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High-throughput analysis of mammalian olfactory receptors: measurement of receptor activation via luciferase activity

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Abstract:	Odorants create unique and overlapping patterns of olfactory receptor activation, allowing a family of approximately 1000 murine and 400 human receptors to recognize thousands of odorants. Odorant ligands have been published for fewer than 6% of human receptors. This lack of data is due in part to difficulties functionally expressing these receptors in heterologous systems. Here, we describe a method for expressing the majority of the olfactory receptor family in Hana3A cells, followed by high-throughput assessment of olfactory receptor activation using a luciferase reporter assay. This assay can be used to (1) screen panels of odorants against panels of olfactory receptors; (2) confirm odorant/receptor interaction via dose response curves; and (3) compare receptor activation levels among receptor variants. In our sample data, 328 olfactory receptors were screened against 26 odorants. Odorant/receptor pairs with varying response scores were selected and tested in dose response. These data indicate that a screen is an effective method to enrich for odorant/receptor pairs that will pass a dose response experiment, i.e. receptors that have a bona fide response to an odorant. Therefore, this high-throughput luciferase assay is an effective method to characterize olfactory receptors—an essential step toward a model of odor coding in the mammalian olfactory system.
Author Comments:	Enclosed please find a resubmission of our article entitled "High-throughput analysis of mammalian olfactory receptors: measurement of receptor activation via luciferase activity." Although the referees were largely positive concerning the manuscript, one describing it as a "detail and accurate procedure that… allows anyone stated in art to reproduce the work," they also had significant comments. We thank the referees for their comments, with which we concurred. In accordance with these comments, we have significantly revised the manuscript. Matching mammalian odorant receptors (ORs) to ligands has seen limited success,

	and the picture is even worse when considering human ORs; ligands have been published for only 22 of the approximately 400 intact human ORs. This lack of data is a critical bottleneck in the field; matching ligands to ORs is essential for understanding the olfactory system at all levels and for building viable models of olfaction. One of the major barriers is the challenge of functionally expressing these ORs in heterologous cell systems. Here, we describe a method for expressing a broad range of ORs in heterologous cells, followed by the high-throughput identification of olfactory receptor ligands and quantification of receptor activation. This assay can be used to screen panels of odorants against panels of ORs, confirm receptor/odor interaction via dose response, and assess how genetic variation affects the level of receptor activation. The large size of both the OR family and the set of odorous molecules requires a high-throughput method for testing odorant/OR combinations. By publishing this assay method in JoVE, we hope to facilitate the use of this method in other laboratories, and accelerate data acquisition in the field. In particular, JoVE's unique format will enable other researchers to become familiar with this type of assay system and potentially use this method to characterize mammalian olfactory receptors.
Additional Information:	
Question	**Response**

TITLE:
High-throughput analysis of mammalian olfactory receptors: measurement of receptor activation via luciferase activity

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SHORT ABSTRACT:

Olfactory receptor activation patterns encode odor identity, but the lack of published data identifying odorant ligands for mammalian olfactory receptors hinders the development of a comprehensive model of odor coding. This protocol describes a method to facilitate high-throughput identification of olfactory receptor ligands and quantification of receptor activation.

LONG ABSTRACT:

Odorants create unique and overlapping patterns of olfactory receptor activation, allowing a family of approximately 1000 murine and 400 human receptors to recognize thousands of odorants. Odorant ligands have been published for fewer than 6% of human receptors.[1–11] This lack of data is due in part to difficulties functionally expressing these receptors in heterologous systems. Here, we describe a method for expressing the majority of the olfactory receptor family in Hana3A cells, followed by high-throughput assessment of olfactory receptor activation using a luciferase reporter assay. This assay can be used to (1) screen panels of odorants against panels of olfactory receptors; (2) confirm odorant/receptor interaction via dose response curves; and (3) compare receptor activation levels among receptor variants. In our sample data, 328 olfactory receptors were screened against 26 odorants. Odorant/receptor pairs with varying response scores were selected and tested in dose response. These data indicate that a screen is an effective method to enrich for odorant/receptor pairs that will pass a dose response experiment, i.e.

receptors that have a bona fide response to an odorant. Therefore, this high-throughput luciferase assay is an effective method to characterize olfactory receptors—an essential step toward a model of odor coding in the mammalian olfactory system.

INTRODUCTION:

The mammalian olfactory system has the ability to respond to a vast number of odorous stimuli, allowing for the detection and discrimination of thousands of odorants. Olfactory receptors (ORs) are the molecular sensors expressed by the olfactory sensory neurons in the olfactory epithelium.[12] Mammalian odor recognition occurs through differential activation of ORs by odorants, and the OR gene family is extensive, with roughly 1000 murine and 400 human receptors.[12–16] Previous functional analyses of ORs in olfactory neurons and in heterologous cells have shown that different odorants are recognized by unique, but overlapping ensembles of ORs.[10,17–20] Matching ligands to ORs is critical for understanding the olfactory code and essential for building viable models of olfaction. Due to difficulties expressing ORs in heterologous systems as well as the large number of both odorants and ORs, this data has been largely absent from the field; indeed, fewer than 6% of human ORs have a published ligand.[1–11] This protocol describes the use of a luciferase assay to characterize odorant/OR interactions. This assay enables the high-throughput characterization of ORs, a task that is essential to understanding odorant/OR interactions as well as developing a model of odor coding.

High-throughput studies of ORs face three major challenges. First, ORs expressed in heterologous cells were retained in the ER and subsequently degraded in the proteasome,[21,22] preventing the ORs from interacting with odorants in the assay system.[23–25] This problem was addressed by the discovery of accessory proteins that facilitate stable cell-surface expression of a broad range of ORs.[19,26,27] Receptor-transporter-proteins 1 and 2 (RTP1 and 2) promote OR cell-surface expression and activation in response to odorant stimulation.[19] Based on this work, HEK293T cells were modified to stably express RTP1 long (RTP1L) and RTP2, receptor expression-enhancing protein 1, and $G_{\alpha olf}$, resulting in the Hana3A cell line.[19,27] In addition, the type 3 muscarinic acetylcholine receptor (M3-R) interacts with ORs at the cell surface and enhances activation in response to odorants.[26] Co-transfection of an OR with RTP1S and M3-R into Hana3A cells results in the robust, consistent, and functional expression of a broad range of ORs at the cell surface.[27] Second, mammalian OR repertoires are quite large. In humans, for example, the OR repertoire is an order of magnitude more numerous than the gustatory receptor repertoire, and two orders of magnitude more numerous than the visual receptor repertoire. Although cloning a single OR is a relatively straightforward protocol, significant up-front effort is required to generate a comprehensive library. Third, although we know that in vision, wavelength translates into color and in audition frequency translates into pitch, the organization of odors is poorly understood, making it difficult for researchers to interpolate from a representative sample of odorants. Although some progress has been made on this front,[10,28] the map of the olfactory landscape remains incomplete. Screening tens of thousands of molecules against hundreds of ORs is a daunting task; high-throughput screens in this domain require carefully defined campaigns. The major remaining challenges are those of logistics and cost rather than problems inherent to the technique. Although heterologous screening has not been widely used to identify ligands by academic groups, a private company has used the same technique to identify ligands for 100 human ORs.[29] Unfortunately, these data remain proprietary.

