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Mimicking nature's noses: From receptor deorphaning to olfactory biosensing

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ABSTRACT

The way in which organisms detect specific volatile compounds within their environment, and the associated neural processing which produces perception and subsequent behavioural responses, have been of interest to scientists for decades. Initially, most olfaction research was conducted using electrophysiological techniques on whole animals. However, the discovery of genes encoding the family of human olfactory receptors (ORs) paved the way for the development of a range of cellular assays, primarily used to deorphan ORs from mammals and insects. These assays have greatly advanced our knowledge of the molecular basis of olfaction, however, while there is currently good agreement on vertebrate and nematode olfactory signalling cascades, debate still surrounds the signalling mechanisms in insects. The inherent specificity and sensitivity of ORs makes them prime candidates as biological detectors of volatile ligands within biosensor devices, which have many potential applications. In the previous decade, researchers have investigated various technologies for transducing OR:ligand interactions into a readable format and thereby produce an olfactory biosensor (or bioelectronic nose) that maintains the discriminating power of the ORs *in vivo*. Here we review and compare the molecular mechanisms of olfaction in vertebrates and invertebrates, and also summarise the assay technologies utilising sub-tissue level sensing elements (cells and cell extracts), which have been applied to OR deorphanisation and biosensor research. Although there are currently no commercial, "field-ready" olfactory biosensors of the kind discussed here, there have been several technological proof-of-concept studies suggesting that we will see their emergence within the next decade.

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Abbreviations: BAW, bulk acoustic wave; BRET, bioluminescence resonance energy transfer; cVA, 11-cis-vaccenyl acetate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CNG, cyclic nucleotide gated; EAG, electroantennagram; EOG, electroolfactogram; EIS, electrochemical impedence spectroscopy; EG, eugenol; EC_{50}, half maximal effective concentration; FET, field-effect transistor; FLIPR, fluorescence imaging plate reader; FRET, fluorescence resonance energy transfer; GC–MS, gas chromatography–mass spectrometry; G-protein, guanine nucleotide binding protein; GPCR, G-protein coupled receptor; Gα, the alpha subunit of the G-protein; Gβ, the beta subunit of the G-protein; Gγ, the gamma subunit of the G-protein; GDP, guanine diphosphate; GTP, guanine triphosphate; GTPγS, non-hydrolysable GTP; GFP, green fluorescent protein; IP_3, inositol triphosphate; LAPS, light-addressable potentiometric sensor; MEA, microelectrode array; OR, olfactory receptor; OSN, olfactory sensory neuron; OBP, olfactory binding protein; QCM, quartz crystal microbalance; SAM, self-assembled monolayer; SAW, surface acoustic waves; SNMP, sensory neuron membrane proteins; SPR, surface plasmon resonance; swCNT, single-walled carbon nanotube.

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1. Introduction

One of the great success stories of biological research has been the discovery of important genes and the functional characterisation of their encoded proteins, which directly regulate the higher order biological processes that we witness daily. Olfaction is a prime example; the discovery of the gene family encoding vertebrate olfactory receptors (ORs) (Buck and Axel, 1991) has led to a relatively detailed understanding of the molecular and neurological bases for how organisms can "smell" volatile compounds. Since the first successful attempt to match an OR to a volatile ligand (Zhao et al., 1998), numerous ligand-binding assays have been developed and used to deorphan a range of ORs (see review by Touhara, 2007). These assays have generally utilised cells expressing recombinant ORs combined with a transduction (reporting) system that allows detection of OR:ligand binding, initially for the purpose of research into olfaction mechanisms. However, the ability to detect volatile ligands at biologically relevant concentrations (approximately nanomolar and below) is crucial for an enormous range of applications, a fact that has seen the expansion of a relatively new field of research, olfactory biosensing. This research has generally utilised characterised OR:ligand interactions to validate a range of sensor platforms and transduction approaches to produce an olfactory biosensor (Lee and Park, 2010). Biosensor research is therefore generally application-driven rather than being driven by pure biological research, with a focus on detection of important ligands in complex environments.

A biosensor can be described as a biological detector or recognition element (e.g. an OR for olfactory biosensing) linked to a physical transduction system (e.g. optical, electrochemical). This definition, however, is rather broad and for olfactory biosensors (also known as bioelectronic noses), could include the use of whole animals or tissues as the biological recognition element. As an example, the use of canaries to detect carbon dioxide in mining applications is legendary (Schmidt, 2009). Dogs have also been widely utilised for detecting people, narcotics or explosives (Furton and Myers, 2001) and it is known that they utilise at least 2-ethyl-1-hexanol and 2,4-dinitrotoluene as cues to detect the latter (Harper et al., 2005). Yale University School of Medicine maintains a website related to use of whole animals as sentinels for human diseases and toxins (http://canarydatabase.org).

Tissue-specific olfactory biosensors have also been produced. Such approaches, such as the electroolfactogram (EOG, for vertebrates) and electroantennogram (EAG, for invertebrates) have been utilised for decades and did not require any knowledge of molecular biology for their implementation (Scott and Scott-Johnson, 2002; Sevonkaev and Katz, 2008). While whole animal and tissue-based recognition elements both utilise ORs for detection at the molecular level, the ability to isolate cells expressing specific ORs or partially purified ORs themselves, has altered the perception of a biosensor to mainly encompass sub-tissue level recognition elements. The use of cells, cell extracts or purified ORs as recognition elements, has a range of advantages such as the level of miniaturisation (and potential transportability) that can be achieved, and the ability to design and control recognition elements to perform specific reporting functions or provide multiplexing. Here we will mainly discuss in detail those olfactory biosensors based on sub-tissue level recognition elements as this is where the main research efforts are directed and where the key advances are being made. In addition, we cover both biosensors used purely for research (e.g. OR deorphaning) and those developed for specific field-based applications or to refine transduction systems using characterised receptors. Both utilise similar technology, albeit for potentially different applications.

This paper reviews the history and developments in the specific area of olfactory biosensors (detecting volatile compounds), however, researchers are developing a much broader range of biosensors that utilise different biological recognition elements. For those interested in biosensing more generally, there are a good number of books and reviews covering the field (Borisov and Wolfbeis, 2008; Cooper and Cass, 2004; Cooper and Singleton, 2007; Knopf and Bassi, 2007; Leifert et al., 2009; Luong et al., 2008; Malhotra et al., 2005; Nakamura and Karube, 2003; Rasooly, 2005; Singh, 2007). While we do deal with some aspects of the detailed mechanisms underlying olfaction, we do not cover this exhaustively, and for further reference a number of detailed reviews have been published on the topic (Chesler and Firestein, 2008; Kaupp, 2010; Nakamura, 2000; Nakagawa and Vosshall, 2009; Silberling and Benton, 2010; Song et al., 2008; Su et al., 2009; Tall et al., 2003; Touhara and Vosshall, 2009; Wicher, 2010). This review aims to provide a glimpse of where these two general areas intersect as olfactory biosensing, with key concepts and techniques discussed.

1.1. Why biosensors?

There is speculation with regard to the worth of biosensor research (Kissinger, 2005), in particular, the applications of the research and the accessibility of alternatives to a "bio"-based sensor. The area of volatile detection is potentially a highly valuable area of biosensor research primarily because the ORs used as biological detectors are orders of magnitude more sensitive in detecting their respective ligands than the most advanced physical approaches such as chemical "noses" or gas-chromatography/mass spectrometry (GC–MS). There is also an extremely diverse range of applications to which a sensitive and specific olfactory sensor could be applied; a few examples include non-invasive disease diagnostics, process monitoring and quality assurance in the food and wine industries, agricultural and environmental monitoring, and detection of biowarfare agents and explosives for security purposes.

Alternative real-time methods to sense volatiles come in the form of electronic/chemical "e-noses", which include conducting polymers and electrochemical sensors (Wilson and Baietto, 2009). As mentioned, a key benefit of bio-based sensors, as opposed to these e-noses, mainly lie in sensitivity and also specificity, however, they are currently limited by the relative lack of stability of the biorecognition element under "field" conditions and lack of transportability. Other alternatives involve the use of a whole organism, however, this approach is not practical or applicable in many targeted applications; the shortcomings of these approaches has driven research towards development of small, tuneable, accurate and fast biosensing devices that maintain the sensitivity inherent in whole organisms, and is worthy of the commercial

research dollar in terms of performing a required application. The holy grail of biosensor research is therefore to adapt the specificity and sensitivity of the sensory components found in the natural world, to a reliable, portable, inanimate device. Research in this area requires knowledge of the not only biological components, but also both the ability to use/modify these components, or synthetic mimics of them, in a manner which exposes them to the analyte to be detected, and the physical mechanism chosen to monitor the biological recognition event. Although here we discuss in detail the use of the actual ORs responsible for sensing events *in vivo*, within a sensing device, we also recognise that alternative, potentially more stable, bio-recognition components could perhaps be developed using knowledge of the OR:ligand interaction. This could take the form of perhaps an aptamer (Hianik and Wang, 2009; Mairal et al., 2008) or other recognition elements (Chambers et al., 2008) for stable integration and storage within a biosensor device.

1.2. Molecular basis of vertebrate and invertebrate olfaction

1.2.1. Vertebrate olfaction

Early research into olfaction involved using whole organisms, or extracted olfactory tissue, and predominantly electrophysiology measurements. A widely utilised example is EOG (reviewed by Scott and Scott-Johnson, 2002), whereby electrodes are used to measure the negative potential generated in olfactory epithelium as a result of odour stimulation of the OR population, and their subsequent signalling. This technique was first utilised to investigate olfaction in dogs (Hosoya and Yoshida, 1937) although the term "electroolfactogram" was not coined for almost 20 years, until EOG was adapted to study olfaction of frogs and rabbits in 1956 (Ottoson, 1956). The first review of the EOG technique was then published in 1967 (Ottoson and Shepherd, 1967) and it has been extensively used since that time to study olfaction biology of a range of vertebrates. While the interaction of a volatile with a specific sub-set of an organism's total OR repertoire (or perhaps a single OR) is the starting point in terms of generating EOG signals, EOG measures the combined, net electrical output from all activated receptors within olfactory epithelium between the electrodes. Therefore it provides no information about the actual OR(s) involved (unless accompanied by additional molecular analyses) or about the molecular bases for olfaction. However, the subsequent development of molecular biology techniques combined with the discovery of the vertebrate OR gene family as a subclass of G-protein-coupled receptors (GPCRs) (Buck and Axel, 1991), allowed the molecular bases for odour detection and associated neural processing to be elucidated. Since that time, bioinformatics has been used to determine the number of putative ORs in the genomes of various vertebrates, and show that their repertoires are highly variable in number (e.g. about 1000 in humans, approximately 10 times more than in fish; Niimura and Nei, 2005).

Vertebrate ORs are known to be expressed in distinct sensory cells (olfactory sensory neurons; OSNs) in the nasal neuroepithelium (Breer, 2003; Buck and Axel, 1991) which is exposed to an external environment that is characterised by a diverse range of volatile compounds (see Fig. 1a). Each bipolar OSN projects a dendrite to the nasal lumen (dendrites contain cilia which contain the ORs and increase the surface area of their exposure to volatiles) and an axon to the olfactory bulb which is involved in neural processing of combined electrical signals propagating from the OSN population (Mori et al., 1999). OR signalling is transferred through the olfactory bulb to the primary olfactory cortex and then to higher order cortical regions and the limbic system; these combined processes lead to perception of single or multiple volatiles and subsequent behavioural responses (Breer, 2003).

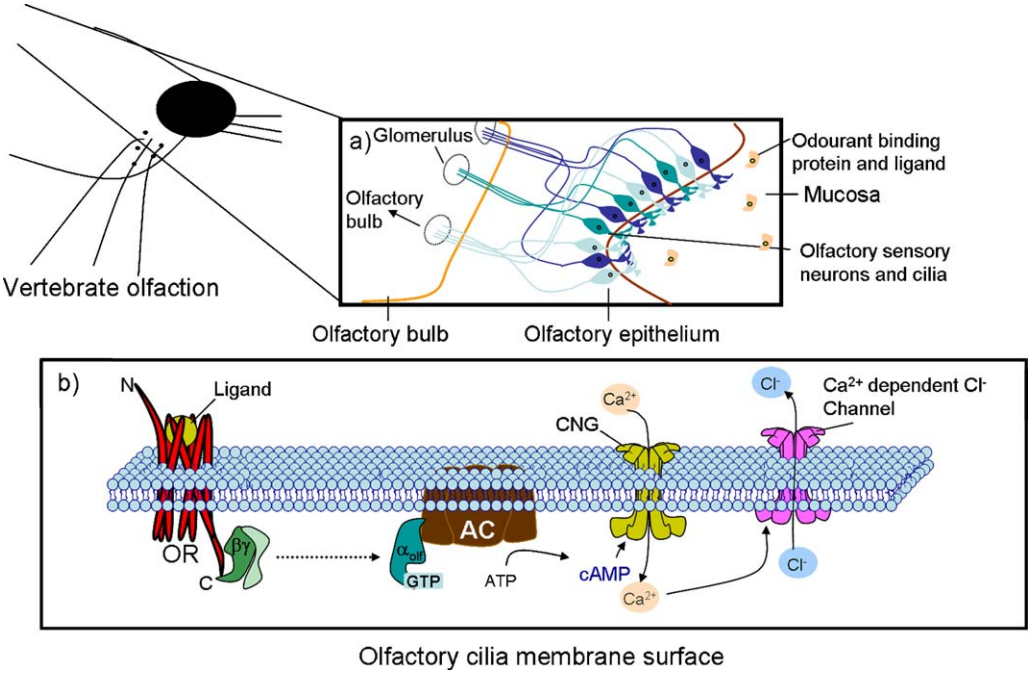

Fig. 1. Vertebrate olfaction through G-protein-coupled receptors – reported mechanisms (reviewed in Buck, 1996; Song et al., 2008; Touhara and Vosshall, 2009). (a) OSNs located in the olfactory epithelium protrude sensory cilia into mucosa containing odourant ligands solubilised by odourant binding proteins. Axons of the OSNs project back into specific glomeruli where they converge with other olfactory neurons and transfer the signal to the olfactory bulb. (b) ORs reside in the cell membrane of the OSN cilia with an extracellular N-terminal region, intracellular C-terminal region and seven transmembrane domains. Ligand-activated ORs have been shown to increase intracellular cAMP levels in the cell through the interaction of the activated $G\alpha_{olf}$ subunit with adenylyl cyclase (AC). Elevated cAMP levels activate CNG ion-channels which increase the flow of Ca^{2+} ions into the cell. Subsequently Ca^{2+}-dependent ion channels can also be triggered in the signalling cascade. *Abbreviations*: OSN, olfactory sensory neuron; OR, olfactory receptor; cAMP, cyclic adenosine monophosphate; CNG, cyclic nucleotide gated; ATP, adenosine triphosphate; GTP, guanosine triphosphate; AC, adenylyl cyclase III; βγ, guanine nucleotide binding protein dimer – beta:gamma; $G\alpha_{olf}$, guanine nucleotide binding protein alpha – olfactory subtype.

Thus far, for each vertebrate tested, each OSN expresses mainly a single type of OR (Touhara and Vosshall, 2009). As "odours" are usually always composed of mixtures of volatiles, perceptions and behavioural responses are necessarily the result of the complex signal resulting from activation of multiple ORs/OSNs simultaneously and furthermore, by the relative amounts of compounds present (Ache and Young, 2005). In order for ORs in the aqueous phase, to access volatile compounds, water-soluble odourant-binding proteins (OBPs) are secreted into the nasal mucosa; there has been speculation that they act to solubilise and transport specific odorants, making them available to the ligand-binding site of ORs (Pelosi, 1994, 1996; Pevsner et al., 1988), but may also function in terminating the signalling response (Vosshall and Stensmyr, 2005). It should be noted that the complete role of OBPs is still unresolved and most studies involving OR signalling *in vitro* do not include OBPs, although odour solubilisation is still required (Krautwurst et al., 1998; Mitsuno et al., 2008). However, using surface plasmon resonance (SPR) on yeast-derived nanosomes containing OR17-40, Vidic et al. (2008) investigated the role of OBP-1F in regulating binding kinetics of the ligand, helional. This study showed that the presence of OBP-1F increased sensitivity at lower concentrations and was required to generate a saturable response. In addition, OBP-1F was thought to be released from a previously occupied OBP-binding site on the OR (indicating that some OBPs might not solubilise ligands), leading to cell-signalling as measured using GTPγS-binding assays. OBPs are better characterised in insects (see Section 1.2.2) although they are apparently not related to mammalian OBPs and have much narrower odour-binding profiles (Hildebrand and Shepherd, 1997). Vertebrate OBPs are small lipocalin-like proteins (Golebiowski et al., 2007; Tegoni et al., 2000), but insect OBPs do not have these structural features (Graham and Davies, 2002).

