Decoding the olfactory map: targeted transcriptomics link olfactory receptors to glomeruli

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TITLE:

Decoding the olfactory map: targeted transcriptomics link olfactory receptors to glomeruli

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26

27 **ONE SENTENCE SUMMARY:**

28 Targeted transcriptomics of olfactory receptor mRNA in olfactory bulb sections determines
29 spatial positions for glomeruli.

30

31 **ABSTRACT:**

32 Sensory processing in olfactory systems is organized across olfactory bulb glomeruli,
33 wherein axons of peripheral sensory neurons expressing the same olfactory receptor co-
34 terminate to transmit receptor-specific activity to central neurons. Understanding how
35 receptors map to glomeruli is therefore critical to understanding olfaction. High-throughput
36 spatial transcriptomics is a rapidly advancing field, but low-abundance olfactory receptor
37 expression within glomeruli has previously precluded high-throughput mapping of receptors to
38 glomeruli. Here we combined sequential sectioning along the anteroposterior, dorsoventral,
39 and mediolateral axes with target capture enrichment sequencing to overcome low-
40 abundance target expression. This strategy allowed us to spatially map 86% of olfactory
41 receptors across the olfactory bulb and uncover a relationship between OR sequence and
42 glomerular position.

43

44 **MAIN TEXT:**

45 The organization of axonal projections from olfactory sensory neurons (OSNs) in the
46 olfactory epithelium (OE) to glomeruli on the olfactory bulb (OB) forms the olfactory map¹⁻⁴. In
47 the mouse, each canonical OSN expresses a single olfactory receptor (OR) or trace amine-
48 associated receptor (TAAR) allele from a repertoire of over 1000 OR and TAAR genes.
49 Insights into the organization of the olfactory map were first obtained using in situ
50 hybridization, where OR transcript probes indicated the convergence of OSN axons into

discrete structures called glomeruli on the OB surface, which range from 50 to 120 μm in diameter^{3,5,6}. Later, this organization was more clearly visualized through the use of gene-targeted mouse lines, which demonstrated that glomeruli are formed from the axonal convergence of OSNs expressing the same OR gene⁷. Together these studies established the convergence of homotypic OSN axons to stereotyped glomeruli whose positional variability ranges from 75 to 270 μm depending on OR identity^{5,8}. Because each glomerulus represents a single OR and a single odorant can bind multiple ORs, odor signals detected in the OE are transformed into a map of glomerular activity on the OB^{9–13}.

To date, glomerular positions for less than 5% of mouse ORs are available, and further progress has been stymied due to the low-throughput, laborious, and time-consuming aspects of currently available methodologies for mapping each OR in the expansive murine repertoire^{7,8,13–29}. Furthermore, the ability to compare locations between multiple glomeruli is limited among these studies due to the lack of reference landmarks on the OB and differences between methodologies. A more efficient approach for mapping OR axon projections to OB glomeruli would serve to generate a more comprehensive and informative map that would serve as a foundation for further functional studies of odor coding and processing. In this study, we demonstrate that target capture enrichment on sequential samples from the OB enables detection of low-abundance OR and TAAR mRNA in the axon termini of OSNs. Using this approach, we map 86% of the 1118 ORs and TAARs along the anteroposterior, mediolateral, and dorsoventral axes and combine these data to generate a three-dimensional model of glomerular positions with a precision of 141 μm . We examine the relationship between OR sequence and OB position, identify the set of ORs and TAARs expressed within dorsal glomeruli accessible to functional imaging, and generate gene-targeted mouse lines for two dorsal glomerular ORs amenable to functional characterization in vivo.