The high-throughput luciferase assay outlined here has several advantages over alternative methods used to assess OR activation. Although the responses of native olfactory sensory neurons have been measured using electrophysiology and calcium imaging, these techniques have difficulty teasing apart which OR leads to a neuron's response due to the overlap in response properties for olfactory neurons. Although knocking-in a GFP-labeled receptor type,[30,31] delivering specific receptors via adenovirus to murine olfactory neurons,[32,33] or performing RT-PCR after recordings[17,24,33] can link recordings to single receptor types, these methods are low-throughput and not suitable for large-scale screens. Heterologous screening systems are more scalable, and two major forms are found in the literature: cAMP pathway reporters and inositol triphosphate (IP3) pathway reporters. Upon odor stimulation, ORs activate a $G_{\alpha olf}$ transduction signaling cascade that results in the production of cyclic AMP (cAMP).[12] By co-transfecting a firefly luciferase reporter gene under the control of a cAMP response element (CRE), luciferase production can be measured as a function of odor response, allowing for the quantification of OR activation. OR activation can also be linked to the IP3 pathway by co-expressing G-proteins such as $G_{\alpha 15/16}$ or a $G_{\alpha 15\text{-}olf}$ chimera.[24,25,34] We have chosen the assay presented here based on three factors: (1) the co-expression of RTP1 with Rho-tagged olfactory receptors improves the expression of olfactory receptors at the cell surface;[19,27] (2) use of a cAMP-responsive reporter gene allows for the measurement of OR activation through the canonical second messenger pathway; and (3) the assay is well-suited to high-throughput screens.

This high-throughput luciferase assay is applicable to a variety of studies valuable to the field of olfaction. First, a large number of ORs can be screened against a single odorant in order to determine the receptor activation pattern for a specific odorant. This type of study identified OR7D4 as the OR responsible for responding to the steroid odorant androstenone.[8] Conversely, one OR can be screened against a panel of odorants in order to determine the receptor response profile.[10] When candidate olfactory odorant/OR pairs are identified via these screens, interaction can be confirmed by conducting a dose response experiment examining the response of the OR to increasing concentrations of odorant. Dose response curves can also assess how genetic variation in an OR affects *in vitro* odorant response,[8,9,11,35] and these studies can be extended to interspecific OR variation, allowing for the examination of receptor evolution across species and causal mutations in evolution.[36,37] Finally, this assay can be used to screen for odor antagonists that are able to antagonize OR response to a particular odorant for a known odorant/receptor pair.[38,39] In summary, this high-throughput luciferase assay is applicable to a range of studies that will help characterize OR activation patterns and provide a better understanding of odor coding in the olfactory system.

PROTOCOL:

1. Culture of Hana3A cells

1.1) Prepare M10 media by supplementing minimum essential medium (MEM) with 10% (vol/vol) FBS.

1.2) Culture maintenance

1.2.1) Maintain cells in M10 media. Note: The expression vectors for RTP1L, RTP2, REEP1, and $G_{\alpha olf}$ confer puromycin resistance to Hana3A cells, but maintaining the cells with this antibiotic does not significantly affect assay results.

1.2.2) Subculture at a ratio of 1:8 in 10 cm dishes every 2-3 days.

1.2.3) Incubate at 37 □C with 5% CO_2.

2. Plating cells for transfection

2.1) Aspirate media from a 100% confluent 10cm dish of Hana3A cells.

2.2) Wash cells by adding 10 ml PBS, swirling the dish, and aspirating the PBS.

2.3) Add 3 ml of 0.05% trypsin/EDTA and wait for cells to dissociate (about 1 min).

2.4) Inactivate trypsin by adding 5 ml M10 and break up cell clumps by triturating roughly 10 times with a 10 ml pipette. Pipette carefully to avoid introducing air bubbles into the media.

2.5) For each 96 well plate, transfer 1 ml of cells into a 15 ml conical tube, centrifuge at 200 x g for 5 min, and aspirate the supernatant without disturbing the cell pellet.

2.6) Resuspend the cells in 6 ml M10 per 1 ml of cells transferred in step 2.5.

2.7) Pipette 50 µl of cells to each well of a 96 well plate and incubate overnight at 37 □C with 5% CO_2.

3. Transfection of olfactory receptors

3.1) Preparation of plasmid DNA

3.1.1) Prepare plasmid DNA via an endotoxin-free protocol. Note: Use plasmid DNA preparation kits designated "endotoxin-free," or add a phenol-chloroform extraction step to the plasmid DNA preparation protocol.

3.1.2) Dilute DNA to a concentration of 100 ng/µl in TE buffer.

3.2) Observe plated cells (step 2.7) to ensure a proper confluency of approximately 30-50% per well and return to incubator. Note: while this confluency is not optimal for the lipid transfection reagent, a confluency of 30-50% at this step is optimal for measuring luciferase activity 24 hours after transfection.

3.3) Preparation of transfection mix

3.3.1) Pipette RTP1S-pCI, M3-R-pCI, pCRE-luc, and pSV40-RL plasmids into MEM medium per the volumes detailed in Table 1 to make the Plasmid mix (volumes indicated are per 96 well plate).

[Place Table 1 here]

3.3.2) For each 96-well plate, dilute 18 µl lipid transfection reagent in 450 µl MEM medium.

3.3.3) Pipette Plasmid mix (from step 3.3.1), rhodopsin-tagged olfactory receptor in pCI plasmid (Rho-OR-pCI), and lipid transfection mix (from step 3.3.2) to make the Complex detailed in Table 2. Mix the solution by trituration and incubate at room temperature for 15 minutes. Stop the reaction by adding M10 according to Table 2. NOTE: this reaction is time-sensitive and should not be allowed to continue for more than 30 minutes. The well+10% calculation is important to ensure sufficient volume for subsequent steps.

[Insert Table 2 here]

3.4) Tap out the media on the cell plates.

