Human Olfaction: From the Nose to Receptors

Methods of deorphanization of olfactory receptors and their significance for increasingly predictive fragrance creation

Alex Veithen, Françoise Wilkin, Magali Philippeau and Pierre Chatelain, TecnoScent S.A.

The discovery of the role of olfactory receptors (ORs) in 1991 by Linda Buck and Richard Axel (Nobel Prize in Physiology or Medicine, 2004) paved the way for better understanding of the sense of smell and its molecular mechanisms. Since then, several studies have demonstrated that ORs play an important role in odorant perception, recognition and discrimination, and that humans can discriminate among an almost infinite spectrum of different odors.

The genes coding for ORs represent the largest family of genes (3% of the whole genome) in the human body dedicated to a single physiological function. (For an explanation of key terms, see **Glossary** on Page 40) Meanwhile, an unexplained 60% of these OR genes are found to be non-active (pseudogenes), thereby leaving humans with about 400 different OR proteins. Each receptor interacts with different molecules, and each odorant molecule can activate more than one OR. Thus, odor perception does not rely on the simple activation of a single OR, but rather on multiple activations of several ORs. An odor (which can be a single molecule or a mixture) is paired with a unique set of activated ORs that are sufficient for its discrimination and characterization. Odorant concentration can dramatically affect the profile of an odor as some additional ORs may be recruited (high concentration) or not activated (low concentration). Therefore, the set of activated ORs will differ for different odor concentrations, leading to varying odor perceptions.

At a Glance

Even as the molecular and cellular bases of human olfaction aid in the immediate conceptualization of industrial applications for the flavor and fragrance industry, full deorphanization of human ORs is expected to generate important data for the study of the least understood sense, smell. In addition, knowledge of the precise mechanisms of odorant/receptor pairings will help in the creation of new odorant designs. Meanwhile, the discovery of OR-blocking molecules may assist malodor counteraction. Relying on the molecular mechanisms of odor recognition will help to rationalize some rules of fragrance creation, allowing that process to become a more predictive science.

