Optimization of Insect Odorant Receptor Trafficking and Functional Expression Via Transient Transfection in HEK293 Cells

Fabio Miazzi1,2, Carolin Hoyer1, Silke Sachse1, Markus Knaden1, Dieter Wicher1, Bill S. Hansson1* and Sofia Lavista-Llanos1*

1Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, 07745, Jena, Germany and 2Present address: Max Planck Institute for Chemical Ecology, Max Planck Research Group Predators and Toxic Prey, Hans-Knöll-Str. 8, 07745, Jena, Germany

Correspondence to be sent to: Fabio Miazzi, Max Planck Institute for Chemical Ecology, Max Planck Research Group Predators and Toxic Prey, Hans-Knöll-Str. 8, 07745, Jena, Germany. E-mail: fmiazzi@ice.mpg.de

*These authors share seniority.

Abstract

Insect odorant receptors (ORs) show a limited functional expression in various heterologous expression systems including insect and mammalian cells. This may be in part due to the absence of key components driving the release of these proteins from the endoplasmic reticulum and directing them to the plasma membrane. In order to mitigate this problem, we took advantage of small export signals within the human HCN1 and Rhodopsin that have been shown to promote protein release from the endoplasmic reticulum and the trafficking of post-Golgi vesicles, respectively. Moreover, we designed a new vector based on a bidirectional expression cassette to drive the functional expression of the insect odorant receptor coreceptor (Orco) and an odor-binding OR, simultaneously. We show that this new method can be used to reliably express insect ORs in HEK293 cells via transient transfection and that is highly suitable for downstream applications using automated and high-throughput imaging platforms.

Key words: Drosophila melanogaster, HCN1, odorant receptors, rhodopsin

Introduction

Insect odorant receptors (ORs) are 7 transmembrane-domain proteins, with an inverted topology compared to G protein-coupled receptors (GPCRs) (Benton et al. 2006; Butterwick et al. 2018), responsible for the detection of a vast number of chemically diverse odorants including pheromones (Hallem and Carlson 2006; Touhara and Vosshall 2009). They form heteromeric cation channels, constituted by an odor-binding receptor (OrX) and a highly conserved coreceptor named Orco (Krieger et al. 2003; Jones et al. 2005; Neuhaus et al. 2005; Benton et al. 2006; Sato et al. 2008; Wicher et al. 2008; Vosshall and Hansson 2011).

Several methods have been used to express insect ORs in heterologous systems in order to characterize their ligand specificity and to study their functional properties (Flescher et al. 2018). For example, the so-called “empty neuron system” allows the ectopic expression of ORs in subsets of olfactory sensory neurons of the vinegar fly Drosophila melanogaster lacking the endogenous OrX, but with a fully functional Orco (Dobritsa et al. 2003; Kurtovic et al. 2007; Gonzalez et al. 2016). Moreover, in vitro expression systems allow the heterologous expression of OR proteins in animal cells including—among others—Xenopus oocytes (Nakagawa et al. 2005; Wang et al. 2010; Nakagawa and Touhara 2013), insect cells (Kiely...
Each of these methods offers several advantages, but also bears disadvantages: the "empty neuron system" allows the expression of OR proteins in an environment that is very similar to their native olfactory sensillum. However, the generation of transgenic flies and the electrophysiological recording tests can be time consuming and are not suitable for high-throughput screening experiments. *Xenopus* oocytes have been successfully used for extensive screenings of odors and are compatible with automated platforms, but the costs associated with *Xenopus* rearing or the purchase of oocytes are prohibitive for very large screenings. Stable and inducible cell lines, on the other hand, originate from cell clones that successfully integrated in their genome an expression cassette containing a tetracycline-dependent promoter driving the expression of Orco and an odor-binding OrX. Such system represents a sort of "gold standard" as cell variability is strongly reduced, compared to transiently transfected cells, by selection of monoclonal populations and non-induced cells constitute an optimal internal negative control. Moreover, the selection of a monoclonal cell population represents—to date—the most effective approach to deal with the limited functional expression of ORs in the plasma membrane in heterologous systems due to an impaired intracellular trafficking of ORs both in insect (German et al. 2013) and mammalian cells (Halley-Leon et al. 2016).

Although a thorough investigation of the bottlenecks in insect OR intracellular trafficking has not been performed yet, their retention within intracellular membranes (German et al. 2013) may be due to the lack of specialized components of the OR release mechanism from the endoplasmic reticulum (ER) and the Golgi apparatus in heterologous systems. Several membrane proteins have been shown to direct their intracellular trafficking through small peptide regions. Among others (Schulein et al. 1998; Ammon et al. 2002), it has been shown that an N-terminal peptide (344VNKFSL) from the human HCN1 channel facilitates the exit of HCN1 proteins from the ER (Pan et al. 2015), and the C-terminal portion of the human Rhodopsin (344QVAPA)—containing the "VxPx" motif—is sufficient to promote the formation of post-Golgi vesicles and their trafficking to the plasma membrane via microtubule-mediated transport (Tay et al. 1999; Deretic et al. 2005; Mazelova et al. 2009; Lodowski et al. 2014).