3.5) Pipette 50 µl of complex to each well and incubate overnight at 37 □C with 5% CO_2.

4. Odor stimulation

4.1) Observe the transfected cells to ensure a proper confluency of 50-80% per well and return to incubator. Note: if cells are less than 50% confluent, firefly luciferase and renilla luciferase readings may be too low for measurement of receptor activation. Consider discarding the plate.

4.2) Prepare 1M stock solutions of each odor in DMSO.

4.3) Prepare odor stimulation solutions in CD293 medium.

4.3.1) For screening experiments, dilute stock solution of odor to 100 µM. Also prepare a no-odor control (CD293 only) in order to control for OR background activation. NOTE: for screening experiments, each OR/odor pair is tested only once per experiment. Because some odor diffusion across wells is possible, it is recommended to stimulate with one odorant for each plate.

4.3.2) For dose response experiments, prepare seven 10-fold serial dilutions of odor stock solution in triplicate starting at 1 mM for each receptor. Also prepare the same odor dilutions in triplicate for empty vector-transfected cells in order to control for odor background activation. NOTE: for dose response experiments, each odor concentration treatment should be conducted in triplicate.

4.4) Tap out the media on the cell plates.

4.5) Pipette 25 µl of odor stimulation solution to each well and incubate for 4 hours.

5. Measuring OR activity via luciferase assay

5.1) Resuspend firefly luciferase substrate per the manufacturer's instructions and store at -80 □C.

5.2) Thaw 1 ml of firefly luciferase substrate per 96 well plate.

5.3) Prepare fresh firefly luciferase reaction quencher and *Renilla* luciferase substrate reagent (5 µl luciferase quencher/*Renilla* luciferase substrate per 1 ml of buffer). NOTE: approximately 1 ml of reagent is needed per 96 well plate.

5.4) Prepare the luminescent microplate reader. Open the microplate reader software. Within the system icon:

5.4.1) Under the "Pre-heating" Tab, check the box for "ON" and set the temperature of the machine to 25 °C.

5.4.2) Under the "Dispenser" tab, prime each dispenser with 1000 µl of 70% ethanol followed by 1000 µl of distilled water. NOTE: use separate aliquots of alcohol and water for each dispenser.

5.4.3) Prime each dispenser with 1500 µl of air (remove dispensers from liquid).

5.5.4) Prime dispenser 1 with 1080 µl of firefly luciferase substrate (from step 5.2). Prime dispenser 2 with *Renilla* luciferase substrate (from step 5.3). NOTE: Be careful not to cross-contaminate the luciferase substrates.

5.5) Remove the lid from the 96-well plate and place the plate in the microplate reader.

5.6) Within the software associated with the microplate reader, under the "File" menu, click on "New Task". Highlight "Protocols" and click on "Create New". In the next window, the circle next to "Standard Protocol" should be selected. Click "OK." Double click on "Procedure" on the left hand side of the screen.

5.6.1) Dispense 10 µl of firefly luciferase substrate to all wells using dispenser 1. Under the "Actions" menu, click "Dispense". In the "Dispense Step" window, set: "Dispenser" to 1, "Priming" to none, "Dispense Volume" to 10 µl and "Rate" to 225 µl/sec. Click "OK".

5.6.2) Shake the plate for 30 sec. Under the "Actions" menu, click "Shake". In the "Shake Step" window, set "Intensity" to Medium and "Duration" to 0:30 MM:SS. Click "OK".

5.6.3) Read the luminescence of all wells for 0.5 sec per well. Under the "Actions" menu, click "Read". In the "Read Step" window, set: "Detection Method" to Luminescence, "Read Type" to Endpoint, "Integration Time" to 0:00:50 MM:SS:ss, "Filter Sets" to 1, "Emission" to Hole, "Optics Position" to Top, "Gain" to 135, and "Read Height" to 1.00 mm. Click "OK".

5.6.4) Dispense 10 µl of *Renilla* luciferase substrate to all wells using dispenser 2. Set the conditions as in step 5.6.1, except set "Dispenser" to 2.

5.6.5) Shake plate for 30 sec. Set the conditions as in step 5.6.2.

5.6.6) Read luminescence of all wells for 0.5 sec per well. Set the conditions as in step 5.6.3.

5.7) Clean the plate reader. From the system icon under the "Dispenser" tab:

5.7.1) Purge 1000 µl of firefly luciferase substrate from the firefly luciferase dispenser into a recovery tube. NOTE: firefly luciferase can be stored at -80 °C and reused.

5.7.2) Prime each dispenser with 1000 µl of distilled water, followed by 1000 µl of 70% ethanol, and finally 1500 µl of air (remove dispensers from liquid).

6. Data Analysis

6.1) Data export

6.1.1) In the microplate reader software, double click on "Report/Export Builders" on the left side of the screen.

6.1.2) Click on the button "New Export to Excel" and click "OK".

6.1.3) Highlight Export1 and click "Edit".

6.1.3.1) Under "Content" check "System Description", "Procedure", "Plate Description", and "Plate Layout Matrix". Include "Raw Data" and "Calculated Data".

6.1.3.2) Under "Workflow", check "Autoexecute on completion of the procedure". Under Export Mode, check "All plates in the same workbook" and "As a new worksheet".

6.1.3.3) Under "File" choose file name format and file location, and click "OK".

6.1.3.4) Close the "Report/Export Builders" window.

6.2) To obtain normalized luciferase values, divide the firefly luciferase luminescence reading for each well (step 5.6.3) by the *Renilla* luminescence reading for each well (step 5.6.6).

REPRESENTATIVE RESULTS:

A primary screen tested 328 ORs against 26 odors at a concentration of 100 µM. This odor concentration has been demonstrated to effectively activate a large proportion of ORs with known ligands.[10] First, normalized luciferase activity was calculated by dividing the firefly luciferase reading by the *Renilla* luciferase reading. Next, baselined values were calculated by subtracting the normalized luciferase readings for the no odor control from the normalized

luciferase readings for each well (Figure 1). Dose response curves were performed on 48 odorant/OR pairs randomly distributed across the range of baselined values, as indicated by colored bars in Figure 1. ORs were treated with 7 concentrations of odorant spanning 1 nM to 1 mM, and the resulting responses were fit to a sigmoidal curve using nonlinear regression. An odorant/OR was considered an agonist if it met three criteria: (1) the standard error of the logEC50 was less than 1 log unit; (2) the 95% confidence intervals for the top and bottom parameters of the curve did not overlap; and (3) the extra sums-of-squares test confirmed that the odorant activated the OR-containing cells significantly more than the control cells, which were transfected with an empty vector. Dose response results are summarized in Table 3.