RESULTS

Targeted capture consistently enriches OR transcripts

Previous studies have detected low levels of OR mRNA in OSN axon terminals, identifying the glomerular positions for specific ORs within histological sections^{5,23,27,30}. To quantify OR and TAAR transcripts in the OB we first performed conventional RNA-Seq on whole-OB tissue from a mouse at postnatal day 21, an age when olfactory glomeruli are fully developed and finalized in their stereotyped positions^{31,32}. Quantification of 25.7 million reads identified 410/1118 (36.7%) intact ORs at an average abundance of 0.06 transcripts per million (TPM) (median OR TPM = 0, 6/15 (40%) TAARs with TPM above 0, mean TAAR TPM = 0.077, median TAAR TPM = 0) (Fig. 1, B and C), confirming the low abundance of OR mRNA in OSN axon terminals^{30,33}.

To enrich sampling for OR transcripts, we designed a target capture array against chemosensory receptor gene families primarily targeting ORs and TAARs³⁴. We applied this target capture array to the previously sequenced OB library and identified 842 of the 1118 ORs (75.3%) and 10 of the 15 TAARs (66.7%) (Fig. 1, A to C). Following targeted capture, these ORs were present in a set of 27.7 million reads at an average abundance of 360.27 TPM (median = 106.74 TPM, mean TAAR TPM = 871.66, median TAAR TPM = 64.62) resulting in a mean fold enrichment of 6005X (mean TAAR fold enrichment = 11320X) (Fig. 1, B and C). Spearman's rho for OR and TAAR transcript abundances between uncaptured and captured samples was 0.71 ($P < 2.2 \times 10^{-16}$) (Fig. 1B). Further, four sets of independently captured technical replicates from two different OBs (two distinct spike-in mRNAs for RNA subsamples from each of the two bulbs with two technical replicates per subsample) exhibited a Spearman's rho of 0.95 ($P < 2.2 \times 10^{-16}$) (Fig. 1D and fig. S1D). The fold enrichment and correlation between pre- and post-capture samples indicates the targeted capture approach enriches the majority of ORs and TAARs in a highly consistent fashion as evidenced by the

technical replicate correlation. The mean pairwise Spearman's rho for three biological replicate OBs was 0.83 ($P < 2.2 \times 10^{-16}$), indicating the relative abundance of OR and TAAR transcripts is conserved between individual animals (Fig. 1E and fig. S1E).

In summary, targeted capture consistently enriched OR and TAAR transcripts to levels that facilitate positional analysis. This encouraged us to conduct targeted capture of ORs and TAARs from sections of OB to determine which ORs were expressed in each section. We sectioned from three directions, dorsoventral (DV), anteroposterior (AP), and mediolateral (ML). We note that these three axes are not precisely orthogonal and not perfectly concordant with the corresponding reference features such as DV zonal boundaries and medial surface of the OB.

Expression of ORs and TAARs in dorsoventral OB sections correlates with OE expression zones

Pioneering investigation established that ORs are expressed in overlapping, continuous zones of the OE along the DV axis^{35,36}. This zonal OE organization further correlates with DV glomerular positions of OR expression in the OB^{7,23,37}, with more recent studies leveraging multiplexed assays and transcriptomics to map an expanded number of ORs to more specific OE zones^{38,39}. To comprehensively assess the relationship of OE-OB DV zonal organization of OR expression, we collected 100 μ m sequential sections along the OB DV axis for targeted transcriptomics to determine which ORs were expressed in each section. Canonically, each OR is expressed in two glomeruli per bulb, both of which are expected to be in similar positions along the DV axis. If enriched OR sequences are from OSN axon terminals, we expect that each OR would be abundant in spatially clustered sections which reflect the OE DV position from which the axons originate.