In this work, we investigated whether insect OR tags targeted at their N-terminus with the peptide "QVAPAGKIPINPLI GLDSTV NKFL" coding for the human Rhodopsin 344QVAPA peptide (minimal Rho tag, abbreviated as "mRho" or "R" tag), a V5 tag (GKPIPNPLIGLDST) and the human HCN1 peptide 344VNKFSL (minimal ER export tag, abbreviated as "mER" or "E" tag)—this construct is hereafter called mRho, V5.mER,E.hOr47a, or R.E.hOr47a—was synthesized and subcloned into the pcDNA3.1(-) vector (Cat. Nr. V79520, Invitrogen) using the XbaI and XhoI restriction sites by Eurofins Genomics GmbH. Constructs bearing only the V5 and mER peptides (V5.mER, hOr47a, or abbreviated E.hOr47a and the V5 tag only (V5,E.hOr47a, or abbreviated hOr47a) were obtained from the mRho,V5.mER, hOr47a construct via PCR using the Advantage 2 PCR kit (Cat. Nr. 639206, Takara) using the E.hOr47a fwd and hOr47a fwd forward primers, and the common (E.)hOr47a_rev reverse primer, respectively. A previously described nonhuman codon optimized version of *D. melanogaster* Orco cloned in pcDNA3.1(-) (Mukunda et al. 2014) was used for cotransfection of the hOr47a constructs in pcDNA3.1(-).

A human codon-optimized version of *D. melanogaster* Orco (hOrco) tagged at the N-terminus with a myc tag (5'-GAACAGAAA CTGATC TCTGAAGAAGACCTG-3') was synthesized by Eurofins Genomics GmbH and subcloned into the pCMV-BI vector using the BamHI and HindIII restriction sites. A vector of hOrco bearing the β-globin/IgG chimeric intron from the pCMVNT vector within the hOrco sixth transmembrane domain was constructed by Phusion polymerase amplification using the hOrcoExon1_fwd and hOrcoExon1_rev, chimeric_intron_fwd, chimeric_intron_rev, hOrcoExon2_fwd and hOrcoExon2_rev primers, respectively. Fragments were assembled using the NEBuilder HiFi DNA Assembly Master Mix (Cat. Nr. E2621, New England Biolabs). A human codon-optimized version of *D. melanogaster* Orco (hOr47a) tagged at the N-terminus with the peptide "QVAPAGKIPINPLI GLDSTV NKFL" coding for the human Rhodopsin 344QVAPA peptide (minimal Rho tag, abbreviated as "mRho" or "R" tag), a V5 tag (GKPIPNPLIGLDST) and the human HCN1 peptide 344VNKFSL (minimal ER export tag, abbreviated as "mER" or "E" tag)—this construct is hereafter called mRho, V5.mER,E.hOr47a, or R.E.hOr47a—was synthesized and subcloned into the pcDNA3.1(-) vector (Cat. Nr. V79520, Invitrogen) using the XbaI and XhoI restriction sites by Eurofins Genomics GmbH. Constructs bearing only the V5 and mER peptides (V5.mER, hOr47a, or abbreviated E.hOr47a and the V5 tag only (V5,E.hOr47a, or abbreviated hOr47a) were obtained from the mRho,V5.mER, hOr47a construct via PCR using the Advantage 2 PCR kit (Cat. Nr. 639206, Takara) using the E.hOr47a_fwd and hOr47a_fwd forward primers, and the common (E.)hOr47a_rev reverse primer, respectively. A previously described nonhuman codon optimized version of *D. melanogaster* Orco cloned in pcDNA3.1(-) (Mukunda et al. 2014) was used for cotransfection of the hOr47a constructs in pcDNA3.1(-).

We created a high-copy number bidirectional expression vector (hence called pCMV-BI) by inserting the bidirectional promoter cassette flanked by the termination regions of the pBI-CMV1 vector (Cat. Nr. 631630, Clontech, in the pCMVNTT (Cat. Nr. L5620, Promega) vector backbone). Both regions were amplified using Phusion high-fidelity polymerase (Cat. Nr. M0530, New England Biolabs) with the pBI-CMV1_fwd, pBI-CMV1_rev, and pCMVNTT_fwd, pCMVNTT_rev primer couples, respectively. Fragments were assembled using the NEBuilder HiFi DNA Assembly Master Mix (Cat. Nr. E2621, New England Biolabs).

A human codon-optimized version of *D. melanogaster* Orco (hOrco) tagged at the N-terminus with a myc tag (5'-GAACAGAAA CTGATC TCTGAAGAAGACCTG-3') was synthesized by Eurofins Genomics GmbH and subcloned into the pCMV-BI vector using the BamHI and HindIII restriction sites. A vector of hOrco bearing the β-globin/IgG chimeric intron from the pCMVNT vector within the hOrco sixth transmembrane domain was constructed by Phusion polymerase amplification using the hOrcoExon1_fwd, hOrcoExon1_rev, chimeric_intron_fwd, chimeric_intron_rev, hOrcoExon2_fwd and hOrcoExon2_rev primers. A new vector called pDmelOR was then created by cloning this intron-containing version of hOrco into the pCMV-BI vector, after digestion with BamHI and HindIII, using the NEBuilder HiFi DNA Assembly kit.

