

A Long-term Culture System for Olfactory Explants with Intrinsically Fluorescent Cell Populations

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Abstract

As a prerequisite for exploring the mechanisms which lead to the formation and maintenance of the precise wiring patterns in the olfactory system, organotypic cultures of olfactory tissue from transgenic mice expressing green fluorescent protein (GFP) under control of the olfactory marker protein promotor have been established. Tissue specimen from embryonic stage 14 were explanted and kept in culture for >1 week. Within the explants, numerous GFP-fluorescent olfactory sensory neurons assembled in an epithelial-like manner during this period. Under optimized culture conditions, strongly GFP-positive axons extended from these explants, fasciculated and formed bundles. When co-cultured with explants from the olfactory bulb, distinct axon populations were attracted by the target tissue; the fluorescent axon bundles invaded the bulbular explants and formed conglomerates at distinct spots. Explants from transgenic mice expressing GFP under control of a given olfactory receptor gene (mOR37A) also comprised labeled neurons that extended intensely fluorescent axonal processes, which all seemed to grow in a common fascicle. The results demonstrate that GFP-labeled olfactory sensory neurons differentiate in the established organotypic cultures, which thus appear to be a useful tool to monitor and to manipulate the processes underlying the axonal wiring between the olfactory epithelium and bulb.

Introduction

The precise spatial organization of the olfactory system is an essential prerequisite for the concept that olfactory coding may involve the transformation of an odorant's molecular features into a spatial map of neuronal activity (Friedrich and Korsching, 1997, 1998; Rubin and Katz, 1999; Meister and Bonhoeffer, 2001). In particular, the convergent projection of olfactory sensory neurons to the olfactory bulb (Jastreboff *et al.*, 1984; Pedersen *et al.*, 1986), where the axons of several thousand cells terminate in a small neuropil, the glomerulus, seems to be essential; ~2000 of these glomeruli are densely packed beneath the bulbar surface (Royet *et al.*, 1988; Meisami and Sendera, 1993). Recent studies employing gene targeting approaches and generation of transgenic mouse lines have greatly improved our insight into the principles underlying the convergent wiring of the olfactory system. Analysis of the intrinsically labeled cells revealed that all of the 10 000–20 000 neurons expressing the same receptor subtype typically converge onto two glomeruli, located at the medial and lateral side of bulb, respectively (Mombaerts *et al.*, 1996; Wang *et al.*, 1988); however, there are exceptions (Strotmann *et al.*, 2000; Pyrski *et al.*, 2001). The cellular mechanisms and molecular elements underlying the formation and maintenance of these precise wiring patterns remain largely unknown. In fact, appropriate experiments for identifying directional

cues which navigate the outgrowing axons to their defined destination as well as attractant and repellent compounds which ultimately allow the axons to contact the appropriate target cells may require suitable *in vitro* culture systems, reminiscent of the previous contribution of retinal explant cultures in the attempts to unravel the molecular network involved in establishing the retinotectal projection (Baier and Bonhoeffer, 1990; Müller *et al.*, 1990; Hoff *et al.*, 1999). The unique possibility to optically identify neurons with a distinct molecular identity combined with the accessibility of an organotypic culture system may enable the monitoring and experimental manipulation of some of the fundamental steps involved in wiring the olfactory system, including axonal outgrowth, fasciculation and guidance, as well as target finding in the olfactory bulb.

Materials and methods

Animals

OMP-GFP mice (Potter *et al.*, 2001) were kindly provided by Prof. Peter Mombaerts; in these mice, green fluorescent protein (GFP) is expressed from the locus encoding the olfactory marker protein (OMP). In mOR37A-ITGFP mice (Strotmann *et al.*, 2000) only cells which express the olfactory receptor gene *mOR37* also express GFP. Embryos

were obtained from timely mated mice; the time of vaginal plug formation was counted E0.