An unrelated sub-family of volatile-binding GPCRs are the vomeronasal type-1 receptors (V1Rs) of mammals, which are expressed in the vomeronasal organ and thought to be involved with detection of compounds such as pheromones (Dulac and Axel, 1995; Mombaerts, 2004; Shirokova et al., 2008; Touhara and Vosshall, 2009), although other types of volatile ligand also appear to be detected (Sam et al., 2001). For a recent review of pheromone biology in vertebrates and invertebrates, see Wyatt (2010).

In the late 1980s, mounting experimental evidence suggested a role for GPCRs as the primary sensing proteins driving higher order olfactory processes. For example, the involvement of guanidine nucleotide binding proteins (G-proteins; which reside in cytoplasm and couple to GPCRs in the cell membrane) were implicated by evidence suggesting that exposure of rat olfactory epithelium to odourants led to stimulation of adenylate cyclase (Pace et al., 1985; Sklar et al., 1986), and increased cellular concentrations of the G-protein related signalling molecules, cyclic adenosine monophosphate (cAMP) and inositol 1,4,5-triphosphate (IP_3) (Boekhoff et al., 1990; Breer et al., 1990). It is generally accepted that increased cAMP concentrations lead to activation of cAMP-gated cation channels (Dhallan et al., 1990; Nakamura and Gold, 1987), which in turn cause membrane potential of OSNs to alter (through influx of Ca^{2+}), generating the electrical signal that is subsequently processed by the olfactory bulb (and which can be measured by EOG) (see Fig. 1b). The discovery of a G-protein specifically expressed in olfactory neurons (named $G\alpha_{olf}$) that showed 88% amino acid identity with $G\alpha_s$, was further strong evidence, as $G\alpha_s$ is known to stimulate adenylate cyclase (Jones and Reed, 1989). These studies paved the way for the discovery of the OR GPCR gene family soon after and this work was published in 1991 (Buck and Axel, 1991).

The activation of multiple G-protein-regulated signalling cascades (i.e. cyclic nucleotide and phosphoinositide pathways) has led to some debate about which G-proteins are involved in olfactory signalling and their specific roles, and the biological significance of activation of multiple/differential pathways (Ache and Young, 2005). These signalling mechanisms are summarised in Fig. 1b. While the role for $G\alpha_{olf}$ in vertebrate olfaction is well understood, the role of $G\alpha_q$ (the G-protein involved in phospholipase C/IP_3 signalling (Kristiansen, 2004)), is less clear (and is omitted from Fig. 1b), and characterised in more detail for invertebrates (Corey et al., 2010; Talluri et al., 1995) (see Fig. 2b), although debate still exists about whether G-proteins are required for invertebrate olfaction (see Section 1.2.2).

In terms of biosensor research, the molecular characterisation of the olfactory system was crucial as it has allowed biological recognition elements for volatile compounds, and their associated signalling proteins/metabolites, to be isolated and used for detection and transduction of OR:ligand binding events. These capabilities were first utilised for deorphaning of OR proteins and further elucidating the processes involved in olfaction. Within the last decade, however, there has been increasing effort to utilise model deorphaned ORs to investigate improved transduction systems (see Section 1.3 and Table 1) and methods for producing recombinant cells/proteins (and maintaining their functional integrity), for potential practical applications involving volatile detection. Biosensors involved in these studies are reviewed in Section 2. As non-olfactory GPCRs have been widely studied for medical applications, there are a range of validated approaches available to measure their activity in a cell or cell-free environment, which could be adapted to olfactory ORs (Leifert et al., 2005; Lundstrom and Svensson, 1998).

1.2.2. Invertebrate olfaction: an evolving paradigm

As for vertebrates, physiological research into invertebrate olfaction was, for a long period, based on measurement of electrical signals in the insect antennal lobes (using EAG), which are the primary olfactory organs for invertebrates (see Fig. 2a), and are analogous to the olfactory bulb of mammals. EAG is similar to EOG (Scott and Scott-Johnson, 2002). Measurement of electrical signals generated in antennae that are exposed to olfactory ligands, is achieved via placement of the antennae into an electrolyte solution and insertion of grounded (reference) and recording electrodes into the antennal tissue. This can be performed on whole invertebrates or isolated heads/antennae (see Fig. 3c). There have been a range of attempts to use isolated insect fragments for biosensing purposes and these, including EAG and some of its derivatives, were reviewed by Sevonkaev and Katz (2008).

Our understanding of vertebrate olfaction is relatively advanced compared to that of invertebrates, particularly with respect to detailed characterisation of individual ORs and the transduction of OR:ligand binding into cellular metabolic changes. Because invertebrates utilise GPCRs for many metabolic functions (including neural functions) and these proteins are often homologous to those in mammals (e.g. biogenic amine receptors as discussed in a review by Brody and Cravchik, 2000), it was expected that the discovery of the vertebrate OR family would quickly lead researchers to putative homologous invertebrate OR genes. However, the relative lack of homology between the ORs of insects and the vertebrates meant that insect ORs were not isolated for almost a decade (Touhara and Vosshall, 2009). Indeed, it took the use of unbiased molecular and bioinformatics approaches to isolate the putative invertebrate OR gene-family from the insect genetic model, the vinegar fly *Drosophila melanogaster* (vinegar fly); these studies were published in 1999 (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). However, once the *Drosophila* OR gene family was identified, the putative OR gene repertoire from each invertebrate genome available, followed relatively soon after i.e. 62 OR genes in vinegar fly *D. melanogaster* (Robertson et al., 2003), 66 in the silkworm moth *Bombyx mori*

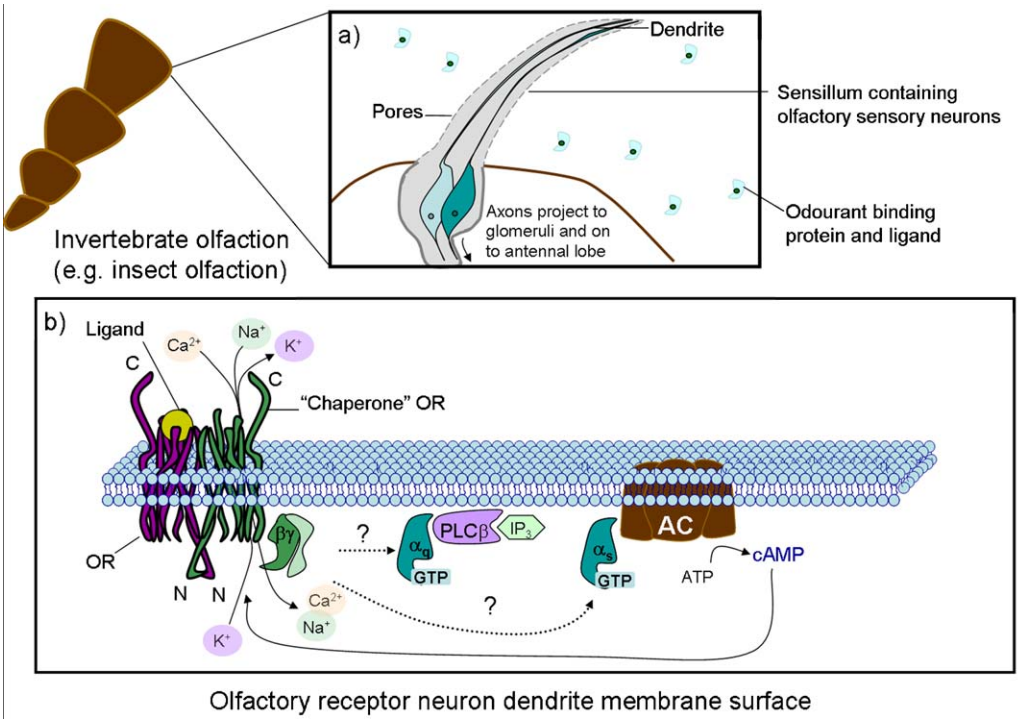

Fig. 2. Insect olfaction through seven-transmembrane olfactory receptors – implicated mechanisms (reviewed by Ha and Smith, 2009; Hansson, 2002; Krieger and Breer, 1999; Nakagawa and Vosshall, 2009; Song et al., 2008; Spehr and Munger, 2009). (a) In insects, OSN dendrites are encapsulated in sensilla within sensory hairs. Odourant molecules (bound to odourant binding proteins) can access the dendritic surfaces through pores in sensilla. OSN axons in the insect tissue antennae converge through glomeruli and transduce the neural signal to the antennal lobe. (b) ORs in the OSN cell membranes are thought to contain an extracellular C-terminal region, intracellular N-terminal region and 7-transmembrane domains. Invertebrate ORs have been shown to signal with the aid of a "chaperone" receptor which also functions to regulate the level of OR in the membrane. G-protein independent ion-channel activity of the "chaperone" protein (or the OR:chaperone complex), and G-protein mediated increases in intracellular cAMP, have both been reported, but the roles and biological significance of these pathways in olfaction is still unresolved (represented by question mark in the schematic). IP_3 pathways in invertebrates are primarily established in the lobster although have been suggested for insects as well (also represented by a question mark due to uncertainty of this pathway's role in insects). Note that some pathways (and specific G-proteins) involved in nematode olfaction are omitted for simplicity. *Abbreviations*: OSN, olfactory sensory neuron; OR, olfactory receptor; cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; AC, adenylyl cyclase III; PLCβ, phospholipase c beta; IP_3, inositol triphosphate; iCa^{2+}, intracellular calcium.

Table 1
List of transduction technologies utilised for olfactory receptor deorphanisation and olfactory biosensing, including the class of the effect being measured, the various techniques used to produce the measurement, and associated literature.

Class	Techniques	Measures	References
Optical	Surface plasmon resonance (SPR) Fluorescence (including FRET) Luminescence Bioluminescence (including BRET) Chemiluminescence Absorbance	Light	Anker et al. (2008), Borisov and Wolfbeis (2008), de Kloe et al. (2010), Dodeigne et al. (2000), Hoa et al. (2007), Homola (2003), Milligan (2004), Roda et al. (2004), Santafe et al. (2010), and Sun et al. (2004)
Resonant	Piezoelectric effect Bulk acoustic wave (BAW) resonator (e.g. QCM) Surface acoustic wave (SAW) resonator Cantilever-based sensors	Mass	Cooper and Singleton (2007), Janshoff et al. (2000), Muramatsu et al. (2002), Lange et al. (2008), and Ferreira et al. (2009)
Electrochemical	Conductometric/impedance EIS Amperometric Potentiometric EOG/EAG Voltage/current/patch clamps Microelectrode array Field-effect transistors (FETs) Light-addressable Potentiometric sensor (LAPS)	Electrical conductance/ resistance Current Ion/pH	Dzyadevych et al. (2003), Ghindilis et al. (1998), Grieshaber et al. (2008), Hianik and Wang (2009), Lisdat and Schafer (2008), Mehrvar and Abdi (2004), Pohanka and Skladai (2008), Sadik et al. (2009), Schoning and Poghossian (2006), Shah and Wilkins (2003), Stein et al. (2004), and Thevenot et al. (2001)

(Touhara and Vosshall, 2009), 79 in the malaria mosquito *Anopheles gambiae* (Hill et al., 2002), 131 in the dengue mosquito *Aedes aegypti* (Kent et al., 2008), 170 in the honeybee *Apis mellifera* (Robertson and Wanner, 2006), 301 in the Jewel wasp *Nasonia vitripennis* (Robertson et al., 2010), 341 in the Red flour beetle *Tribolium castaneum* (Engsontia et al., 2008) and 41 in the nematode *Caenorhabditis elegans* (Troemel et al., 1995). Given that few of these genes have yet to be proven to be functional ORs *in vivo* (see Table 3), these figures should be treated with caution; the level of pseudogeneity is thought to be high and quite variable (Touhara and Vosshall, 2009). It is not clear why two closely related mosquito species with similar biology (*Anopheles gambiae* and

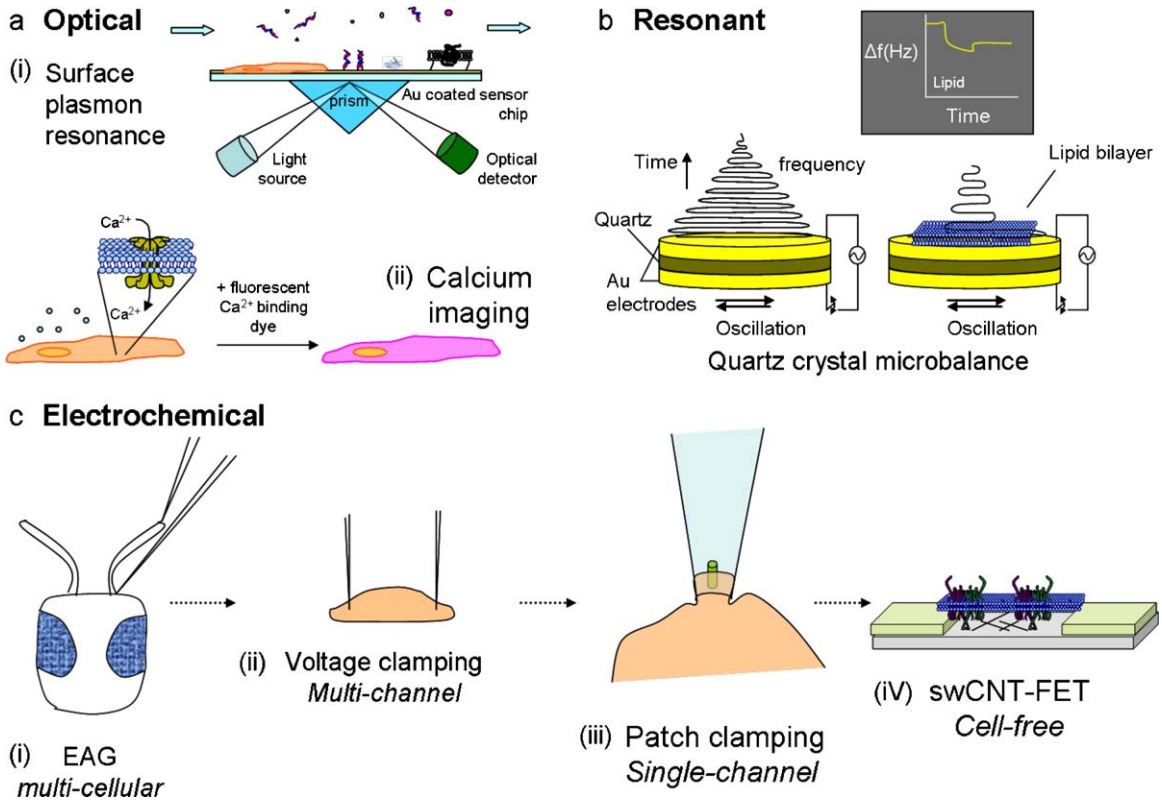

Fig. 3. Schematic examples of transducer technologies used in olfactory biosensor research. (a) Optical transduction – (i) SPR – biomolecules such as cells, DNA and proteins can be immobilised on a sensor surface. Analyte flows over biomolecules and subsequent interactions can be monitored. Measurements of shifts in the critical angle of incidence (at which surface plasmons are generated) caused by interactions on the sensor surface, are used to detect an interaction (for relevant literature see Table 1); (ii) calcium imaging – intracellular calcium dyes can monitor the influx of calcium into a cell caused be odourant-induced ion-channel activation, (b) resonant transduction–QCM schematic showing alternating current applied to gold (Au) electrodes attached to a quartz crystal. The piezoelectric properties of the crystal cause oscillation of the sensor, the frequency of which is reduced with additional mass (e.g. an adsorbed lipid bilayer shown here) on the crystal surface. Inset plot – change in frequency (Δf) versus time. Arrow indicates addition of lipid to the QCM surface resulting in a change in resonant frequency. The frequency curve plateaus after unbound lipid is washed away. (c) Electrochemical – potentiometric techniques such as (i) EAG, whereby electrodes contact insect antennae; (ii) voltage clamping whereby electrodes are positioned within a cell to measure ion-currents across a membrane at a controlled voltage; (iii) patch clamping which uses a micropipette attached to the cell membrane to allow recording from a single ion-channel and (iv) single walled carbon nanotubes are used as semiconductors in field effect transistor devices to monitor ligand-activated ORs in a membrane preparation, removing the need for whole cells (image modified from Kim et al., 2009). *Abbreviations*: OR, olfactory receptor; SPR, surface plasmon resonance; QCM, quartz crystal microbalance; EAG, electroantennogram; swCNT-FET, single-walled carbon nanotube-field effect transistor; Δf, change in frequency.