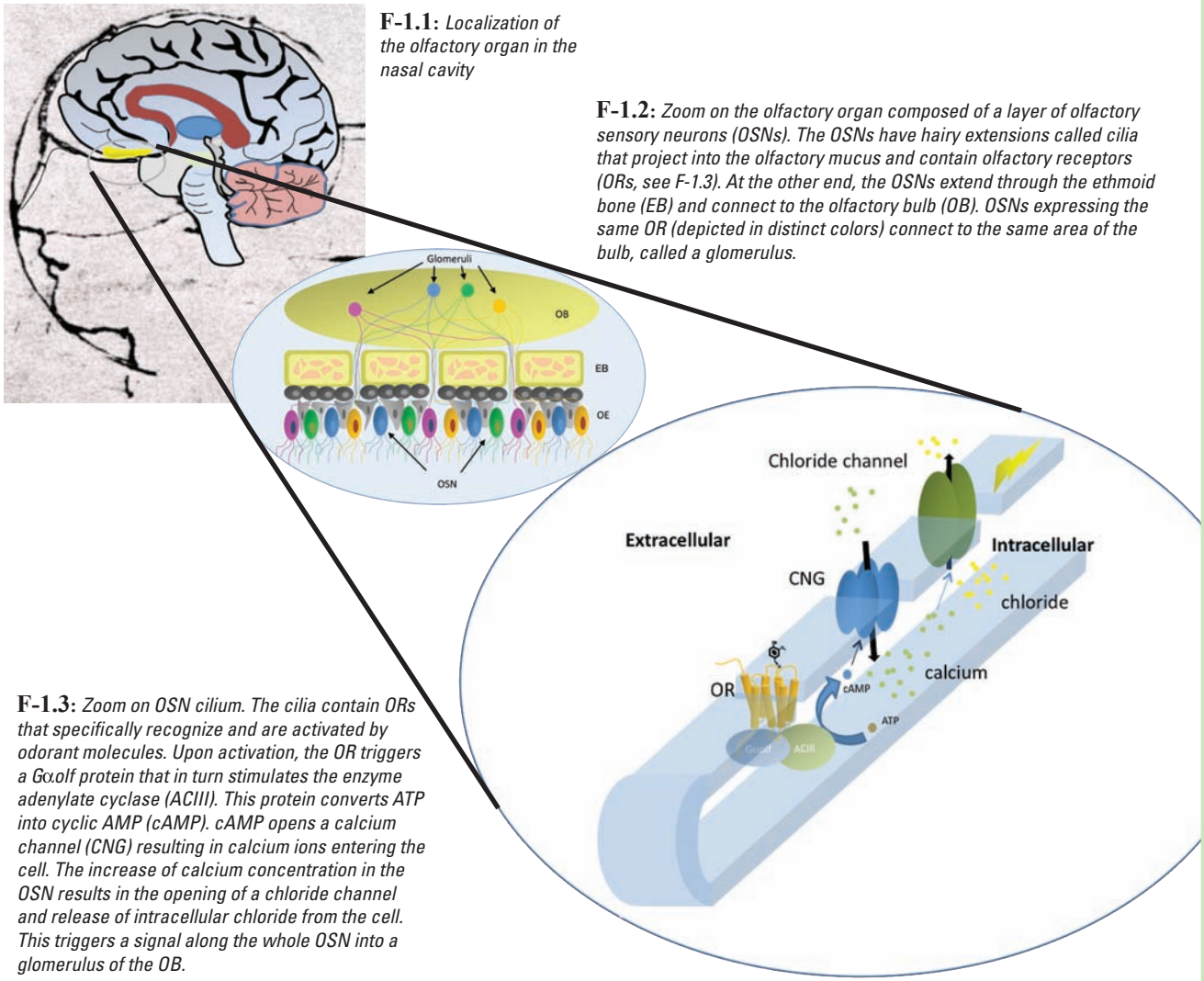
With a pool of 400 ORs, the number of possible combinations is almost infinite, thus explaining the outstanding discrimination properties of the olfactory system.

Even as the molecular and cellular bases of human olfaction aid in the immediate conceptualization of industrial applications for the flavor and fragrance industry, full deorphanization of human ORs is expected to generate important data for the study of the least understood sense, smell. In addition, knowledge of the precise mechanisms of odorant/receptor pairings will help in the creation of new odorant designs. Meanwhile, the discovery of OR-blocking molecules may assist malodor counteraction. Relying on the molecular mechanisms of odor recognition will help to rationalize some rules of fragrance creation, allowing that process to become a more predictive science. The deorphanization (i.e. identification of at least one activator for each OR) of all human ORs and their characterization (structure–activity relationships, i.e. the identification of several agonists and antagonists) represents an important challenge for the flavor and fragrance industry. This article reviews the different approaches used to deorphanize ORs and highlights their significance in large deorphanization projects.

How Does Olfaction Work?

Odorant receptors are expressed in specialized olfactory sensory neurons (OSNs) located at the top of the nasal cavity in a small area that constitutes the olfactory epithelium (**F-1.1**). Filiform extensions at one end of these cells contain the ORs on their surface and float in the nasal mucus where the odorants are dissolved. At the opposite end, the OSN extends its axon across the ethmoid bone at the base of the cranium to connect to the olfactory bulb—a small region of the brain dedicated to the integration of the olfactory stimuli (**see F-1.2**).

An outstanding feature of the tens of millions of OSNs scattered throughout the olfactory epithelium is that each one expresses only one of the 400 OR genes available in the human genome. The OSNs expressing the matching gene connect their axons to the same subregion of the olfactory bulb forming a structure called a glomerulus. It is from this organization of OSNs that the coding of an odor by a specific set of activated ORs is translated geographically in the bulb by a corresponding pattern of activated glomeruli. This information is further transmitted to the olfactory area of the cortex where it is decoded and analyzed.



