Olfactory Sensory Neuron: Immunohistochemical Markers, Technique of Flat-Mounted Mucosa and Cell Count

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Abstract

A classification of olfactory sensory neuron (OSN) markers, a simple and robust technique of immunostaining on flat-mounted olfactory epithelium (OE) and a reliable quantification of the density of mature and immature OSNs are three crucial tools to study the pathophysiology of olfactory dysfunction.

Using the rat model, we first categorized the main OSN markers by immunohistochemistry (IHC) on cross sections of OE. The OSN markers were divided into 3 groups: mature OSNs (OMP), immature OSNs (Tuj-1, DCX, OLIG2) and both (N-cadherin, LHX2, PGP9.5). The subcellular localization of each marker was also described.

Tuj-1, OMP, DCX, PGP9.5 and N-cadherin were selected for immunostaining on flat-mounted OE because of their staining of OSN dendrites. We were able to successfully label OE with all the 5 markers using a simple technique for flat-mounted OE. In addition, this technique allowed the first mapping of the OE directly on the ethmoid turbinates.

Finally, we quantified the mature (OMP+, Tuj-1-), immature (OMP-, Tuj-1+), transitory (OMP+, Tuj-1+) and total OSN density which were respectively 42080 ± 11820 , 49384 ± 7134 , 14448 ± 5865 and 105912 ± 13899 per mm^2 (mean $\pm$ SD). The transitory population was quantified for the first time.

Introduction

Smell disorders can markedly affect the quality of life. Indeed, olfaction allows not only the full appreciation of the flavor and the palatability of foods but also serves as an early warning system against gas leak, fire or spoiled food products.

Frequency of smell disorders in the general population is still a matter of debate¹. Subjective complaints do not always accurately reflect the chemosensory disturbance experienced by a patient. In 2016, the National Health and Nutrition Examination Survey (NHANES) indicated that the rate of olfactory dysfunction in general population was around 4% at age 40 to 49 years, 10% at age 50 to 59, 13% at age 60 to 69, 25% at age 70 to 79, and 39% for those over 80 years of age. 14–22% of patients over 60 years reported anosmia². Olfactory disturbance has many possible causes: naso-sinusal diseases, head trauma, degenerative pathology such as Alzheimer disease³. However, even with the recent advances, the pathophysiology of olfactory disorders remains poorly understood⁴. Recently, the SARS-Cov-2 pandemic has led to a new resurgence of olfactory disorders. Indeed, sudden loss of olfactory functions in SARS-Cov-2 affected patients became one of the most reported symptoms at the onset of the pandemic⁵. However, the exact pathophysiology of this dysfunction on the olfactory epithelium (OE) is still a matter of debate^{6–8} and the lack of recovery for some patients is always a thorny issue⁹. Likewise, the significant decline of olfactory function with age in apparent contrast to the extreme regenerative capacity of the OE is the subject of current studies^{9,10}.

Immunostaining is one of the major tools used to describe the OE, its cell population and its regenerative capacity. In this technique, the selection of an appropriate marker is an essential step for a simple, reliable and reproducible work. Numerous markers of olfactory sensory neurons (OSNs) are reported in literature but their tissue and cellular localization according to the stage of maturity of the OSNs, are not clearly described, nor well classified.

The most frequently used technique of immunostaining on olfactory mucosa is immunohistochemistry (IHC) on cross sections, due to different reasons: the technique's maturity, the low tissue consumption, the high number of antibodies available for IHC application, and the pseudo-stratified structure of the OE. Immunostaining on flat-mounted OE, an unusual approach, is especially interesting to explore the mapping and the boundaries of the OE and is appropriate for the evaluation of the OSN cell density.

On each side of the nasal cavity, the olfactory cleft is enclosed by the medial flat septum and the lateral ethmoid turbinates' convolutions. Because of these convolutions, the turbinates' olfactory mucosa cannot be easily separated from its underlying bony structure. In the literature, immunostaining on flat-mounted olfactory mucosa has been therefore mainly performed on separated septal mucosa, that represents a rather flat portion of mucosa^{11,12}. The only study describing the turbinates' OE used transgenic mice that co-expressed the green fluorescent protein (GFP) with Olfactory Marker Protein (OMP)¹³. This technique is however not transferable to human. To our knowledge, immunostaining applied directly on whole ethmoid turbinates has never been described.