After weighting and normalization across individual mice, the localization pattern of each OR and TAAR was limited to a single spatial cluster in a series of neighboring sections for a majority of the capture-enriched transcripts (Fig. 2, A, B, and C). Uniform Manifold Approximation and Projection (UMAP)⁴⁰ visualization of data from the three DV replicate mice (22 sections per replicate) placed sequential sections from replicate animals in an ordered, non-clustered path, indicating that spatially related sections have similar transcriptional profiles (Fig. 2D). Replicate heatmaps were similar to each other (mean $RV_{adj} = 0.1803$), which supports the stereotyped targeting of glomeruli to local domains^{41,42}. To assess concordance of OB and OE positions along the DV axis, we constructed an expression-weighted mean DV position for each OR from the average of all three DV replicate mice, which we found correlated with the published OE DV positions of each OR (Spearman's rho of mean position and OE index = 0.775, $P < 2.2 \times 10^{-16}$, Spearman's rho = -0.690, $P < 2.2 \times 10^{-16}$) (Fig. 2E and fig. S2A)^{38,39}. Along the DV axis, we found dorsal OE ORs, Class I ORs, and TAARs primarily located in the dorsal OB sections, while Class II ORs were distributed evenly along the DV axis, in agreement with previous mapping studies (dorsal vs ventral $P = 4.292 \times 10^{-95}$, Class I vs Class II $P = 5.912 \times 10^{-20}$, TAAR vs OR $P = 0.0001$, Mann-Whitney U-test) (Fig. 2F)²⁹.

Anteroposterior spatial sections reflect stereotyped targeting of mirror-symmetric glomeruli

Using the same approach as for the DV axis, we examined 100 μ m sections along the AP axis of the OB to further assess the precision and reproducibility of our method and the stereotypical patterning of OR glomeruli. Prior studies have shown that each OR typically has two glomeruli located in distinct, yet spatially linked AP and mediolateral (ML) positions, as each OB is organized into half bulbs along a non-orthogonal symmetry line^{8,43,44}. This

150 symmetry typically leads to more posterior positioning of the medial glomerulus for an OR.
151 However, in cases where the target location of an OR is close to the symmetry line, both
152 glomeruli may appear in the same AP plane or only a single glomerulus may form⁴⁵. Based
153 on these studies, we hypothesized that a majority of ORs would exhibit a bimodal expression
154 pattern along the AP axis.

155 Spatial expression patterns for 966 ORs and 14 TAARs, measured by the normalized
156 transcript abundances, were consistent across replicates with stereotyped AP glomerular
157 positions across OBs^{8,46}. When sorted by position of mean expression, ORs primarily
158 displayed two peaks of expression, consistent with published studies for labeled ORs
159 displaying the medial glomerulus in a more posterior location relative to the lateral glomerulus
160 (Fig. 3, A and B, and fig. S3A)⁸. Compared to the distribution of normalized OR expression
161 across the DV axis, ORs along the AP axis were distributed bimodally (Fig. 3C). Similar to the
162 DV axis, UMAP projections of gene expression values from the six AP replicate mice (23
163 sections per replicate) revealed correlated expression patterns and AP positions across each
164 OB (Fig. 3D). Across the AP axis, we found Class I ORs biased to the anterior set of sections,
165 while TAARs tended to be localized to the central portion of the axis (Class I vs Class II $P =$
166 2.017×10^{-26} , TAAR vs OR $P = 0.7882$, Mann-Whitney U-test) (Fig. 3E), consistent with
167 previous studies examining glomeruli labeled in gene-targeted mice^{29,47}. We also examined
168 our data for concordance against the set of 32 ORs cloned from the anterior, middle, and
169 posterior sections of an OB from Nakashima et al.⁴⁸. The ORs cloned from the anterior and
170 middle OB ($n = 13$) on average occupied a significantly more anterior position in our data than
171 ORs cloned from the posterior OB ($n = 18$) (anterior + middle OB cloned ORs vs posterior OB
172 cloned ORs $P = 0.001$, Mann-Whitney U-test) (Fig. 3F and fig. S4A). We further divided these
173 cloned ORs across dorsal OE ($n = 11$) and ventral OE ($n = 20$) zones and found that both sets
174 displayed concordance with our AP data, with ORs cloned from the anterior and middle OB

175 positions having a lower AP mean position than ORs cloned from the posterior OB (dorsal
176 OE: anterior and middle OB cloned ORs vs posterior OB cloned ORs $P = 0.1636$, ventral OE:
177 anterior and middle OB cloned ORs vs posterior OB cloned ORs $P = 0.0117$, Mann-Whitney
178 U-test) (fig. S4, B and C). Our high-throughput mapping of OR positions along the OB AP axis
179 not only displays concordance with the largest available dataset of AP positions, but expands
180 the available information from 32 to 966 ORs.