F-1.1: *Localization of the olfactory organ in the nasal cavity*

F-1.2: *Zoom on the olfactory organ composed of a layer of olfactory sensory neurons (OSNs). The OSNs have hairy extensions called cilia that project into the olfactory mucus and contain olfactory receptors (ORs, see F-1.3). At the other end, the OSNs extend through the ethmoid bone (EB) and connect to the olfactory bulb (OB). OSNs expressing the same OR (depicted in distinct colors) connect to the same area of the bulb, called a glomerulus.*

F-1.3: *Zoom on OSN cilium. The cilia contain ORs that specifically recognize and are activated by odorant molecules. Upon activation, the OR triggers a Gαolf protein that in turn stimulates the enzyme adenylate cyclase (ACIII). This protein converts ATP into cyclic AMP (cAMP). cAMP opens a calcium channel (CNG) resulting in calcium ions entering the cell. The increase of calcium concentration in the OSN results in the opening of a chloride channel and release of intracellular chloride from the cell. This triggers a signal along the whole OSN into a glomerulus of the OB.*

From this coding process, it is clear that determination of selectivity and specificity of human ORs is a prerequisite to the development of an accurate model that would allow prediction of the odor quality of a newly synthesized molecule. There are various approaches to trying to match odorant molecules with the receptors that respond to them. These methods vary from investigations that use whole animals and in vitro techniques, which are reviewed next.

Determination of selectivity and specificity of human ORs is a prerequisite to the development of an accurate model that would allow prediction of the odor quality of a newly synthesized molecule.

The Whole Organism

Perfumers and flavorists have been characterizing the scent of molecules for decades, or even centuries. However, despite the considerable knowledge acquired by flavor and fragrance specialists over the years, the scent of a given molecule cannot be ascertained by considering only its chemical structure; nor does there exist a well-established relationship between chemical structures and odor families. A combination of genetic analysis and sensory assay, however, may provide an interesting approach to understand the interaction of a receptor with a molecule.

Further Reading

- L Buck and R Axel, A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell,* 65(1) 175–187 (1991)
- B Malnic, Searching for the ligands of odorant receptors. *Mol Neurobiol,* 35(2) 175–181 (2007)
- M Zarzo, The sense of smell: molecular basis of odorant recognition. *Biol Rev Camb Philos Soc,* 82(3) 455–479 (2007)

Specific anosmia is a well-known phenomenon that refers to an individual's inability to perceive a given odorant molecule. This odor "blindness" can result from the absence or alteration of one or more ORs, thus causing an inability to interact with the odorant. In addition, there exist different versions (or alleles) of each OR gene, and some of these alleles might correspond to nonfunctional proteins. Humans rely less on olfaction than do most mammals; thus, the inability to smell a particular molecule is not considered crucial in some cases—this might explain the higher frequency of nonfunctional genes in humans. Since an odorant molecule may interact with several different ORs, the loss of functionality of one of these receptors would not necessarily result in the complete loss of perception. The probability of an individual acquiring nonfunctional alleles of ORs involved in the perception of an odorant molecule is inversely proportional to the number of these ORs. Interestingly as a result, molecules for which a specific anosmia is frequently observed are probably recognized by only a few ORs; possibly just one.

Although the genetic analysis of anosmia could lead to the establishment of a link between odorant molecules and ORs, further studies by a large panel of volunteers and sufficient data collection would be required to correlate a specific anosmia with a particular receptor. Moreover, despite this, there could still be a molecule without any corresponding anosmia. Therefore, it would be better to use such analyses to confirm involvement of an OR in the perception of odorant molecules—after the interaction has been demonstrated by some other approaches.