In this study, we first classified the different olfactory markers according to their expression in mature and immature OSNs and to their subcellular localization, using the IHC technique on cross sections. We then developed and validated a simple technique of immunostaining on flat-mounted septal OE and we selected a panel of olfactory markers usable in these conditions. In addition, we quantified OSNs cell density on flat septal OE, with a differentiation between mature, immature and transitory OSNs. Finally, this technique was successfully applied on entire lateral portions of the olfactory cleft.

Results

IHC on cross-sections of OE

Markers of the mature and immature OSNs: OMP was used as the reference marker of mature OSNs to define the localization of the other markers (Figure 1). The neuron-specific mouse anti Tuj-1 (β -III tubulin) was chosen as the reference marker for immature OSNs (Figure 2).

The cell bodies of the OSNs located in the upper layers of the OE were strongly labelled with the antibody against OMP: these OSNs located in the upper layers of the OE were mature OSNs. Tuj-1, DCX, and OLIG2 stained OSNs located at the lower layers of the OE, that corresponded to the location of immature OSNs. PGP9.5, LHX2, and N-cadherin stained both mature and immature OSNs located in different layers of the OE. Besides this, N-cadherin also stained the basal cells, especially the horizontal basal cells.

OSN dendrite markers: to enable immunostaining on flat-mounted mucosa, we looked for and determined the olfactory markers located in the dendrites or "dendritic knob", associated with a strong labelling of the most superficial OE layer. Five proteins were identified: OMP, Tuj-1, DCX, N-cadherin and PGP9.5 (Figure 1, 2).

Olfactory nerves (axons) and sensory nerves: four OSN markers (OMP, Tuj-1, DCX and PGP9.5) were associated with a strong labelling of the olfactory nerve fascicles (OSN axons). The N-cadherin was also detected in the olfactory fascicles, but with a weak staining signal. OLIG2 and LHX2 labelling was only observed in the OSN nuclei and not in the OSN dendrites or axons (Figure 1, 2). We found that GAP43 and G β labeling was present in the olfactory nerves located in the lamina propria but their expression was not detected in the OSN cell body located in the OE (**Supplementary Figure 1a, c**). The 3 neurofilaments (NF) subunits (NF-L, NF-M and NF-H) and peripherin were not expressed in olfactory but trigeminal nerves (**Supplementary Figure 1b, d**).

Immunostaining on flat-mounted septal mucosa: technique and validation of the preselected markers

In this study, we developed a simple technique for immunostaining on flat-mounted septal mucosa: fixation of the isolated septal mucosa in 0.5% PFA preceded by pretreatment of the rat head in 1% PFA for 1 hour.

As described above, we identified 5 markers of the OSN dendrite: OMP, Tuj-1, DCX, N-cadherin, and PGP9.5. We used these markers on flat-mounted septal mucosa.

To ensure specificity of OSN labeling, a double staining was achieved with one of the olfactory markers and a respiratory epithelium marker. Three cellular cilia markers of the respiratory epithelium were tested: tubulin α , acetylated tubulin α , and tubulin β IV. They were tested first by IHC on cross sections. Tubulin β IV was chosen because of its specificity and strong staining (**Supplementary Figure 2**). This double staining was especially noticed on the transition zone between the olfactory and the respiratory epithelium (Figure 3 and **Supplementary Figure 3**).

Tuj-1, DCX, N-cadherin, PGP9.5 and tubulin β IV were easily stained on the flat-mounted tissue fixed in 0.5% PFA. A treatment of the tissue in 0.5% SDS for 10 minutes at RT after fixation was necessary to reveal the OMP staining. This treatment had no obvious effect on the labelling of other markers. We noted that the boundary between OE and respiratory epithelium was rather clear and unambiguous on septal mucosa (Figure 3). No respiratory metaplasia or interpenetration was observed in the OE. This sharp transition between olfactory and respiratory epithelium was not only observed in immunostaining on flat-mounted mucosa but also in IHC on cross sections (**Supplementary Figure 3**).