The mRho,V5.mER,E.hOr47a construct was inserted in the pDmelOR vector after linearization with EcoRI, using the BI-R.E.hOr47a_fwd and BI-R.E.hOr47a_rev using the NEBuilder HiFi DNA Assembly Master Mix (pDmelOR-mRho,V5.mER, hOr47a, or pDmelOR-R.E.hOr47a). Moreover, a human codon-optimized version of *D. melanogaster* Or56a (hOr56a) was synthesized by Eurofins Genomics GmbH and inserted in the pDmelOR vector after linearization with EcoRI, together with the mRho,V5.mER tag using the following set of primers: hOr56a_fwd, hOr56a_rev, hOr56aTag_fwd, hOr56aTag_rev (pDmelOR-mRho,V5.mER,hOr56a, or pDmelOR-R.E.hOr56a).

All sequences were verified by Sanger sequencing (by Eurofins Genomics GmbH and the Department of Entomology, Max Planck Institute for Chemical Ecology, Jena). The CMV enhancer region of the pCMV-BI empty plasmid was sequenced using the pBI-CAG_for and pBI-CAG_rev primers. Sequencing of CMV promoter and termination regions from pCMV-BI and related constructs required a preliminary digestion with restriction enzymes, band isolation and purification after gel electrophoresis and possibly amplification of target sequences due to the presence of 2 very similar CMV promoter and SV40 polyA regions. The pCMV-BI empty plasmid was

**Materials and methods**

**Constructs**

A human codon-optimized version of *D. melanogaster* Or47a (hOr47a) tagged at the N-terminus with the peptide "QVAPAGKIPINPLI GLDSTV NKFL" coding for the human Rhodopsin 344QVAPA peptide (minimal Rho tag, abbreviated as "mRho" or "R" tag), a V5 tag (GKPIPNPLIGLDST) and the human HCN1 peptide 344VNKFSL (minimal ER export tag, abbreviated as "mER" or "E" tag)—this construct is hereafter called mRho, V5.mER,E.hOr47a, or R.E.hOr47a was synthesized and subcloned into the pcDNA3.1(-) vector (Cat. Nr. V79520, Invitrogen) using the XbaI and XhoI restriction sites by Eurofins Genomics GmbH.
digested with DraII-HF (Cat. Nr. R3510, New England Biolabs) and Ncol-HF (Cat. Nr. R3193, New England Biolabs). The pdmELOR vector was sequenced after digestion with XhoI and Pvu-HF (Cat. Nr. R3150, New England Biolabs); constructs with inserted Or47a and Or56a genes were cut with XhoI and NaeI (Cat. Nr. R0190, New England Biolabs). If required, the inserted Orco gene was subsequently amplified from the Orco-bearing purified plasmid fragment with the pBl-Orco_for and pBl-Orco_rev primers, and the tuning receptor gene with the pBl-OrX_fwd, pBl-OrX_rev primers using the Advantage 2 PCR kit and the resulting PCR products were sequenced. Primer sequences are listed in the Supplementary Table 1.

Full sequences for the reported constructs and plasmid availability information are accessible at Addgene (https://www.addgene.org) with the following reference numbers: pdNA3.1(-)-mRho.V5.mEr.hOr47a: #126472; pdNA3.1(-)-V5.mEr.hOr47a: #126473; pdNA3.1(-)-V5.hOr47a: #126474; pCMV-BE: #126475; pdmELOR: #126476; pDMELOR-mRho.V5.mEr.hOr47a: #126478; pdmELOR-mRho.V5.mEr.hOr56a: #126479.

**Transient expression in mammalian cells**

HEK293 cells (DSMZ no. ACC 305) were purchased from the Leibniz Institute DSMZ GmbH and grown in DMEM/F12 1:1 medium (Cat. Nr. 11320, Gibco, Life Technologies) supplied with 10% fetal bovine serum. Zero Ca\(^{2+}\) (Cat. Nr. 9183.1, Carl Roth), and F12 (Gibco, Cat. Nr. 21765) in a 1:1 mixture, supplemented with 10% fetal bovine serum. DMEM was preferred to standard DMEM in a 1:1 mix with F12, and F12 (Gibco, Cat. Nr. 21068) supplemented with 1% Roti-Cell glutamine solution normalized to the \([Ca^{2+}]_i\) before each stimulus application. Statistical analysis was performed using parametric or nonparametric tests (according to the data distribution) and \(P\)-values were corrected for multiple comparisons using Holm’s correction. Software plugin and packages used are listed in the [Supplementary Table 2](#).