Cell culture conditions

Cultures were grown in glass-bottomed dishes which were coated with 10 µg/ml laminin (Invitrogen, Karlsruhe, Germany) in Hank's balanced salt solution (HBSS; Invitrogen) for 3 h at 37°C and 5% CO₂. The dishes were subsequently washed with HBSS and Neurobasal-A^{supp} medium (Brewer *et al.*, 1993; Brewer, 1995): Neurobasal-A (Invitrogen) supplemented with 0.8 mM L-glutamine (Invitrogen), 0.4% methylcellulose (Sigma, Taufkirchen, Germany), 10 mM HEPES, pH 7.5, 5 µg/ml Gentamycin (Invitrogen) and 0.02 × B27-supplement (Invitrogen).

Tissue preparation

Pregnant mice were killed by cervical dislocation; embryos were decapitated and the heads stored in ice-cold HBSS. Dissection of the olfactory epithelium from the nasal septum and the turbinates was performed under a stereomicroscope in L-15 medium (Invitrogen). The cellular layer containing olfactory sensory neurons (OSNs) was removed from the underlying lamina propria using a lancet. Olfactory bulbs were removed from the bony capsule after cutting the olfactory nerves. The bulbs were cut into small pieces in a black microscope-tray filled with Neurobasal-A^{supp}.

About 20–30 explants were transferred to the center of a culture dish, where four metal blocks formed a small basin of 10 mm × 10 mm × 2 mm, filled with 200 µl of Neurobasal-A^{supp}. The metal blocks were subsequently pulled apart to generate a thin film of medium covering the explants and pressing them down to the bottom.

Explants were cultured at 37°C, 5% CO₂ and 95% humidity. Four to six hours after dissection, 1 ml of sterile H₂O was added to the outer rim of the culture dish to avoid evaporation of the medium. After the explants had attached to the culture dish (4 h), 200–500 µl of Neurobasal-A^{supp} was added to the cultures; half of the medium was replaced every other day.

Microscopy and photography

Cultures were observed using an inverted microscope (Olympus IX70; Olympus, Hamburg, Germany) with fluorescence optics. Images were taken using a digital camera (Micromax 5 MHz, Princeton Scientific Instruments, Inc., Monmouth Junction, NJ).

Results

The nasal epithelium of OMP-GFP mice (Potter *et al.*, 2001) appeared to be most suitable for establishing an organotypic culture system of the olfactory epithelium to monitor OSNs under controlled and manipulable conditions *in vitro*. In these mice, GFP is expressed from the

locus encoding the OMP, which is expressed at high levels and selectively in mature OSNs (Margolis, 1972). Since the onset of OMP expression occurs around embryonic stage 14 (E14) (Monti-Graziadei *et al.*, 1980), the olfactory epithelia (OEs) from mice of this stage were dissected and small pieces were cultured under conditions as described in Materials and methods. After 2 days *in vitro*, intensely green fluorescent cells were visible within the OE explants (Figure 1a,b); they seemed to be arranged randomly. The majority of the cells showed the typical morphology of OSNs, with a dendrite extending from one pole of the cell body ending in a small swelling, the dendritic knob (Figure 1a), and a thin axonal process extending from the opposite pole of the soma (Figure 1b).

After 6 days in culture, fluorescent OSNs within the OE explants were arranged in a characteristic parallel, epithelial-like manner (Figure 1c); this is quite reminiscent of their arrangement *in situ*, where OSNs form a pseudo-stratified epithelium with their dendrites in a palisade-like parallel orientation. In small explants, the dendrites of GFP-positive cells formed a spherical arrangement with a central cavity (Figure 1d). The center of the cavity was not fluorescent and thus most likely represented a liquid-filled space.

After 9 days in culture, the OE explants contained numerous GFP-fluorescent cells displaying a morphology typical for OSNs (data not shown). These results thus demonstrate that, under the optimized culture conditions, explants from an embryonic OE maintained and probably generated mature olfactory neurons, which formed a characteristic epithelial-like assembly.