In contrast to humans, animal models allow experimental approaches to be performed at the whole organism level. The first reliable identification of an activating molecule for a mammalian OR was made by infecting the olfactory epithelium of rats with a virus containing the rat OR-I7 gene. In this way, a large majority of OSNs were forced to express this OR; eventually, electrophysiological measurements upon puffing odorant molecules into the rats' noses led to the identification of octanal as a ligand. Alternatively, it is possible to generate transgenic animals that express the same OR in virtually all of their OSNs; this has been recently demonstrated with the acetophenone receptor M71.[1] Despite their effectiveness in revealing the activity of a desired OR, viral infection of a rat's nose or the development of transgenic animals that overexpress one OR may hardly be considered core procedures for a large-scale deorphanization program. These experiments are too time consuming to be repeated with large libraries of molecules, much less with several hundred OR genes to be expressed.

Highly demonstrative studies have analyzed the pattern of glomerulus activation at the olfactory bulb level.[2] In addition, imaging techniques have helped to prove that a unique activation pattern corresponds to an individual molecule. For example, it has been demonstrated that stereoisomers induce distinct patterns and may therefore be discriminated. One could, therefore, imagine classifying odorant

molecules based on their activation pattern in the olfactory bulb. Such mapping of activation might be of interest, but it would not give information about the OR involved in molecule recognition. Moreover, this approach involves surgical opening of the cranium, thereby requiring sacrifice of the animal.

Another way of finding odorant molecule-OR couples consists of identifying the OR from a responding OSN upon activation by the odorant. This approach relies on ex vivo cultures of OSNs. The responses of OSNs may be recorded by tracking modifications occurring in the cell when the odorant is bound to the expressed OR. For example, modification of cell membrane potential or measurement of calcium increases are commonly used to measure OR activity in OSNs (**see F-1.3**). Responding OSNs are then harvested one at a time; the respective expressed OR genes are identified using the technique of single cell RT-PCR. In addition to being used for deorphanization of several mouse ORs, this approach was first used to demonstrate that one receptor may interact with more than one molecule and that a single molecule may trigger more than one OR. However, regardless of its technical difficulty, this procedure is not suitable for use with the desired system for human OR deorphanization, since human olfactory epithelium is unavailable in sufficient quantities. One team of researchers has, therefore, proposed to use this approach on mice and then transpose the results to humans. According to this approach, once a ligand is found for a mouse OR, the most closely related human receptor is assumed to interact with the same odorant. Nevertheless, this approach requires much caution since, for some receptors, minor differences between two OR sequences may lead to major alterations in their ability to interact with a given odorant. Hence, such an approach cannot be considered generic.

In vitro Expression of ORs

Cultured cell lines have been widely used to characterize and study receptors of interest in both academic and industrial contexts. This approach involves introduction of the corresponding gene into the cells, and subsequent promotion of its stable or transient overexpression. The activity of the receptor can be monitored using a functional assay (see **Measurement of OR Activity**). The use of easy-to-culture cell lines along with easy-to-perform functional assays facilitates several thousand measurements per day. Typically, in the pharmaceutical industry, it is common to test libraries of 1,000,000 compounds per day on non-olfactory receptors. This process, also known as high throughput screening (HTS), is

Glossary

Acellular: Does not contain living or intact cells.

Allele: Version of a gene. Different versions of a gene may exist within a population, generally with minor differences. Each individual has two copies of the same gene and these copies may or may not correspond to two different alleles.

Anosmia: Inability of an individual to smell. Specific anosmia corresponds to the inability to smell a particular odor.

Axon: Long extension of a neuron that facilitates its connection with another neuron.

Cell culture: Process that allows cells extracted from an organism to be maintained alive, and possibly to multiply, under controlled conditions.

Cell line: Cultured cells taken from an organism that have been immortalized and can thus be indefinitely maintained and multiplied under appropriate conditions.

Cyclic AMP (cAMP): Cyclic adenosine monophosphate, a small molecule that serves to transmit information inside the cells.

Deorphanization: Process that consists of identifying, for the first time, at least one activator for a receptor for which no activator was previously known.