Imaging experiments using an automated platform were performed on a BD Pathway 855 (BD Biosciences) controlled by the AttoVision software (version 1.6/855). Excitation at 340 (for 150 ms) and 380 nm (for 250 ms) was performed using the BD Pathway 855 settings and filters for Fura-2 with a 5 s interval between frames. Stimuli consisted of 20 µL solutions of odor at the desired concentration (Figure 3), or 20 µL of 100 µM VUA1 in SES. The odor solution was presented at the 30th frame. After background subtraction, cells were segmented using the built-in tools of AttoVision and mean ROI intensity values were exported as text files for subsequent analysis.
Figure 1. Optimization of OR trafficking to the plasma membrane in HEK293 cells. (a) The human HCN1 receptor sequence 106VNKFSL111 encoding a minimal endoplasmic reticulum release signal ("mER") or "E") and the human Rhodopsin sequence 344QVAPA348 encoding a minimal Rho tag ("mRho", or "R") were used to enhance the functional expression of insect ORs by promoting their exit from the endoplasmic reticulum and the transport of post-Golgi vesicles to the plasma membrane, respectively. (b) Schematic representation of the constructs tested. HEK293 cells were cotransfected by electroporation with 2 plasmid constructs. The first, with the insertion of a human codon-optimized version of the D. melanogaster Or47a (hOr47a) tagged at the N-terminus with: a peptide composed of the mRho, mER and a V5 tag—to detect the receptor via immunochemistry if necessary—(R.E.hOr47a construct), or tagged only with the mER and V5 peptides (E.hOr47a) or the V5 tag alone (hOr47a). The second construct was constituted by a noncodon-optimized version of D. melanogaster Orco coreceptor. As controls, cells were cotransfected with the Orco coreceptor together with the empty vector backbone (EV) instead of the hOr47a construct, or with the empty vector alone. (c) Changes in intracellular calcium concentration over time (Δ[Ca2+]i) in HEK293 cells transfected with the constructs shown in panel (b), following stimulations with 100 µL of 100 µM of the Or47a agonist pentyl acetate (PA) at 50 s and the synthetic Orco agonist VUAA1 at 350 s. Graphs represent mean ± SD. n = 5 for each panel. Each n = 1 represents the mean of all imaged cells coming from each independent electroporated cuvette (see Methods). (d) Intensity of calcium responses following a pentyl acetate or a VUAA1 stimulation in cells transfected with the constructs described in panel (b). Values were extracted 50 s after a stimulation with 100 µL of 100 µM pentyl acetate, and 20 s after a stimulation with 100 µL of 100 µM VUAA1. Mean ± SD values for pentyl acetate: EV, -0.07 ± 0.63; Orco, 0.11 ± 0.64; hOr47a + Orco, 0.35 ± 0.61; E.hOr47a + Orco, 4.44 ± 0.66; R.E.hOr47a + Orco, 6.89 ± 1.41. Statistical analysis for pentyl acetate: R.E.hOr47a + Orco versus E.hOr47a + Orco: t = 3.52, P = 0.0014; R.E.hOr47a + Orco versus hOr47a + Orco: t = 9.52, P < 0.001; R.E.hOr47a + Orco versus Orco: t = 9.81, P < 0.001. Mean ± SD values for VUAA1: EV, -0.68 ± 0.24; Orco, 9.52 ± 4.11; hOr47a + Orco, 15.88 ± 5.29; E.hOr47a + Orco, 45.02 ± 7.02; R.E.hOr47a + Orco, 61.32 ± 13.29. Statistical analysis for VUAA1: R.E.hOr47a + Orco versus E.hOr47a + Orco: t = 2.42, P = 0.051; R.E.hOr47a + Orco versus hOr47a + Orco: t = 7.10, P = 0.0015; R.E.hOr47a + Orco versus Orco: t = 3.38, P = 0.015; R.E.hOr47a + Orco versus EV: t = 10.16, P = 0.0015. Mean ± SD values for pentyl acetate: EV, 0.18 ± 0.16; Orco, 0.64 ± 0.50; hOr47a + Orco, 2.90 ± 1.06; E.hOr47a + Orco, 21.61 ± 4.16; R.E.hOr47a + Orco, 30.51 ± 6.56. Statistical analysis for pentyl acetate: R.E.hOr47a + Orco versus E.hOr47a + Orco: t = 2.56, P = 0.039; R.E.hOr47a + Orco versus hOr47a + Orco: t = 9.29, P = 0.0015; R.E.hOr47a + Orco versus Orco: t = 10.16, P = 0.0015. Mean ± SD values for VUAA1: EV, 0.06 ± 0.14; Orco, 24.12 ± 9.10; hOr47a + Orco, 30.24 ± 7.21; E.hOr47a + Orco, 61.48 ± 5.95; R.E.hOr47a + Orco, 71.76 ± 3.29. Statistical analysis for VUAA1: R.E.hOr47a + Orco versus E.hOr47a + Orco: t = 3.38, P = 0.015; R.E.hOr47a + Orco versus hOr47a + Orco: t = 11.71, P < 0.001; R.E.hOr47a + Orco versus Orco: W = 25, P = 0.016 (Wilcoxon rank test). Unless otherwise stated, tests are unpaired 2-tail Welch’s t-test. P values corrected for multiple comparisons using Holm’s correction. Bar plots represent mean ± SD, n = 5 for each treatment. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.
in R (see Supplementary Code). Briefly, for experiments shown in Figure 3d–h, ROIs showing high base level fluorescence standard deviation (> 0.05 × R [340/380 nm] ratio) or a high intracellular Ca$^{2+}$ (> 2 × R [340/380 nm] ratio) in frames 1–9 (before odor stimulus application), or showing no or very small VUAA1 responses (local max after VUAA1 application < 1.5 × baselevel R [340/380 nm] ratio) or showing an increasing monotonic VUAA1 response (i.e., no peak in the VUAA1 response before the end of the experiment) were excluded from analysis. For control experiments (Figure 3i), only ROIs with high base level fluorescence standard deviation, or with high intracellular Ca$^{2+}$ before odor stimulus application were deleted. ROIs from wells subjected to the same treatment within the same plate were averaged ($n$ = 1) in order to calculate the experimental time series and the responses following stimuli application. Statistical analysis was performed using parametric or nonparametric tests (according to the data distribution) and $P$-values were corrected for multiple comparisons using Dunnett's correction. Software plugin and packages used are listed in the Supplementary Table 2.

