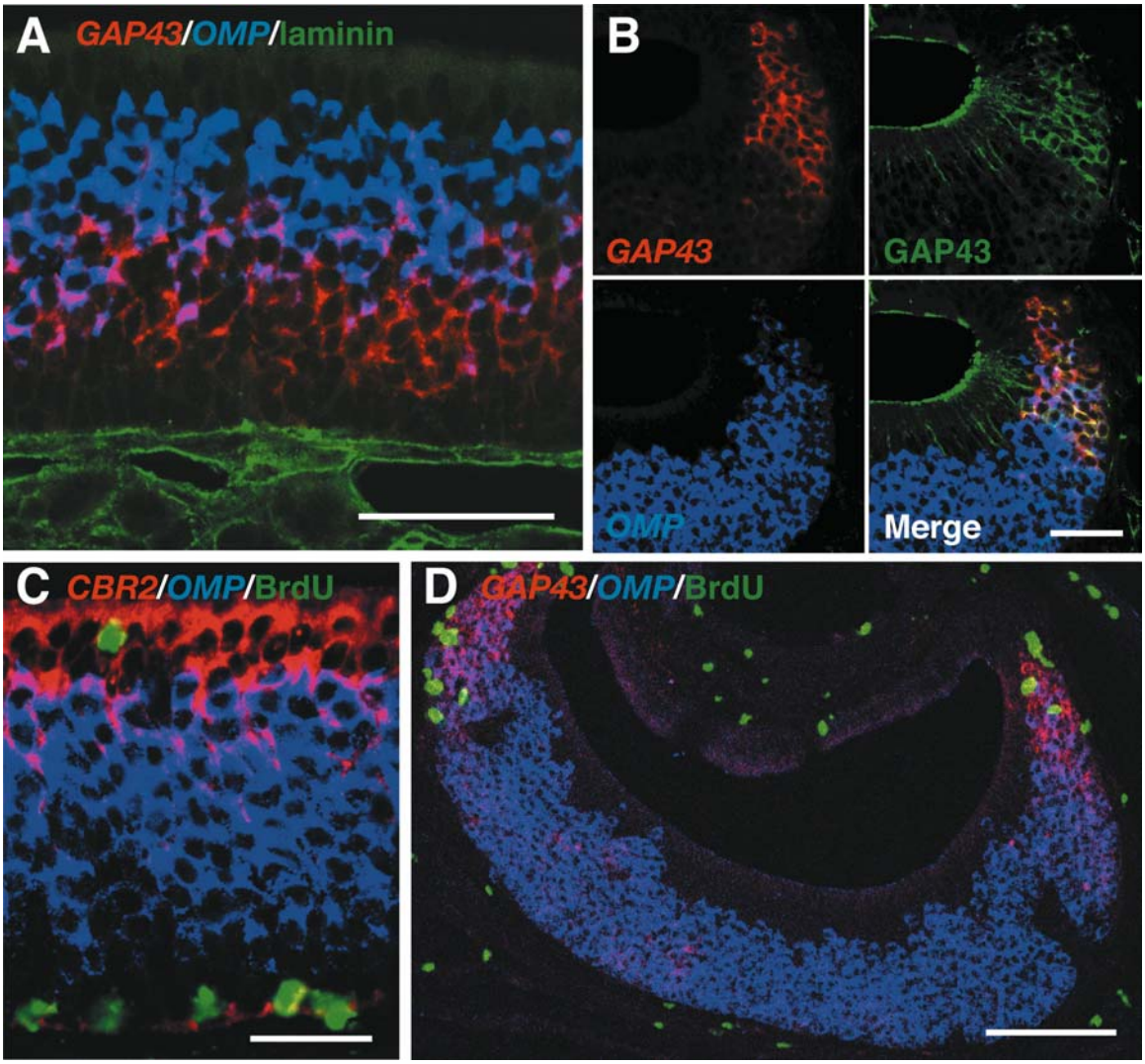

Fig. 4. Two-color RNA ISH combined with immunohistochemistry or a BrdU-DNA detection in the MOE and VNE. cRNA probes for the detection of *GAP43* (DIG, red) and *OMP* (FLU, blue) are used in combination with the anti-laminin antibody for the MOE section (A) or anti-GAP43 antibody for the VNO section (B). The anti-laminin antibody labels the basal lamina of the MOE. BrdU-incorporated DNA is detected in a 2-week old mouse dissected 24 hours after intraperitoneal administration of BrdU, together with the RNA ISH detection of *CBR2* (DIG, red) and *OMP* (DNP, blue) in the *MOE* (C), or *GAP43* (DIG, red) and *OMP* (DNP, blue) in the VNE (D). Bars = 50 μm in (A) and (B), 30 μm in (C) and 100 μm in (D).