Aedes aegypti) should have such a large difference in the number of expressed ORs. ORs have also been isolated/deorphaned from other (non-sequenced) pest insects such as *Epiphyus postvittana* (Light brown apple moth) (Jordan et al., 2009), *Culex pipiens* (southern house mosquito) (Pelletier et al., 2010), *Diaphania indica* (cucumber moth), *Mythimna separata* (northern armyworm), *Plutella xylostella* (diamondback moth) (Mitsuno et al., 2008) and *Ostrinia* spp. (Miura et al., 2009, 2010) (see Table 3).

The olfactory system of the nematode worm *C. elegans* (another class of invertebrate), is quite well understood and differs from other invertebrates in a number of ways (see reviews by Bergamasco and Bazzicalupo, 2006; Troemel, 1999). Unlike mammals and insects, the nematode expresses multiple OR genes in a single OSN (Ache and Young, 2005); the only deorphaned OR being the diacetyl receptor ODR-10 (Sengupta et al., 1996). ODR-10 deorphaning was performed using chemotaxis assays combined with use of mutant nematode lines and expression analysis. Since then, sequencing of the *C. elegans* genome (*C. elegans* Sequencing Consortium, 1998) has unveiled hundreds of putative ORs. The transduction pathways implicated in *C. elegans* signalling, similarly to other olfaction systems, works to activate ion-channels which results in changes in membrane potential of the cells. However, the mechanisms by which these ion-channels are activated differ somewhat to other invertebrates. G-protein signalling is implicated in nematode olfaction, using $G\alpha_i$-like proteins linked specifically to chemoreception including the GPA and ODR proteins (O'Halloran et al., 2006). In addition, a protein thought to regulate of G-protein signalling (RGWS-3) is thought to be involved in regulation of *C. elegans* odour responses (Ferkey et al., 2007). The nematode chemosensory system also involves receptor guanylate cyclases and cGMP (as opposed to cAMP)-gated ion channels and cGMP-dependent protein kinase (EGL-4) (L'Etoile et al., 2002). A lipid signalling pathway has also been implicated to activate the transient receptor potential vanilloid-related (TRPV) channel in some chemosensory events (for further discussion see Bergamasco and Bazzicalupo, 2006).

The lack of sequence homology between the vertebrate and the insect ORs suggests that their OR gene families are independently evolved (Wistrand et al., 2006), and this is reflected in several key differences in the structure and function of the respective proteins. A major difference was discovered during research into *Drosophila* ORs, that being the presence of a "chaperone" receptor, named Or83b. This receptor, in contrast to other ORs, is expressed in all OSNs, and apparently does not bind volatile ligands but instead forms dimers with all other ORs (see Fig. 2b) to provide the functional ligand-detecting receptor complex (Benton et al., 2006; Larsson et al., 2004; Neuhaus et al., 2005). In 2005, Neuhaus et al. used *Drosophila* ORs to investigated the role of the co-receptor Or83b by expressing Or43a and Or22a in HEK cells, either alone or co-expressed with Or83b. By co-expression with Or83b, the

sensitivity of Or43a to cyclohexanone was increased from millimolar to micromolar and the percentage of responsive cells was increased from <1% to 10–15%. The same phenomena occurred with the response of recombinant cells expressing the Or22a constructs, for the ligand ethyl butyrate. Bioluminescence resonance energy transfer (BRET) was utilised to show that Or83b formed heterodimers with the co-expressed receptors.

Or83b is also the only receptor for which clear orthologues exist in other species from different insect orders (Krieger et al., 2003); sequence homology has facilitated its isolation from various species (Jones et al., 2005; Malpel et al., 2008; Miura et al., 2010) and it has been co-expressed with other ORs in recent studies investigating invertebrate olfaction mechanisms (Mitsuno et al., 2008; Miura et al., 2009, 2010; Nakagawa et al., 2005; Neuhaus et al., 2005; Pelletier et al., 2010; Sakuri et al., 2004; Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008). These studies have produced some intriguing results which appear to highlight other key differences with vertebrate ORs (compare Figs. 1b and 2b). Firstly, the membrane topology of the "chaperone" Or83b and the odourant-binding Or22a receptors, have been investigated using bioinformatics, enzyme mediated colorimetry and fluorescent tags (Lundin et al., 2007; Smart et al., 2008; Wistrand et al., 2006). These studies suggest that insect ORs display an inverted membrane topology compared to vertebrate GPCRs and non-OR invertebrate GPCRs, in that the C-terminus is extracellular and N-terminus is cytoplasmic (see Fig. 2b).

In 2008 a series of reports investigating invertebrate olfactory signalling were published, with apparently conflicting data, and this raised new questions about how invertebrate OR signalling is mediated (Chesler and Firestein, 2008). Sato et al. (2008), investigated transduction of three insect OR:chaperone complexes: *Drosophila* Or47a:Or83b, *Bombyx* BmOr1:BmOr2 and *Anopheles* AgOR2:AgOR7 complexes (see Table 3). The research used a combination of patch clamp experiments to generate electrical transduction of binding of the relevant ligands, pentyl acetate, 2-methyl phenol and bombykol, respectively. These experiments appeared to show that the OR:chaperone complexes could act as ligand-gated ion channels, independently of G-protein signalling, and raised questions as to whether invertebrate ORs do indeed couple to G-proteins as expected, and if G-proteins play any role in olfactory signalling. G-protein independent signalling was also reported for *Drosophila* Or43a expressed in *Sf*9 insect cells in that G-protein inhibitors had no effect on ligand-induced calcium influx (Smart et al., 2008). Wicher et al. (2008) also investigated transduction of insect OR activation by expressing *Drosophila* Or22a:Or83b complex in mammalian HEK-293 cells, and using patch clamping to generate an electrical signal due to ethyl butyrate application. Their data suggested that the chaperone Or83b could act alone as an ion channel but that the OR complex did indeed signal through a G-protein mediated pathway (Wicher et al., 2008).

In addition to a range of older observational studies implicating G-protein associated second messengers (Breer, 2003), further recent evidence implicates G-proteins in invertebrate OR signalling. For example, *Drosophila* mutants targeting the $G\alpha_q$ pathway (associated with phosphoinositide signalling) had significantly reduced EAG responses to multiple odourants, with $G\alpha_q$-knockouts being rescued by expression of a dominant-active $G\alpha_q$ (Kain et al., 2008). However, this was contradicted by Yao and Carlson (2010) who used a range of techniques to target different $G\alpha$ proteins *in vivo* and reported that none affected odour sensitivity. Interestingly, they did find that $G\alpha_q$ specifically, was important for sensitivity of *Drosophila* CO_2 receptors (from the gustatory receptor family, Gr) expressed in olfactory neurons. A detailed expression analysis of 6 $G\alpha$ genes (as well as several $G\beta$ and $G\gamma$ genes) was inconclusive but showed that $G\alpha_s$ and $G\alpha_i$ (involved with stimulating and inhibiting cAMP transduction, respectively) co-localised at the base of olfactory sensilla (Boto et al., 2010). $G\alpha_q$-related signalling appears to be important in lobster olfactory neurons (Corey et al., 2010), however, it is not clear how this relates to insect ORs as lobsters live entirely in an aqueous environment and clearly do not need to sample undissolved volatiles. As previously mentioned, olfaction in *C. elegans* has been shown to be mediated through G-protein pathways using G-protein subtypes specific to the OSNs of the nematode (O'Halloran et al., 2006). Currently, the role of G-protein signalling in insect olfaction remains in question with various groups working on different investigative approaches. The lack of a clear resolution to this question is problematic in terms of utilising traditional G-protein-based methods of generating olfactory signal transduction in biosensors (see Section 1.3), such as cAMP assays. In an editorial in 2010, Wicher proposed that invertebrate ORs may signal through G-proteins at low ligand concentration (similarly to mammalian ORs), activating their dimeric OR complexes (which act as ion channels; these molecules are separated in vertebrates) and that this ion channel activity may also be driven "directly" by high ligand concentrations (independent of G-proteins). For more detail, there are several recent discussions that summarise the data and questions surrounding signal transduction in invertebrate olfaction, and the comparison with vertebrate olfaction (Ha and Smith, 2009; Kaupp, 2010; Nakagawa and Vosshall, 2009; Silberling and Benton, 2010; Su et al., 2009; Wicher, 2010). Fig. 2b summarises the OR signalling mechanisms that have been associated with insect olfaction.

Another peculiarity of insect olfaction is the highly developed use of semiochemicals, including pheromones, for various aspects of biology including food-, host- and mate-finding (Howard and Blomquist, 2005; Wyatt, 2010). For this reason, insect pheromone receptors will likely be highly utilised for biosensing applications involved with pest management and food quality. The sex pheromone system is most highly characterised in moths, for which hundreds of pheromones have been identified, but is known to be utilised by many other insects (see database at www.pherobase.com). Male moths often show marked sexual dimorphism in their antennal structure; generally males have significantly greater surface area and sensitively detect female-produced volatiles. It also appears that the expression pattern for some pheromone receptors may be sexually biased, and this has been utilised in expression studies to detect putative pheromone receptors (Grosse-Wild et al., 2010; Mitsuno et al., 2008; Sakuri et al., 2004; Wanner et al., 2007a,b). Additionally, several studies have determined that pheromone receptors may also reside in sex-specific olfactory tissue and drive sex-specific neuronal circuits (Datta et al., 2008; Kanzaki et al., 2003). These circuits could be thought of as analogous to the vomeronasal (pheromone reception) organ of vertebrates (mentioned briefly in Section 1.2.1). It has been shown in *Drosophila*, that the sex pheromone 11-cis-vaccenyl acetate (cVA) regulates differential mating behaviours in male and female flies, which both express the receptor in olfactory organs (Kurtovic et al., 2007). This was mediated through a specific class of neuron, in which the cVA receptor was expressed, and supported a long held belief that pheromone detecting neurons are fundamentally different to those detecting other odourants (Hildebrand and Shepherd, 1997). By replacing the cVA receptor (Or67d) with BmOR1 (bombykol receptor of silkworm moth) in adult *Drosophila*, similar behaviours were elicited when flies were exposed to the moth pheromone (Kurtovic et al., 2007). This suggests that specific neuronal wiring regulates behavioural aspects, rather than an inherent property of a given ligand. This has also been shown in *C. elegans* where then same receptor may produce attraction or repulsion depending on which neuron it is expressed in (Bargmann, 1998; Bargmann et al., 1993; Milani et al.,

2002; Wes and Bargmann, 2001). There is emerging evidence that different sub-classes of chemosensory receptor, which may be linked to specific signalling pathways, are responsible for detection of specific odourant types such as food volatiles, 'general' volatiles and pheromones (Silberling and Benton, 2010). Biosensing approaches have been utilised to determine the ORs that bind to known pheromones from *B. mori* (Sakuri et al., 2004), *Drosophila* (Ha and Smith, 2006; Kurtovic et al., 2007) and *A. mellifera* (Wanner et al., 2007b); these methods are discussed in Section 2.3 (see also Table 3).

Although the accessory proteins (non-ORs) involved in invertebrate olfaction (see review by Vogt, 2005) are not widely utilised for biosensing, they may become important for improving the signal-to-noise ratio, providing feedback on metabolic consequences of an interaction or to produce biosensors that behave more like *in vivo* olfactory systems. As discussed for vertebrates (above), OBPs have also been characterised in insects, in which they are highly conserved (Vogt et al., 1999; Vosshall, 2000). They were first discovered as small, secreted molecules that were present in fluid that bathed pheromone-sensitive OSNs (see Fig. 2a) and originally termed "pheromone binding proteins" (Vogt and Riddiford, 1981). Since that time, a range of OBPs have been discovered and placed into three broad classes; pheromone-binding proteins, general odourant-binding proteins classes 1 and 2 (Wang et al., 2003). *Drosophila* is known to express at least 35 OBPs (Vosshall and Stensmyr, 2005), with the most highly characterised being OBP76a (known as LUSH) (Laughlin et al., 2008). It appears that *in vivo*, LUSH is required for neuronal sensitivity to the sex pheromone, cVA, and appeared to regulate neuronal responses at high ligand concentrations (Xu et al., 2005).

Another group of accessory olfaction proteins are the antennal sensory neuron membrane proteins (SNMPs), which are similar to CD36 proteins from mammals, first discovered in moth antennae (Rogers et al., 1997) and later found in the genomes of *Drosophila* and *C. elegans* (Rogers et al., 2001). In *Drosophila*, SNMP-1 is expressed in trichoid sensilla and colocalises with cVA receptor within dendrites of T1 neurons (the only neurons that express cVA receptor) (Ha and Smith, 2006; Kurtovic et al., 2007). Interestingly, a modified LUSH OBP was found to stimulate T1 neurons through Or67a:SNMP, without cVA being present, apparently by mimicking the cVA-bound LUSH conformation; SNMP presence had a small effect in enhancing sensitivity (Laughlin et al., 2008). These findings suggest that *in vivo*, OBP conformation may be altered by odourant-binding and may play a role in binding to OR ligand pockets. A further type of accessory invertebrate olfactory proteins is pheromone-degrading enzymes, also discovered in moth antennae (in males of *Antheraea polyphemus* (Vogt and Riddiford, 1981)). This first example was shown to be an esterase that could degrade the pheromone (6E,11Z)-hexadecadienyl acetate; it was proposed that these pheromone-degrading enzymes act to improve signal-to-noise ratio by modulating the OR response to pheromone build-up within olfactory tissue (Vogt et al., 1985). Since then, several other sex-independent pheromone-degrading enzymes have been isolated from moths, including aldehyde oxidases from *A. polyphemus*, *B. mori* and *Manduca sexta* (Rybczynski et al., 1989, 1990). The next decade of research is likely to significantly improve our understanding of invertebrate olfactory signalling, and consequently drive production of invertebrate OR biosensors.

The clear differences between the vertebrate and the insect receptors have direct implications for the development of OR-based biosensors as they may require different approaches to transduction of ligand-binding events. The invertebrate species chosen for genome sequencing reflects the application-driven nature of invertebrate OR research (they are generally agricultural or medical pests; one is a biocontrol agent of a pest) as opposed to the more "pure", olfaction biology-driven nature of vertebrate research that generally uses organisms that are models of human biology.

1.3. Detecting and interpreting the signal: biosensor transducers

As described above, olfactory processing is mediated by a series of complex protein–protein interactions and their associated metabolic pathways. It not only requires the recognition components (i.e. the receptor and associated machinery), but also requires coordinated connections of neuronal axons towards the olfactory cortex. This complex translation from a molecular binding event to a perceived odour is the pathway analogous to the transducer system of a biosensor although a biosensor may require only part of this transduction pathway for purposes of detecting a ligand binding event. As previously mentioned, tissue-level electrical measurements have been extensively used in olfaction research (e.g. EAG and EOG; for further reading on these techniques, see Scott and Scott-Johnson, 2002; Sevonkaev and Katz, 2008), but here we describe some more recent developments in transduction research, perhaps more amenable to a commercial biosensor platform. A recognition signal can be measured as a change in weight, light, sound, heat, chemical composition or electrochemical signal. These measurements can be made using a variety of techniques (Table 1) including SPR, quartz crystal microbalance (QCM) and field-effect transistors (FET). In a biosensor device, the signal produced using these techniques requires conversion to a "readable" form to enable interpretation. Below we provide some of the key examples of transducer systems utilised in recent studies describing olfactory biosensor research.

From techniques such as EOG on the whole animal and tissue, to using methods such as voltage clamping on single cells or patch clamping to monitor single channels (Sakmann and Neher, 1984), the electrophysiological approach to these techniques forms the basis for a range of biosensor transduction mechanisms (see Table 1 and Fig. 3c). Researchers have used EOG, and particularly EAG, to act as a sensor transducer component, e.g. Ziesmann et al. (2000) used EAG on a female *B. mori* antenna to assess odourous contaminants in a laboratory. However, due to the technical expertise required for some of these techniques, in addition to the desire for miniaturization, stability and portability in a biosensor device, transducer systems utilizing planar microelectrodes (microelectrode arrays) to monitor electrophysiological changes in the active cells are becoming more popular (e.g. see Liu et al., 2010a). Below is a brief discussion regarding some of the techniques used to detect biological events for olfactory biosensing applications.

