Video Article

Whole Mount Labeling of Cilia in the Main Olfactory System of Mice

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Abstract

The mouse olfactory system comprises 6-10 million olfactory sensory neurons in the epithelium lining the nasal cavity. Olfactory neurons extend a single dendrite to the surface of the epithelium, ending in a structure called dendritic knob. Cilia emanate from this knob into the mucus covering the epithelial surface. The proteins of the olfactory signal transduction cascade are mainly localized in the ciliary membrane, being in direct contact with volatile substances in the environment. For a detailed understanding of olfactory signal transduction, one important aspect is the exact morphological analysis of signaling protein distribution. Using light microscopical approaches in conventional cryosections, protein localization in olfactory cilia is difficult to determine due to the density of ciliary structures. To overcome this problem, we optimized an approach for whole mount labeling of cilia, leading to improved visualization of their morphology and the distribution of signaling proteins. We demonstrate the power of this approach by comparing whole mount and conventional cryosection labeling of Kirrel2. This axon-guidance adhesion molecule is known to localize in a subset of sensory neurons and their axons in an activity-dependent manner. Whole mount cilia labeling revealed an additional and novel picture of the localization of this protein.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52299/>

Introduction

The mouse olfactory epithelium in the nasal cavity comprises 6-10 million bipolar olfactory sensory neurons¹. Each olfactory neuron chooses one of 1,200 odorant receptor genes for expression. Detection of odorants starts by odorant binding to an olfactory receptor², which then activates adenylyl cyclase type-III (ACIII)³ via the olfactory specific G protein $G_{\alpha_{olf}}$ ⁴. The resulting rise in cyclic adenosine monophosphate (cAMP) opens a cyclic nucleotide-gated (CNG), nonselective cation channel leading to influx of Ca^{2+} and Na^{+} , and subsequently Ca^{2+} influx leads to opening of a Ca^{2+} activated Cl^{-} channel^{5,6}. The resulting outward Cl^{-} flux is facilitated by a high intracellular Cl^{-} concentration maintained by steady Cl^{-} uptake, likely via the $Na^{+}/K^{+}/Cl^{-}$ cotransporter NKCC1, the Cl^{-}/HCO_3^{-} exchanger SLC4A1, and maybe additional yet to be identified transporters⁶⁻⁸.

Bipolar olfactory neurons have single, unbranched axons that project directly to the olfactory bulb, and a dendrite that extends to the surface of the epithelium and ends as a specialized compartment, the dendritic knob. From this knob, 10-30 cilia, which can reach a length of up to 50-60 μm , emanate into the mucus covering the epithelial surface⁹. Proteins of the canonical signal transduction cascade are mainly localized in the membrane of these cilia. The increased sensory surface of the epithelium amplifies the ability to detect odorants. Due to the density of sensory neurons, cilia extending from neighboring dendritic knobs intermingle. This intermingling results in a random mixture of cilia from different neurons, expressing different types of olfactory receptors, on the surface of the epithelium. The detection and cellular allocation of ciliary proteins which are only present in a subset of sensory neurons is therefore difficult in cryosections. In addition, the precise localization of such proteins along the cilia is barely possible, since cryosections are typically thinner than the average length of the cilia.

To enable investigation of ciliary localization of so far uncharacterized membrane proteins in olfactory neurons, we optimized an *en face* preparation technique which allows the detailed analysis of protein localization in cilia. Briefly, the mouse is sacrificed and the head split near the midline. Turbinates, nasal, and frontal bones are removed to expose the septum. The septum with the olfactory part of the lining epithelium is loosened by cutting all connections to the nasal cavity. After putting the septum into a petri dish filled with Ringer's solution, the epithelium is peeled off und transferred to a coated glass slide. Following a short fixation step, immunostaining procedures can be performed if handling is as gentle as possible to avoid damage of the fragile tissue. We demonstrate the achievable resolution by comparing the staining of two different membrane proteins in olfactory cilia in classical cryosections and in the *en face* preparation described.

Protocol

NOTE: All animal procedures were handled at the Charité or University Clinic Jena in accord with German Animal Care laws avoiding any undue suffering of animals.

1. Preparing Solutions and Dissection Workplace

1. Solutions

NOTE: Prepare the following solutions before starting dissection of the epithelium.