Single-, double- and triple-positive cells were observed for three genes of this family, *M9, M11* and *M10.4*, detected simultaneously (Fig. 3C–I). Importantly, all combinations of expression of the three *H2-Mvs* can be examined in a single experiment, as summarized in Fig. 3J. Thus, complex expression patterns in overlapping populations of cells can be revealed with multi-color ISH.

TWO COLOR *in situ* HYBRIDIZATION COMBINED WITH IMMUNOHISTOCHEMISTRY

For the simultaneous detection of mRNA and protein expression, the detection of the DNP-labeled probe in three-color RNA ISH was replaced with immunohistochemistry using anti-laminin or anti-GAP43 antibodies, combined with a fluorescent dye-conjugated secondary antibody (Fig. 1).

In the MOE, *OMP* and *GAP43* mRNAs were visualized by two-color RNA ISH and laminin protein was detected simultaneously by immunohistochemistry (Fig. 4A). Laminin is concentrated in the basal lamina underneath the OE, and thus serves as a basal lamina marker. In the VNE, *OMP* and *GAP43* mRNAs were visualized by RNA ISH, and GAP43 protein was detected immunohistochemically (Fig. 4B). GAP43-positive cells are preferentially localized at the edge of the VNE, but some cells reside in the intermediate region of the VNE (data not shown) (Giacobini *et al.*, 2000). RNA ISH signals for *GAP43* were restricted to

the cell bodies, while immunostaining with anti-GAP43 antibody also visualized the dendrites of the same cells.

TWO COLOR IN SITU HYBRIDIZATION COMBINED WITH BrdU-DNA DETECTION

The MOE and VNO exhibit neuronal replacement continuously throughout adult life. BrdU was used to visualize newly born cells that incorporated nucleotides during DNA replication. Mice were injected with BrdU and dissected 24 h later, to label the cells that incorporated significant amount of nucleotides into their DNA within a 24 h time frame. Anti-BrdU-POD was used instead of the combination of FLU-labeled probe and anti-FLU-POD in the method of three-color RNA ISH (Fig. 1).

BrdU-DNA detection was performed in combination with RNA ISH using *CBR2* and *OMP* probes for MOE, and RNA ISH using *GAP43* and *OMP* probes for VNE. In the MOE,BrdU-positive signals were predominantly found basally (below the OMP-positive cells) and apically (sustentacular cells) (Fig. 4C). In the VNE, BrdU signals were detected at the edge of the VNO (Fig. 4D). Our observations are consistent with the literature (Giacobini *et al*., 2000; Martinez-Marcos *et al*., 2000; Matarazzo *et al*., 2004; Weiler & Farbman, 1997; Weiler *et al*., 1999), and reflect the sites of ongoing neurogenesis in postnatal animals.

Discussion

MULTI-COLOR ISH COMPARED TO IMMUNOHISTOCHEMISTRY

The generation of specific RNA probes is straightforward, now that the sequence of the mouse genome is available. A specific probe can be made possibly for any gene in a sequenced genome. By contrast, immunohistochemistry requires a specific antibody which takes a few months to raise. Antibodies cost much more than ISH probes, and generation of specific antibodies may be very difficult or impossible.

ISH is suitable for co-expression studies. Double staining for co-expressed genes results in clear overlap of the signals, because mRNA is detected in the cell body. In contrast, co-expression can be obscured in immunohistochemical studies due to differential subcellular localization.

Finally, cellular ISH signals provide excellent spatial resolution within a tissue. The distribution of cells expressing a given gene within a tissue can be visualized directly, but this is not possible for single cell RT-PCR. In immunohistochemical studies the distribution of cells expressing a given gene might not be revealed as clearly if the epitope is localized to cellular appendages such as dendrites or axons.

APPLICATIONS OF MULTICOLOR ISH IN OLFACTORY EPITHELIA

The MOE can be divided into two regions based on the expression of *OCAM* (Alenius & Bohm; 1997; Yoshihara *et al*., 1997), *O-MACS* (Oka *et al*., 2003) or *NQO1* (Gussing & Bohm, 2004), but it is not clear whether all neurons expressing a particular *OR* are positive for *OCAM*, *O-MACS* or *NQO1*.