Differentiation: Process of transforming a cell that has not yet acquired a defined function into a more specialized cell type that plays a defined function in an organ.

Electrophysiology: Science that studies the electrical properties of cells (most often nerve cells) or tissue.

Expression (gene expression): Process that consists of producing the protein corresponding to a gene.

Functional expression: Expression into a cell of a receptor that can be activated by its ligand.

G protein: A protein that directly interacts with and is activated by receptors.

Gene: Sequence of DNA that encodes a protein.

Genome: The entirety of an organism's genes.

Glomerulus: Small area at the surface of the olfactory bulb where the axons of olfactory neurons expressing the same olfactory receptor converge.

Ligand: Molecule that binds to a receptor. A ligand may be either an activator or an inhibitor of the receptor.

Olfactory bulb: Small part of the brain in direct connection with the olfactory epithelium that serves to integrate olfactory signals.

Olfactory epithelium: Small area at the top of the nasal cavity that contains the olfactory neurons and that serves to detect odorant molecules.

Oocyte: Female reproductive cell (also called egg).

RT-PCR: Technique that allows the detection of a gene that is expressed in a cell line, a tissue or an organ.

Sequence (of a gene or of a protein): Succession of nucleotides (for a gene) or of amino acids (for a protein) that compose a gene or a protein.

Transgenic: Organism in which the genome has been modified by the artificial insertion or deletion of one or more genes.

applied to ORs and is used at TecnoScent. In the aftermath of OR discovery, several attempts were made to express the OR in the cell lines suitable for the expression of non-olfactory receptors, but they remained largely unsuccessful. The reason for such a setback can be found, not in the failure of the cell to produce the receptor, but rather in its inability to send the receptor to the surface of the cell. Several techniques/methods have been adopted to circumvent this problem.

The first method uses cell lines originating from the olfactory epithelium. There are several examples of such lines, but none of them corresponds to a real differentiated OSN. Indeed, while differentiating into a neuron, the cell loses its ability to multiply and can no longer be maintained in long-term culture. Therefore, the olfactory cell lines correspond to progenitors that keep their ability to grow in vitro and can be differentiated into OSNs by adding appropriate factors to the culture medium. Although this approach has met some success in expressing functional ORs, it has not yet been widely used due to several issues. To begin with, the introduction of exogenous DNA works poorly on olfactory cells when using conventional methods for this purpose. This lowers the OR expression efficiency below a level suitable for deorphanization experiments. Moreover, it is difficult to properly achieve the differentiation of the growing cell into an OSN, and some attempts to improve this process are ongoing. Beyond these technical hurdles, a more fundamental issue that arises when using olfactory cells as a model for exogenous OR functional expression is that the cell also expresses its own OR during its transformation into an OSN. In this case, the ligand identification is likely to be attributed to the wrong OR.

The other method of improving expression of ORs in a cell line consists of modifying the receptor. Adding the first 20 amino acids of the bovine rhodopsin sequence upstream to the sequence of ORs has, in several cases, improved both their targeting to the cell surface and activity. Similarly, alternative sequences, corresponding to signal peptides of other non-olfactory receptors, have also been used successfully. A few ORs can be readily expressed without any sequence modification in conventional cell lines; activating odorants have been identified for some of them. In this case, comparisons of responses of both a modified and an unmodified version have shown that the added sequence does not influence the way the receptor interacts with its ligand. Nevertheless, such engineering of receptors does not seem to be the ultimate solution to the problem of OR functional expression in conventional cell lines; cell surface expression of such modified ORs has been assessed for more than 100 human ORs, with less than 20% being detected at the cell periphery.