**OR agonists**

The following odors and agonists were used: pentyl acetate (Sigma-Aldrich, Cat. Nr. 109584, 99%), butyl acetate (Fluka, Cat. Nr. 45860, ≥99.5% (GC), ACS Reagent), methyl hexanoate (Fluka, Cat. Nr. 21599, 99.8%, analytical standard grade), propyl acetate (Sigma-Aldrich, Cat. Nr. 133108, 99%; Fluka, Cat. Nr. 40858, ≥99%, analytical standard grade), 3-methylthio-1-propanol (Sigma-Aldrich, Cat. Nr. 318396, 98%; Sigma-Aldrich, Cat. Nr. W341509, ≥98% synthetic, FG grade), hexyl acetate (Sigma-Aldrich, Cat. Nr. 108154, 99%), isobutyl acetate (Fluka, Cat. Nr. 94823, 99.8%, analytical standard grade), 2-heptanone (Sigma-Aldrich, Cat. Nr. W254401, 98% synthetic, FG grade), 3-octanol (Sigma-Aldrich, Cat. Nr. 218405,
100 mM solutions in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Chemical Ecology (Jena, Germany). Stimuli stocks consisted of VUAA1 (CAS Nr. 525582-84-7) was synthesized by the group 99%), (±)-geosmin (Sigma-Adrich, Cat. Nr. UC18, ≥98% [GC]), χ² = 50.148, df = 9, Kruskall–Wallis rank sum test: χ² = 10.088, df = 2, χ² = 27.115, df = 12, Kruskall–Wallis rank sum test: χ² = 2.45·10–5 (1.83 × 10–5; 3.29 × 10–5). Values of the data fit (mean ± st. error) for pDmelOR-R.E.hOr47a: slope, −2.89 ± 0.42; upper limit, 30.80 ± 1.28; EC₅₀ (log), −4.61 ± 0.06. EC₅₀ values expressed in Molar (estimate, 2.5% and 97.5% confidence intervals): 2.45·10–5 (1.83 × 10–5; 3.29 × 10–5). Values of the data fit for pDmelOR-R.E.hOr56a: slope, −2.46 ± 0.58; upper limit, 11.93 ± 0.92; EC₅₀ (log), −6.14 ± 0.13. EC₅₀ values expressed in Molar (estimate, 2.5% and 97.5% confidence intervals): 7.19 × 10–7 (3.97 × 10–7; 1.30 × 10–6). Plot shows estimate ± SE. For 5 ≤ n ≤ 6 (b) and 4 ≤ n ≤ 10. The red box highlights 2 odors whose response intensities do not follow the expected trend, i.e., 3-methylthio-1-propanol and pentyl acetate. (The effect of propyl acetate is consistent across commercial stocks with different purity. The response to pentyl acetate was significant compared to the DMSO control when working solutions were prepared from a 99% purity stock odor (Sigma-Aldrich, Cat. Nr. W341509), but not from a ≥98% food/pharmaceutical (FG) grade stock odor (Sigma-Aldrich, Cat. Nr. D8418); working solutions were prepared fresh diluting 100 mM stocks in SES to the desired concentration just before the start of the experiment. Negative controls consisted of the equivalent maximum volume of DMSO used to prepare odor stimuli solutions diluted in SES.

Figure 3. Analysis of insect OR agonist sensitivity and specificity by means of an automated imaging platform. (a) Normalization of the odor response respect to the VUAA1 internal control. After calculating the ratio (R) between the emission light at 340 nm and 380 nm, base levels were subtracted from peak agonist responses to obtain the odor, e.g., pentyl acetate (PA), and the VUAA1 net responses (Δx and Δy, respectively). The ratio ϱ (rho) between the odor and VUAA1 net responses expressed in percentage (ϱ = (Δx/Δy)*100) can be used to account for the cell-specific OR expression level due to the transient transfection protocol. (b,c) Example showing how normalization of the odor response to the VUAA1 internal control can reduce response variability. (b) Time series of R (340/380 nm) ratios for 2 regions of interests (RO1 and RO2) showing different response intensities to the same odor and VUAA1 stimulation. (c) Time series of normalized ϱ values for the odor responses of the same RO1 and RO2 as shown in panel (b). ϱ values reveal that both RO1 and RO2 show a very similar maximal response to the PA stimulation. (d,e) Dose–response curve for HEK293 cells transfected with pDmelOR-R.E.hOr47a and stimulated with pentyl acetate (d), or with pDmelOR-R.E.hOr56a and stimulated with geosmin (e). Data were fitted to a 3-parameter logistic function using the drc package in R (see Supplementary Code). Values of the data fit (mean ± std. error) for pDmelOR-R.E.hOr47a: slope, −2.89 ± 0.42; upper limit, 30.80 ± 1.28; EC₅₀ (log), −4.61 ± 0.06. EC₅₀ values expressed in Molar (estimate, 2.5% and 97.5% confidence intervals): 2.45·10–5 (1.83 × 10–5; 3.29 × 10–5). Values of the data fit for pDmelOR-R.E.hOr56a: slope, −2.46 ± 0.58; upper limit, 11.93 ± 0.92; EC₅₀ (log), −6.14 ± 0.13. EC₅₀ values expressed in Molar (estimate, 2.5% and 97.5% confidence intervals): 7.19 × 10–7 (3.97 × 10–7; 1.30 × 10–6). Plot shows estimate ± SE. For 5 ≤ n ≤ 6 (b) and 4 ≤ n ≤ 10. The red box highlights 2 odors whose response intensities do not follow the expected trend, i.e., 3-methylthio-1-propanol and pentyl acetate. (The effect of propyl acetate is consistent across commercial stocks with different purity. The response to pentyl acetate was significant compared to the DMSO control when working solutions were prepared from a 99% purity stock odor (Sigma-Aldrich, Cat. Nr. W341509), but not from a ≥98% food/pharmaceutical (FG) grade stock odor (Sigma-Aldrich, Cat. Nr. D8418); working solutions were prepared fresh diluting 100 mM stocks in SES to the desired concentration just before the start of the experiment. Negative controls consisted of the equivalent maximum volume of DMSO used to prepare odor stimuli solutions diluted in SES.