1.3.1. Optical transduction

Measurement of fluorescence, bio- and chemi-luminescence, and absorbance are some of the techniques used in standard optical assays which have been developed for monitoring cellular activation events such as olfaction. There are molecules that become fluorescent under certain conditions such as the presence of specific metal ions, e.g. calcium (Roe et al., 1990), a common indicator of cellular activity (see Fig. 3aii). Fluorescent methods also include Förster Resonant Energy Transfer (FRET) reporting, which utilises specific changes in conformation or interaction of fluorescently labelled molecules as a result of a biological process, to produce an increase or decrease in fluorescence (Jares-Erijman and Jovin, 2003; Ko and Park, 2007). Such interactions can be measured within, or independently of, a cell. Other methods include bioluminescent proteins or enzymes (commonly luciferase) that convert a substrate to a bioluminescent form produced as a reporter for cellular activity (Fan and Wood, 2007). These methods (more of which were reviewed by Lalonde et al., 2008) are

attractive due to low limits of detection. These methods do however require a suitable light source and detection equipment for transduction and readout (which is often expensive). Some also require the labelling of biological elements within the system, which can add to the preparatory steps involved in sensing and can potentially lead to associated problems with changes in native function of interacting components.

Label-free optical techniques such as surface plasmon resonance (SPR) have become popular transducer technologies for studying biomolecular interactions (Homola et al., 1999) (see Fig. 3ai). SPR relies on changes in light produced by chemical or physical interactions at the sensor surface, as a result of alterations in refractive index at the sensor surface. Surface plasmons are electromagnetic waves that occur in close proximity to, and propagate in a parallel direction to, the sensor surface. An incident light source is directed at a thin film of an inert metal (usually gold), which results in the generation of surface plasmons at a critical incident angle for a given surface composition. At this critical angle, total internal reflection of the incident light no longer occurs, the incident light energy is instead converted to surface plasmons and there is a resultant reduction in the measured reflected light intensity. The angle of incidence at which the reduction occurs is dependent on the refractive index of the medium next to the metal film. Therefore, a shift in the critical angle of incidence (as measured by reflected light intensity), indicates a change in the composition of the sensor surface (e.g. biomolecules such as protein or DNA may have become attached to the surface). The SPR technique is an established and reliable method which is label-free, meaning the bio-recognition components do not have to be altered to contain a fluorescent probe to enable monitoring. However, SPR, as yet, does not provide a cheap, portable transducer solution although efforts are being made in this area (Kurita et al., 2006; Vala et al., 2010; Naimushin et al., 2003). In addition, it often requires complex control measurements to be performed and pre-fabrication of a defined surface monolayer (often termed a self-assembled monolayer or SAM) on the inert sensor chip, for attachment of biological entities. Indeed, the development of suitable SAMs is a key part of modern biosensor research, both for SPR and other transduction approaches (Benilova et al., 2008; Gomila et al., 2006; Hou et al., 2007; Karlsson and Lofas, 2002; Lee et al., 2006; Lee et al., 2009b; Liu et al., 2006; Marrakchi et al., 2007; Rodriguez Segui et al., 2006; Sung et al., 2006; Vidic et al., 2006a,b, 2007). SPR is also often used to confirm the SAM construction and initial attachment of biological components (Barton et al., 2007; Karlsson and Lofas, 2002; Lee et al., 2006, 2009b; Rodriguez Segui et al., 2006; Santafe et al., 2010; Vidic et al., 2006a, 2007), as well as transducing ligand-binding events.

Ligands in the field of olfaction generally refer to small volatile chemical compounds with a molecular weight of less than 300 g mol^{-1} (many of which can be found in the database created by Dunkel et al., 2009). Instruments using the SPR technique, such as the Biacore (GE Healthcare), have been limited with regard to detection of very low molecular weight compounds, one reason being that the ligands were too small to cause measurable changes in refractive index on the substrate chip surface, given previous sensitivity limits. However, SPR instrumentation is becoming increasingly sensitive to smaller surface changes (as discussed in Karlsson, 2004). Resonance changes measured using current Biacore models, such as the T100, have been used to monitor compounds with molecular weights between 157 and 341 g mol^{-1} (Papalia et al., 2006), and fragments of drug compounds down to 100 g mol^{-1} (de Kloe et al., 2010). However, when monitoring interactions of such small molecules using SPR, factors such as temperature have been shown to contribute to inaccuracies in the measurement (Moreira et al., 2008; O'Brien et al., 1999; Xiao et al., 2010). It is factors such as these which need to be overcome in the development of a truly portable biosensor device using SPR for low molecular weight molecules such as odourants.

1.3.2. Resonant transduction

Resonant sensors based on acoustic waves (Table 1) such as the bulk acoustic wave (BAW) and surface acoustic wave (SAW) resonators, are techniques that are sensitive to mass and viscosity changes, making them a useful tool to study biomolecular interactions (for detailed reviews, see Benes et al., 1998; Lange et al., 2008; Marx, 2003). The propagating acoustic wave is generated by an applied electric field over a substrate with piezoelectric properties, such as the quartz crystal. A wave that propagates through the substrate is called a bulk wave. If the wave propagates along the surface of the substrate, it is known as a surface wave. As the acoustic wave propagates through or along the surface of the material, any changes that occur to the characteristics of its path (e.g. adsorbed/bound materials on the substrate) affect the frequency and/or amplitude of the wave, which can be monitored.

The measurement of mass changes using QCM, a bulk acoustic wave sensor, involves inducing a resonance in a quartz crystal by the application of an alternating electric field. This crystal oscillates at a tuned frequency, which changes in accordance with the mass on the crystal. If a binding/dissolution event occurs on the crystal, increasing/decreasing its mass, the frequency of oscillation alters and this change can be measured. QCM devices are now also capable of measuring dissipation (dampening) values which are measurements of the oscillation decay every time the driving electric field is removed from the crystal. This can aid in determining the type or perhaps shape of attached material as the dampening of crystal movement occurs more rapidly when a softer or more viscous layer is present on the surface (see Fig. 3b). As the viscosity of the surface increases (e.g. with the adsorption of a lipid bilayer), the dissipation value increases, and *vice versa*.

Cantilevers, which are most commonly known for their application in atomic force microscopy, also provide a platform for biosensor applications (Lavrik et al., 2004). Similarly to the acoustic wave devices, cantilevers rely on the precise changes in piezoelectric crystals. Two different modes of measurement exist with cantilevers, the deflection of the cantilever caused by mechanical stresses such as adsorption-induced surface deformation, and changes in resonant frequency of the cantilever. The resonance frequency of a microcantilever shifts due to mass changes on the cantilever surface, as is the case with acoustic wave sensing.

1.3.3. Electrochemical transduction

Due to their specific biocatalytic activities which are regulated through electron transfer, enzymes involved in redox reactions are the bio-recognition elements to which electrochemical transduction is most often applied e.g. glucose oxidase in a glucose sensor (Wilson and Turner, 1992). In general, electrochemical transducers either measure a current (amperometric), a potential or charge accumulation (potentiometric), or the conductive properties of a medium between electrodes (conductometric) (Grieshaber et al., 2008; Mehrvar and Abdi, 2004, #172; Thevenot et al., 2001, #228). An example of a conductometric system is electrochemical impedance spectroscopy (EIS), which is a tool that measures the electrical resistance of a system and changes that occur in this resistance due to alterations at a transducer surface (Lisdat and Schafer, 2008). Commonly used within the field of olfaction due to the involvement of electrogenic cells (neurons), potentiometric studies including EOG, EAG, voltage clamping, patch clamping, and micro-electrode arrays, are used to monitor the activity of ion channels and the subsequent changes in membrane potential (see Fig. 3c).

Another example of a device using potentiometric measurements, and which have become of interest to research in the field of olfactory-based biosensing, are field effect transistors (FETs) (Kimura and Kuriyama, 1990). These transistors can measure ion concentrations and hence, are also known as ion-sensitive field-effect transistors. Used in biological applications such as biosensing, bio-field-effect devices have been utilised for measuring pH or ion-concentration change, adsorption of charged macromolecules, and potential changes coming from living biological systems (Schoning and Poghossian, 2006). Electrogenic cells, such as neuronal cells and muscle cells that upon activation, undergo a change in potential relative to the extracellular environment, can be monitored through modulations in the source-drain current of the FET sensor. Techniques such as these could also be used to monitor ion transfer across a membrane in the absence of the cell.

Light addressable potentiometric sensor (LAPS) technology belongs to the same family as field-effect devices, and measures photocurrent generated when a site-directed light source is applied (Owicki et al., 1994). The photocurrent is related to the composition of the analyte in the local area of the light beam, so changes in extracellular potential due to cell activity can generate corresponding fluctuations in the photocurrent signal that can be measured. A focused laser source allows for the interrogation of individual cells with LAPS (Stein et al., 2004).

Recently, research has concentrated on the use of nano-components in these FET devices with single-walled nanotubes being one such element applied in the field of olfactory biosensing (see Figs. 3c and 4a). These components are attractive for use in these devices due to their size and electrical properties. For more information on carbon nanotubes and their applications in electrochemical sensing see reviews by Rivas et al. (2007) and Hu et al. (2010).

2. Olfactory biosensors

2.1. Deorphaning the vertebrate ORs of interest

This section describes techniques that have been used to study olfaction and define particular receptor-ligand pairs (deorphan). Research into deorphaning ORs has involved assays utilising either *in vivo* expression such as whole tissue (e.g. olfactory epithelium; Liu et al., 2010b) or dissociated OSNs, or *in vitro* expression using recombinant heterologous cells or cultured cell-lines derived from olfactory epithelium. Here we will focus on sub-tissue level recognition elements and subsequently, much of the research we describe utilises cell-based assays (see summary in Table 2). While not often focused on biosensor production *per se*, deorphaning assays generally utilise the same techniques applicable to the production of a biosensor targeted to a defined application. It should be noted that several radiometric assays have been developed for olfaction studies and OR deorphanisation (Pevsner et al., 1985; Raming et al., 1993; Shirokova et al., 2005), however, we do not discuss these methods as they are not likely to see widespread use (especially for field applications) due to health and safety issues.

As mentioned, cell-based assays involve the use of ORs expressed in either their native OSNs (which can be isolated and tested) or expressed in heterologous systems. Due to ethical issues and other difficulties associated with use of human olfactory epithelium and/or OSNs, much of the *in vivo* expression has been performed on rat and mouse receptors (also bullfrog ORs (Wu, 1999)); virus-mediated expression is often used to express a given OR along with a fluorescent reporter, usually green fluorescent protein (GFP), which allows recombinant OSNs to be targeted for measurement (Boschat et al., 2002; Bozza et al., 2002; Corcelli et al., 2010; Touhara et al., 1999; Zhao et al., 1998). Indeed, the first OR deorphaned was rat ORI7; recombinant isolated OSNs were used and were obtained by infecting rats with adenovirus expressing ORI7 and GFP (Zhao et al., 1998). GFP co-expression was used to monitor the upregulated OR expression by recombinant adenovirus infection of rat olfactory epithelium (Belluscio et al., 2002; Zhao et al., 1998). Subsequently, this technique was also used to select OSNs for EOG recordings and calcium imaging, to investigate ligand specificity of ORI7 (Araneda et al., 2000).

In terms of heterologous expression, functional ORs have been produced in a range of eukaryotic cells types including yeast (*Saccharomyces cerevisiae*), amphibian (*Xenopus laevis* oocytes and melanophores), insect (*Sf*9 ovary-derived cells) and mammalian (including HeLa, HEK-293, PC12 and CHO cells); see Table 2. For vertebrate receptors, HEK-293 and *S. cerevisiae* are the most widely utilised cell lines. Transduction pathways in olfaction involve cellular responses such as calcium influx through nucleotide-gated channels (for review see Zufall *et al.* (1994)), which provide measurable changes within recombinant cells, that indicate ligand binding and subsequent OR-mediated signalling. This has been exploited extensively in the field of olfaction research to determine odour-receptor pairs, but could also be adaptable to a sensor platform.

Fluorescent dyes such as Fura-2 and Fluo-4 are the most commonly used reporter of OR activation; they are used to monitor calcium levels (calcium imaging) within a cell, have been used in experiments on most ORs and have been instrumental for OR deorphaning (for references see Table 2). Calcium imaging has been used on individual cells and also on cell populations, such as for fluorescent imaging plate reader (FLIPR) assays. Krautwurst et al. (1998) used a library-based approach to deorphan three mouse receptors, I-C6, I-D3 and I-G7 by coexpressing them with $G\alpha_{15}$ and $G\alpha_{16}$ in HEK-293 cells. Chimeric receptors were constructed to contain "generic" flanking sequences into which only the transmembrane regions tested ORs were inserted. These flanking regions consisted of the 5′ untranslated region and first 19 amino acids of rhodopsin (commonly called a Rho-tag), and the 3′ region of the mouse olfactory receptor M4. Specific responses were recorded for I-C6, I-D3 and I-G7 with (−) citronelal, carvone and limonene, respectively (Krautwurst et al., 1998). Using a similar approach, mouse mOR912–93, expressed as chimeras containing only the transmembrane regions III-VII of the tested receptor, was deorphaned using mammalian expression in HEK-293 (and modified HEK-293 (pEAKrapid)) cells (Gaillard et al., 2002). Again, the ORs were co-expression with $G\alpha_{15}$ and a $G\alpha_{q_0}G_{\gamma}$ to provide appropriate signal transduction. Fura-2 measurement of calcium influx to 2-heptanone (1 μM and 10 nM), 2-butanone (1 μM only) and 2-decanone (1 μM and 10 nM) was reported.

Shirokova et al. (2005) utilised this established library of chimeric mouse ORs (Krautwurst et al., 1998) to deorphan two new mouse ORs. They utilised the previously characterised chimeric receptor Olfr43 (then known as IC-6 (Krautwurst et al., 1998)) as a positive control for an approach utilising recombinant HeLa/Olf cells and a Fluo-4 based FLIPR assay. This work showed that Olfr49, and MOR267-1 both detected (−)citronellal with Olfr49 being the more sensitive (EC_{50} of 2.1 μM) (Shirokova et al., 2005). Fluorescent (Fura-2) reporting was also used in combination with COS-7 cells for rat ORI7 expression, and HEK-293 cells for expression OR17-40 (human) (Wetzel et al., 1999) and mOR-EG (mouse) (Oka et al., 2004; Kajiya et al., 2001, #322; Katada et al., 2003, #317), which elicited responses to helional and eugenol, respectively. Oka et al. also detected antagonists of the mOR-EG receptor by monitoring the reduction in normal calcium response in the presence of certain compounds. Two antagonists were found; methyl isoeugenol (MIEG) was the stronger antagonist with weaker antagonism from isosafrole (ISF) Sanz et al. (2005) also

Table 2
Summary of the various sub-tissue level approaches to utilising vertebrate olfactory receptors as a biological sensing element, either for receptor deorphanisation or development of an olfactory biosensor (bioelectronic nose).