These data were then used to determine how well assay measurements in a primary screen predict results from the dose response curve. Blue bars in Figure 1 correspond to pairs that were classified as agonists in a full dose response experiment, while red bars did not meet our three criteria outlined above. Values from the primary screen predicted results from the full dose response experiment (area under the receiver operating characteristic curve (AUC) = 0.68, $p < 0.01$, Mann-Whitney U test), indicating that our primary screen is a useful method to enrich for odorant/OR pairs that will be classified as agonists in a full dose response experiment (Figure 2).

Figure Legends:

Figure 1. Frequency of baselined luciferase values for a screen with a panel of olfactory receptors and odorants. Histogram of the frequency (Count) of baselined luciferase values calculated for each odorant/OR pair in the primary screen. As odorant/receptor activation pairs are sparse, the majority of the values are centered at zero and the large central distribution estimates the noise distribution for this assay. Colored bars indicate odorant/receptor pairs chosen for dose response analysis; blue bars are pairs that were classified as agonists based on the full dose response, and red bars are pairs that were not classified as agonists.

Figure 2. ROC curve for the odorant/receptor screen. 48 odorant/receptor pairs were classified as being agonists or as not being agonists. True positive rate (sensitivity) was then plotted against the false positive rate (1-specificity) using the R statistical package.[40] The area under the curve (AUC) is 0.68, indicating that odorant/receptor pairs with higher luciferase screen values are more likely to pass dose response than those with lower values.

Table 1. Plasmid mix components. Per well and per 96-well plate volumes of RTP1S-pCI, M3-R-pCI, pCRE-luc, and pSV40-RL, and MEM.

Table 2. Complex components. Per well and per well + 10% volumes of plasmid mix (Table 1), olfactory receptor plasmid (Rho-OR-pCI), and lipid transfection mix. M10 is added to quench the reaction following a 15 minute incubation at room temperature.

Table 3. Olfactory receptor/odor pairs tested in dose response. Baselined luciferase values and dose response results (pass or fail) for 48 OR/odor pairs chosen from the screen. For 30 pairs tested in the screen twice, both baselined luciferase values are included.

DISCUSSION:

Odorant identity is encoded by olfactory receptor activation patterns, but receptor activation patterns, including which receptors are activated and to what degree, are known for fewer than 6% of human olfactory receptors.[1–11] Efforts to characterize olfactory receptors have been limited by their labor-intensive methods or applicability to only a subset of the olfactory receptor family.[17,23,24,33,34] The Hana3A heterologous expression system supports the robust expression of the majority of tested olfactory receptors, and can be used in conjunction with a cAMP-responsive luciferase reporter system to monitor olfactory receptor activation.[19,26,27] Performance of this assay in a 96-well format supports a number of high-throughput experimental designs, including screens to determine likely candidates for odorant/olfactory receptor pairs and dose-response curves to confirm interactions and assess how receptor activation levels are affected by intra- and interspecific variations. Odorant/receptor pairs with higher activity values in a screen are more likely to demonstrate a significant dose response. These data suggest that this screening method is able to enrich for odorant/receptor pairs that will pass dose response, thereby facilitating the identification of odorant ligands and olfactory receptor activation patterns.

The success of this assay optimized for olfactory receptor analysis is dependent on several factors. All plasmid DNA must be prepared via an endotoxin-free protocol. Consistent olfactory receptor expression at the cell surface is critical. The Hana3A cell line stably expresses several accessory proteins that aid in OR expression, but co-transfection of RTP1S and M3-R enhances receptor expression and activation, respectively.[27] This combination of accessory protein expression results in the reliable expression of most olfactory receptors, allowing the comparison of OR activation among experiments and receptors. In addition, monitoring of cell confluency is important for obtaining consistent results. Assuming the cells in the original $10cm^2$ dish are roughly 100% confluent, following the protocol described herein will result in reliable cell confluency throughout the experiment. Importantly, sufficient cells will be plated to obtain a measurable luciferase reading, but cells will not be over-grown, a condition which may affect receptor activation following odorant stimulation. Normalizing for constitutive renilla expression further controls not only for cell density, but also for transfection efficiency. A renilla luciferase reading more than 2.5 standard deviations below the mean may indicate cell loss. Cells should be plated uniformly to avoid dense plaques that detach more easily from the plate surface than sparser cells, and transfection and odorant solutions should be added gently to the side of the well to avoid detaching cells. Cell loss could also be due to cell death caused by odorant toxicity, a problem that may be circumvented by lowering odorant concentration, or excessive DMSO, which can be avoided by keeping DMSO concentrations below 0.5%. Finally, treating each receptor-expressing cell population with 1 μM forskolin, an adenylyl cyclase activator that causes luciferase reporter expression from the cAMP-responsive promoter, can serve as a positive control for the assay.

Although the assay described herein represents an improvement over alternative methods, including a high-throughput format and more general applicability to the mammalian olfactory receptor family, it has limitations. First, our *in vitro* assay lacks many components of an *in vivo* olfactory system, including odorant binding proteins, a mucosal layer, intracellular molecules and sniffing behaviors. Second, this method relies on a luciferase reporter system to measure olfactory receptor activation in contrast to common alternative methods that utilize calcium

imaging. Recent work suggests that these two methods can produce conflicting results; indeed, a few olfactory receptors respond to a particular odorant when examined via calcium imaging but not luciferase assay.[41] Whether one assay type is more relevant to studies of human olfactory perception remains unclear, but both methods could be useful depending on context and receptor type. Third, while this functional expression system has successfully been used to express the majority of tested mammalian olfactory receptors, some ORs may not be amenable to expression using this system. If previously uncharacterized receptors fail to respond to an odor, it may be due to lack of expression at the cell surface rather than a lack of interaction between odorant and receptor. Receptor cell surface expression can be examined via immunofluorescence before drawing conclusions from negative assay results.[27,42] Finally, due to low background luciferase activity in no-odor conditions, our assay is not designed to detect inhibitory responses. To determine odor antagonists for olfactory receptors,[38,39] most receptors must first be stimulated with an odor in order to observe a reduction in luciferase activity.

Despite these limitations, this assay system has the ability to greatly increase data acquisition in the field of olfaction. First, the high-throughput 96-well format makes large-scale receptor and/or odor screens feasible. Second, its heterologous expression system is applicable to a variety of mammalian olfactory receptors. Third, luciferase activity can be used to measure olfactory receptor activation, which is valuable in describing the receptor activation patterns for a particular odorant. Fourth, previous results from similar *in vitro* assay systems predict human olfactory perception.[8,11,35] These characteristics are particularly important given the large size of the mammalian olfactory receptor family and our limited knowledge regarding the OR activation patterns elicited by specific odors. Broad application of this assay system optimized for olfactory receptor analysis will contribute to a more comprehensive picture of olfactory receptor/odorant interaction and the molecular basis of odor coding.