99%), (±)-geosmin (Sigma-Aldrich, Cat. Nr. UC18, ≥98% [GC]), VUAA1 (CAS Nr. 525338-84-7) was synthesized by the group Mass Spectrometry/Proteomics of the Max Planck Institute for Chemical Ecology (Jena, Germany). Stimuli stocks consisted of 100 mM solutions in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat. Nr. D8418); working solutions were prepared fresh diluting 100 mM stocks in SES to the desired concentration just before the start of the experiment. Negative controls consisted of the equivalent maximum volume of DMSO used to prepare odor stimuli solutions diluted in SES.

99%), (±)-geosmin (Sigma-Aldrich, Cat. Nr. UC18, ≥98% [GC]), VUAA1 (CAS Nr. 525338-84-7) was synthesized by the group Mass Spectrometry/Proteomics of the Max Planck Institute for Chemical Ecology (Jena, Germany). Stimuli stocks consisted of 100 mM solutions in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat. Nr. D8418); working solutions were prepared fresh diluting 100 mM stocks in SES to the desired concentration just before the start of the experiment. Negative controls consisted of the equivalent maximum volume of DMSO used to prepare odor stimuli solutions diluted in SES.
Results

Targeting of insect ORs to the plasma membrane

We first tested whether the 344QVAPA348 (minimal Rho tag, abbreviated as “mRho” or “R” tag) and the 106VNKFSL111 (minimal ER expression cassette of the pH-CMV1 vector, in order to guide the expression of 2 genes simultaneously (see Methods). We first inserted a human codon-optimized version of D. melanogaster Orco (hOrco) tagged at the N-terminus with a myc tag in correspondence of the multiple cloning site 1 of the vector. As the CMV promoter can initiate expression in Escherichia coli (Lewin et al., 2005) and Orco forms leaky ion channels, the unintended expression of Orco during cloning may reduce the viability of successfully transformed bacterial colonies. To avoid such inconvenience, we inserted a β-globin/inG chimeric intron within the Orco sixth transmembrane domain. In this way, only mammalian cells can splice out the intron, leading to the production of functional Orco ion channels. The resulting plasmid, named pDmelOR, is intended to serve as a plasmid backbone to insert one of the 61 (including splice variants) tuning ORs of D. melanogaster (Robertson et al., 2003), and the same principle can be adapted to optimize the functional expression of ORs belonging to any insect species.

In order to evaluate the performance of the bidirectional pDmelOR vector against a standard cotransfection protocol, we compared the response profile of HEK293 cells transfected with pDmelOR-R.E.hOr47a to that of cells cotransfected with an Orco and an R.E.hOr47a construct inserted in a pcDNA3.1(-) each (Figure 2a–c). HEK293 cells transfected with pDmelOR-R.E.hOr47a showed a significantly higher Δ[Ca2+] in response to both pentyl acetate and VUAA1 stimulation, with respect to cells cotransfected with R.E.hOr47a and Orco in pcDNA3.1(-) (Figure 2d). Moreover, a significantly higher number of cells responded to pentyl acetate and after transfection with pDmelOR-R.E.hOr47a compared to cells cotransfected with R.E.hOr47a and Orco in pcDNA3.1(-) (Figure 2e). These results propose the pDmelOR vector as an efficient transfection tool for the expression of insect OR fusion constructs with higher efficacy than standard plasmid cotransfection procedures.

Optimization of transient transfection for automated imaging platforms

Finally, we took advantage of the high level of functional expression reached combining the increased OR trafficking to the plasma membrane using the mRho and mER tags, together with the optimization of the transfection protocol due to the pDmelOR vector, to validate our method using an automated imaging platform (Figure 3).

A monoclonal stable cell line guarantees a highly homogeneous level of transgene expression within the cell population, which minimizes variability in functional assays. On the other hand, the expected variability in a population of transiently transfected cells is much higher, due to the variation in plasmid copy number between cells that results in different levels of functional expression of the gene of interest. To control for this phenomenon, we stimulated tested cells with VUA1 100 s after the presentation of the odor stimulus, in order to use the intensity of the response to this synthetic OR agonist as a proxy of the OrX/Orco functional expression level for each cell. In this way, we could remove from downstream analyses those cells that were not expressing ORs at sufficient levels (see Methods), and we could normalize the odor response for each cell to the intensity of the VUA1 response, thus minimizing the variability induced by the transient transfection protocol (Figure 3).