Since the established culture conditions allowed the survival and epithelial arrangement of olfactory sensory neurons *in vitro*, we next asked whether these neurons may form axons and whether these processes may contain sufficient GFP to visualize fluorescent fibers. Figure 2a shows a representative OE explant after 4 days in culture. In this overview, fluorescent somata of OSNs are visible in the explant (Figure 2b), they direct their dendrites towards the center of this structure; in addition, several intensely green fluorescent fiber bundles are extending from the OE explant, projecting in different directions. At certain distances (100–150 µm) from the explant, these bundles defasciculated into smaller bundles and finally into what appear to be individual fibers. At the points of defasciculation, groups of axons initially growing in the same direction now change their trajectory. High magnification of their distal termini (Figure 2c) revealed typical filopodia-like structures reminiscent of growth cones, suggesting that these processes are indeed axons of OSNs. Thus, the results demonstrate that even in the most distal compartment of the cells the level of GFP was high enough to visualize and monitor the growth cones of olfactory axons *in vitro*.

Since the organotypic culture conditions allowed the OSNs to maintain their morphological as well as their

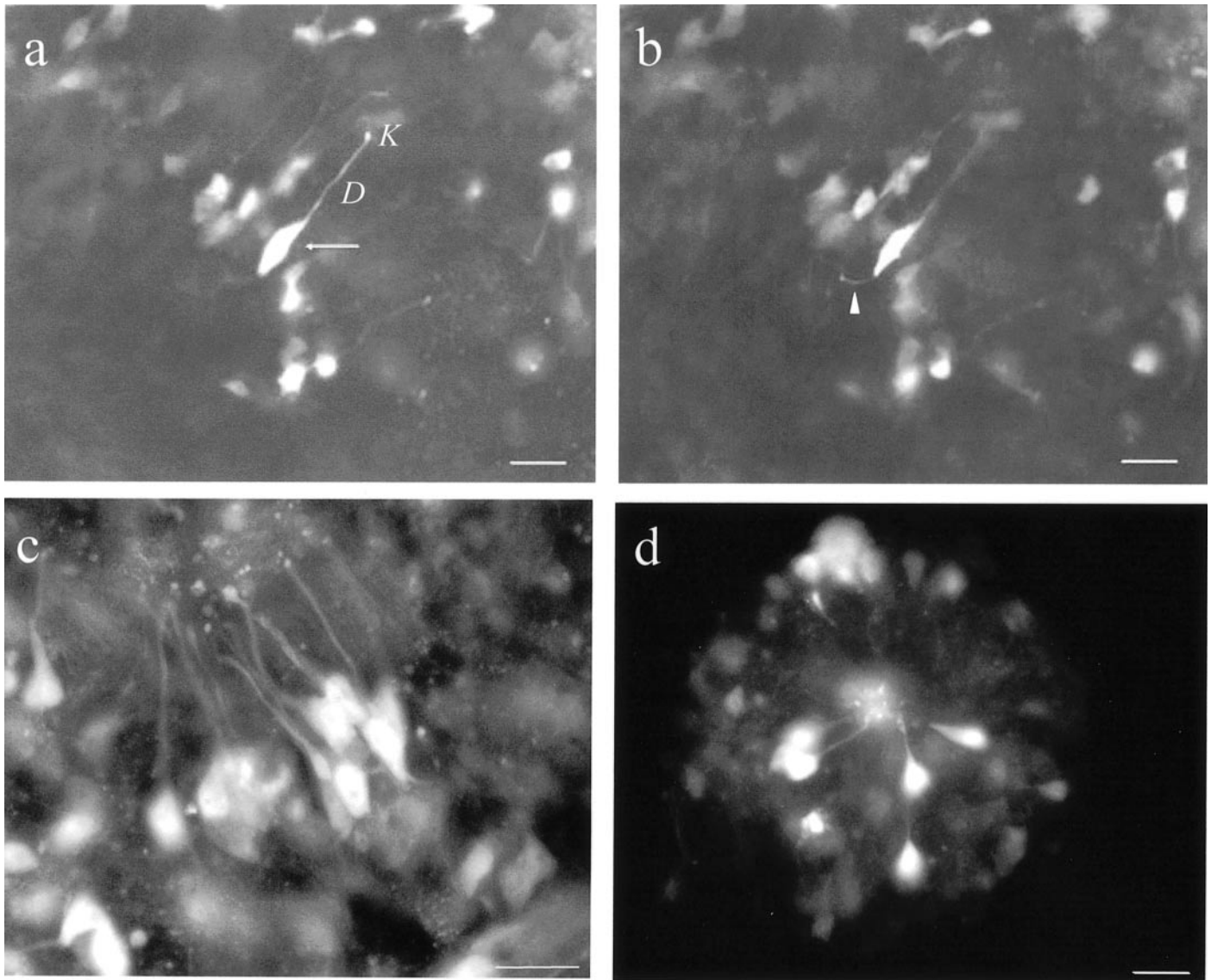


Figure 1 OE explants from an OMP-GFP mouse at embryonic stage 14. **(a)** After 2 days in culture, numerous strongly GFP-fluorescent cells are detectable, some (arrow) exhibiting the typical morphology of mature olfactory sensory neurons with a dendrite (D) and the olfactory knob (K). **(b)** In a slightly different focal plane, a small axonal process is visible extending from the cell soma (arrowhead). **(c)** After 6 days in culture, GFP-fluorescent cells arrange in a parallel, epithelial-like manner. **(d)** Small OE explants form spherical structures with the dendrites of cells directed towards a central cavity. Scale bars: 20 μ m.