Cell density of mature, immature and transitory OSNs

OMP was the only marker for mature OSNs. Tuj-1 was chosen for labelling the immature OSNs as it had a better staining signal than DCX. They were thus used for cell density evaluation. Double labeling with

OMP and Tuj-1 was performed on septal OE of 3 animals (4 week-old). Five pictures at different locations of the OE were acquired for each animal. In total, 15 images of 44859 μm^2 area were analyzed. We counted an average of 4751 ± 623 OSNs per image.

The density of OMP+ OSNs was $56528 \pm 9294/\text{mm}^2$, the density of Tuj-1+ OSNs was $63832 \pm 8174/\text{mm}^2$, and the density of OMP+/Tuj-1+ was $14448 \pm 5865/\text{mm}^2$. We further distinguished OSNs in three categories based on the double labelling pattern: mature OSNs (OMP+, Tuj-1-) with a cell density of $42080 \pm 11820/\text{mm}^2$; immature OSNs (OMP-, Tuj-1+) with a cell density of $49384 \pm 7134/\text{mm}^2$, and transitory OSNs (OMP+, Tuj-1+) with a cell density of $14448 \pm 5865/\text{mm}^2$. The total OSN density was $105912 \pm 13899/\text{mm}^2$ (Figure 4).

Direct immunostaining on ethmoid turbinates

The immunostaining of whole ethmoid turbinates showed that the OE was clearly labeled by Tuj-1 (Figure 5). The staining specificity was verified by comparing to a negative control and confirmed by coherent mapping of the olfactory zone. Dendrite staining was also verified under an epifluorescence microscope. This staining was repeated in 3 independent experiments, and the results were well reproducible. Similar to septal mucosa, the boundary between OE and respiratory epithelium was also clear and unambiguous on turbinate mucosa (Figure 5e,f).

Discussion

Characterization of different olfactory markers by IHC

The classification of different olfactory markers in function of their expression in mature and immature OSNs can be a useful guide for investigators to choose an appropriate marker. OMP is known to be a critical protein in OSN maturation process¹⁴. We chose it as the reference marker to localize mature OSNs. Tuj-1 expression decreases during OSN maturation¹⁵ and it is an usual marker for immature OSNs in adult rodents¹⁶. In our study using rats of 3 to 5-week old, we observed its localization in immature OSNs by double staining assay with OMP as reported in adult rats. In addition, Tuj-1 showed a very strong labeling signal. As a consequence, Tuj-1 was chosen as the reference marker of immature OSNs.

Nine olfactory markers were characterized in this study (Figure 6): OMP was the only marker of mature OSNs. Tuj-1, DCX, and Olig2 were the markers of immature OSNs whereas N-cadherin, LHX, and PGP9.5 were detected in both populations. GAP43 and G β seemed specific of the OSN nerve fascicles. Immature OSNs were localized to the lower layers of the OE, and mature OSNs were localized in the upper layers. N-cadherin was the only marker that was strongly expressed in horizontal basal cells.

GAP43, a nervous system-specific protein that plays an important role in OSN axon regeneration growth¹⁷, is expressed in both OSN cytoplasm and axon¹⁸ or only in axon¹⁹. In this study, we observed GAP43 expression preferentially in the olfactory nerve fibers of the lamina propria. Two hypotheses could explain these differences in subcellular locations: 1. The expression of GAP43 in the cytoplasm of the

OSN may not be constant during lifetime but decreases rapidly with the age of the animal²⁰. 2. A single antibody may not recognize its antigenic site in all forms of the protein due to possible antigenic site concealment in particular subcellular location of the protein. Dominant expression of Gβ1, a component of the heterotrimeric G proteins, was found in OSN axons by IHC as previously reported²¹. These two markers could be useful to study the olfactory nerve fiber network on a flat-mounted nasal mucosa without being hindered by OE staining.

In the lamina propria which is a thin layer of loose connective tissue under the respiratory and olfactory epithelium, we observed two distinct types of nerve fiber fascicles: OSN nerve fibers/axons and nerve fibers/axons from the peripheral nervous system (PNS). These two types of nerve fibers had a very different morphology (**Supplementary Figure 1**) and staining. NF (L, M, and H) and peripherin were mainly observed in the nerve fibers of the PNS. These thin regular bundles were very different from the swollen fascicles of the olfactory nerve. These PNS nerve fiber bundles have been described in the literature as trigeminal nerves^{19,22}.

