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.