growth characteristics, we next analyzed whether the direction of axonal outgrowth may be influenced by explants from the olfactory bulb. To this end, OE explants were co-cultured with explants from developing bulbs (E14) which were placed a suitable distance from the OE explants. Typical results of such a co-culture experiment are shown in Figure 3a,b. After 4 days of co-culturing, fluorescent fiber bundles extended radially from the OE explants, growing in all directions. Fiber bundles which grew in directions without bulb tissue seemed unaffected; in contrast, fiber bundles which grew in the direction of bulb explants apparently entered the target tissue. Certain fibers seemed to be redirected toward the bulb explant (arrows in Figure 3a,b). In most cases, bundles initially passing the bulb

explant tangentially, defasciculated at a distinct position, where some axons changed their direction and now grew towards the bulb, whereas other axons continued to grow along the original route (Figure 3b). These results demonstrate that, under these *in vitro* conditions, outgrowing axons seem to be attracted by bulb tissue. This targeting process seemed to be selective; only a subpopulation of axons appeared to enter the bulb explant, while most of the visible axons grew along the surface of the bulb (Figure 3c) but did not invade the tissue.

Since the convergence of olfactory axons to two or more glomeruli may represent one of the fundamental prerequisites for olfactory coding, we next examined whether OSNs converge *in vitro* to distinct spots in their target tissue.