Species	Receptor(s)	Expression system	Volatile giving significant response	Transduction system	Literature
Bullfrog	Multiple unknown	*In vivo;* partially purified extracts from sensory tissue	n-Capronic acid, β-ionone, n-octyl alcohol, n-decyl alcohol and isoamyl acetate	Resonant; piezoelectric crystal electrode	Wu (1999)
Mouse	mORI7 and M71	*In vivo*; use of transgenic mice expressing OR with GFP reporter	Acetophenone and benzaldehyde (M71); heptanal, octanal, *trans*-2-octenal, (+) and (−) citronellal, hexanal, nonanal and hydroxycitronellal (mORI7)	Optical; fluorescence (Ca^{2+} imaging of recombinant OSNs dissociated from epithelium)	Bozza et al. (2002)
	mORI7 and mOR912-93 (chimeras containing TMIII-VII of these ORs)	Mammalian; HEK-293 and modified HEK-293 ($pEAK^{rapid}$)	Heptanal (mORI7); 2-heptanone, 2-butanone, 2-decanone and isoamyl acetate (mOR912-93)	Optical; fluorescence (Ca^{2+} imaging)	Gaillard et al. (2002)
	mORI7, I-C6, I-D3, I-G7	Mammalian; HEK-293	Heptanal (mORI7), (−)-citronelal (I-C6), carvone (I-D3) and limonene (I-G7)	Optical; fluorescence (Ca^{2+} imaging)	Krautwurst et al. (1998) and Shirokova et al. (2005)
	mORI7, Olfr49, I-C6 (Olfr43), and MOR267-1	Mammalian; HeLa	(−)-Citronellal; Olfr43 also bound helional, (*E*)-4-decenal, octanal, heptanal, β-citronellol	Optical; fluorescence (Ca^{2+} imaging in single cells and/or using FLIPR)	Shirokova et al. (2005)
	mOR912-93 (Olfr154)	Mammalian; HeLa	2-Heptanone	Optical; fluorescence (Ca^{2+} imaging in single cells and using FLIPR)	Shirokova et al. (2005)
	MOR23	*In vivo*; utilised tissue-printed mouse epithelial cells	Lyral	Optical; fluorescence (Ca^{2+} imaging)	Touhara et al. (1999)
	MOR23, mOR-EG, mOR-EV	Mammalian; HEK-293	Lyral (MOR23); eugenol, vanillin, ethyl vanillin and 4-hydroxy-3-methyl benzaldehyde (mOR-EG); ethyl vanillin and vanillin (mOR-EV)	Optical; fluorescence (Ca^{2+} imaging)	Kajiya et al. (2001) and Oka et al. (2006)
	mOR-EG	*In vivo*; utilised isolated OSNs	Eugenol, isoeugenol, vanillin and 4-hydroxy-3-methyl benzaldehyde	Optical; fluorescence (Ca^{2+} imaging)	Oka et al. (2006)
		Mammalian; HEK-293, COS-7, CHO-K1 and PC12h	Eugenol, isoeugenol, vanillin and 4-hydroxy-3-methyl benzaldehyde	Optical; fluorescence (Ca^{2+} imaging of HEK-293), bioluminescence (luciferase reporting of cAMP in HEK-293 and PC12h) and chemiluminescence (phosphatase reporting of cAMP in HEK-293)	Katada et al. (2003), Oka et al. (2006), and Saito et al. (2004)
		Mammalian; HEK-293 and HeLa	Eugenol (antagonism by methyl isoeugenol and isosafrole)	Optical; fluorescence (Ca^{2+} imaging)	Oka et al. (2004)
		Amphibian; *Xenopus leaevis* oocytes	Eugenol	Electrochemical; whole cell voltage clamp	Katada et al. (2003)
		Mammalian; HeLa	Eugenol	Electrochemical; whole cell patch clamp	Sato et al. (2008)
	mOR-EG, Ors46	Mammalian; HEK-293	Eugenol, vanillin and ethyl vanillin (mOR-EG); decanoic acid and nonanoic acid (Ors46)	Optical; bioluminescence (luciferase reporting of cAMP) and chemiluminescence (phosphatase reporting of cAMP)	Saito et al. (2004)
	mOR-EG, Ors6/Ors79, Ors18, Ors46, Ors50, MOR23-1, MOR31-4, MOR31-6, MOR32-5, MOR32-11, MOR203-1 and Olfr62	Mammalian; Hana3A	mOR-EG (eugenol); Ors6/Ors79 & Ors50 (nonanedioate); Ors18 (pentanoate and hexanoate); Ors46 (nonanoate and decanoate); MOR23-1 (heptanaote–nonanoate); MOR31-4 (hexanoate–decanoate); MOR31-6 (pentanal, pentanoate and isovaleric acid); MOR32-5 (decanoate); MOR32-11 (octanoate–decanoate); MOR203-1 (nonanoate) and Olfr62 (coumarin, 2-coumaranone, piperonal, benzaldehyde and 4-chromanone)	Optical; bioluminescence (luciferase reporting of cAMP)	Saito et al. (2004)

	Ors1, Ors3, Ors6, Ors18, Ors19, Ors25, Ors41, Ors46, Ors50, Ors51, Ors79, Ors83, Ors85 and Ors86	*In vivo*; utilised isolated OSNs	Hexanoate (Ors19); heptanoate (Ors18, Ors19, Ors41, Ors51 and Ors79); octanoate (Ors1, Ors18, Ors19, Ors41, Ors46, Ors51 and Ors83); nonanoate (Ors1, Ors18, Ors19, Ors41, Ors46, Ors51, Ors83 and Ors86); hexanol, (Ors3 and Ors25); heptanol (Ors3, Ors19 and Ors25); octanol (Ors18, Ors19, Ors41 and Ors51); nonanol (Ors18, Ors19, Ors41, Ors51 and Ors83); bromobutanoate and bromopentanoate (Ors85); bromohexanoate (Ors19, Ors41 and Ors85); bromooctanoate (Ors1, Ors18, Ors19, Ors41, Ors46, Ors51, Ors83 and Ors85); hexanedioate–octanedioate (Ors85) and nonanedioate (Ors6, Ors51, Ors79 and Ors85)	Optical; fluorescence (Ca^{2+} imaging)	Malnic et al. (1999)
	Ors86	Mammalian; HeLa	Nonanoic acid and octanoic acid (antagonist)	Optical; fluorescence (Ca^{2+} imaging by FLIPR)	Shirokova et al. (2005)
	Ors6	Mammalian; HeLa	Nonanedioic acid and octanoic acid (antagonist)	Optical; fluorescence (Ca^{2+} imaging in single cells and using FLIPR)	Shirokova et al. (2005)
	MOR204-34, MOR224-5, MOR224-9, MOR224-13 and MOR31-2	Mammalian; HEK-293	Methyl isoeugenol, methyl eugenol and aceto isoeugenol (MOR204-34); eugenol, isoeugenol, methyl isoeugenol, methyl eugenol and guaiacol (MOR224-5); eugenol and methyl isoeugenol (MOR224-9); eugenol, isoeugenol, methyl eugenol, aceto isoeugenol and vanillin (MOR224-13); isovaleric acid (MOR31-2)	Optical; fluorescence (Ca^{2+} imaging)	Oka et al. (2006)
	MOR204-34	Mammalian; HEK-293	Methyl isoeugenol, methyl eugenol and aceto isoeugenol	Optical; chemiluminescence (phosphatase reporting of cAMP)	Oka et al. (2006)
	mTAAR5	Amphibian; *Xenopus leavis* melanophores	Triethylamine (TMA)	Optical; absorbance	Suska et al. (2009)
	V1rb2 (vomeronasal)	*In vivo*; use of transgenic mice expressing V1r-family receptor with GFP reporter	2-Heptanone	Optical; fluorescence (Ca^{2+} imaging of recombinant VSNs dissociated from epithelium)	Boschat et al. (2002)
		In vivo; use of transgenic mice expressing V1r-family receptor with GFP reporter	2-Heptanone	Electrochemical; whole cell voltage clamp of recombinant VSNs dissociated from epithelium	Boschat et al. (2002)
Rat and rat:mouse chimeras	Rat: ORI7 and ORI7:Olfr226 fusion. Rat:mouse chimeras ORI7:Olfr74 and ORI7:IC-6	Fungal; yeast (*Saccharomyces cerevisiae* strain WIF-1α)	Octanal and heptanal (ORI7), vanillin (Olfr74 chimera), (−)-citronellal (IC-6 chimera) and 2,4-dinitrotoluene (DNT) (Olfr226 fusion)	Optical; fluorescence (cAMP-mediated GFP-expression)	Radhika et al. (2007)
Rat	ORI7	*In vivo*; adenovirus-mediated expression in infected rats	C_7–C_{10} saturated aldehydes, particularly octanal	Electrochemical; whole cell voltage clamp of isolated recombinant neurons	Zhao et al. (1998)
	ORI7	*In vivo*; use of transgenic mice expressing OR with GFP reporter	Heptanal, octanal, *trans*-2-octenal, (+) and (−) citronellal, hexanal, nonanal and hydroxycitronellal (citral as antagonist)	Optical; fluorescence (Ca^{2+} imaging of recombinant OSNs dissociated from epithelium)	Araneda et al. (2000) and Bozza et al. (2002)

Table 2 (*Continued*)

Species	Receptor(s)	Expression system	Volatile giving significant response	Transduction system	Literature
		Mammalian; HEK-293	Octanal	Optical; SPR and fluorescence utilising FRET and Ca^{2+} imaging (single cells and FLIPR)	Krautwurst et al. (1998)
		Mammalian; HEK-293	Octanal (strong response); heptanal, hexanal, nonanal, and decanal (weak responses)	Optical; fluorescence (Ca^{2+} imaging)	Ko and Park (2006)
		Mammalian; COS-7	Heptanal, octanal and nonanal	Optical; fluorescence (Ca^{2+} imaging)	Levasseur et al. (2003)
		Mammalian; HeLa	Octanal and heptanal	Optical; fluorescence (Ca^{2+} imaging in single cells and using FLIPR)	Shirokova et al. (2005)
		Fungal; yeast (*Saccharomyces cerevisiae* strain MC18)	Octanal	Optical; SPR and bioluminescence (luciferase reporting of Gβγ activation)	Minic et al. (2005) and Vidic et al. (2006a)
		Mammalian; HEK-293	Octanal (strong response); heptanal and hexanal (weak responses)	Resonant; QCM	Ko and Park (2005)
		Mammalian; HEK-293	Octanal	Electrochemical; potentiometric – MEA	Lee et al. (2009a)
		Fungal; yeast (*Saccharomyces cerevisiae* strain MC18)	Octanal, heptanal and helional	Electrochemical; EIS of membrane fraction	Alfinito et al. (2010a, 2010b), Gomila et al. (2006) and Hou et al. (2007)
	U131	Mammalian	Enanthic acid and pelargonic acid	Optical; fluorescence (Ca^{2+} imaging)	Murrell and Hunter (1999)
	Multiple unknown	Mammalian; cultured OSN's derived from tissue	Mixture containing acetic acid, octanal, cineole, hexanal, 2-heptatone	Electrochemical; potentiometric – LAPS	Wu et al. (2009b)
		In vivo; pig OSNs isolated from olfactory epithelium	Specific responses generated by different OSNs to various compounds used in explosives, i.e. toluene, trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Also specific responses to styrene and phenol	Optical; fluorescence (Ca^{2+} imaging)	Corcelli et al. (2010)
Human	OR17-40	Mammalian; HEK-293 and rat olfactory epithelium-derived ODORA cells	Helional (ODORA and HEK-293); heliotroplyacetone (HEK-293 only)	Optical; fluorescence (Ca^{2+} imaging)	Levasseur et al. (2003) and Wetzel et al. (1999)
		Mammalian; HeLa	Helional	Optical; fluorescence (Ca^{2+} imaging in single cells and using FLIPR)	Shirokova et al. (2005)
		Fungal; yeast (*Saccharomyces cerevisiae* strain MC18)	Helional and analogues cassione, piperonyl acetate, 3,4-methylenedioxy phenyl acetate, 3,4-methylenedioxy propiophenone	Optical; SPR and bioluminescence (luciferase reporting driven by Gβγ)	Akimov et al. (2008), Benilova et al. (2008), Minic et al. (2005), Vidic et al. (2008), Vidic et al. (2007), and Vidic et al. (2006a)
		Fungal; yeast (*Saccharomyces cerevisiae* strain MC18)	OBP-1F (odorant-binding protein)	Optical; SPR	Vidic et al. (2008)
		Amphibian; *Xenopus leaevis* oocytes	Helional	Electrochemical; whole cell voltage clamp	Wetzel et al. (1999)
		Fungal; yeast (*Saccharomyces cerevisiae* strain MC18)	Helional	Electrochemcial; conductometric electrodes	Marrakchi et al. (2007)
	hOR2AG1	Mammalian; HEK-293	Amyl butyrate	Optical; fluorescence (Ca^{2+} imaging)	Neuhaus et al. (2006)
		Bacterial (*Escherichia coli*)	Amyl butyrate	Electrochemical; potentiometric – FET utilising membrane fraction	Kim et al. (2009) and Yoon et al. (2009)
	OR 17-209 and OR 17-210	Insect; *Sf*9	Ester mix and isoamyl acetate (OR 17–209). Ketone mix and acetophenone (OR 17–210).	Optical; fluorescence (Ca^{2+} imaging)	Matarazzo et al. (2005)

OR52D1	Mammalian; HEK-293	Wide range of medium-level responses with strongest agonists being methyl octanoate, phenyl methanol and 1-nonanol	Optical; fluorescence (Ca^{2+} imaging)	Sanz et al. (2005)
OR1G1	Mammalian; HEK-293	Wide range of responses. Strong and medium agonists were 8–10 carbon molecules (9 was optimum). Strongest were 2-ethyl-1-hexanol, 1-nonanol, ethyl isobutyrate, γ-decalactone and nonanal	Optical; fluorescence (Ca^{2+} imaging)	Sanz et al. (2005)
OR7D4	Mammalian; Hana3A	Androstenone and androsta-4,16-dien-3-one (androstadienone)	Optical; bioluminescence (luciferase reporting of cAMP)	Keller et al. (2007)
VN1R1, VN1R3 and VN1R4 (vomeronasal)	Mammalian; HeLa/Olf	Varying sensitivity to long chain aliphatic aldehydes (C9–C10 were most potent)	Optical; fluorescence (Ca^{2+} imaging by FLIPR)	Shirokova et al. (2008)
VN1R2 (vomeronasal)	Mammalian; HeLa/Olf	Range of long chain alcohols (C9–C10 were most potent)	Optical; fluorescence (Ca^{2+} imaging by FLIPR)	Shirokova et al. (2008)
VN1R5 (vomeronasal)	Mammalian; HeLa/Olf	Range of alcohols and aldehydes (C9–C10 again most potent); also linalool and (−)-carveol	Optical; fluorescence (Ca^{2+} imaging by FLIPR)	Shirokova et al. (2008)
hOR-17-4 (testicular OR)	Mammalian; HEK-293	Bourgeonal, cyclamal, canthoxal, lilial, floralazone, 3-PPA (3-phenylpropionicaldehyde), 4-PBA (4-phenylbutyraldehyde, PAA (phenylacetaldehyde), 3-PBA (3-phenylbutyraldehyde) and 4-BPAA (4-*tert*-butylphenoxy acetaldehyde)	Optical; SPR and fluorescence (Ca^{2+} imaging)	Cook et al. (2009), Neuhaus et al. (2006), and Spehr et al. (2003)
Multiple unknown	Mammalian; cultured OSNs	Acetic acid	Electrochemical; potentiometric – LAPS	Liu et al. (2006)

Abbreviations: EIS, electrochemical impedance spectroscopy; FET, field-effect transistor; FLIPR, fluorescent imaging plate reader; FRET, Forster resonance energy transfer; GFP, green fluorescent protein; OR, olfactory receptor; OSN, olfactory sensory neuron; LAPS, light addressable potentiometric sensor; MEA, micro-electrode array; QCM, quartz crystal microbalance; SPR, surface plasmon resonance; VSN, vomeronasal sensory neuron.

employed calcium imaging in recombinant HEK cells (co-expressing $G\alpha_{16}$) to develop and compare broad ligand profiles for two phylogenetically distinct human ORs, OR52D1 and OR1G1. In addition, dose-dependent antagonism of γ-lactonone-induced OR1G1-mediated calcium response was demonstrated, and several other potential antagonists of OR1G1 were detected. Similar experiments were conducted to deorphan mouse Ors86 and Ors6 (binding nonanoic and nonanedioic acids, respectively), and to identify antagonising compounds for each OR (Shirokova et al., 2005). In addition, FLIPR assays have been utilised to determine ligand profiles for all five of the known human V1R receptors (Shirokova et al., 2008). Recently, a novel microfluidics approach was reported whereby 20,000 cultured OSNs were exposed to volatiles, and calcium imaging utilised to detect up to 2900 responsive cells simultaneously (Figueroa et al., 2010).

Murrell and Hunter (1999) produced a specialised cell line (ODORA cells) derived from neurons taken from rat olfactory epithelium, for functional OR expression. This cell line was used for fluorescent reporting of calcium influx due to ligand interactions with the previously isolated but uncharacterised rat receptor U131 (both enanthic and pelargonic acids produced increased fluorescence) (Murrell and Hunter, 1999). ODORA cells were also exploited for OR17-40 expression and Fura-2 reporting of helional induced activation (Levasseur et al., 2003). Insect, (*Sf*9) cells were utilised in one study whereby two human ORs were co-expressed individually with $G\alpha_{16}$, and ligand activation (down to nanomolar concentrations) monitored through Fura-2 calcium imaging. Results showed that OR 17-209 responded to an ester mix and isoamyl acetate (from the ester mix), and OR 17-210 responded to a ketone mix and acetophenone (from the ketone mix). Interestingly, micro- and millimolar concentrations gave no response (Matarazzo et al., 2005), perhaps indicating that there was some inhibitory effect and/or that ligand concentration plays a part in dictating which receptors are activated. Fura-2 has also been utilised to image calcium in neurons isolated from mouse epithelium to deorphan MOR23 to find its cognate ligand, lyral (Touhara et al., 1999) and V1rb2, a mouse vomeronasal receptor, which was shown to be activated by 2-heptanone at nanomolar concentrations (Boschat et al., 2002). Calcium imaging of heterologous cells has also been used to transduce ligand-binding to MOR23 (Touhara et al., 1999), mOR-EV (Kajiya et al., 2001) and the human testicular OR, hOR-17-4 (Spehr et al., 2003). Interestingly, sperm cells could also be adapted to a novel chemotaxis assay to investigate ligand interactions with hOR-17-4 (Spehr et al., 2003). Recently, a second mouse vomeronasal receptor (V2Rp5) was shown to bind exocrine gland-secreting peptide 1, although this data was generated using a calcium imaging of intact epithelium combined with behavioural and electrophysiological studies (Haga et al., 2010).