We believe that this characterization of OSNs markers developed in this study, according to their cellular and subcellular localizations (Figure 6), will facilitate the choice of appropriate markers in future studies on olfactory mucosa.

Immunostaining on flat-mounted septal mucosa

The olfactory mucosa is a pseudo-stratified epithelium located on the surface of the nasal cavity. It is therefore possible to perform immunostaining on flat-mounted olfactory mucosa.

However, observations of OE with this technique have been rarely reported in literature: 9 publications over a period of 23 years were identified and summarized in **Table 1**^{9,11,12,23-28}. Among these studies, 5 are from the same team, directed by Professor James Schwob in Boston, supporting that this approach is not mastered by numerous teams, possibly due to technical pitfalls.

First, considering the technique, there is no consensus. Each team used a different protocol and even in a same team, different protocols are reported and adapted to each situation. Yet, tissue fixation with Formaldehyde 4% (equivalent to PFA 4% or formalin 10%) represent a common step in almost all these protocols. It is a conventional fixative and the main component of the different fixatives used (**Table 1**). Post-fixation treatment, consisting in antigen retrieval and cell permeabilization, was reported in most cases. Glycosidase (enzyme), SDS (denaturant), and ethanol (denaturant) can all be used for antigen retrieval. Sonication, freeze/thaw, Ethanol or SDS may play a role in cell permeabilization like triton, but can be also responsible for damages of the tissular microstructure.

Only 3 studies out of 9 used immunofluorescence, in spite of many advantages compared to immunoenzymology. One of the main causes could be the important autofluorescence of the olfactory mucosa which is opaque. For this reason, Child *et al.* added a tissue clarification step to make the olfactory mucosa more transparent in order to decrease autofluorescence⁹.

The immunostaining technique on flat-mounted cornea, we developed previously, highlighted the fact that cells on a flat-mounted tissue are more susceptible to overfixation with a 4% concentration of PFA, compared to cells in culture²⁹. Using a low concentration of PFA, 0.5% or 1%, can greatly improve the results of immunostaining on the flat-mounted corneal tissue and still allows an overall tissue view and an accurate subcellular marker localization^{30,31}. In addition, overfixation by formaldehyde promotes additional autofluorescence of the tissue. By applying the same technical approach to the nasal tissue, we succeeded in developing a simple and easily reproducible immunostaining technique on flat-mounted olfactory mucosa. Four of the five OSN markers selected by IHC (Tuj-1, DCX, N-cadherin and PGP9.5) were detectable directly without any antigen retrieval process. Only the OMP marker needed antigen retrieval with SDS. This antigen retrieval had also shown its effectiveness for some difficult staining in the case of cornea²⁹.

We listed here the 5 olfactory markers suitable for immunostaining on flat-mounted septal mucosa: OMP, Tuj-1, DCX, N-cadherin and PGP9.5. We speculate that other olfactory markers that are able to stain OSN dendrites would also be suitable for immunostaining on flat-mounted olfactory mucosa. From a technical point of view, we noted in this study that antibodies validated by IHC (frozen) were also applicable for immunostaining on flat-mounted tissue.

In this study, we provided a simple and efficient protocol and described the 5 more relevant OSN markers for immunostaining on flat-mounted septal mucosa. By combining one of the 5 OSN markers with Tubulin β IV (a marker of the respiratory epithelium), the boundary between the two epithelia was clearly defined. This precise visualization of the transition zone between olfactory and respiratory epithelium may offer many opportunities and applications in the future, especially in research on respiratory metaplasia of the OE.

Density of mature, immature and transitory OSNs

Few studies have been devoted to evaluate the density of OSNs, yet this data is important for the analysis and understanding of olfactory dysfunction.