181

182 ***Relationship of OR sequence and glomerulus position***

183 Gene-targeting approaches have identified a couple of examples in which closely
184 related ORs target to nearby^{49,50}. Our dataset with a majority of ORs assigned to specific AP
185 positions allowed us to systematically interrogate whether ORs with similar sequences exhibit
186 similar glomerular positions by computing pairwise alignments for all ORs. To assess this
187 relationship in all dimensions, we additionally generated a ML dataset (3 replicates, 22
188 sections per replicate) (fig. S5, A to E). Due to the combined presence and sequence diversity
189 of Class I ORs, Class II ORs, and TAARs on the dorsal surface of the OB, we separated our
190 analysis into three groups: 1) Class I dorsal OB ORs, 2) Class II dorsal OB ORs, and 3) Class
191 II ventral OB ORs. To assess OR similarity, we calculated pairwise protein sequence identities
192 and pairwise distances between the mean expression position for ORs along each dimension
193 and compared the results for OR pairs using percent identity cutoffs corresponding to the
194 level of homology used to classify OR genes as belonging to a family (40%) (fig. S6A)⁵¹.
195 When comparing OR pairs above and below the family-level threshold, we found both Class II
196 dorsal and ventral ORs above the family level threshold displayed significant lower mean
197 interglomerular distances across the AP, DV, and ML axes, suggesting topological relationship
198 between the glomerular positions and family-level OR similarities for Class II ORs (Fig. 4A). In
199 contrast, Class I ORs did not display a consistent relationship between sequence similarity

200 and mean expression position along the DV and ML axes (Fig. 4A and Table S6). We further
201 examined this relationship by comparing ORs using the 60% (subfamily level ORs) and 80%
202 (highly similar) protein sequence identity thresholds^{51,52}. Comparisons between family (40-
203 60%), subfamily (60-80%), and highly similar ORs (>80%) revealed that both dorsal and
204 ventral Class II OR glomerular positions along all axes typically become more similar or do
205 not change with increasing sequence similarity (Fig. 4B). Class I ORs as a whole displayed
206 largely non-significant differences in position except for comparisons against highly similar
207 ORs along the DV axis (Fig. 4B). Altogether our comprehensive analysis generally agrees
208 with a model in which overall similarities of ORs influence the relative glomerular locations.

209 We next sought to determine if any specific OR amino acid residues correlated with AP
210 position. Due to the different relationships between sequence similarity and OB position for
211 dorsal and ventral ORs, we examined Class II ORs using different cutoffs for the sets of the
212 most anterior and most posterior ORs included in the analysis (20%, 27.5%, 35%) to identify
213 amino acid residues correlating with AP position (Fig. 4C and fig. S7A, B, C). We identified 22
214 residues whose physicochemical properties differed from all ventral Class II ORs (Fig. 4D and
215 fig. S8C and S9)⁵³. Notably, four consecutive residues that correlated with AP position were in
216 the third intracellular loop, which has been shown to interact directly with the G protein during
217 Class A GPCR activation (Fig. 4, D to F, and fig S8, A and B)⁵⁴. Additionally, the phenylalanine
218 within the KAFSTC_xSH motif is sandwiched between four residues involved in G-protein
219 binding⁵⁵⁻⁵⁷. These findings indicate residues that are at or near the sites of G-protein
220 interactions are critical in determining glomerular position, which is consistent with the
221 hypothesis that ligand-independent basal activity of ORs influences glomerular
222 targeting^{45,48,58-60}.