As alluded to previously, optical detection of olfactory receptor activation has not only been demonstrated by calcium dyes, but also by the induced expression the bioluminescent reporter luciferase. Although luciferase has been utilised mainly for biosensor research using deorphaned ORs (see Section 2.2), it was also used in the initial series of experiments to show that mouse mOR-EG could bind eugenol (Katada et al., 2003). The same group confirmed this result by phosphatase-mediated chemiluminescent reporting of cAMP increase due to OR signalling in HEK-293 cells, arising from eugenol addition. A similar method was used to deorphan mouse MOR204-34, which was shown to bind MIEG, methyl eugenol (MEG) and aceto isoeugenol (AIEG) (Oka et al., 2006). cAMP-mediated luciferase expression was also utilised in combination with a specialised cell-line based on HEK-293T, for functional OR expression (Saito et al., 2004). Given the name Hana3A, the puromycin-resistant cell-line co-expressed $G\alpha_{olf}$ and several membrane proteins (RTP1, RTP2 and REEP1) that were shown to enhance functional cell-surface expression of ORs. The usefulness of the Hana3A cells were confirmed using known OR:ligand interactions and then further utilised to deorphan seven new mouse ORs (Table 2; Saito et al., 2004). Hana3a cells were also subsequently used to show that steroids androstenone and androstadienone, were ligands for the orphan human receptor OR7D4 (Keller et al., 2007). In addition to changes in fluorescence/bioluminescence, changes in optical absorbance have also been used to monitor receptor activation. Changes in optical absorbance at 630 nm, indicating melanosome dispersion, were measured when ligand (triethylamine) was added to melanophores of *X. laevis* expressing the mouse trace amine-associated receptor (mTAAR5) (Suska et al., 2009).

Electrophysical measurements have often been employed in olfaction research (discussed in Section 1.3.1). These techniques can be used to deorphan ORs by expressing the OR of interest in a cell commonly used for electrical measurements, most often *Xenopus* oocytes and HEK-293 cells, but also including yeast cells or isolated OSNs (see Table 2). The most commonly used methods are the patch clamp (Fig. 3ciii) and 2-electrode voltage clamp (see Fig. 3cii). Zhao et al. (1998) used voltage clamping of isolated recombinant mouse OSNs to deorphan ORI7, showing that the receptor responded to C7-C10 saturated aldehydes, most sensitively to octanal. Human OR17-40 was similarly deorphaned using *X. laevis* oocytes and shown to bind helional (Wetzel et al., 1999). The mOR-EG:eugenol interaction was also confirmed using voltage clamp of recombinant HeLa cells, as a control in experiments investigating olfactory signalling in insects (Sato and Touhara, 2009). Fluorescent reporting has been used in some studies to select cells for patch/voltage clamping experiments. For example, Boschat et al. created a transgenic line of mice in which expression of vomeronasal OR, V1rb2, was linked to GFP expression, and intact GFP-expressing OSNs were isolated from mouse epithelium for voltage clamping showing 2-heptanone binding (Boschat et al., 2002). Other electrochemical methods (such as use of LAPS, FET and MEA technologies; see Table 1 and Section 1.3.3) have also been utilised although only for transduction research for biosensor production (see Section 2.2), rather than deorphaning.

2.2. Vertebrate biosensors: use of characterised receptor:ligand pairs to develop a biosensor platform

Here we review the current approaches to develop specific biosensor platforms. It is clear that molecular characterisation of OR activity is required for successful development of OR-based biosensors for specific "field" applications. This is because, once an OR is deorphaned, the key shortfall is availability of transduction technologies as robust, transportable formats, which maintain the integrity of the biological components within. Therefore, only a few highly studied receptors have been utilised specifically for biosensor development, *viz.*: ORI7 (Hou et al., 2007; Lee et al., 2009a; Ko and Park, 2005; Segui et al., 2006; Vidic et al., 2006a & b), and OR17-40 (Vidic et al., 2006a; Cook et al., 2009). Recombinant cells have been utilised as biological recognition elements, however, in contrast to deorphanisation assays, there is increasing use of partially purified receptors, contained in membrane fractions isolated from recombinant cells. The use of a living cell in a biosensor device adds to its complexity, primarily because cells require specific conditions to maintain their health and function. There are also many reactions occurring in a cell and other elements that may interfere with the signal being monitored. Additionally, the size of the sensor itself is limited to the dimensions of a cell (~10 μm in diameter). Cell-free sensing technology is based upon utilising active components of the sensing mechanism of the cell, which have been purified to some degree and then used outside of the cellular environment. This technology has challenges involving the maintenance of functional cellular components in an extracellular surround, linking these

recognition elements to a transducer, and providing a measurable signal.

Protein interactions can tell us a great deal about cellular metabolism, without having to monitor the whole cell itself. The ability to produce sub-cellular sensing components facilitates device miniaturization, and parallel screening (multiplexing). A key challenge in producing a cell-free biosensor is displaying the recognition component on a platform in a way that maintains their functional integrity and is conducive to transduction of recognition events. This involves the immobilisation or encapsulation of the sensing component such as a receptor protein or enzyme. This challenge illustrates the importance of surface chemistry for surface attachment of sub-cellular entities to a given sensor (or culture of cells for cell-based approaches). Due to their role in receiving and relaying signals, membrane proteins such as olfactory receptors, stand out as useful targets for biosensor technology. Because many important receptors are membrane-bound, advances in lipid purification, manipulation and surface-attachment are also likely to become increasingly important for biosensor production (Leifert et al., 2009). The term "nanosomes" was used for lipid structures (obtained by sonication of membrane fractions) containing either ORI7 or OR17–40 receptors that were captured by onto a Biacore L1 sensor chip surface (for use in SPR experiments) by Vidic et al. (2006a). The chip surface was functionalised using carboxymethyl-modified dextran polymer hydrogel on which glucose moieties were grafted with lipophilic alkyl chains which efficiently captured the "nanosomes" (Vidic et al., 2006b). Similarly, Segui et al. (2006) used the term "nanosomes" to describe the structures containing ORI7 that they immobilized onto a QCM surface, although function of these receptors was not shown.

For some biosensing applications, ligand detection may be the only requirement, with metabolic consequences perhaps being irrelevant. This is in stark contrast to deorphanisation or signalling research which require that ligand-mediated cell-signalling is achieved (hence need for cellular assays), rather than just a ligand-binding event. In practice this means that sensing elements could be modified or simplified to better achieve this purpose, (e.g. an OR ligand-binging pocket may be sufficient for detection of its ligand). However, currently, only use of "complete" ORs has been reported although (unlike for deorphaning) bacterial expression has been used (Kim et al., 2009; Yoon et al., 2009) which is generally considered as not able to produce fully functional GPCRs that will efficiently couple to their respective signalling systems.

Electrochemical transduction is by far the most utilised method for biosensor development due to the existence of a range of technologies that can be adapted to biological applications (e.g. FET, LAPS, EIS, MEA; see Table 1). Microelectrode-based sensing has been investigated for use in olfactory biosensing in combination with *S. cerevisiae* cells expressing the human OR17-40, as detectors (Marrakchi et al., 2007). These cells were immobilised onto the surface of interdigitated thin film microelectrodes and odour-specific changes in conductance were measured. Octanal-mediated responses by ORI7-expressing HEK-293 cells have also been monitored by changes in field potential using a micro-fabricated planar electrode (Lee et al., 2009a). As a way of amplifying the signal for the biosensor, the HEK cells were produced coexpressing ORI7 with a cyclic nucleotide gated (CNG) channel, providing the opportunity for increased calcium influx from outside the cell upon octanal binding, therefore resulting in a larger change in field potential. In addition, this group also demonstrated that the application of an external electrical stimulation of the cell, produced a significant increase in cellular response (Lee et al., 2009a).

Isolated OSNs have been used in several studies to monitor odour induced changes in extracellular potential using LAPS (Liu et al., 2006; Wu et al., 2009a,b). Liu et al. (2006) attempted to make an OSN-based biosensor using human OSNs cultured on a LAPS chip coated with 1:1 mixture of poly-L-ornithine/laminin, which showed an acetic acid stimulated odour response. Wu et al. (2009a) used cultured rat olfactory sensory neurons and the LAPS technique to detect mixed odourants acetic acid, octanal, cineole, hexanal, 2-heptatone (see Fig. 4c). These studies demonstrate the importance of surface chemistry technologies in conjugating biological entities to the electrode surface, as discussed previously.

As mentioned EIS and FET technologies have also been adapted to olfactory biosensing in recent studies. Hou et al. (2007) used cell extracts to coat a gold electrode and transduce an odourant response. Yeast-expressed ORI7 receptor was prepared as a membrane fraction and attached to a gold electrode with functionalised SAM presenting biotinyl sites (using biotinylated anti-ORI7 polyclonal antibody raised against 15 N-terminal amino acids). Responses from octanal, heptanal and helional were recorded using EIS. More recently, the same interactions were monitored using EIS to measure differences in polarisation resistance associated with inactive and ligand-bound states of the membrane-bound ORI7 (Alfinito et al., 2010a,b) FETs utilising single-walled carbon nanotubes (swCNT)-FETs have been used to transduce responses from GST-tagged hOR2AG1 expressed in bacteria (Kim et al., 2009; Yoon et al., 2009). A silicon dioxide surface was used to attach a single layer of swCNT-FETs, onto which were placed electrodes and the bacterial membrane containing the receptors (see Fig. 4a). This method was able to electrochemically transduce specific detection of 10^{-13} M of the ligand amyl butyrate (Kim et al., 2009). The same bacterially expressed hOR2AG1 was also investigated in combination with another electrical FET-based transduction system utilising carboxylated polypyrrole (a conducting polymer rather than an inorganic semiconductor) nanotubes (i.e. CPNT-FET). Bacterial membranes containing ORs were obtained by detergent extraction and centrifugation, and then attached to the CPNT surface. The biosensor could apparently detect the cognate ligand amyl butyrate applied at 40 fM. In this system, current is thought to be generated by ligand-mediated structural rearrangement of the ORs which lead to increased OR-based cysteine residues entering a negatively charged state (Yoon et al., 2009).

As for deorphaning assays, optical transduction methods are also widely applied to biosensor research. Calcium imaging assays and luciferase have been commonly utilised, however, in contrast to deorphaning assays, there is increasing use of SPR for optical transduction (see Tables 1 and 2; also Section 1.3.1) as well as FRET-based methods. SPR has been used in a range of studies utilising SPR to transduce ligand-binding to the ORI7 and OR17-40 receptors (Akimov et al., 2008; Benilova et al., 2008; Minic et al., 2005; Vidic et al., 2006a, 2007, 2008). These experiments showed that ORs could be functionally immobilised onto the surface of an SPR chip.

Vidic et al. (2006a) reported changes in SPR signal resulting from the separation of an activated G-protein (Gα) from the immobilised nanosome-bound OR complex due to odourant induced receptor activation. In later experiments, the same group functionalised the sensor chip with biotin groups to capture neutravidin, which was in turn able to capture a biotinylated antibody to the OR protein (Vidic et al., 2007). Benilova et al. (2008) also described the use of antibodies to capture ORs on SPR surfaces and postulated that the SPR changes induced by ligand binding were due to not only Gα dissociation in the presence of GTPγS, but perhaps also receptor rearrangement (see Fig. 4b). Interestingly, the same approach was successful in detecting binding of human OBP, OBP-1F (Vidic et al., 2008). These research groups are within a European consortium known as the SPOT-NOSE (Single PrOTein-NanObioSEnsor grid array) project (Akimov et al., 2008), which has

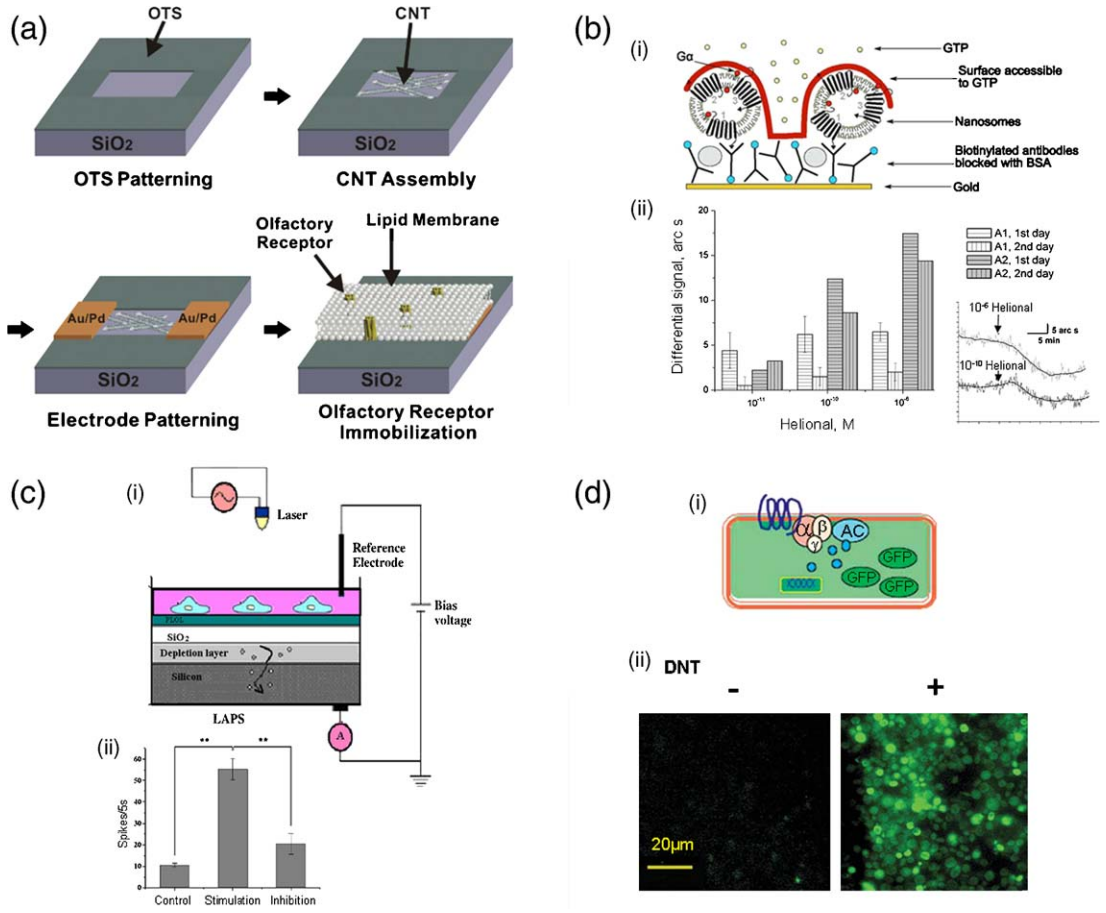

Fig. 4. Olfactory biosensing research: (a) schematic – fabrication of OR sensing platform developed by Kim et al. (2009) using swCNT-FET. An OTS-based SAM was patterned onto a SiO_2 surface using photolithography. Single-walled carbon nanotubes were adsorbed onto the bare SiO_2 regions. Gold (Au) on palladium (Pd) electrodes were fabricated using conventional lithography. Bacterial membranes containing human ORs (hOR2AG1) were spread over swCNT and vacuum dried. The technique showed odourant specific changes in current (Kim et al., 2009). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Kim et al. (2009). (b) (i) Schematic of SPR platform built by Benilova et al. (2008) using human OR17-40 in lipid "nanosomes" and immobilised antibodies on a gold substrate; (ii) helional induced changes in SPR signal, comparison of 2 surfaces (A2 surface shown in schematic) over 2 days at 3 helional concentrations. Reproduced with permission from Springer Science + Business Media from Benilova et al. (2008). (c) (i) Schematic of the LAPS-based biosensing system used by Wu et al. (2009a) to monitor changes in extracellular potential due to odourant stimulation of rat OSNs by measuring photocurrent fluctuations. (ii) Extracellular recordings from OSNs stimulated by a mixture of odours and inhibited by the addition of an adenylyl cyclase inhibitor. Reprinted from Wu et al. (2009a) with permission from Elsevier. (d) Yeast cells containing rat ORs (Olfr226) were used as a 2,4-dinitrotoluene (DNT) biosensor platform by Radhika et al. (2007). (i) GFP expression was used as a fluorescent reporter for activation of the olfactory signalling system. (ii) GFP fluorescence was visible in cells exposed to DNT. Reprinted with permission from Macmillan publishers Ltd. Nat. Chem. Biol. from Radhika et al. (2007) (copyright 2007). *Abbreviations*: OTS, octadecyltrichlorosilane; SAM, self assembled monolayer; OSN, olfactory sensory neuron; OR, olfactory receptor; SPR, surface plasmon resonance; swCNT-FET, single-walled carbon nanotube-field effect transistor; QCM, quartz crystal microbalance; EAG, electroantennogram; swCNT-FET, single-walled carbon nanotube-field effect transistor; GFP, green fluorescent protein; DNT, 2,4-dinitrotoluene; LAPS, light-addressable potentiometric sensor.

been well represented in the literature in the field of olfactory biosensor development. Cook et al. also demonstrated that it was possible to monitor the binding of odourants to the testicular hOR-17-4 using SPR transduction (Cook et al., 2009). Detergent solubilised receptor (with C-terminal Rho-tags) was captured on anti-Rho mAb bound to dextran SPR chip by amine coupling. The SPR response was induced by lilial and floralozone (Cook et al., 2009).