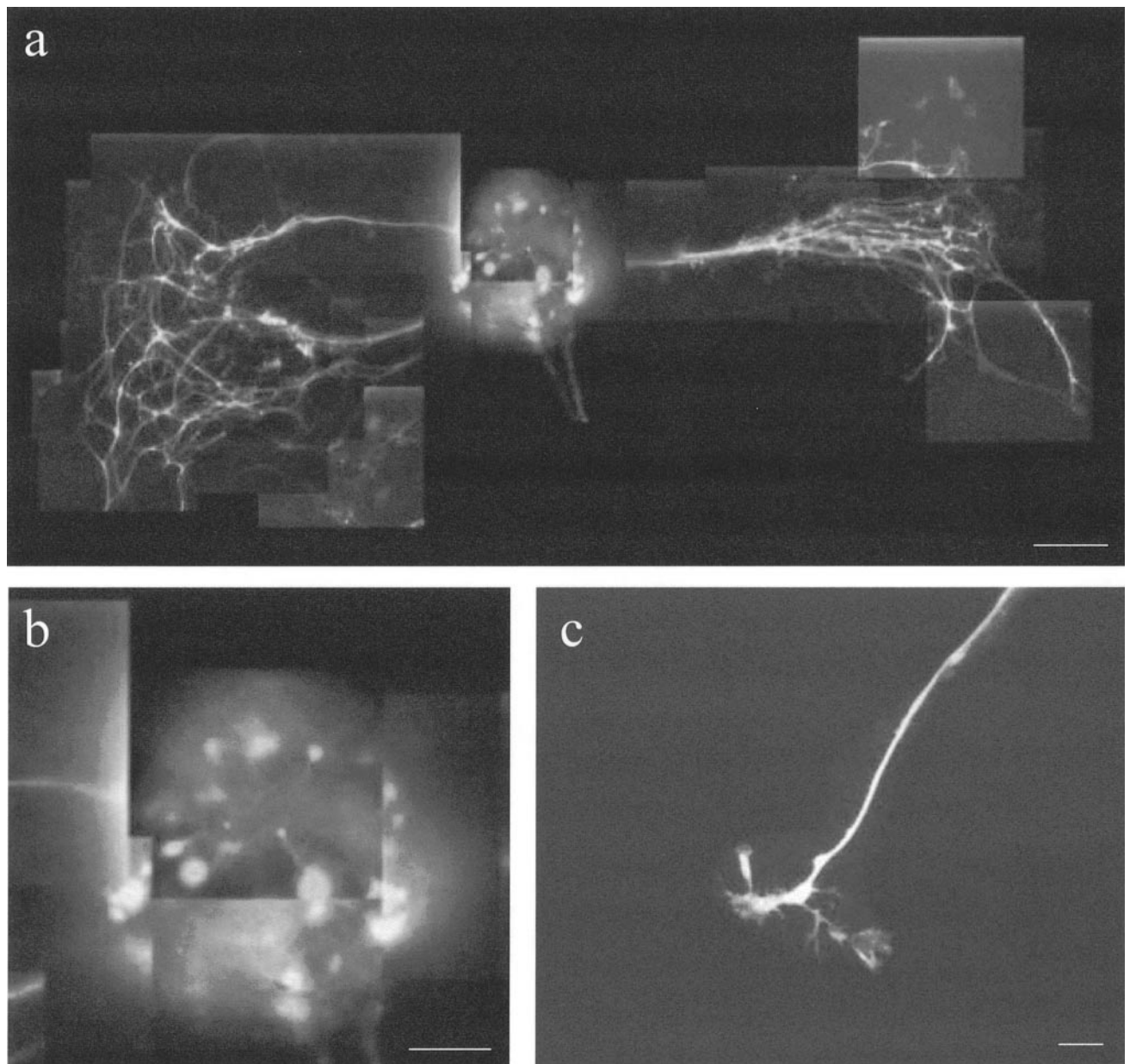


Figure 2 OE explants from an OMP-GFP mouse at embryonic stage 14. **(a)** After 4 days *in vitro*, strongly GFP-fluorescent fiber bundles extend from the OE explant in different directions. Individual regions were photographed at high magnification and arranged for an overview. **(b)** High magnification of the OE explant shows its spherical arrangement of GFP-positive cells, with their dendrites arranged towards the center of the structure. **(c)** High magnification of the distal terminus of a single fiber with a typical growthcone-like appearance. The entire structure is strongly fluorescent. Scale bars: (a) 50 μm ; (b) 25 μm ; (c) 10 μm .

It was found that in co-culture preparations, after 6 days, GFP-fluorescent axons extended from the epithelium and entered the bulb tissue (Figure 3d). Many axons apparently condensed into a particular spot (Figure 3e), whereas the remaining bulb tissue seemed to receive only a few ingrowing axons. Similar structures were observed in a variety of bulb explants; the size of such axonal conglomerates was in the range of $\sim 50 \mu\text{m}$.

To analyze a more definite OSN subpopulation, explants from the transgenic mouse line mOR37A-GFP (Strotmann

et al., 2000) were employed. In this mouse line olfactory neurons expressing the receptor subtype OR37A also express GFP. Since the onset of receptor expression for this receptor type occurs early in development (Conzelmann *et al.*, 2001), OE explants from E14 embryos were dissected from those regions of the epithelium where OR37-expressing neurons are located. After 2 days in culture, GFP-fluorescent cells were detectable in the explants (Figure 4a), usually located within slightly different focal planes; as expected, this contrasts to the large number of