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DISCLOSURES:

The authors have nothing to disclose.

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*Figure

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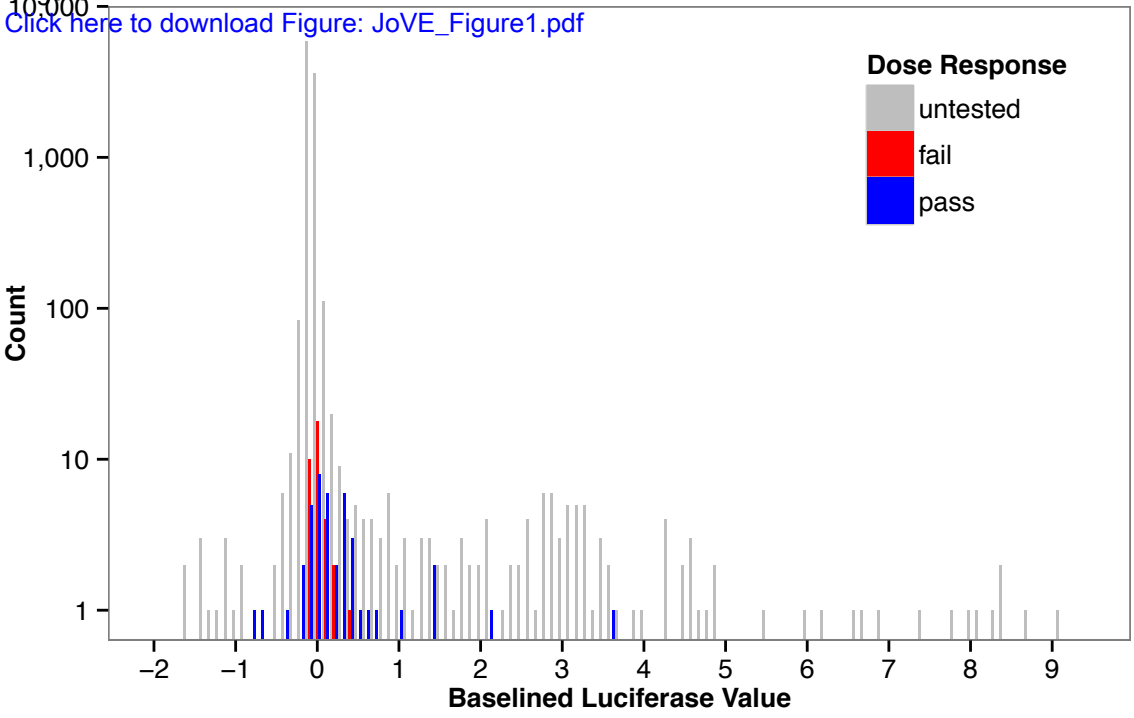

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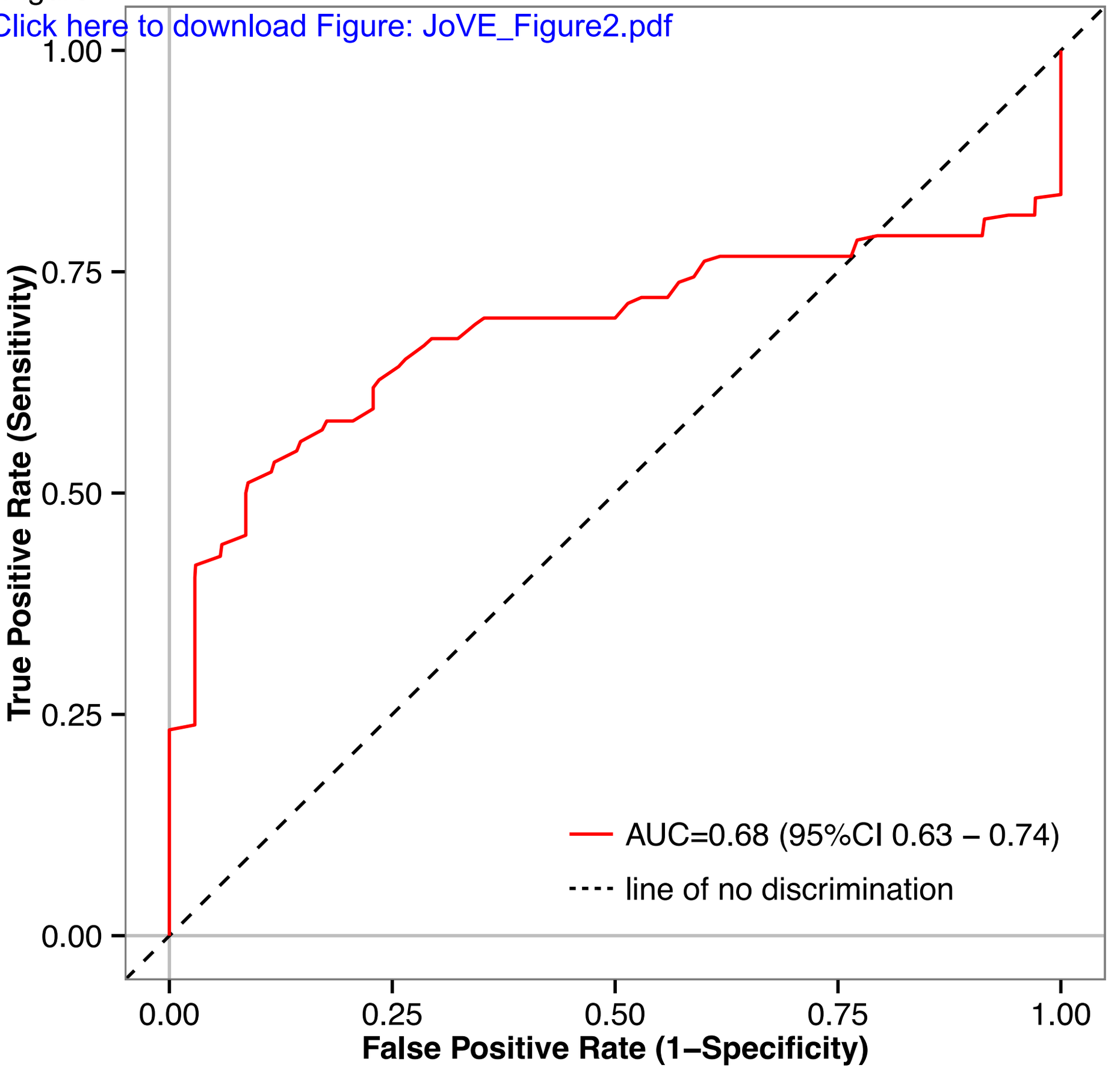