A third technique aimed at improving the functional expression of ORs requires engineering a conventional cell line to make it suitable for OR expression. In fact, it had long been suspected that correct expression and targeting of the OR at the cell surface requires an OSN-specific intracellular machinery that is absent in a non-olfactory cell line. Thorough analysis of the expression in OSNs revealed two members of a new family of proteins that are specific to

this sensory cell. When co-introduced into a conventional cell line along with a model OR, the so-called receptor transport proteins 1 and 2 (RTP1 and RTP2) enhanced both the cell surface expression and the response of the receptor to its cognate odorants.[3] The precise mode of action of these chaperone proteins is not yet clear; they could possibly be just elements of even more complex machinery dedicated to the export of ORs from the intracellular compartment to the cell surface. The discovery of RTP1 and RTP2 alone does not considerably improve the cell surface expression of a majority of OR; however, when combined with the addition of a leader sequence to the OR, it represents an important advancement for the set up of an in vitro expression system for these receptors.

Measurement of OR Activity

In addition to the issue of cell surface expression of ORs, the method used for detection and quantification of OR activation by odorant molecules is significant in the development of a deorphanization system for ORs. In OSN, triggering of the OR promotes the activation of an olfactory-specific G protein (Gαolf) that stimulates a type III adenylate cyclase to produce cyclic AMP; this plays the role of a second messenger (**see F-1.3**). Upon binding to a cAMP-gated cation channel, this messenger induces the entry of calcium into the cell. Calcium causes the opening of another channel that promotes the exit of chloride ions, and hence triggers an action potential of the neuron.

Measurement of intracellular calcium has been widely used to demonstrate the activation of an OR expressed in a model cell line due to its high degree of resolution. Also, by using calcium-sensitive dyes, it is possible to follow the response of each individual cell with a microscope. To allow the calcium-based functional response of an OR in a heterologous cell line, it is necessary to introduce additional elements to promote the intracellular release of calcium from the signal initiated by the receptor activation. The co-introduction of a cAMP-gated cation channel along with the OR of interest helps to successfully measure calcium as an output for monitoring OR activity. Alternatively, Gα15 and Gα16 proteins have been used to promote the coupling of the receptor activity to the release of calcium from intracellular stores. These G proteins originating from blood cells can readily couple to a large variety of receptors, and have been shown to interact with some ORs. They do not naturally occur in conventional cell lines, but can be co-expressed with ORs. Interestingly, the coupling efficiency between the promiscuous Gα15 or Gα16 and ORs can be reinforced by replacing the last 47 amino acids of these G proteins by the corresponding domain of the olfactory-specific Gαolf.

The resulting Gα16olf47 chimeric protein has also been successfully used to deorphanize human ORs.

As an alternative to calcium measurement, cAMP measurement can also be used to quantify the activity of ORs. In the OSN, the OR preferentially binds to Gαolf, but this G protein is not mandatory for promotion of an OR-induced increase of cAMP. In conventional cell lines, where Gαolf is not expressed or weakly expressed, triggering of exogenous ORs results in a measurable increase of cyclic AMP. A variety of immunoassays allow the direct detection of this second messenger.

The production of cAMP may also be detected by an indirect approach that consists of the use of a reporter gene. This gene is placed under the control of a cAMP-inducible promoter and is expressed only upon induction by cAMP. Different genes can be used for this purpose, but one of the most popular ones encodes the light-producing protein luciferase. While cleaving its substrate, luciferin, this enzyme releases light that is readily detected and quantified. The intensity of light emitted reflects the amount of luciferase produced, which is proportional to the cAMP increase and therefore directly related to the activity of the receptor. One of the advantages of reporter gene assays is dependent upon the signal amplification between receptor activation and reporter production. This makes the assay particularly sensitive to weak responses that can hardly be detected by other functional assays.

The different functional assays described above are all compatible with automation processes that allow testing of several hundred or even thousands of ligands per day.