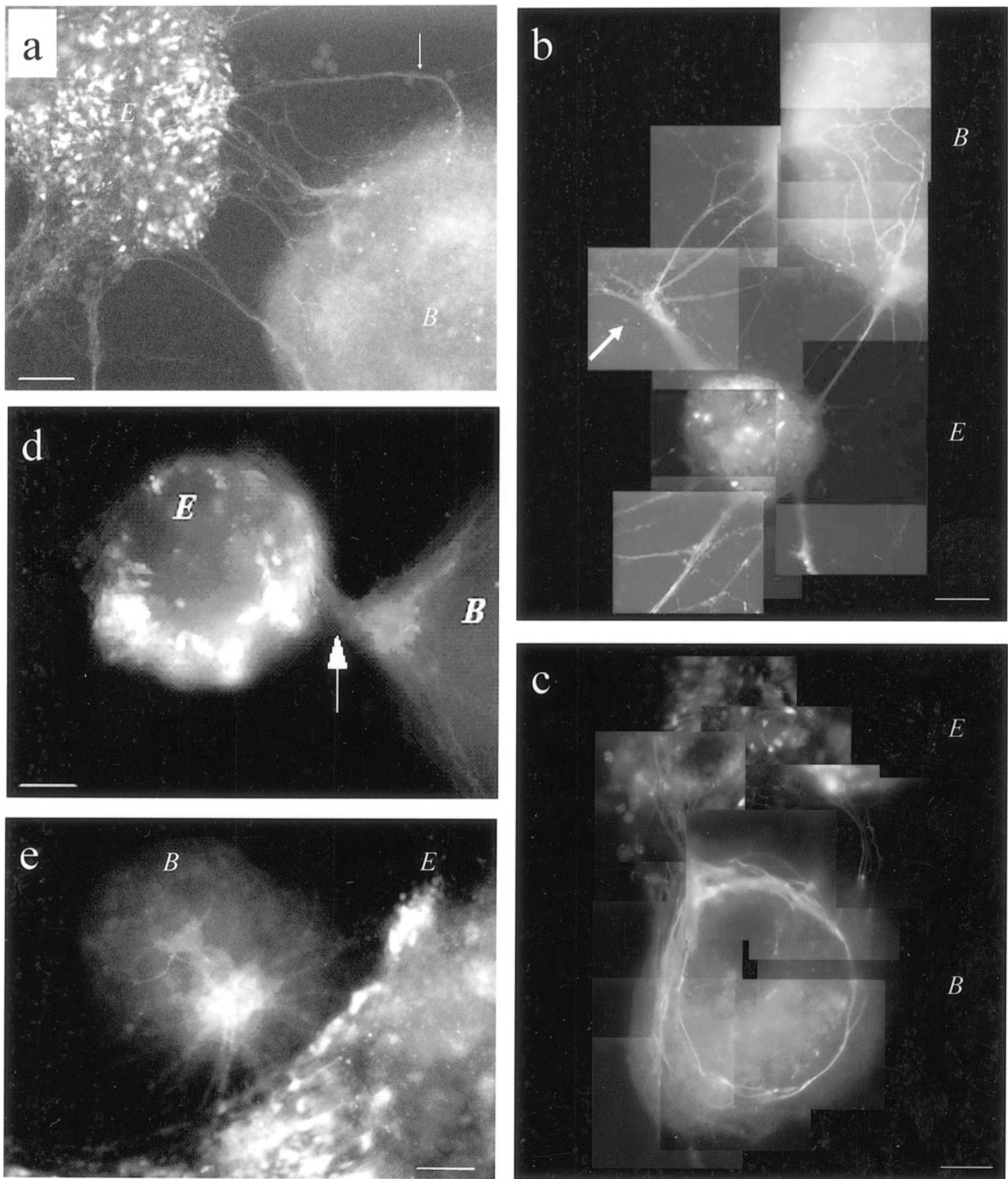


Figure 3 Olfactory epithelium explants (*E*) from an OMP-GFP mouse at embryonic stage 14 co-cultured with bulb tissue (*B*) of the same age for 4 days (a–c) and 6 days (d, e), respectively. **(a)** GFP-fluorescent fibers extend from the OE explant in all directions. Individual fiber bundles growing in close vicinity to the bulb tissue are re-directed towards it (arrow). **(b)** Distinct fiber bundles extending from OE explants defasciculate and some fibers change their direction of growth towards the bulb (arrow), whereas others are not re-directed. Overview is composed of pictures photographed at high magnification. **(c)** Only a fraction of GFP-positive axons growing towards the bulb explant invade the target tissue; the majority of axons grows along the tissue surface. Overview is composed of pictures photographed at high magnification. **(d)** GFP-fluorescent axons extend the OE explant (arrow) and invade the bulb tissue at distinct positions. **(e)** GFP-fluorescent axons condensed at a distinct spot within the bulb explant. Scale bars: (a) 100 μm ; (b–e) 50 μm .