Using this method, we built dose–response curves for 2 D. melanogaster ORs, namely the broadly tuned receptor Or47a and the narrowly tuned Or56a, stimulated with their main agonists,
did not quantify how the mRho and mER epitopes affected the cell fluorescence base level (Figure 3i). However, we further investigated whether odor purity could have influenced our results. To do so, we tested 2 odors (propyl acetate and 3-methylthio-1-propanol) whose relative potency differed from the expected pattern (compare Figure 3f with Supplementary Figure 2). For each odor, we tested 2 aliquots with different chemical grade. Interestingly, while the responses to propyl acetate were not affected by the odor chemical grade (Figure 3g), the responses to 3-methylthio-1-propanol were significantly affected by this factor, indicating that 3-methylthio-1-propanol might not be an actual agonist of Or47a and the source of OR activation might originate from impurities within the extract (Figure 3h).

**Discussion**

Functional expression in heterologous systems represents a key method to elucidate the function and structure of membrane protein. When confronted with the choice of which expression system to use, there are several factors to consider: from the codon of the gene of interest and necessary post-translational modifications (Gomes et al. 2016), to the type of downstream applications and the level of automation required. HEK293 cells represent a well-understood and versatile choice: their transcriptome has been extensively profiled (Sultan et al. 2008; Richard et al. 2010) providing fundamental information regarding possible cross-talk between heterologous and native proteins. Furthermore, an extensive set of molecular tools has already been optimized to support protein functional expression (for an overview, see Baser and van den Heuvel 2016), and these tools are amenable to a vast array of downstream applications, from imaging to electrophysiology, that can be performed with automated and high-throughput systems (Mattiazi Usaj et al. 2016; Obergrussberger et al. 2018).

On such a basis, we decided to implement a fast, inexpensive, and versatile method for the expression of insect ORs in HEK293 cells. In order to achieve this result, we here tackled 2 main problems: the poor surface localization of OR proteins in mammalian cells and the limits imposed by the cotransfection of 2 genes (Orco and an odor-binding receptor) to obtain functional odor-gated ion channels. To improve the surface localization of insect ORs, we prepared fusion constructs carrying at the N-terminus small epitopes from the human HCN1 (mER) and Rhodopsin (mRho) that are known to facilitate the release of membrane proteins from the ER and the targeting to the plasma membrane, respectively. Using this method, we obtained a nearly 20-fold increase in the mean response (Figure 1d) and a 10-fold increase in the number of cells responding (Figure 1e and Supplementary Figure 1) to odor stimulation. Although we did not quantify how the mRho and mER epitopes affected the abundance of OR proteins in the plasma membrane, a correlation between an increased amount of OR proteins that successfully reach the cell plasma membrane and the increase in the mean Ca^2+ response and the number of responding units is the most parsimonious explanation. Then, by adopting a new high-copy number vector based on a bidirectional expression cassette (Figure 2), we significantly improved the expression efficiency of ORs, showing that three-quarters of cells were stimulated with an overall 100-fold increase in the mean ∆[Ca^2+]i, in response to an odor stimulation, when compared to a standard cotransfection protocol with ORs lacking the HCN1 and Rhodopsin-derived tags (Figures 1 and 2 and Supplementary Figure 1). Finally, thanks to the high expression level and the possibility to use synthetic Orco agonists as internal stimulus controls to account for intercell variability, we showed that such a system is amenable to be used with automated platforms (Figure 3) and can be consequently used for high-throughput screenings.

Although we proved the effectiveness of such a system for 2 D. melanogaster ORs with different properties—a broadly tuned receptor as Or47a and the narrowly tuned Or56a—it remains to be shown how generalizable such approach is. Mammalian ORs are affected by similar problems regarding an incorrect intracellular trafficking in heterologous expression systems. Although specific classes of proteins have been shown to support the trafficking of mammalian ORs in native olfactory neurons and in heterologous systems (Sato et al. 2004; Mainland and Matsunami 2012), conserved OR residues linked to in silico structural stability were shown to impact their functional expression (Ikegami et al. 2019); we cannot exclude that insect ORs are subject to similar structural constraints.

Taken together, our results show that by optimizing the intracellular trafficking and transfection conditions of insect ORs, it is possible via transient transfection to achieve expression levels in HEK293 cells that are comparable to more time and resource-demanding methods such as the establishment of mammalian stable cell lines. Hence, we hope that such method can advance the study of insect ORs structure and function even in nonmodel organisms.

**Supplementary material**

Supplementary data are available at Chemical Senses online.

**Funding**

This work was supported by the European Union’s Horizon 2020 research and innovation program under the Grant Agreement No. 662629 (F.M. and S.L.L.) and the Max Planck Society (D.W., S.S., M.K., and B.S.H.).

**Acknowledgments**

The authors thank Sabine Kaltofen for help in culturing and transfecting HEK293 cells, Sascha Buchs for help with the BD Pathway 855 setup, Kerstin Wenger and Anna Späthe with chemical profile analysis, Alois Svaros and Jerrit Weißflog for the synthesis of VUAA1 and Domenica Schnabelrauch for the in-house Sanger sequencing. The authors thank Antonella di Pizio (Leibniz-Institute for Food Systems Biology at the Technical University of Munich, Germany) for fruitful discussion.

**Author contributions**

F.M. and S.L.L. designed the study; D.W. and B.S.H. contributed to the study design; S.S., M.K., and B.S.H. obtained funding for the project; F.M. and C.H. created the constructs; F.M. performed the imaging experiments, analyzed the data, and wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.
Conflict of Interest

The authors declare no competing interests.