With a direct security application in mind, several groups have used cell-based systems to demonstrate biosensing developments targeting explosive compounds. Radhika et al. (2007) utilised a fluorescent cell-based assay in which OR stimulation initiated the translation and transformation of GFP proteins (see Fig. 4d). The application of this biosensing transduction system was in the detection of 2,4-dinitrotoluene (DNT), an explosive residue mimic, and utilised a range of wild-type, fusion and chimeric rat receptor constructs. A more recent demonstration of sensor development for explosives detection was published this year by Corcelli et al. (2010). This group isolated rat OSNs and exposed them to compounds associated with explosives; they demonstrated a specific response to toluene, trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by calcium imaging (Corcelli et al., 2010), although the ORs responsible were not known.

A novel method for fluorescent transduction involved HEK-293 cells stably coexpressing ORI7 and yellow cameleon-2 protein to report calcium influx. Cameleon-2 is a calcium-binding motif (calmodulin-binging protein) separating two GFP variants which are spectrally matched to perform FRET. Binding of calcium brings the GFP variants within the Forster radius, allowing FRET to occur and reporting Ca^{2+} levels. This system was used to detect the known ligand octanal (Ko and Park, 2007). Another optical transduction strategy, bioluminescent transduction, has been used (by researchers involved in the SPOT-NOSE consortium; see above) to engineer a yeast-based odourant sensing platform (Minic et al., 2005). These experiments made use of luciferase expression linked to ligand-mediated dissociation of yeast G-protein subunits (coupled to the recombinant ORs when ligand is not bound).

Resonant transduction techniques have not been utilised for deorphaning but they have found limited use in biosensor

research. For example, Ko and Park (2005) immobilised HEK cells expressing ORI7 onto the surface of a QCM (see Section 1.3.2) and produced a dose-dependent response to the receptor's cognate ligand, octanal. QCM has also been used to monitor odourant responses using partially purified cellular extracts containing multiple unknown ORs from bullfrog (Wu, 1999). Piezoelectric crystal electrodes were coated with the cell extracts and used to detect n-capronic acid, β-ionone, n-octyl alcohol, n-decyl alcohol and isoamyl acetate; the coated crystals reacted differently to each volatile, giving a characteristic fingerprint. This shows the potential to use cell/tissue extracts for multiplexed biosensing if the transduction method can discriminate in this way. Another advantage of coating sensor surfaces with crude extracts containing olfactory receptors is that sub-cellular sized electrodes/crystals (e.g. swCNTs) can be utilised and proteins need not be highly purified and reconstituted; GPCRs are notoriously difficult in this regard due to their complex hydrophobic structure and sensitivity to detergents. There are several reports of purification of ORs heterologously expressed in insects cells and bacteria (Cook et al., 2009; Nekrasova et al., 1996; Song et al., 2009). Importantly, using SPR, Cook et al., were able to demonstrate binding of the known agonists lilial and floralozone to detergent-solubilised hOR-17-4. Full functionality of a solubilised OR (in terms of its ability to couple to specific signalling pathways) is yet to be reported.

2.3. Deorphaning the invertebrate olfactory receptors

The diversity of invertebrates, which is orders of magnitude above that of all other multicellular land organisms combined, and the fact that olfaction is the primary method of sensing the environment for invertebrates (which often have poor sight), means that the repertoire of ligands that are potentially detectable by invertebrate ORs, dwarfs that detectable by vertebrate ORs. Furthermore, many olfactory ligands that are important for invertebrate biology are similarly important in commercial applications, especially those associated with human detection by parasites, food/agriculture, and the environment. Therefore, through sheer weight of numbers, invertebrate receptors will likely become the most widely utilised recognition elements for olfactory biosensors.

From a strategic perspective, olfaction research in entomology contrasts with that involving vertebrates in that ligands are the primary focus. Deorphaning of insect ORs has essentially involved the search for ORs that bind known ligands of medical and commercial importance. Examples include pheromones (Mitsuno et al., 2008; Miura et al., 2009, 2010; Nakagawa et al., 2005; Sakuri et al., 2004; Wanner et al., 2007a) and mosquito semiochemicals (Hughes et al., 2010; Pelletier et al., 2010; Sato et al., 2008; Wang et al., 2010). The rationale for targeting these receptors is to develop control methods for pest insects, including vectors of disease in humans, livestock and crops. An exception to this is *Drosophila*; the level of genetic and neural characterisation, combined with proven genetic manipulation tools has seen the species utilised heavily for "pure" olfaction research including the determination of ligand specificities for previously identified ORs (see Table 3). The rationale for much of this research is to develop control options for invertebrates.

The first invertebrate OR deorphaned was ODR-10 from *C. elegans*, and although a biosensor-based approach was used in part of the process, it required whole recombinant/mutant animals being subjected to chemotaxis assays (Sengupta et al., 1996). Deorphaning assays for insect ORs invariably employ either patch/voltage clamping for electrochemical transduction, or calcium imaging for optical transduction (Table 3). As for mammalian ORs, *Xenopus* and mammalian cells are mostly used for heterologous OR expression (see Table 3). A range of pheromone ORs have been deorphaned using electrochemical transduction. The earliest example was a sex pheromone receptor *B. mori*, by utilising electrochemical transduction via voltage clamping of recombinant *Xenopus* oocytes expressing BmOr-1 and various levels of $BmG\alpha_q$. Recombinant cells responded to bombykol and response was proportional to level of $BmG\alpha_q$ expression (Sakuri et al., 2004). Subsequently, a series of similar experiments (Nakagawa et al., 2005) were utilised to investigate the role of the orthologous insect chaperone receptors (see Fig. 2b and section 1.2.2) and also to deorphan another *B. mori* sex pheromone receptor (BmOr-3). BmOr-3 showed a strong response to bombykal and a weak response to the reduced form, bombykol. Interestingly, co-expression of previously deorphaned BmOr-1 with chaperone BmOr-2 increased sensitivity and number of cells responding to bombykol showing the importance of the co-receptor. Detection could also be mediated through co-receptors from other species (Or83b and HR2) but not through other insect ORs. The Or47a:Or83b complex from *Drosophila* was used as a control to detect pentyl acetate and 2-heptanone (Nakagawa et al., 2005).

Mitsuno et al. (2008) also used recombinant *Xenopus* oocytes to express a putative pheromone receptor and the relevant Or83b homologue for three pest lepidopteran species, these being *Diaphania indica* (cucumber moth), *Mythimna separata* (northern armyworm) and *Plutella xylostella* (diamondback moth). Voltage clamping was again used for detection of the main pheromone component for each species, i.e. (*E*)-11-hexadecenal (EC_{50} = 0.95 μM), (*Z*)-11-hexadecenyl acetate (EC_{50} = 0.45 μM) to (*Z*)-11-hexadecenal (EC_{50} = 0.88 μM), respectively. The detection limit was approximately 100 nM for each compound.

A similar approach was used to deorphan pheromone receptors from two species of *Ostrinia*. Each species OR was expressed with its relevant Or83b orthologue and both receptor complexes gave large response to a single pheromone component of *O. latipennis* ((*E*)-11-tetradecenol) but were relatively insensitive in this assay (EC_{50} ~1 μM). Both complexes also gave small response to (*Z*)-11-hexadecenyl acetate (Miura et al., 2009). The same group subsequently examined *O. scapulalis* ORs in more detail by the same method. OscaOR3–OscaOR8 were each individually coexpressed with OscaOR2 (Or83b orthologue) and pheromone binding analysed (Miura et al., 2010). Data indicated that OscaOR3 responded to (*E*)-11- and (*Z*)-11-tetradecenyl acetates which are the two pheromone components for this species. OscaOR3 also responded to (*Z*)-9-, (*E*)-12- and (*Z*)-12-tetradecenyl acetates which are pheromone components or behavioural antagonists of other *Ostrinia* spp. In contrast, OscaOR4 responded strongly only to (*E*)-11-tetradecenyl acetate and weakly to some other positional and stereo isomers. OscaOR5 responded only weakly to (*E*)-12- and (*Z*)-12-tetradecenyl acetates and (*Z*)-11-hexadecenal. OscaORs 6-8 did not respond to any of the compounds tested. Honeybee ORs AmOr10, AmOr11, AmOr18 or AmOr170, were utilised in similar assays (Wanner et al., 2007b). These ORs had shown male bias in their expression patterns and were also expressed with their Or83b homologue AmOr2. The main component of queen retinue pheromone, 9-oxo-2-decenoic acid (9-ODA) was applied to recombinant oocytes and experiments indicated that only the AmOr11:AmOr2 complex responded to 9-ODA and is likely the receptor that mediates detection of this compound and the subsequent behavioural affects (Wanner et al., 2007b).

Research into mosquito olfaction has generally employed the same method, i.e. voltage clamping of recombinant oocytes. CquiOR2 (from the southern house mosquito) was expressed with the chaperone CquiOR7 and used to detect indole with an EC_{50} of 280 nM (Pelletier et al., 2010). The receptor complex also responded to other methylindoles (from 1 to 6 methylindole) with EC_{50} values ranging from 3 to 20 μM, and to 2-methylphenol with EC_{50} of 7.3 μM. 3-methylindole (skatole) and 2-methylphenol

Table 3
Summary of the various sub-tissue level approaches to utilising invertebrate olfactory receptors as a biological sensing element, either for receptor deorphanisation or development of an olfactory biosensor (bioelectronic nose).

Species	Receptors	Cellular expression system	Volatile giving significant response	Transduction system	Literature
Caenorhabditis elegans; nematode	ODR-10	Bacterial (*Escherichia coli*)	Diacetyl (2,3-butanedione)	Resonant; QCM	Sung et al. (2006)
		Mammalian; HEK-293	Diacetyl (2,3-butanedione)	Optical; SPR and fluorescence (FRET)	Ko and Park (2007) and Lee et al. (2006)
Anopheles gambiae; malaria mosquito	AgOR2:AgOR7 complex	Mammalian; HeLa and HEK-293T	2-Methyl phenol	Electrochemical; whole cell voltage clamp of HeLa cells and outside-out patch clamp of HEK-293 membranes	Sato et al. (2008)
		Amphibian; *Xenopus leaevis* oocytes	2-Methyl phenol	Electrochemical; outside-out patch clamp	Sato et al. (2008)
		Mammalian; HeLa	2-Methyl phenol	Optical; fluorescence (Ca^{2+} imaging)	Sato et al. (2008)
	Panel of 37 ORs expressed individually as complexes with AgOR7	Amphibian; *Xenopus leaevis* oocytes	Each OR complex tested against 81 individuals odorants; each OR had different ranges of specificity	Electrochemical; whole cell voltage clamp	Wang et al. (2010)
	Compelxes of AgOR7 with each of AgOR1, AgOR2, AgOr6, AgOr10, AgOR28, AgOR34, AgOR37, AgOR40 and AgOR48	Amphibian; *Xenopus leaevis* oocytes	4-Methyl phenol (AgOR1); 2-methyl phenol, 2-ethyl phenol, benzaldehyde, indole, 3-methyl indole (AgOR2); acetophenone, 2-acetylthiophene (AgOR6); acetophenone, 4-methyl phenol, benzaldehyde, indole, 3-methyl indole (AgOR10); acetophenone, 2-acetylthiophene, 2,4,5-trimethylthiazole (AgOR28); 4-ethyl phenol (AgOR34); acetophenone, benzaldehyde, 2-acteylthiophene, 2-ethoxythiazole (AgOR37); 2-ethyl phenol, N-diethyl-m-tolumaide, fenchone, Henkel 100 (AgOR40); phenethyl acetate, decanolactone, 1-octanol, (S)-1-octen-3-ol (AgOR48)	Electrochemical; whole cell voltage clamp	Xia et al. (2008)
Apis mellifera; honeybee	AmOr11:AmOr2 complex	Amphibian; *Xenopus leaevis* oocytes	9-Oxo-2-decenoic acid (9-ODA)	Electrochemical; whole cell voltage clamp	Wanner et al. (2007b)
Bombyx mori; silkworm moth	BmOr-1 singly and in complexes with BmOr-2, Or83b and HR2	Amphibian; *Xenopus leaevis* oocytes	Hexadecadienol (bombykol) and very weak response to hexadecadienal (bombykal)	Electrochemical; whole cell voltage clamp	Sakuri et al. (2004) and Nakagawa et al. (2005)
	BmOr-3:BmOr-2 complex	Amphibian; *Xenopus leaevis* oocytes	Hexadecadienal (bombykal) and very weak response to hexadecadienol (bombykol)	Electrochemical; whole cell voltage clamp	Nakagawa et al. (2005)
	BmOR19	Insect; *Sf*9	Linalool	Optical; fluorescence (Ca^{2+} imaging)	Anderson et al. (2009)
	BmOR45	Insect; *Sf*9	Benzoate, 2-phenylethanol, benzaldehyde, ethyl benzoate and methyl bezoate	Optical; fluorescence (Ca^{2+} imaging)	Anderson et al. (2009)
	BmOR47	Insect; *Sf*9	Benzoate, 2-phenylethanol and benzaldehyde	Optical; fluorescence (Ca^{2+} imaging)	Anderson et al. (2009)
Culex pipiens quinquefasciatus; southern house mosquito	CquiOR2:CquiOR7 complex	Amphibian; *Xenopus leaevis* oocytes	Indole (nM sensitivity); 1-methylindole to 6-methyindole, and 2-methylphenol (μM sensitivity)	Electrochemical; whole cell voltage clamp	Pelletier et al. (2010)
	CquiOR10:CquiOR7 complex	Amphibian; *Xenopus leaevis* oocytes	3-Methylindole (skatole) and weaker responses to indole, 2-methylphenol and other (1–7)-methylindoles	Electrochemical; whole cell voltage clamp	Hughes et al. (2010)
Diaphania indica; cucumber moth	DiOR1:DiOr83 complex	Amphibian; *Xenopus leaevis* oocytes	(*E*)-11-Hexadecenal	Electrochemical; whole cell voltage clamp	Mitsuno et al. (2008)

Table 3 (*Continued*)