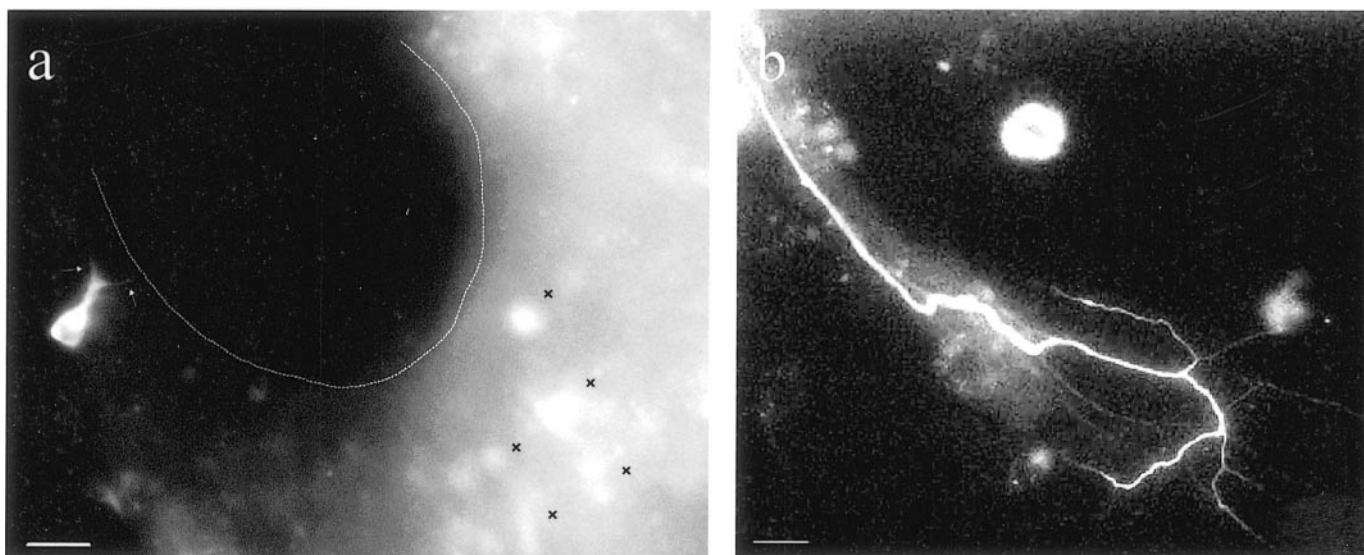


Figure 4 Olfactory epithelium explant from a mOR37A-GFP mouse at embryonic stage 14 after 2 days in culture. **(a)** GFP-positive mOR37A-GFP neurons are visible, one in focal plane, others out of focus (crosses). GFP-fluorescent initial segments of cilia are visible extending from the knob region (arrows). The dotted line delineates the border of a cavity along which the cells are arranged. **(b)** A single GFP-positive fiber bundle extends from the OE explant and defasciculates. Scale bars: 20 μm .

OMP-GFP positive cells. All fluorescent cells exhibited the typical morphology of OSNs (Figure 4a); somata, dendrites and even the initial segments of cilia were visible due to their intense fluorescence.

Since the fluorescent axons from the large OMP-GFP-labeled neuron population appeared to extend from the explants in distinct fiber bundles (see Figure 3a), it is of particular interest whether the axons of a defined neuron subpopulation sharing the same receptor subtype may grow out in a common fascicle or in distinct bundles. The examination of four independent cultures of epithelial explants containing mOR37A-GFP-expressing neurons revealed in all cases only a single fluorescent fiber bundle extending from the explant (Figure 4b). At distinct distances from the explant, a defasciculation of the bundle was observed; individual fibers deviated from the main fascicle. These results suggest that axons of a receptor-specific neuron population initially grow together in one fiber bundle. It is currently unknown whether axons of subpopulations expressing other receptor types are also part of these bundles.