223

224 ***A three-dimensional model of OR glomerular positions reflects established features***

225 To date, a systematic 3D model of OR glomerular positions has yet to be made for a
226 majority of the OR repertoire, but our high-throughput sequencing of spatial sections along
227 the 3 cardinal axes may be combined to achieve such a result. UMAP plots of the mean
228 position of ORs in each dimensional replicate indicated that ORs that are clustered on the OB
229 surface are also clustered within the UMAP positions (Fig. 5A and fig. S10, A and B).

230 To account for the location of OR glomeruli on the outer surface of the OB, we
231 extracted coordinates from a diffusion tensor imaging (DTI) model of the mouse OB to
232 represent the approximate glomerular layer that would have been sampled by each section
233 along each dimension of our targeted transcriptomics data⁶¹. We based our 3D reconstruction
234 in a statistical approach to integrate our orthogonal sequencing data to determine which
235 voxels on the OB surface where the probability for a given OR is highest^{62,63}. We then
236 developed an algorithm for the systematic reduction of positional probabilities across the
237 whole OB surface into predicted OR glomerular positions. Lastly, we filtered out predictions
238 with posterior median values below 0.0005 to account for ORs with low expression. Posterior
239 median summaries for the resulting set of 709 ORs and TAARs predicted glomeruli positions
240 as well as the raw positional probabilities for all 980 ORs and TAARs in all voxels are freely
241 viewable as interactive 3D maps at kanazian.shinyapps.io/obmap/.

242 We assessed our algorithm by comparing predictions for Class I and II ORs, dorsal and
243 ventral OE ORs, and the set of ORs examined via transgenic mouse lines by Zapiec et al.⁸.
244 Predicted glomerular positions for Class I ORs, Class II ORs, and dorsal and ventral ORs
245 were consistent with expectations based on OE zone, OR class, and our single-dimension
246 data (dorsal vs ventral $P = 9.188 \times 10^{-98}$, Class I vs Class II $P = 5.96 \times 10^{-14}$, TAAR vs OR $P =$
247 2.682×10^{-6} , functional imaging surface enriched vs not-enriched $P = 1.75 \times 10^{-24}$ (see
248 below), Mann-Whitney U-test) (Fig. 5, B, C and F and fig. S11, B and C). Additionally, the
249 distribution of predictions for the sets of Class I ORs and TAARs matched previously

published findings for target domains (Fig. 5C and fig. 11B). Our current model predicts glomerular positions for 700 ORs and 9 TAARs, with predicted positions outperforming randomly selected ORs from the same DV zone and medial/lateral side for the subset of ORs with known positions with a median error of 141 μm (Fig. 5, D and E)⁸. The relative positions of the Olfr1377 and Olfr881 glomeruli in the gene-targeted mouse lines (see below) were also consistent with those predicted from the spatial transcriptomic data (Fig. 5D and figs. S11D, S12D, S13 and S14), providing further support for our three-dimensional glomerular predictions based on the spatial transcriptomic data. In summary, we found our three-dimensional reconstruction of OR glomerular positions to be both in agreement with established OR spatial features and to show greater concordance with these established features than the sets of single-dimension target capture sequencing data alone. Collectively, our results thus provide the first large-scale, unified, and systematic database of OR glomerular positions for the mouse OB.

263

264 ***Identification of ORs within the dorsal functional imaging window***

265 We sought to validate and extend our findings by examining specific ORs that map to
266 glomeruli on the dorsal-central OB surface, which has been extensively characterized by
267 functional imaging in vivo^{9,11,12,46,64}. To define the set of ORs accessible under standard
268 functional imaging approaches, we collected tissue samples from OBs from C57BL6 mice (2
269 male, 2 female). Each OB was dissected into two parts, one containing the approximate
270 dorsal-central imaging area and the other containing the remainder of the OB (fig. S12A).
271 These 16 samples were processed for target capture sequencing and differential expression
272 analysis to identify ORs enriched in the functional imaging area.