Table 1

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Plasmid mix		
	per well	**per 96-well plate**
MEM		500 µl
RTP1S-pCI	5 ng	480 ng
M3-R-pCI	2.5 ng	240 ng
pCRE-luc	10 ng	960 ng
pSV40-RL	5 ng	480 ng

Table 2

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Complex		
	per well	**per well+10%**
Plasmid mix	4.2 µl	4.58 µl
Rho-OR-pCI	0.05 ng	0.06 ng
Lipid transfection mix	4.2 µl	4.58 µl
M10	41.7 µl	45.83 µl

Table 3
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Baselined Value	Dose Response
0.051793067	fail
0.006376956	fail
0.331936398	pass
0.591006519	pass
0.049093369	pass
0.396788976	pass
-0.013655743	pass
0.011080217	pass
0.004203349	fail
0.003975049	fail
-0.077935718	pass
-0.084488317	pass
0.030236078	fail
-0.042963576	fail
0.031466406	fail
0.025897747	fail
-0.030434651	fail
-0.004122795	fail
-0.010075533	fail
0.028883452	fail
0.019402373	fail
0.047508749	fail
0.00255344	fail
0.017221449	fail
0.340216655	pass
-0.026912181	fail
0.037140428	fail
0.467763017	pass
0.097665337	fail
0.080657267	pass
0.172819211	pass
0.05568393	pass
-0.106721064	pass
0.136614849	pass
0.457839849	fail
0.211751741	fail
0.1581464	pass
-0.62099155	pass
-0.066949491	pass
-0.78712035	pass

0.752503007	pass
1.433407558	pass
0.475431098	pass
1.457936815	pass
0.048652537	fail
0.027196782	fail
0.129599842	fail
-0.069781272	fail
0.016450039	fail
-0.025639207	fail
0.158152141	fail
-0.032570055	fail
0.140139926	fail
-0.052030276	fail
0.657140133	pass
1.040410297	pass
0.164647156	pass
0.399588712	pass
0.188094387	pass
0.039371424	pass
0.016784352	pass
0.229959571	pass
0.238381997	fail
0.074118909	fail
0.423901128	pass
0.152621022	pass
-0.109048046	pass
0.075301806	pass
0.395233972	pass
0.261892958	pass
0.156693306	fail
2.163418147	pass
3.649862104	pass
0.025716169	pass
-0.033258008	pass
-0.026984127	fail
-0.338441868	pass
0.37398618	pass

*Excel Spreadsheet- Table of Materials/Equipment
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Hana3A cells	Avaiable from the Matsunami Laboratory upon request		
RTP1S-pCI	Avaiable from the Matsunami Laboratory upon request		
M3-R-pCI	Avaiable from the Matsunami Laboratory upon request		
pCRE-luc	Agilent	**219076**	LUC
pSV40-RL	Promega	**E2231**	RL
Minimum Essential Media, Eagle	Sigma Aldrich	M4655	MEM
FBS	Life Technologies	16000-044	FBS
PBS (without Ca2+ and Mg2+)	Cellgro	21-040-CV	PBS
Trypsin (0.05% Trypsin EDTA)	Life Technologies	25300	Trypsin
CD293	Life Technologies	11913-019	CD293
96 well PDL white/clear plate	BD BioCoat	356693	plates
Lipid transfection reagent: Lipofectamine 2000	Life Technologies	11668-019	Lipofectamine
Firefly luciferase substrate, firefly luciferase quencher/Renilla luciferase substrate: Dual-Glo Assay	Promega	E2980	dual glo
Synergy S2	BioTek	SLAD	BioTek S2
Microplate reader software: Gen5 Data Analysis Software	BioTek	Gen5	Gen5
BIOSTACK	BioTek	BIOSTACK2WR	BioStack
Multiflo	BioTek	MFP	MultiFlo
300ul GripTips	Integra	4433	GripTips
12.5ul GripTips	Integra	4414	GripTips

300ul GripTips ViaFlo96	Integra	6433	XYZ tips
12.5ul GripTips 384 XYZ	Integra	6403	XYZ tips
384ViaFlo	Integra	6030	384ViaFlo
TE buffer	Macherey Nagel	740797.1	
DMSO	Sigma Aldrich	D2650-100ML	DMSO
forskolin	Enzo Life Sciences	BML-CN100-0010	FOR

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Dear Dr. Cherny,

Enclosed please find our revised manuscript. Although the referees were largely positive concerning the manuscript, one describing it as a "detail and accurate procedure that… allows anyone stated in art to reproduce the work," they also had significant comments. We thank the referees for their comments, with which we concurred. In accordance with these comments, we have significantly revised the manuscript.

Reviewers' comments:

Reviewer #1:
Minor Concerns:
1. Under Section 1 (Culture of Hana3A cells) of the Protocol, the authors should specify the type and frequency of antibiotics used to maintain the stable cell line.
We have added a note in this section explaining that although these cells are resistance to puromycin, we do not find that selecting with this antibiotic significantly affects the results of our assay.

2. In Discussion, Line 366, note that coexpression of M3 is not known to significantly enhance the cell surface expression of olfactory receptors.
The wording of this sentence was changed to: "co-transfection of RTP1S and M3-R enhances receptor expression and activation, respectively."

3. In Discussion, Line 375, the authors mentioned that "Low renilla luciferase readings (roughly 150) may indicate cell loss." Again, this could be an arbitrary number that is specific to the plate reader used. Low readings could be better defined as mean minus a certain number times standard deviation.
"roughly 150" has been removed from the text, as this number is indeed dependent on the plate reader being used. We've changed the text to note that an RL value more than 2.5 standard deviations below the mean may indicate cell loss.

Reviewer #2:
Major Concerns:
1- A paper describing a method that is highly related to this method has been recently published in JoVE (Assaying surface expression of chemosensory receptors in heterologous cells. (Dey S, Zhan S, Matsunami H.) J Vis Exp. 2011 Feb 23;(48). The authors should mention this article and discuss the differences and advantages of the present method.
Dey et al. describe a method to assess the cell-surface expression of olfactory receptors; in contrast, here we describe a method to measure receptor activation. This reference is indeed quite relevant as a complement to this manuscript and has been added on page 9 where we suggest checking cell-surface expression of the OR if it fails to respond in this assay.