Discussion

In the present study conditions were designed and optimized to allow for the first time the culture of nasal explants with GFP-labeled OSNs for more than 1 week *in vitro*. Due to their fluorescence, the cells could be visualized and monitored for a considerable period of time. It was found that, under adequate *in vitro* conditions, the olfactory neurons established organotypical features, and the differentiation processes mimicked those observed during development *in vivo*. OMP-expressing, and thus mature,

OSNs have been maintained for >9 days *in vitro*, a period significantly longer than in cultures reported previously (Calof and Chikaraishi, 1989; Chuah *et al.*, 1991; Mahantappa and Schwarting, 1993; Gong *et al.*, 1996; Cunningham *et al.*, 1999; McEntire and Pixley, 2000); this emphasizes that the culture conditions seem to be suitable for OSNs *in vitro*. The observation that, even after >1 week *in vitro*, numerous GFP-positive cells were detectable within the OE explants even in the absence of their target tissue strongly suggests that these cells were newly generated from basal cells in the explant. It has previously been reported that basal cells can differentiate into mature OSNs even without the bulb (Carr *et al.*, 1998); however, for survival, they depend on trophical support from bulb tissue (Schwob *et al.*, 1992). Thus, the presence of GFP-positive cells showing the characteristic morphological features of mature OSNs in long-term cultures of OE explants indicates that these cells went through the full program of differentiation *in vitro*.

In this study, for the first time, conditions have been established which allowed us to monitor the expression of an OR gene *in vitro*, an important prerequisite for manipulating transcription control mechanisms. The results emphasize that 'turn on' of receptor gene expression is an intrinsic feature of the epithelium, and thus are supportive of previous studies demonstrating that receptor expression is not initiated by the target tissue (Strotmann *et al.*, 1995; Sullivan *et al.*, 1995).

The culture conditions allowed an extensive outgrowth of multiple OSN processes from the explants, which, due to their intense green fluorescence, could be visualized even in the most distal regions, the growth cones. The GFP fluor-

escence of axonal processes from OSNs expressing a distinct OR gene even allowed us to assess a distinct subpopulation of processes which *in vivo* share a common destination. These preparations now offer the possibility to employ time-lapse techniques and monitor the outgrowth and pathfinding of axonal processes; in addition, it may be possible to experimentally interfere with the process by employing appropriate agents. These manipulatory approaches (inhibitory antibodies, antisense oligos, RNA interference) may eventually lead to the identification of attractant and/or repellent cues for outgrowing olfactory axons. Diffusible factors secreted from the bulb which may attract OSN axons have been proposed in the past (Ressler *et al.*, 1994; Vassar *et al.*, 1994); however, experimental evidence is still elusive. In addition, the identification of putative guidance molecules in the olfactory tissue (Key and Akeson, 1990; Puche and Key, 1996; Kafitz and Greer, 1997, 1998; Yoshihara *et al.*, 1997; Tisay and Key, 1999; Schwarting *et al.*, 2000; St John *et al.*, 2000) has so far not been supplemented by functional evidence demonstrating their direct involvement in axon outgrowth and pathfinding. The observation that distinct axons appear to be attracted to and grow towards the bulb tissue (see Figure 3) seems to support the view. Using GFP-fluorescent OSNs, it has been possible for the first time to demonstrate that axons are capable of converging onto distinct positions within the bulb *in vitro*. The axon bundles tended to defasciculate at distinct distances from the OE explants, mimicking the re-sorting of olfactory axons which occurs when they reach the outer nerve layer of the bulb (Key and Akeson, 1993; St John and Key, 2001) [for a recent review see (St John and Key, 2002)].

In sum, the results demonstrate that GFP-labeled OSNs in organotypic cultures appear to be a useful tool for monitoring and manipulating the processes underlying the axonal wiring between the olfactory epithelium and bulb.

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