1. Solutions for the dissection procedure:

1. Prepare Ringer's solution (pH 7.4) with concentrations of 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM glucose.
2. Prepare a PBS^{-/-} solution (pH 7.4) with concentrations of 2.68 mM KCl, 1.47 mM KH₂PO₄, 136 mM NaCl, and 8.1 mM Na₂HPO₄.
3. Prepare a fixative solution (pH 7.2) with concentrations of 1x PBS^{-/-}, 0.2 mM CaCl₂, 4% sucrose, and 4% paraformaldehyde. Sucrose in the fixative solution improves cryosectioning and pick-up of cryosections. Store at -20 °C.

2. Solutions for the staining procedure

1. Prepare a PBS^{+/+} solution with concentrations of 1x PBS^{-/-}, 0.48 mM MgCl₂, 0.9 mM CaCl₂.
2. Prepare a PBST^{+/+} solution with concentrations of 1x PBS^{+/+} and 0.1% Triton X-100.
3. Prepare a blocking solution with concentrations of 1x PBST^{+/+} and 1% gelatine.

2. Workplace

1. For the dissection workplace, use a dissecting microscope with bright illumination, as well as a liquid-blocker pen.
2. Prepare a petri dish, filled with Ringer's solution, and adhesive glass slides.
3. Obtain the following surgical instruments: a pair of surgical scissors with one sharp and one blunt tip, a pair of spring scissors with straight tip shape, two forceps with fine curved tip shape and a razor blade (**Figure 1A**).

2. Preparation of the Nasal Septum

1. House animals in approved cages with regular access to food and water and appropriate day/night cycle.
2. Perform anesthesia in a closed receptacle containing gauze soaked with 100% isoflurane and monitor analgesia by testing rear foot reflexes. Since cervical dislocation can cause blood in nasal cavities, directly decapitate anesthetized mice.
3. Remove the skin to expose the bone of the entire skull and nose, and wipe away the remaining blood and tissue thoroughly using a paper towel. Remove the lower jaw and the front teeth.
4. Incise the dorsal bone of the nose bilaterally in 1-2 mm distance parallel to the suture line to separate one side of the septum (medially) from the maxilla (laterally). Split the nose with a single cut. If bone remnants and turbinates are still attached to the septum, remove these carefully to expose the septum completely without touching it.
5. Remove the dorsal nasal bone by sliding the fine curved tip of a forceps along the dorsal side of the septum, between septum and nasal bone. Apply slight pressure on the bone, push it up and remove it.
6. To provide access to the two septal tissues, one from each cavity, carefully remove the maxilla of the other side of the head. When preparing older animals (P > 28), remove the tip of the frontal bone covering the olfactory bulbs.
7. Identify the olfactory epithelium by its slightly yellow color (**Figure 1B**). The bordering respiratory epithelium is white and shows movement of motile cilia that can be seen under the dissecting microscope. Cut along the border between olfactory and respiratory epithelium with a pair of fine spring scissors.
8. Extend the cut and separate the septum from the ventral connection to the vomer bone. Then cut along the border between the septum and the lamina cribrosa of the Os ethmoidale. The septum is now completely isolated from all connections to the head. Use the cutting positions shown in **Figure 1C**.

3. Isolation of the Olfactory Epithelium for Immunostaining

1. Place an adhesive glass slide in the petri dish filled with Ringer's solution. Place it onto the edge of the petri dish, so that one half of the glass lies in the Ringer's solution (**Figure 1A**).
2. Use a forceps to carefully transfer the ethmoid bone with the olfactory epithelium to the petri dish. Lift it without grabbing it with the forceps tips.
3. Grab the perpendicular plate of the ethmoid bone with one forceps and use a second one to carefully remove the epithelium from one side of the septum. Slide the curved tip between the perpendicular plate and epithelium to peel the epithelium off.
4. Be always sure to identify the ciliary side, in case the epithelium flips in the Ringer's solution. If the epithelium flips, it is hardly possible to identify the ciliary side afterwards. Mostly, the epithelium rolls up with the ciliary surface inside.
5. Compare the enrolled tissue shape with the cutting positions shown in **Figure 1C**. Grab the epithelium at a position at the border and pull it onto the glass slide. Do not touch the ciliary side with the forceps to avoid lesions of the tissue.
6. Turn the septum and repeat the procedure with the epithelium from the other side.
7. Dry the glass slide around both pieces of olfactory epithelium with a paper towel and encircle the tissue with a liquid-blocker pen.