References

Ammon C, Schafer J, Kreuzer OJ, Meyerhof W. 2002. Presence of a plasma membrane targeting sequence in the amino-terminal region of the rat somatostatin receptor 3. Arch Physiol Biochem. 110:137–145.

Baser K, van den Heuvel J. 2016. Assembling multi-subunit complexes using mammalian expression. In: Vega MC, editor. Advanced technologies for protein complex production and characterization. Cham: Springer International Publishing. p. 225–238.

Benten R, Sachse S, Mischnak SW, Vossahl LB. 2006. Atripal membrane topology and heteromeric function of Drosophila odorant receptors in vivo. PLoS Biol. 4:e20.

Bokhob JD, Jones PL, Wang G, Pits RJ, Pask GM, Zwiebel LJ. 2011. Conservation of heterologous rodent olfactory receptors in mosquitoes reveals an ancient olfactory trait. Chem Senses. 36:149–160.

Butterwick JA, Del Marmol J, Kim KH, Kalnins MA, Rogow JA, Walz T, Ruta V. 2018. Cryo-EM structure of the insect olfactory receptor Orco. Nature. 560:447–452.

Corcoran JA, Jordan MD, Carracher C, Newcomb RD. 2014. A novel method to study insect olfactory receptor function using HEK293 cells. Insect Biochem Mol Biol. 54:22–32.

Deretic D, Williams AH, Ransom N, Morel V, Hargrave PA, Arendt A. 2005. Rhodopsin C terminus, the site of mutations causing retinal disease, regulates trafficking by binding to ADP-ribosylation factor 4 (ARF4). Proc Natl Acad Sci USA 102:3301–3306.

Dobritsa AA, van der Goes van Naters W, Warr CG, Steinbrecht RA, Carlson JR. 2003. Integrating the molecular and cellular basis of odor coding. The Drosophila antenna. Neuron. 37:827–841.

Fleischer J, Preigracter P, Beer H, Krieger J. 2018. Access to the odor world: olfactory receptors and their role for signal transduction in insects. Cell Mol Life Sci. 75:485–508.

German PF, van der Poel S, Carracher C, Kalriek AK, Newcomb RD. 2013. Insights into subunit interactions within the insect olfactory receptor complex using FRET. Insect Biochem Mol Biol. 43:138–145.

Gomes AR, Byeegowda SM, Veregowda BM, Balamurugan V. 2016. An overview of heterologous expression host systems for the production of recombinant Drosophila melanogaster odorant receptor ion channels. J Neurosci Methods. 271:149–153.

Halty-deLeon L, Miazzi F, Schulze HC, Kaltofen S, Hansson BS, Wicher D. 2019. Low Ca2+ levels in the culture media support the heterologous expression of insect odorant receptor proteins in HEK cells. J Neurosci Methods. 312:122–125.

Kurtovic A, Widmer A, Dickson BJ. 2007. A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature. 446:542–546.

Lewin A, Mayer M, Chusainow J, Jacob D, Appel B. 2005. Viral promoters can initiate expression of toxin genes introduced into Escherichia coli. BMC Biotechnol. 5:19.

Lodowski KH, Lee R, Ropolewski P, Nemer I, Tian G, Imanishi Y. 2013. Signals governing the trafficking and misfolding of a ciliary GPCR, rhodopsin. J Neurosci. 33:13621–13638.

Mainland J, Matsunami H. 2012. RAMP like proteins: RTP and REEP family of proteins. Adv Exp Med Biol. 744:75–86.

Mattiazzi Usaj M, Styles EB, Verster AJ, Friisen H, Boone C, Andrews BJ. 2016. High-content screening for quantitative cell biology. Trends Cell Biol. 26:598–611.

Mazelova J, Astuto-Gribble L, Inoue H, Tam BM, Schontech E, Prekeris R, Monitz OL, Randazzo PA, Deretic D. 2009. Ciliary targeting motif VxPx directs assembly of a trafficking module through Arf4. EMBO J. 28:183–192.

Mazia F, Schulze H, Zhang L, Kaltofen S, Hansson BS, Wicher D. 2019. Conservation of an insect olfactory trait. J Comp Physiol A Neurobiol Sens Neural Behav Physiol. 189:519–526.
Sultan M, Schulz MH, Richard H, Magen A, Klingenhoff A, Scherf M, Seifert M, Borodina T, Soldatov A, Parkhomchuk D, et al. 2008. A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science*. 321:956–960.

Tai AW, Chuang JZ, Bode C, Wolfrum U, Sung CH. 1999. Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. *Cell*. 97:877–887.

Touhara K, Vosshall LB. 2009. Sensing odorants and pheromones with chemosensory receptors. *Annu Rev Physiol*. 71:307–332.

Tsitoura P, Andronopoulou E, Tsikou D, Agalou A, Papakonstantinou MP, Kotzia GA, Labropoulou V, Swevers L, Georgoussi Z, Iatrou K. 2010. Expression and membrane topology of *Anopheles gambiae* odorant receptors in lepidopteran insect cells. *PLoS One*. 5:e15428.

Vosshall LB, Hansson BS. 2011. A unified nomenclature system for the insect olfactory coreceptor. *Chem Senses*. 36:497–498.

Wang G, Carey AF, Carlson JR, Zwiebel LJ. 2010. Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA* 107:4418–4423.

Wicher D, Schäfer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS. 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature*. 452:1007–1011.