In 1981, Hinds *et al.* calculated the density of OSNs using electron microscopy, based on the count of olfactory knobs on a section passing through the middle of the rat's septal OE. They estimated the density of all OSNs between 60,000 and 120,000/mm² ³². In 1989, Meisami *et al.* calculated the density of total OSNs from cross-sections of OE by counting the number of non-specifically counterstained nuclei. They estimated the OSNs cell density at $94170 \pm 3950/\text{mm}^2$ in 25-day-old rats³³. In 1996, Loo *et al.* described a count of mature and immature OSNs based on the immunostaining technique on flat-mounted olfactory mucosa²⁴. They counted olfactory dendrites/buttons of mature OSNs stained by OMP and immature OSNs by GAP43. The technique for labeling revelation was immunoenzymology with the tracer DAB (3, 3'-diaminobenzidine). They found $30022 \pm 5311/\text{mm}^2$ mature OSNs (OMP+) and $8443 \pm 1012/\text{mm}^2$ immature OSNs (GAP43+) on the septal OE of 7-month-old rats. Double staining was impossible using immunoenzymology, so the transitory OSNs were not detected.

In this study, we found the density of total OSNs per millimeter square was 105912 ± 13899 which is consistent with the two first studies previously cited^{32,33}. We found $56528 \pm 9294/\text{mm}^2$ of OMP+ OSNs and 63832 ± 8174 of Tuj-1 + OSNs. In general, the density of mature and immature OSNs evaluated by this study is higher than the OSNs density reported by Loo *et al.*²⁴. One of the reasons that could explain these differences could be to the sensitivity of immunofluorescence compared to immunoenzymology (DAB tracer system). Although, it can explain the difference of mature (OMP+) OSN density (56528 ± 9294 vs 30022 ± 5311), this cannot be a valuable explanation for the huge difference in immature OSNs density (63832 ± 8174 (Tuj-1+) vs 8443 ± 1012 (GAP43+)). Another reason might be the weaken and even absence of expression of GAP43 in adult rat²⁰. In their study, Loo *et al.* used 7 month-old rats to count immature OSNs stained by GAP43²⁴. In addition, younger rats we used could have more immature OSNs than older rats.

Thanks to double staining inside dendrites/knobs with both OMP and Tuj-1, we were able to identify a population of transitory OSNs that express OMP and Tuj-1 at the same time. This transitory OSN population density was $14448 \pm 5865 /\text{mm}^2$ in 3-5 week-old rats, representing about 14% of total OSNs. We noticed that this population co-expressing OMP and Tuj-1 was not easy to detect by IHC on cross sections in the OSN soma (neuron body). Indeed, very few OSNs co-expressed these two proteins in the soma (**Supplementary Figure 4**). This difference in co-expression of OMP and Tuj-1 in soma and dendrites was already observed in a previous study³⁴. On the contrary, the co-staining of these two proteins seemed much more evident in the OSNs axons (Figure 1). This contrast of co-staining in the soma, dendrites and axons indicates that the retrograde transport of proteins in OSNs may be very slow.

Due to double labeling with fluorescent tracers on flat-mounted mucosa, we were able to quantify for the first time a population of transitory OSNs that express both OMP and Tuj-1 in their dendrites. We believe that this technique of counting mature, immature and transitory OSNs would be a good tool for studying pathophysiology of OE.

Direct immunostaining on ethmoid turbinates

Once finalized, the protocol of immunostaining on flat-mounted tissue was successfully transferred to the ethmoid turbinates. The high intensity of Tuj-1 staining allowed us to clearly identify the mapping of the OE on the ethmoid turbinates despite the fact that the image was acquired using a fluorescence microscope that were not able to filter the autofluorescence of the tissue (connective tissue and bone) below the OE. We observed that the boundary between the respiratory and olfactory epithelium was clear and unambiguous on both septal and turbinate mucosa in 3 to 5 weeks old rats.

To the best of our knowledge, the only study showing the turbinates' OE used transgenic mice in which the OSNs expressed GFP¹³. This transgenic technique is not transferable to human. In this study, we have, for the first time, demonstrated OE mapping directly on rat ethmoid turbinates using the immunostaining technique. We believe that this technique would be applicable for OE mapping on human turbinates. We could transpose this technique directly to the nasal cavities of human donors to

allow the mapping of the olfactory zones using the technology of fluorescence video microscopy with an optic fiber.