8. Fix the tissue with 150 μ l fixative solution for 10 min at RT. Concerning cilia stability and integrity, use short fixation times for a variety of tested antibodies. Within these 10 min cilia are fixed, but probably not the whole epithelium tissue. Thus, immediately visualize with the microscope subsequent to the staining procedure. However, fixative times might vary for individual antibodies.

4. Staining Protocol

NOTE: Handle the tissue very carefully to preserve ciliary structures of the olfactory sensory neurons. Remove solutions by pipetting. Do not drop any solutions directly onto the epithelium, as mechanical forces could disrupt the fine cilia. Perform all steps at RT if not stated otherwise.

1. Remove the fixative solution and wash 2 times with PBS^{+/+}.
2. Incubate the epithelium for at least 1 hr with blocking solution.
3. Prepare primary antibody solution by dissolving the antibody in blocking solution (1:200 for the antibodies used in this study) and centrifuge for 5 min at full speed to remove any precipitates.
4. Incubate the epithelium with antibody solution in a humid chamber at 4 °C O/N.
5. Wash epithelium with PBS^{+/+} for 5 min at least 3 times.
6. Prepare secondary antibody solution by dissolving the antibody in blocking solution (1:500) and centrifuge for 5 min at full speed.
7. Incubate the epithelium with secondary antibody solution for 1 hr in a dark chamber.
8. Wash epithelium with PBS^{+/+} for 5 min at least 3 times.
9. Remove the liquid-blocker with a razor blade or paper towel. Wash the epithelium with distilled water for 5 s.
10. Preserve the tissue in antifade mounting reagent.

NOTE: Background fluorescence increases rapidly within days; microscopical analysis not later than 2 days after embedding in mounting medium is therefore recommended. Special care has to be taken not to squeeze the tissue when searching the correct focal plane, since it can severely damage the preparation. Due to out-of-focus fluorescence, further investigation with confocal microscopy is recommended.

Representative Results

Olfactory epithelium *en face* preparations can be used to investigate localization of proteins in the cilia of sensory neurons, allowing the detailed investigation of proteins whose localization is unclear after analysis of cryosections. This problem can be exemplified in the case of the staining for Kirrel2 (also called Neph3). Kirrel2 is a member of the immunoglobulin (Ig) superfamily of membrane proteins and functions as a homophilic adhesion protein. It was shown to play a role in axon-guidance in the olfactory system, and to be expressed in a subset of olfactory neurons in an activity dependent manner¹⁰.

Immunostaining for Kirrel2 in a typical cryosection through olfactory epithelium revealed localization of Kirrel2 in axons, soma and dendrites (**Figure 2A**). All solutions were prepared as described above for the *en face* preparation technique. Although some labeling appears on top of the layer composed of the dendritic knobs, ciliary localization remained uncertain after analysis of cryosections. Some neurons exhibited staining in and around their olfactory knob, but single cilia were difficult to identify. In contrast, *en face* preparation of the olfactory epithelium performed according to the above described protocol unambiguously showed ciliary localization of Kirrel2 (**Figure 2B**). Interestingly, Kirrel2 was only expressed in cilia of a relatively small subset of olfactory neurons, although analysis of stained cryosections showed expression in a much larger percentage of neurons. Moreover, the *en face* preparation did not just demonstrate ciliary staining in general, but revealed variations in ciliary expression patterns ranging from neurons with many long cilia to neurons with only few short cilia. In some cells, Kirrel2 was detected in the dendritic knob, but not in cilia extending from this knob. The cause and functional relevance of this finding remains to be elucidated, but it illustrates the potential of the *en face* preparation to provide new insights into protein localization in the olfactory system.

In addition, we performed an immunostaining for the olfactory receptor mOR-EG, that exhibits strong ciliary expression¹¹. The receptor is already clearly identifiable in the ciliary layer in cryosections (**Figure 3A**). Nevertheless, analysis of the detailed characteristics such as the number of cilia per knob or the length of individual cilia is not possible. Using the *en face* preparation, identification of cilia from neurons expressing the olfactory receptor is easily possible (**Figure 3B, C**). The *en face* preparation can be performed with animals of different ages. Even cilia from prenatal animals were successfully stained and visualized (**Figure 3D**).

En face preparations therefore represent a tool well suited to analyze the ciliary morphology and the localization of well-known ciliary proteins in detail.