273 A total of 121 ORs, including 27 Class I ORs and 94 Class II ORs, were consistently
274 enriched in the imaging surface ($\text{FDR} \leq 0.05$ and $\text{LogFC} > 0$) (Fig. 5B, Fig. S12, B, E and F),

275 with 96% of these ORs known to localize in dorsal OE zones³⁸. We also found nine of the 15
276 TAARs enriched in the imaging surface, with no TAARs enriched in the remaining OB tissue
277 (fig. S12G), consistent with previous functional imaging of some TAAR glomeruli⁴⁷. To
278 anatomically and functionally validate our expression analysis, we chose two ORs as targets
279 for the generation of receptor-tagged gene-targeting mouse lines, based on their enrichment
280 in the functional imaging area (Olfr881: FDR = 0.007, LogFC = 2.55 and Olfr1377: FDR =
281 0.021, LogFC = 2.14) (fig. S12B) and their robust response to the odorant 4-
282 methylacetophenone in pS6-IP RNA-Seq experiments (Olfr1377: FDR = $3.43e^{-26}$, LogFC =
283 3.18, Olfr881: FDR = $7.37e^{-23}$, LogFC = 3.18) (fig. S12C)⁶⁵. Using *Easi*-CRISPR⁶⁶, we
284 inserted *IRES-mKate2* cassettes following the CDS of each OR to create Olfr1377-IRES-
285 mKate2 and Olfr881-IRES-mKate2 mice, in which OSNs expressing either Olfr1377 or
286 Olfr881 also express the cytosolic fluorescent marker mKate2, labeling cell bodies in the OE
287 and glomeruli in the OB (fig. S12D). Whole-mount confocal imaging of the OB in gene-
288 targeted mice revealed mKate2-labeled glomeruli within the dorsal-central OB surface (Fig.
289 6A and fig. S13, A and B).

290 Examination of additional whole-mount epifluorescence images allowed us to further
291 assess position and variance of both Olfr1377 and Olfr881 (fig. S14, Fig. 5C). Consistent with
292 the singular expression of most ORs as two mirror-symmetric glomeruli, both gene-targeted
293 mouse lines labeled two glomeruli per OB. The lateral Olfr1377 glomerulus (n = 10) was
294 positioned centrally along on the AP axis and central-laterally within the ML axis imaging area,
295 while the medial glomerulus (n = 6) was more posterior, ventral, and medial. The lateral
296 Olfr881 glomerulus (n = 9) was positioned centrally along the ML axis and relatively posterior
297 within the imaging area, while the medial glomerulus (n = 8) displayed a more variable
298 position across the medioposterior quarter of the dorsal surface. The lateral Olfr1377
299 glomerulus displayed nearly twice (196.8%) the positional variance than the lateral Olfr881

glomerulus while the medial Olfr1377 glomerulus was distributed in an area nearly half the size (52.8%) of its Olfr881 counterpart.

Functional imaging of dorsal ORs

Expression of long-wavelength mKate2 as an OR-specific marker allowed for functional characterization of Olfr1377 and Olfr881 glomeruli by crossing the generated mouse lines to OSN-specific driver lines expressing a GCaMP Ca^{2+} reporter. For maximal imaging sensitivity, we crossed each mKate2 line to the OMP-IRES-tTA driver line⁶⁷ and the tetO-GCaMP6s reporter line⁶⁸. In the resulting triple crosses, we readily located the lateral mKate2-tagged glomeruli on the dorsal functional imaging surface (Fig. 6A) and imaged odorant-evoked GCaMP6s signals from these and neighboring glomeruli using dual-wavelength two-photon imaging in anesthetized mice. Consistent with our pS6-IP in vivo data (fig. S12C), both Olfr1377 and Olfr881 exhibited robust responses to low concentrations of 4-methylacetophenone, with Olfr1377 exhibiting a stronger response than Olfr881 (Fig. 6, B and C). In addition to 4-methylacetophenone, we also tested a large odorant panel including multiple cyclic ketones structurally related to 4-methylacetophenone, as well as more diverse odorants, all at relatively low concentrations. From this panel, we identified multiple new, high-affinity ligands for each OR, including many cyclic ketones, and ultimately uncovered overlapping but distinct response spectra for Olfr1377 and Olfr881 (Fig. 6C, fig. S15C). For example, Olfr1377 showed strong responses to p-anisaldehyde, acetophenone, and the aliphatic ketone 4-methyl-3-penten-2-one, while Olfr881 proved unresponsive to these odorants.