Conclusion

We were able to characterize different olfactory markers according to their expression in mature and immature OSNs and their subcellular locations. These data should be very useful to select appropriate olfactory markers in future studies. The technique of immunostaining on flat-mounted septal mucosa by simply lowering the concentration of PFA fixative to 0.5% seems to be very effective and has a tremendous benefit of simplifying the existing protocols. All the 5 markers with dendrite location identified by IHC (Tuj-1, OMP, DCX, PGP9.5 and N-cadherin) were appropriate for immunostaining on flat-mounted tissues. This technique was relevant to determine the olfactory areas directly on turbinates. Finally, we were able to count the density of total ($105912/\text{mm}^2$), mature ($42080/\text{mm}^2$), immature ($49384/\text{mm}^2$) and transitory ($14448/\text{mm}^2$) OSNs in rats.

Methods

Collection and pretreatment of rat nasal tissue

The head of 3- to 5-week-old rats (Wistar Han) was recovered after the animals were sacrificed. This is not animal experiments according to the European and French regulations because the rats were not treated in any way while alive. To limit the number of animals used in research studies, the remaining rat organs were shared with other laboratories. The rats were first anesthetized with isoflurane and then killed by an overdose of pentobarbital. To further protect the surface of the nasal mucosa, the rat head was immersed in 2% PFA for 1h at room temperature (RT) in the case of immunohistochemistry (IHC) on cross-sectional tissue, or 1% PFA for 1h at RT in the case of immunostaining on flat-mounted tissue. After this pretreatment, the nasal cavities with surrounding bone were isolated. The soft tissues (skin, connective tissue) around the bone and the teeth of the upper jaw were removed.

IHC on cross sections of OE

After dissection in order to isolate the nasal cavity with the surrounding bone, samples were placed back into 1% PFA overnight at 4°C to enhance fixation. After 3 rinses in PBS, the samples were incubated with 0.5M EDTA (E5134, Sigma Aldrich), pH adjusted to 7.4 for 24 hours at RT under gentle agitation in order to soften the bone and facilitate cutting.

After 3 rinses in PBS, the samples were immersed in increasing concentrations of sucrose (10%, 20%, 30%) for one hour each. After immersion in optimum cutting temperature compound (OCT, TissueTek, Sakura Finetek, USA), the samples were quickly frozen in liquid nitrogen and then stored at -20°C until processing. Frozen tissue cross sections of 10 μm thickness were performed using a cryostat (CM1520, Leica).

Frozen sections were rehydrated in water for 5 minutes (min) at RT. After 5-min incubation in 0,5% Triton, the saturation of non-specific sites was done by 30-min incubation in saturation buffer (2% BSA and 2% goat serum in PBS) at 37°C. Incubation with the primary antibody (1/500 dilution) in the saturation buffer was performed at 4°C for one night. After 3 rinses in PBS, the incubation with the secondary antibody (1/500 dilution) was done at 37°C for one hour. Cell nuclei were highlighted by incubation with 2µg/ml DAPI in PBS for 5 min at RT. The used primary antibodies are listed in **Table 2**. The different secondary antibodies used in this study are all highly cross-adsorbed. The combined fluorophores are Alexa Fluor 488 or 555 (A21121, A21127, A21131, A21137, A21141, A21147, A21422, A32723, A32727, A32731, A32732, A32766, A32790, A32814, A32816, Invitrogen/Thermo Fisher Scientific). After placing a drop of Fluorescence mounting medium (NB-23-00158-2, Neo Biotech) on the tissue section, a glass coverslip was placed and attached with adhesive tape to both ends of the slide.

When one of the primary antibodies was from goat, such as OMP or OLIG2 (**Table 2**), the saturation buffer was 4% BSA (goat serum suppression) and the secondary antibodies were from donkey. As antibodies anti-OLIG2 and anti-OMP were both from goat: therefore, they were not suitable for double staining. The positive staining of each marker was repeated at least 3 times independently in 3 different rats (4-week-old). Negative controls consisted in replacing primary antibodies by a non-specific mouse, rabbit or goat IgG.