2- In terms of materials required for the protocol, it is important to know from where the Hana3A cells can be obtained.
We have changed the Material list to state that the cells can be obtained through the Matsunami laboratory upon request.

3- In table 2, the amount of OR plasmid in ng m(mass) should be indicated (not in <mu>l).
Table 2 has been updated.

4- Figure 1: what is plotted in the y axis? Explain in the legend.
The y-axis of figure 1 represents the count of the number of wells that fall in a particular bin range. Text has been added to the legend of Figure 1 to reflect this.

Minor Concerns:
Line 153: should specify what kind of pipette is used to triturate cells. Done.
Line 156: 15 ml conical tube. Done.
Line 60: incubate overnight at 370C with 5% CO2? Done.
Line 166: explain what endotoxin free protocol means. A note has been added to this step.
Line 193: incubate overnight at 370C 5% CO2. Done.
Line 202: in CD293 Medium. Done.

Reviewer #3:
Manuscript Summary:
Title: strictly speaking, the manuscript does not deal with any quantification of the number of receptor expressed per cell,.. nor of the receptor activation overwise quantification of the concentrations levels of..) cAMP should be included by means of calibration curves,...This is not the case and hardly achievable with gene reporter assays. Thus the use of .. quantification.. in the tittle is somewhat misleading.
The title has been changed to "High-throughput analysis of mammalian olfactory receptors: measurement of receptor activation via luciferase activity."

Major Concerns:
The manuscript intends to propose a screening system that would allow the identification of activators (agonists) for mammalian olfactory receptors (OR).
The subject is of interest since, as emphasized by the authors, the lack of information about the range of activation of ORs in mammalian species (including human) hampers the development of an accurate modeling of odor perception. This is clearly explained in the introduction.

A detail experimental protocol is provided that describes how to express OR in a dedicated cell line (Hana3A), how to challenge these ORs with potential agonists and how to record specific activation of the ORs tested. This experimental process has already been described in Zhuang and Matsunami, 2007 and 2008 and in Li and Matsunami, 2011. The authors provided here a detail and accurate procedure that, in combination with the "Material/Equipment" list, allows anyone stated in art to reproduce the work. Some minor comments about the protocol are provided below .
In the section "Representative results", the authors describe a screening of 26 odors on 328 ORs that is supposed to fish out new OR/odorant couples. Each odorant is tested at a single concentration of 100 µM. The way results are normalized and baselined is well explained. Figure 1 discloses the frequency distribution of baselined values from the screeing (comments on the correctness of Figure one are provided below). Likewise, the procedure of validation by dose-response experiments and the criteria of acceptation of an agonist for an OR are well stated.

Nevertheless, the procedure of selection of OR/odorant couples to be validated is only vaguely evoked while it is a central step in screening process. Indeed, the screening method and analysis should be conceived to limit possible cases of : 1) the selection of too many false positives for validation process which represent a waste of time and means, especially when

the screening endows a large array of OR tested with library of several hundreds of compounds and 2) the non-selection of true activators (false negatives). To address the first point, a set of 48 odorant/OR pairs randomly distributed are selected for a validation with dose-response experiment. The true positives emerging this analysis are disclosed on Figure 1 with blue bars, but this representation is not well readable.
The central purpose of the manuscript is to relate the protocol for collecting data from a high-throughput luciferase assay. The sample data is one of many experiments that can be conducted using this protocol.

Because many of the baselined luciferase values chosen for dose response analysis are concentrated around 0, it is difficult to graphically represent these results and have them be easily visible. We have included a table (Table 3) of baselined values with corresponding dose response results to facilitate the viewing of this data. Also, we have modified Figure 1 to make the baselined values more readable.

Based on this validation, a ROC curve is computed (see comment C13 below) and intend to show that odorant/OR pairs from the screening that have a higher luciferase value are more likely to be true agonists (as validated by dose-response experiments). This is rather obvious and is a ROC analysis required to show it ? Unfortunately, what is not clearly provided here, neither by the ROC analysis nor by the baselined value distribution, is a well-defined procedure to determine a threshold for the selection of odorant /OR couples to be validated. After having read the manuscript, we still don't know how to do this selection, although it is a crucial step in a screening process. This is a major objection to the present work. Authors should absolutely address this issue by proposing a clear procedure for selecting couples to be validated.
We have chosen to include an ROC curve in this manuscript in order to allow the reader to determine the best threshold for his/her own experimental needs. For example, choosing a very high threshold baselined luciferase value such as 1 should theoretically result in OR/odor pairs that will all pass dose response (DR), but will exclude many OR/odor pairs that will also pass DR. Choosing an extremely low cutoff value such as -1 means you will not miss any OR/odor pairs that will pass DR, but you will also test many pairs that will fail DR. The appropriate tradeoff between type I and type II errors will depend on the specific requirements of your experiment. The ROC curve allows the reader to tailor their experimental design and threshold choice when presented with data from an actual screening experiment that illustrates the chances of OR/odor pairs with particular baselined values passing dose response. Again, the central focus of the manuscript is on the technique for collecting data, not on the analysis of the data in the context of a single sample experiment.

Concerning the second point (false negatives), it has not been discussed or evoked in the manuscript. However, the figure 1 seems to show (although it is not very clear from the figure ; a table with the baselined values and the "pass" or "fail" statute for the 48 couples would have been more readable) that roughly half of the true positives have a baselined value close to or under 0. Since values at 0 or under mean no difference or lower value obtained when stimulating the OR with the odorant compared to OR incubated in an odorant-free medium. It means that the screening method described here is not very robust for detecting true OR activation. This is a major objection.
Table 3 with baselined luciferase values and dose response results has now been added to the manuscript in order to facilitate the visualization of this data. We have also modified Figure 1 to make the baselined values more readable. While it is true that a number of OR/odor pairs that passed dose response had baselined luciferase values around 0, a greater number of OR/odor pairs

that failed to pass dose response had baselined luciferase values around 0. Obviously, this screening method is not a perfect way to determine which OR/odor pairs will pass dose response, but the ROC curve shows that luciferase values from a screen allow for the significant enrichment of OR/odor pairs that go on to respond in dose response.