Figure 1. Preparation of the mouse head. (A) Arrangement of the dissection workplace. Petri dish is filled with Ringer's solution and holds an adhesive glass slide. The surgical instruments are recommended for *en face* preparation. (B) After removing one half of the mouse head, the septum is exposed. The septum is coated with olfactory epithelium (OE) and respiratory epithelium (RE). OB: olfactory bulb, V: vomeronasal organ. (C) To isolate the part of the septum lined with olfactory epithelium, cut the septum along the demonstrated lines with a pair of fine scissors. [Please click here to view a larger version of this figure.](#)

Figure 2. Comparison of Kirrel2 immunostaining in cryosections and *en face* preparations. (A) Confocal image (maximum projection) of a postnatal day 60 (P60) cryosection immunostained for Kirrel2 reveals protein localization in olfactory sensory neurons. Single olfactory knobs show strong staining and Kirrel2 localization in cilia is assumed (asterisk), but uncertain. (B) Confocal image (maximum projection) of a P65 olfactory epithelium *en face* preparation exhibits clear Kirrel2 staining in cilia of a subset of olfactory sensory neurons. (Scale bars, 10 μ m) [Please click here to view a larger version of this figure.](#)

Figure 3. Comparison of mOR-EG immunostaining in cryosections and *en face* preparations. (A) Confocal image (maximum projection) of a P60 cryosection immunostained for the olfactory receptor mOR-EG. Ciliary localization is already detectable. (B) Confocal image of a P65 *en face* preparation immunostained for the olfactory receptor mOR-EG. mOR-EG is strongly expressed in cilia of a subset of olfactory sensory neurons. In contrast to cryosections, cilia characteristics concerning length, number of cilia per knob and exact protein localization can be analyzed. (C) Higher magnification of the boxed area in (B). (D) Confocal image of an embryonic day 18 (E18) *en face* preparation immunostained for the olfactory receptor mOR-EG. Staining of cilia can clearly be seen. (Scale bars, 10 μ m) [Please click here to view a larger version of this figure.](#)

Discussion

The *en face* preparation technique described in this protocol provides a powerful tool for the detailed analysis of the olfactory system. So far, most studies characterizing the localization of signaling proteins use immunostainings of cryosections. They present a good overview of the olfactory epithelium, and protein expression in distinct cell types or regions can be easily identified. However, expression in olfactory cilia is sometimes hard to detect. Even if ciliary localization is obvious, cryosections offer a very limited view on the morphology of the ciliary layer. *En face* preparations provide an additional perspective onto the epithelium.

The olfactory epithelium preparation described here is especially useful for the investigation of proteins that are expressed in a small subset of olfactory neurons. In this case, single knobs are well identifiable, so that localization in knobs and extending cilia can easily be analyzed. In contrast to staining of signaling proteins in cryosections, it is possible to determine the amount of cilia per knob and the length of the cilia. Moreover, *en face* preparations allow the exact localization of proteins along the cilia. It is also possible to analyze whether proteins are distributed homogeneously along the ciliary membrane. Moreover, changes in protein localization upon application of odorant ligands can be investigated. Another aspect is the investigation of developmental changes in protein localization. We experienced good results not only for *en face* preparations from adult mice, but also from younger animals, up to E18.

Limitations for this technique are the analyses of highly abundant signaling proteins that exhibit strong expression in (almost) all neurons of the olfactory epithelium. In this case, distinguishing single cilia is difficult due to the strong labeling of many intermingled cilia. Another aspect that should be considered is the zonal expression of certain proteins in the olfactory system. It is necessary to analyze the distribution of neurons expressing a protein of interest to figure out where in the epithelium cells are located. We describe the preparation of olfactory epithelium from the nasal septum, but if proteins would only be expressed on specific turbinates such as mOR262 (mOR37) family receptors¹², the protocol for tissue preparation must be adapted.

We successfully demonstrate that the *en face* preparation technique can be used to gain additional insights into the protein organization in cilia of the olfactory system. Using *en-face* preparations we uncovered that Kirrel2, a membrane protein which has previously been shown to be important for axon sorting, is also localized to the cilia of sensory neurons. Moreover, ciliary localization was inhomogeneous and not observed in all sensory neurons expressing Kirrel2, indicating that ciliary localization of Kirrel2 is regulated.

Taken together, *en face* preparations can help to understand the organization of the olfactory system by providing a different view on whether certain signaling proteins are localized to the ciliary compartment of sensory neurons. Many ciliopathies affect the olfactory system^{13,14}, the assessment of the anatomy and the molecular composition of olfactory cilia may therefore be used as a tool for improved analysis of the associated defects.

Disclosures

The authors have nothing to disclose.

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