Immunostaining on flat-mounted tissue (isolated septal mucosa)

The initial step of head removal was similar to previously described. The septum was removed from the remaining of the nasal cavity. The septal mucosa was carefully isolated under an operating microscope (OPMI 6-CFR, ZEISS) and immersed in 0.5% PFA for 2 hours at RT to enhance fixation. After rinsing in PBS, the samples were permeabilized and saturated in saturation buffer (2% BSA, 2% goat serum, 0.5% Triton and 0.02% sodium azide in PBS) for 30 min at 37°C. The sodium azide was added in order to avoid microbial contamination. Incubation with the primary antibody diluted to 1/300 in saturation buffer was done for 3h at 37°C or overnight at RT or 4°C. After 3 rinses in PBS, incubation of the secondary antibody diluted to 1/500 was performed at 37°C for 2 hours. Cell nuclei were highlighted with incubation with 2µg/ml DAPI added directly into the secondary antibody mix. The primary antibodies were selected from the antibodies validated by IHC (**Table 2**). The different secondary antibodies were the same as those used for cross-sectional IHC. The isolated septal mucosa was placed on a glass slide epithelium side up under an operating microscope. 2 to 3 drops of Fluorescence mounting medium were added on the tissue. The glass slide was placed on top and fixed with adhesive tape.

When the primary antibody was the goat anti-OMP antibody, a treatment of the tissue with 0.5% SDS for 10 minutes at RT was necessary. 5 washes in PBS to remove the SDS residue, were done before saturation in PBS containing 4% BSA, 0.02% sodium azide. The secondary antibody was from donkey. The immunostaining of each marker was repeated at least 2 times independently in 2 different rats.

Direct immunostaining on turbinate

Unlike the nasal septum on which the mucosa is rather flat and can be easily separated from the underlying cartilaginous and bony structures, the mucosa on turbinates is very difficult to separate from the underlying bones. The only way to observe an intact OE on ethmoid turbinates is to perform immunostaining directly on turbinates without dissection.

The protocol for direct immunostaining was similar to the protocol for immunostaining on flat-mounted septal mucosa. Briefly, after 1h of incubation of the rat's head in 1% PFA at RT, the lateral wall of the nasal cavity (connective tissue and bone included) was separated from the remaining of the cavity. Further fixation in 0.5% PFA for 2 hours at RT was done. The primary antibody chosen was anti-Tuj-1 antibody from mouse (IgG2a) because of its strong staining in IHC and on flat-mounted mucosa. The protocol of permeabilization, saturation, incubation of the primary antibody and counterstaining was the same as the protocol for immunostaining on the flat-mounted mucosa. To increase staining intensity, we used simultaneously two secondary antibodies both targeting the antibody anti-Tuj-1: 1/1000 Alexa Fluor 488 goat anti-IgG (H+L) (A32731, Invitrogen) and 1/1000 mouse anti IgG2a (A21131, Invitrogen). The images of immunostaining of whole ethmoid turbinates were taken by a fluorescence microscope.

The results were repeated 3 times independently in 3 rats. Negative controls consisted in replacing primary antibody anti-Tuj-1 by a non-specific mouse IgG.

Image acquisition

For IHC applied on tissue cross sections, an epifluorescence microscope (IX81, Olympus, Japan) equipped with CellSens software (Olympus, Germany) was used. The objectives were 4X, 10X and 40X. A confocal laser scanning microscope (Fluoview FV1200, Olympus, Japan) equipped with FV10-ASW4.1 imaging software (Olympus, Germany) was used for immunostaining on flat-mounted mucosa. It was also used for IHC on cross-sections only at high magnification (60X Objective). Because of the large size and irregularity of the tissues, a fluorescence microscope (MVX10, Olympus, Japan) equipped with CellSens software (Olympus, Germany) was used for direct immunostaining on ethmoid turbinates.

OSN density counting using Fiji Macro

Evaluation of OSN density was performed by counting the number of dendrites. The images were acquired using a confocal microscope with an objective 60X. Only the upper surface of tissue, where every OSN dendrite was highly discernible, was pictured for OSN count (Figure 4). In order to measure the mature and immature OSNs density, we performed double immunostaining (OMP for mature OSNs, Tuj-1 for immature OSNs) on flat-mounted septal mucosa. The cell density of OSNs was defined as the number of dendrites (Mean $\pm$ SD) per millimeter square. In total, 15 images were analyzed (3 rats, 5 images per rat).