Species	Receptors	Cellular expression system	Volatile giving significant response	Transduction system	Literature
Drosophila melanogaster; vinegar fly	Or22a	Insect; *Sf*9	Ethyl butyrate	Optical; fluorescence (Ca^{2+} imaging)	Kiely et al. (2007)
	Or22a and Or22a:Or83b complex	Mammalian; HEK-293	Ethyl butyrate	Optical; fluorescence (Ca^{2+} imaging)	Neuhaus et al. (2005)
	Or22a:Or83b complex	Mammalian; HEK-293	Ethyl butyrate	Electrochemical; whole cell voltage clamp and outside-out patch clamp	Wicher et al. (2008)
	Or43a:Or83b complex	Mammalian; HEK-293	Cyclohexanone, benzaldehyde, isoamyl acetate, cineole and cyclohexanol	Optical; fluorescence (Ca^{2+} imaging)	Neuhaus et al. (2005)
	Or43b:Or83b complex	Mammalian; HEK-293	Ethyl butyrate	Electrochemical; whole cell voltage clamp	Smart et al. (2008)
		Insect; *Sf*9	Ethyl butyrate	Optical; fluorescence (Ca^{2+} imaging)	Smart et al. (2008)
		Mammalian; HEK-293	Ethyl butyrate	Optical; fluorescence (Ca^{2+} imaging)	Smart et al. (2008)
	Or47a:Or83b complex	Mammalian; HeLa and HEK-293T	Pentyl acetate	Electrochemical; whole cell patch clamp (HeLa) and outside-out patch clamp of membranes (HEK-293)	Sato et al. (2008)
		Amphibian; *Xenopus leaevis* oocytes	Pentyl acetate and 2-heptanone	Electrochemical; whole cell voltage clamp and outside-out patch clamp of membranes	Nakagawa et al. (2005)
		Mammalian; HeLa	Pentyl acetate	Optical; fluorescence (Ca^{2+} imaging)	Sato et al. (2008)
Epiphyas postvittana; light brown apple moth	EpOR1	Insect; *Sf*9	Methy salicylate and geraniol	Optical; fluorescence (Ca^{2+} imaging)	Jordan et al. (2009)
	EpOR3	Insect; *Sf*9	Citral (range of other compounds gave response at higher concentrations)	Optical; fluorescence (Ca^{2+} imaging)	Jordan et al. (2009)
Mythimna separata; northern armyworm	MsOR1:MsOR83 complex	Amphibian; *Xenopus leaevis* oocytes	(*Z*)-11-Hexadecenyl acetate	Electrochemical; whole cell voltage clamp	Mitsuno et al. (2008)
Ostrinia scapulalis	OscaOR1:OscaOR2 complex	Amphibian; *nopus leaevis* oocytes	(*E*)-11-Tetradecenol; lesser response to (*Z*)-11-hexadecenyl acetate	Electrochemical; whole cell voltage clamp	Miura et al. (2009)
	OscaOR3:OscaOR2 complex	Amphibian; *Xenopus leaevis* oocytes	(*E*)-11- and (*Z*)-11-tetradecenyl acetates. Also (*Z*)-9-, (*E*)-12- and (*Z*)-12-tetradecenyl acetates	Electrochemical; whole cell voltage clamp	Miura et al. (2010)
	OscaOR4:OscaOR2 complex	Amphibian; *Xenopus leaevis* oocytes	(*E*)-11-Tetradecenyl acetate; weaker response to some positional and stereo isomers	Electrochemical; whole cell voltage clamp	Miura et al. (2010)
	OscaOR5:OscaOR2 complex	Amphibian; *Xenopus leaevis* oocytes	Weak response to (*E*)-12- and (*Z*)-12-tetradecenyl acetates, and (*Z*)-11-hexadecenal	Electrochemical; whole cell voltage clamp	Miura et al. (2010)
Ostrinia latipennis	OlatOR1:OlatOR2 complex	Amphibian; *Xenopus leaevis* oocytes	(*E*)-11-Tetradecenol; weaker response to (*Z*)-11-hexadecenyl acetate	Electrochemical; whole cell voltage clamp	Miura et al. (2009)
Plutella xylostella; diamondback moth	PxOR1:PxOR83 complex	Amphibian; *Xenopus leaevis* oocytes	(*Z*)-11-Hexadecenal	Electrochemical; whole cell voltage clamp	Mitsuno et al. (2008)

Abbreviations: FRET, Forster resonance energy transfer; OR, olfactory receptor; QCM, quartz crystal microbalance; SPR, surface plasmon resonance.

are oviposition attractants for this species. Later, the assay was used to show that 3-methylindole was the key ligand for CquiOR10 to which it showed extreme sensitivity (EC_{50} = 90 nM), and that the receptor was also activated by indole (EC_{50} = 2.4 μM), 2-methylphenol (EC_{50} = 41 μM) and a range of other (1–7)-methylindoles (Hughes et al., 2010).

Malaria mosquito (*A. gambiae)* ORs were examined similarly. In 2010, Wang et al. published a large scale study of 37 ORs from *A. gambiae*, each expressed with the chaperone AgOR7 in *Xenopus* oocytes. Each was tested against a panel of 81 odourants from different classes such as acids, ketones, aromatics, heterocyclics and alcohols. Electrochemical transduction (voltage clamp) was used to show that some ORs were "narrowly tuned" and responded strongly to a single odourant (e.g. AgOR1, AgOR5, AgOR8, AgOR26, AgOR31, AgOR34 and AgOR65) while some were very "broadly tuned"; AgOR30 and AgOR5 responded strongly to 14 and 15 chemically diverse odourants, respectively. A previous study had used a combination of patch/voltage clamp transduction approaches to detect *A. gambiae* AgOR2 complex (expressed with chaperone) binding to 2-methyl phenol (Sato et al., 2008). In a similar way to other studies mentioned in this section (see above), this was performed using recombinant oocytes. However, 2-methyl phenol was also utilising mammalian HEK and HeLa cells for electrochemical transduction experiments, and HeLa cells for calcium imaging. The ORs mediating larval mosquito behavioural responses to a range of olfactory cues was investigated using voltage clamping of recombinant oocytes co-expressing individual candidate larval ORs with the AgOR2 chaperone (Xia et al., 2008). This led to deorphanisation of nine ORs (see Table 3), including the important interaction between the AgOR40 and the mosquito-repelling compound N,N-diethyl-m-toluamide (DEET). C. Liu et al. (2010) recently proposed a new class of *Anopheles* chemosensory receptors (called variant ionotropic receptors; AgIRs) which also occur in larvae and are regulated through a distinct signalling pathway. Expression analysis, gene-silencing and larval chemotaxis assays were combined to identify AgIR-mediated affects and show that they were involved in AgOR40-independent behavioural responses to DEET.

Investigations into *Drosophila* olfaction have utilised mammalian, *Xenopus* and insect cells for heterologous OR expression to transduce ligand detection (see Table 3). Research into the effect of OR83b (see Section 1.2.2) utilised HEK cells in combination with calcium imaging to detect ligands of Or22a and Or43a (Neuhaus et al., 2005). Ligand-binding to HEK-expressed Or22a:OR83b complex has also been analysed using patch and voltage clamping (Wicher et al., 2008). Or22a has also been expressed without the chaperone OR in *Sf*9 insect cells and calcium imaging used to detect ligand addition (Kiely et al., 2007). Transduction mechanisms of *Drosophila* Or43b:Or82b complex were investigated using calcium imaging of transiently infected HEK and insect *Sf*9 cells, to detect ethyl butyrate (Smart et al., 2008). This study also utilised voltage clamping of the HEK cells for electrochemical transduction of ethyl butyrate binding. Another *Drosophila* complex, OR47a:or83b, was studied with several biosensing approaches to detect the ligands pentyl acetate and 2-heptanone (Nakagawa et al., 2005; Sato et al., 2008). Sato et al., used whole mammalian cells (HeLa) and cell membranes (HeLa and HEK) for electrochemical transduction, and in addition, calcium imaging was performed on HEK cells. *Xenopus* oocytes and membranes thereof, were also produced for patch/voltage clamp experiments (Nakagawa et al., 2005; Sato et al., 2008).

Calcium imaging of recombinant insect cells has also been used to deorphan two ORs (EpOR1 and EpOR3) from light brown apple moth, a polyphagous pest of a range of crops. EpOR1 responded to low concentrations of plant volatile methyl salicylate ($EC_{50} = 1.8 \times 10^{-12}$ M) and also geraniol ($EC_{50} = 5.8 \times 10^{-11}$ M), while EpOR3 responded to low concentrations of the monoterpene citral ($EC_{50} = 1.1 \times 10^{-13}$) (Jordan et al., 2009). A range of other compounds also gave response at higher concentrations. This method was also utilised by Anderson et al. (2009) to demonstrate that various *B. mori* receptors with female-biased expression patterns (BmOR19, BmOR45 and BmOR47) were activated by plant volatiles; some of these volatiles (such as benzoate and linalool) had previously been used in EAG experiments indicating that female moths could detect them using sexually dimorphic sensilla that were enlarged in females (Heinbockel and Kaissling, 1996).

2.4. Biosensing with invertebrate olfactory receptors

The potential to use invertebrate ORs as biosensing devices has been investigated for over 50 years (long before the discovery of the ORs and attempts to deorphan them) since the development of the EAG (Butenandt et al., 1959); some of this research has already been discussed in Section 1.2.2. EAG was first used on isolated antennae of male adult B. *mori* and used to measure an electrical response to volatiles emitted from adult female glands which produce sex pheromone; only male antennae responded (Butenandt et al., 1959). Subsequently, the technique was used to show the pheromone (first known as bombykol) was hexadecadienol (Schneider, 1975, 1998). There have been a group of studies utilising EAG, attempting to improve the technique for biosensing applications, dealing with many of the same issues as for modern (sub-tissue level) sensors. Some examples are field adaptability (Baker and Haynes, 1989; Sauer et al., 1992), signal-to-noise improvement through judicious electrode placement (Park and Baker, 2002; van Geissen et al., 1994) or series connection of multiple antennae (Park and Baker, 2002), and multiplexing (Park et al., 2002; Schroth et al., 2001; Schutz et al., 1999). EAG is still highly utilised, often to characterise genetic mutants (e.g. Kain et al., 2008). Currently, the three most advanced forms of EAG modify the original approach by either coupling the EAG to GC–MS (a technique known as electroanntenographic detection or EAD) (Cork, 1994; Cosse et al., 1995; Weissbecker et al., 2004), use of FET to generate the electrical signal (Schoning et al., 2000; Schroth et al., 1999a,b, 2001; Schutz et al., 2000) and the ability to take readings from individual olfactory sensilla, which provides a mechanism of matching sub-sets of ORs to potential ligands (de Bruyne et al., 2001). Recently, single sensillum recordings were used to mine for potential candidate *Drosophila* ORs capable of detecting illicit compounds such as drugs (or drug precursors) and explosives (Marshall et al., 2010). In addition, the technique was used to show that *Drosophila* OR7a could sensitively detect the silkworm moth pheromone bombykol (Syed et al., 2010). The mechanisms regulating specific OR expression in specific sensilla are currently being elucidated (e.g. Miller and Carlson, 2010) and promise to increase the utility/resolution of single sensillum recordings.

Although our primary focus is sub-tissue level biosensors, it is worth mentioning that in a similar way to dogs and canaries, invertebrates have also been used as whole organism biosensors. A well known example of a whole-animal, invertebrate biosensor is the use of the proboscis extension response of honeybees, whereby bees can be trained to extend their proboscis when they detect a specific volatile to which they have been previously exposed along with sugar water (Bitterman et al., 1983; Takeda, 1961). This has generally been employed to understand the behaviour and neurobiology of bees (Gronenberg and Couvillon, 2010), however, it is being investigated as a field based biosensor for compounds such as explosives (King et al., 2004). In addition, it has been used in conjunction with measurement of electrical signals generated by the M-17 muscle which operates the proboscis extension, to characterise this response at a more detailed level (Smith and

Menzel, 1989); this is referred to as an electromyogram and was first reported by Rehder (1987). The use of nematodes in chemotaxis assays (discussed briefly in Sections 1.2.2 and 2.3), and also mosquito larvae (see 2.3), are other examples of whole-animal biosensors (C. Liu et al., 2010; Milani et al., 2002; Sengupta et al., 1996; Wes and Bargmann, 2001; Xia et al., 2008).

The relatively late discovery of insect ORs combined with the relative lack of characterisation of their signalling cascades (compared to vertebrate and nematode ORs) has meant that invertebrate biosensor research has been slow to emerge and until recently (see Misawa et al., 2010), there were no reports of biosensor-specific research (i.e. focussed on transduction improvement and surface attachment) utilising partially purified insect ORs or recombinant cells. Most research (Sung et al., 2006; Lee et al., 2006) has utilised the highly characterised ODR-10:diacetyl interaction from *C. elegans* (in which signalling is better understood; see Section 1.2.2). Bacterial and mammalian systems have been used to express ODR-10, and subsequently to investigate novel methods for transducing the signal from diacetyl-binding to the OR (Ko and Park, 2007; Lee et al., 2006; Sung et al., 2006). Similar to the FRET-based ORI7 biosensor utilising "cameleon" proteins mentioned in Section 2.2, ODR-10 expressed in mammalian cells were monitored through FRET-mediated reporting of calcium. The same recombinant cells were also cultured on poly-L-lysine coated, gold sensor chip and used to detect diacetyl via another optical method, SPR (Lee et al., 2006). Resonant transduction of diacetyl-binding was also investigated by obtaining the membrane fraction of recombinant bacteria and using it to coat the gold surface of a QCM. Diacetyl addition gave a large frequency response, with an insignificant response being obtained from addition of the aldehydes hexanal–decanal, as expected (Sung et al., 2006).

With regard to insect ORs, Misawa et al. (2010), recently reported on a portable, microfluidics-based biosensor utilising recombinant oocytes co-expressing insect pheromone receptors BmOr1, BmOr3 and PxOR1, or *Drosophila* OR85b (non-pheromone OR), with their relevant chaperones. Using voltage clamping of trapped oocytes, binding of known cognate ligands for each receptor could be simultaneously detected and gave a dose-dependent response.

3. Discussion and future outlooks

Here we have reviewed the biological process of olfaction in both vertebrates and invertebrates. The distinction between the two was made for three reasons: (1) the different modes of action for olfaction in the two groups, (2) the difference in volatile targets that each of these groups would operate to detect, and (3) the fact that insect olfactory signalling processes are still much debated. We have also discussed the concept of a biosensor, and introduced some of the transduction techniques that have been used in the context of volatile detection, either for receptor deorphaning (generally using standard transduction techniques such as calcium imaging or voltage clamping) or biosensor research (often involving "experimental" transduction techniques such as SPR, QCM, LAPS and FET). We primarily focussed the review by discussing only methodological approaches using sub-tissue biological recognition components, however, some relevant tissue-based and whole organism assays have also briefly been mentioned, particularly in a historical context. The ability to detect volatile compounds with the same sensitivity and specificity as nature's olfactory machinery, is applicable to a bewildering array of applications, most obviously in environmental monitoring, medicine, security and agriculture.

Olfactory biosensing with sub-tissue level detectors is a relatively new area of research, however, some early trends have been established which we expect to see accelerate over the next decade. We would expect to see deorphanisation of a range of ORs from multiple organisms, with increasing speed. This is likely to be most marked for invertebrates due to the large number of commercially and/or medically important species present, and the importance of the ligands they detect. A range of cloning and deorphaning/screening strategies have been investigated to increase the throughput of deorphanisation assays; large percentages of the mouse, human and *Drosophila* OR repertoires are now cloned into specially adapted cell lines that are optimised for functional OR expression and high-throughput ligand screening (Figueroa et al., 2010; Keller et al., 2007; Krautwurst et al., 1998; Marshall et al., 2010; Saito et al., 2004; Touhara, 2007). In addition, due to the current focus on insect olfaction by a range of research groups with access to advanced technologies, we will likely see some of the signalling mechanisms unravelled. This will in turn lead to the development of the first insect OR-based biosensors utilising cells or cellular components.

Currently we are unaware of any commercial biosensors of the type we have discussed here. As in all biosensor technologies, the fragility of the biological components used for recognition will continue to be a hurdle in the development of a truly portable, stable and reusable olfactory biosensing device. However, advances in microfluidic technologies are likely to aid in bridging the bio-electronic interface, providing a constant fluid and therefore more stable, environment for the biological components. Additionally, further knowledge regarding odourant binding sites, and those of associated proteins such as OBPs, could facilitate development of alternative/synthetic, more stable, binding site mimics for use as the recognition components in olfactory sensing devices. For the development of biosensors where simple detection is the focus (rather than characterisation or detection of subsequent metabolic activity), the use of sub-protein components (e.g. ligand binding regions or "synthetic peptides") is likely to increase.

Olfactory biosensor production has also been hampered by the lack of a small, portable transduction system. Indeed, although much of the research into biosensor development aims to produce technology that is transportable and able to be used with confidence in a commercial setting, transduction systems generally still utilise relatively complex technologies that are laboratory-based (such as patch clamping or SPR). A transduction system capable of sensitive and selective detection, combined with minimal sample preparation (e.g. labelling), ability to multiplex, and stability for operational requirements, will be required for development of successful OR-based biosensors. The use of nanotechnologies such as swCNT-based platforms, shows great promise in providing portability and sensitivity, and their use is another trend we expect to see accelerate. Their size provides the potential for highly miniaturized integration, which is important for a portable biosensing platform. The electrical properties of the CNTs have been shown to be very sensitive to environmental changes in the FET devices, a property which could be utilized to monitor biological binding events or localized ionic changes in solution. As more receptors are deorphaned, olfactory signalling is further unravelled and transduction technologies are improved and simplified, more potential biosensing applications will be attempted and the first commercially applicable biosensors will undoubtedly emerge.

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