It could possibly be due to the fact only a single concentration of 100 µM of odorant is tested. This concentration of 100 µM could be too low to detect the activation of a particular OR by a given odorant. Indeed, there is a nice series of examples of dose-response curves of OR/odorant couples given in Saito et al. (2009 ; cited by in the present manuscript) for which no activation is seen at 100 µM but is well detected for higher concentrations. Authors should at least consider verifying that false negatives from their screening correspond to that type of OR/odorant couples. By the way, no justification of the choice of this concentration is given in the text. In High throughput screenings for non-olfactory GPCRs, a concentration of 100 µM is often used, but accumulating results on ORs pharmacology tend to show that OR/odorant couples have higher EC50 value (i.e. are less sensitive) than those usually found for non-sensory GPCRs. From this and from the results presented in figure 1, it can be suspected that the 100 µM concentration is not appropriated for the screening and higher concentrations should be considered (e.g. 1 mM). Ideally, more than one concentration should be used in the screening process. If authors have performed screenings with a higher concentration, they should disclose it here. Otherwise, they should consider doing it.
We chose this particular concentration based on the data in the Saito et al. paper. While a screening concentration of 100 µM may miss some of the reported receptors, it remains a good compromise at which a large proportion of receptors (though admittedly not all) can be activated, while still being a reasonable concentration to use in large screens.

We have added a justification of this odorant concentration to the manuscript.

Editorial comment:
[Please keep JoVE's protocol guidelines and length requirements in mind while addressing reviewer comments(use short steps, imperative tense, proper spacing, etc).]

The discussion is well constructed although some assertions required to be clarified (see comments below).

Minor Concerns:
Minor, specific comments (in order of their occurrence in the text).
C1. In the long abstract (p1 lne 37) it stated "Odorant ligands have been published for fewer than 6% of human receptors". Authors should either state that this count is from the scientific literature (published articles in peer reviewed journals) or take into account deorphanization published in patents and abstracts in addition. According to a recent counting, at least 44 human ORs would have found at least one agonsit. Taking into account a set of 400 hORs, it represents more than 10%, a more positive view on human deorphanization statute.
References have been added to the abstract to reflect how we made our calculation.

C2. In introduction (p2, line 83), "the dimensions of olfactory stimulus" is an unusual concept. What do the authors mean?
The wording has been changed to state our meaning more clearly.

C3. In Protocol (p3, line 132) : could the authors mention where Hana3A can be obtained ? It is not said in the Material/Equipment list.
We have changed the Material list to state that the cells can be obtained from the Matsunami laboratory upon request.

C4. In protocol, (p4, line 156), it is not very clear that 1 ml of cells will serve to the seeding of one 96 well plate. An alternative formulation such as "2.5) For the seeding of one 96 well plate, transfer 1 ml of cells into a 15 ml conical tube..."
The wording of step 2.5 has been changed to be clearer.

C5. (p4, line 160) 50 µl instead of 50 ul. Done.

C6. (p3, line 170) a proper confluency of 30-50% is not in agreement with recommendations provided for lipofectamine 2000 as transfection agent. Indeed, a 90-95% of confluency at the time of transfection is mentioned as an important guideline in the user manual.
A note has been added to the manuscript explaining that this confluency is optimal for this assay system.

C7. (p4, line 175) The vector backbone for RTP1S and M3-R is not mentioned. Same comment for olfactory receptor plasmid (same page, line 183)
We have changed the text to note that all plasmids are in the pCI backbone.

C8. (p5, line 197) What if the cells are not at the expected confluence. Do the authors recommend to discard the plate ?
A note has been added regarding the importance of the confluency.

C9. (p5, line 209) the starting concentration for dose response is 10 µM but it said in the "Representative Results" section that the range of the dose response is from 1 nM to 1 mM. Authors, please correct it. Done.

C10. (p6, line 275) The step 5.7.2 is identical to step 5.4.2 + step 5.4.3. Why doing it twice?
The dispensers in the plate reader need to be cleaned both before reading (5.4.2, 5.4.3) and after reading (5.7.2). Also please note that the order of liquids being primed is slightly different between the steps.

C11. Representative Results (p7). It is not clear how the screening is done. Is it a different OR in each well of the 96 well plate? or there only one OR per plate ? or 3 ORs per plate tested each with a batch of 26 odorants ?
Step 4.3.1 in the Protocol notes that for screening experiments, each OR/odor pair is tested only once per experiment. We've added a note describing that the preferred method of screening is one odorant per plate due to potential odor diffusion.

C12 (p8, line 331) "that" is repeated twice. Done.

C13 (p8, line 333) The way the ROC curve and AUC value are computed is not mentioned.
Text has been added to the legend for Figure 2 indicating that the R statistical package was used to make the ROC curve and calculate AUC.

C14. In discussion, (p8, line 353) it is stated : "the majority of olfactory receptors". This is misleading since there is no report of an expression study where all (or a majority) of mammalian or even human ORs would have been tested in Hana3A. It should be more correct to say "the majority of tested olfactory receptors" (that in fact represents a very small portion of the whole human or mouse olfactome).
The text has been changed to reflect this comment.

C15. (p8, line 358) ...interspecific variations (with a "s"). Done.

C16. (p9, line 381) The receptor is not treated with forskolin. I've never heard about an activation of an OR with this AC activator... It would be more correct to say that "... assay performance can be assessed by stimulating each cell population expressing an OR with 1 µM forskolin..."
The text has been changed to reflect this comment.

C17. (p9, line 395) It would probably be more appropriated to say " while this functional expression system" instead of "while this transfection system" since it is not only transfection that is important here.
The text has been changed to reflect this comment.

C18. (p9, line 396) same comment as C14. The text has been edited.

C19. (p9, line 399) Is there an obvious link between cell surface expression and functional expression (especially when it is assessed with the luciferase assay) ?
Yes, if a receptor is shown to respond to an odor, it is being functionally expressed at the cell surface. However, a lack of response may indicate that the receptor is unable to respond to odor or that the receptor is unable to be expressed at the cell surface via this assay system. This is applicable only to receptors that have not been previously shown to respond in this system, as mentioned in the text. We feel it is important to mention this limitation to readers.

C20. (p9, line 400) It would make sense to seek for antagonist using a unstimulated OR only if a physiologically relevant high constitutive activity of the OR can be demonstrate. In addition, due to the nature of the gene reporter assay, a high basal activity would result in an accumulation of luciferase even before addition of the antagonist. Therefore, there are very few chances to see a significant reduction of luciferase production during the 4 hours of the assay with antagonist. If the conclusion that one needs to stimulate the OR with a cognate agonist in order to find antagonist is quite true, I do not think it is a matter of floor effect.
The text has been changed to reflect this comment.

C21. Figure 1. The legend of the Y axis mentions "Log(Count)". If so, a value at 1000 on this scale would mean 101000. That's quite a lot! Normally, there must be no more than 8528 counts?
Figure 1 has been modified to make viewing the distribution of baselined luciferase values easier. The y-axis now represents counts of baselined luciferase values, and the axis title and figure legend have been changed.