Alternative Systems for Functional Expression of ORs

In addition to conventional cell lines, other functional expression systems have been explored. One of these systems consists of injecting the DNA or mRNA of the receptor into the oocyte of the African clawed frog, *Xenopus laevis*.[4] This unfertilized egg corresponds to a giant cell that can be easily manipulated for microinjection and electrophysiological measurements. A measurable depolarization of the egg membrane occurs upon the OR activation if a cAMP-sensitive chloride channel is co-injected into the oocyte. This technique is routinely used in several labs and has already led to deorphanization of ORs; it is, however, difficult to adapt for extensive screening of odorant molecules. Expression of ORs in yeast has also been assessed.[5] One of the advantages of using this easy-to-manipulate organism is that it relies on few requirements for culturing. It fits particularly well with receptor activity detection based on systems using reporter genes such as luciferase, and is therefore particularly convenient for large-scale assays. The major hurdle is the evolutionary distance that exists between the intracellular signaling components in a yeast cell, compared to that of a vertebrate cell. It is therefore necessary to either import the different elements that transduce the OR activation signal or to engineer the yeast to promote the coupling of the signal with yeast transduction pathways. While two research teams have reported some success in functionally expressing ORs in yeast, so far these systems are not being widely used for OR deorphanization.

Finally, another technique which has been considered for deorphanization of ORs consists of using an acellular system. In this case, the receptor is produced by cultured cells, but it does not go to the cell surface. In fact, either the intracellular or cell surface membranes are purified and placed on a pre-treated surface. The activation of the OR is therefore measured by physical techniques (such as surface plasmon resonance or single-walled carbon nanotube field effect transistors). Such systems present the tremendous advantage of being miniaturizable, and might therefore constitute a base for biosensor development. At this stage, however, they are still under development and their efficiency for deorphanizing ORs remains to be determined.

Conclusion

From the discovery of ORs 18 years ago, considerable progress has been made in the understanding of their functioning. The recent advances in OR functional expression have brought with them the reasonable hope of seeing a majority of the human ORs deorphanized in the coming years. Although the technological aspects in this field are still evolving, the system gathering N-terminal modified ORs and use of ancillary proteins favoring OR expression has already led to the deorphanization of several dozens of these receptors. The systematic screening of odorant libraries is therefore becoming possible, and the ambitious project of deorphanizing all human ORs has already been started by several groups. Interaction of ORs and odorant molecules, and its potential industrial applications will be discussed in our next article, titled "Olfactory Receptors: From Basic Science to Applications in F&F," appearing in the December issue of *P&F* magazine.

Acknowledgements

The authors are grateful to Charles Sell (Givaudan) for his suggestions, comments and manuscript revision, and to Boris Schilling (Givaudan) and Christian Van Osselear (TecnoScent) for their critical review of the manuscript.

Address correspondence to Alex Veithen, TecnoScent S.A., 802 Route de Lennik, 1070 Brussels, Belgium; ave@chemcom.be.

References

1. A Fleischmann, BM Shykind, DL Sosulski, KM Franks, ME Glinka , DF Mei, Y Sun, J Kirkland, M Mendelsohn, MW Albers and R Axel, Mice with a "monoclonal nose": perturbations in an olfactory map impair odor discrimination. *Neuron*, 60(6) 1068–1081 (2008)
2. BD Rubin and LC Katz, Spatial coding of enantiomers in the rat olfactory bulb. *Nat Neurosci,* 4(4) 355–356 (2001)
3. H Zhuang and H Matsunami, Evaluating cell-surface expression and measuring activation of mammalian odorant receptors in heterologous cells. *Nat Protoc*, 3(9) 1402–1413 (2008)
4. T Abaffy, A Malhotra and CW Luetje, The molecular basis for ligand specificity in a mouse olfactory receptor: a network of functionally important residues. *J Biol Chem,* 282(2) 1216–1224 (2007)
5. J Minic, MA Persuy, E Godel, J Aioun, I Connerton, R Salesse and E Pajot-Augy, Functional expression of olfactory receptors in yeast and development of a bioassay for odorant screening. *FEBS J,* 272(2) 524–537 (2005)