The VNE is divided into two layers, apical and basal, based on the expression of *Gαi*2 and *Gαo*. Several other genes show a differential expression in the basal and apical VNE: a high expression of *Gγ*2 (Runnenburger *et al*., 2002), *OCAM*(Alenius & Bohm, 1997; Yoshihara *et al*., 1997), *neuropilin-2*(Cloutier *et al*., 2002; Walz *et al*., 2002), *PDE4A* .(Lau & Cherry, 2000), *Eph-A5* (Knoll *et al*., 2001), *slit-1* (Cloutier *et al*., 2004), *RGS3* (Norlin & Berghard, 2001), and *NQO1* (Gussing & Bohm, 2004) in the apical layer; and of *Gγ*8 (Runnenburger *et al*., 2002), *PDE4D*(Cherry & Pho, 2002) and *robo-2* (Knoll *et al*., 2003) in the basal layer (Halpern & Martinez-Marcos, 2003). The expression of these genes is assigned to one of these two layers by position, but uniform co-expression with either *Gαi*2 or *Gαo* has not yet been evaluated.

It is particulary difficult to identify precisely the cell type for populations of differentiating neurons because of their continuously changing nature. If a specific region, layer, or tissue looks positive by single-color RNA ISH, it can be difficult to show conclusively that all cells express this gene. To resolve such issues, multi-color RNA ISH can be performed using a probe for the target gene along with marker gene probes, for example, *OMP*, *CBR2* and *GAP43*, and genes involved in neurogenesis such as *Mash1* (Cau *et al*., 2002; Guillemot *et al*., 1993), *Neurogenin1* (Cau *et al*., 2002; Ma *et al*., 1996) and *Lhx2* (Hirota & Mombaerts, 2004) in the MOE, or *Gαi*2 and *Gαo* in the VNO. This comparison allows for an unambiguous answer as to whether overlap is complete or partial, such as between *Gαo* and *β*2- *microglobulin* in the basal VNE (Ishii *et al*., 2003).

Multi-color RNA ISH is convenient to characterize the expression of multigene families with differential expression of its members. In the MOE and VNO, OR and VR genes are thought to be expressed as one gene per cell—but perhaps not always (Mombaerts, 2004b). Co-expression of two OR genes has been reported in rat by two-color RNA ISH (Rawson *et al*., 2000), and the rat *V2R* gene *V2R2*, is co-expressed with other *V2R*s (Martini *et al*., 2001). The generality of the one receptor-one neuron rule can further be evaluated by multi-color RNA ISH. To study the mechanisms that regulate expression of OR and VR genes, multi-color RNA ISH will be useful in combination with genetic manipulations. For instance, the phenotype of a *OR* coding region deletion has been analyzed by the combination of RNA ISH and immunohistochemistry (Serizawa *et al*., 2003; Shykind *et al*.,

2004). Expression of the nine *H2-Mv* genes of the MHC shows a variegated pattern in the basal VNE. There are preferred combinations of co-expression among family members and also non-random co-expression between *H2-Mvs* and *V2Rs*, as we have shown with two-color RNA ISH (Ishii *et al*., 2003). Cadherin-related neuronal receptor/protocadherin a (CNR/Pcdha) genes show unusual expression patterns in the brain (Esumi *et al*., 2005). The complex expression pattern of CNR/Pcdha genes in neurons of the olfactory bulb was documented by two-color RNA ISH (Kohmura *et al*., 1998).

Single-color RNA ISH using a radioisotope-labeled probe may be more sensitive, and will continue to be useful for certain applications.

COMBINATION OF RNA ISH AND IMMUNOHISTOCHEMISTRY/BrdU-DNA DETECTION

RNA ISH and immunohistochemistry are compatible techniques. Cell bodies can be identified by detection of mRNA with RNA ISH, and other aspects of the cell by detection of protein with immunohistochemistry. It is also useful to compare mRNA and protein expression when the protein is unstable and degraded quickly, or conversely, when it is very stable and outlives mRNA long after it has disappeared (Shykind *et al*., 2004). It can also be helpful to evaluate the specificity of an antibody, by comparing expression of mRNA and protein. We caution that, in our hands, the conditions for RNA ISH cannot be changed very much. Because epitopes may be destructed by the proteinase K treatment, not all antibodies will work well.

While non-fluorescent detection of RNA ISH and BrdU has been reported (Ishii *et al*., 2000), fluorescent detection yields clearer signals. BrdU-labeled cells can differentiate to GAP43 or OMP-positive neurons (Matarazzo *et al*., 2004). Because probes for RNA ISH are easier to generate than antibodies, BrdU-DNA detection combined with RNA ISH can be applied to virtually any cell type.

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