Counting was done semi-automatically with a macro created in Fiji. To summarize, the counting of OMP+ OSNs and Tuj-1+ OSNs was done on flat-mounted septum immunostained with OMP and Tuj-1 respectively. The transitory OSNs were defined by the yellow spectrum in which the Tuj-1 (green) and

OMP (red) were merged (**supplementary Figure 5**). The counting of particles was done from particles with a minimum surface of 0.3 μm^2 . This area corresponds to a diameter of 0.6 μm .

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Figures

Figure 1

Co-localization of different OSN markers with OMP, a mature OSN marker. Immunohistochemistry (IHC) on cross sections of olfactory mucosa. The tested markers were colored in green and their name were noted on the left of each line of images. Due to the identical animal production between the anti-OMP antibody and anti-OliG2, we could not double label these two markers. The nuclei were stained in blue using DAPI. The images in the 3 left columns were acquired using an epifluorescence microscope, with a

40X objective. The images in the right column were acquired from a confocal microscope with a 60X objective.

Figure 2

Co-localization of different OSN markers with Tuj-1, an immature OSN marker. IHC on cross sections of olfactory mucosa. The tested markers were colored in green and their name were noted on the left of each line of images. The nuclei were stained in blue using DAPI. The images of the 3 left columns were

acquired using an epifluorescence microscope, with a 40X objective. The images of the right column were acquired from a confocal microscope with a 60X objective.

Figure 3

Immunostaining on flat-mounted septal mucosa using pre-selected OSN markers. Five OSN markers (Tuj-1, OMP, DCX, N-cadherine and PGP9.5) were selected because of their staining of the OE's surface (OSN dendrites). The co-localization with a respiratory epithelium marker, Tubulin β IV was realized on

transition zones in order to show the specificity of the OSN markers. The images were acquired using a confocal microscope and presented as 3D projection. For each marker, the top pictures showed face view using a 20X objective; the bottom pictures showed a tangential view.

Figure 4

OSN density evaluation using immunostaining of dendrites on flat-mounted septal OE. a) Double immunostaining was done on the separated septal mucosa. The mature OSNs were stained in red using

OMP and the immature OSNs were stained in green using Tuj-1. The co-stained OSN dendrites (Tuj-1+, OMP+) which appear in yellow/orange, were indicated by arrow heads. Images were acquired using a confocal microscope with a 60X objective. b) The horizontal black line illustrates the Z level of OSN where the image was acquired. c) and d) In total, 105912 ± 13899 OSN/mm² were detected by this method: 49384 ± 7134 /mm² were immature OSNs (Tuj-1+, OMP-), 42080 ± 11820 /mm² were mature OSNs (Tuj-1-, OMP+), and 14448 ± 5865 /mm² were transitory OSNs (Tuj-1+, OMP+). The percentage in the table corresponds to the cell number ratio compared to the total number of OSNs (105912). n=15 (3 animals and 5 different fields per animals).

Figure 5

Direct immunostaining on ethmoid turbinates. The morphology of whole ethmoid turbinates was visible on bright field (BF) (a), and by Dapi staining (b). The OE was highlighted by immunostaining with Tuj-1 (c and e). The merge of Tuj-1 (green) and DAPI (blue) with or without BF (gray) indicated the olfactory boundary (d and f). The photos were acquired using a fluorescence macroscope with 1.6X objective for (a-d) and 5X objective for (e, f).

	Olfactory epithelium (OE)			Lamina propria
	Dendrites of OSN	Upper cell layers (Mature OSNs)	Lower cell layers (Immature OSNs)	Olfactory nerve fascicles (Axons of OSNs)
OMP	+	+(C)		+
Tuj-1	+		+(C)	+
DCX	+		+(C)	+
OLIG2			+(N)	
N-cadherin	+	+(M)	+(M)	*+
LHX2		+(N)	+(N)	
PGP9.5		+(C)	+(C)	+
Gβ				+
GAP43				+

Figure 6

Summary of the localization of the different neuronal markers within the olfactory mucosa. +: Evident staining. C: Cytoplasm. N: Nucleus. M: Membrane. Images of staining with GAP43 and Gβ, are presented in Supplementary Figure 1. * N-cadherin stained weakly olfactory fascicles.

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