Interestingly, Olfr1377 (but not Olfr881) exhibited an exceptionally strong response to 4-methoxyacetophenone, with a brief (2 s) presentation of ~0.4 nM 4-methoxyacetophenone eliciting long-term activation and desensitization (fig. S15, A and B). Additional concentration

325 screening suggested an in vivo response threshold of $\sim 10^{-13}$ M 4-methoxyacetophenone for
326 the Olfr1377 glomerulus (Fig. 6D and fig. S15D). To complement our in vivo imaging and
327 pS6-IP RNA-Seq analyses and further evaluate the sensitivity of Olfr1377 to 4-
328 methylacetophenone and 4-methoxyacetophenone, we additionally expressed Olfr1377 in
329 Hana3a cells and examined luciferase responses to different concentrations of 4-
330 methoxyacetophenone, 4-methylacetophenone, 2'-hydroxyacetophenone, and
331 acetophenone^{69,70}. Reinforcing our in vivo results, Olfr1377 responded to all four odorants
332 (Fig. 6E). Moreover, Olfr1377 responded to 4-methoxyacetophenone at just 1 nM and
333 saturated at 100 nM in vitro, a response orders-of-magnitude more sensitive than the
334 response to any other odorant tested. Collectively, these findings identify 4-
335 methoxyacetophenone-Olfr1377 as a ligand-receptor interaction with exceptionally high
336 affinity.

337

338 **DISCUSSION**

339 A central goal of neurobiology is to understand how sensory systems interpret the external
340 world⁷¹. The visual and auditory sensory maps feature topographic maps which organize and
341 process stimuli such as light position and sound frequency. In vertebrates, the organization of
342 the olfactory topographic map remains largely unknown, and understanding how OR
343 glomeruli are organized can reveal how inputs are transformed into output signals for the
344 olfactory cortex as well as which aspects of olfactory input are important^{72,73}. In *Drosophila*,
345 glomeruli have been individually linked to specific ORs, allowing for a comprehensive
346 understanding of how sensory information is organized within the first olfactory relay^{73–75}. This
347 key map has served as a foundation for critical studies regarding sensory processing,
348 olfactory receptor neuron-targeting factors, the propagation of information to higher-order
349 neurons, and the cellular composition of the antennal lobe^{76–79}.

350 Here, we demonstrate a unique application of target capture sequencing, enriching low-
351 abundance target transcripts found in the axon terminals of OSNs and comparing post-
352 enrichment abundances across samples. Our high-throughput approach enabled us to
353 generate the first repertoire-scale dataset for OR glomeruli positions. This approach also
354 allowed us to create a three-dimensional positional estimate for the glomeruli of a majority of
355 the OR repertoire with precision within the ranges previously observed for positional variability
356 between animals^{5–8,23}.

357 With a systematic, anatomical mapping of OR identities to OB glomeruli, we can begin to
358 understand which features of sensory information impact organizational strategies utilized
359 within the mouse OB, the central circuit of olfaction. We identified functional odor-OR
360 relationships at the peripheral interface using the in vivo pS6-IP RNA-Seq deorphanization
361 approach. By identifying the set of ORs within functionally imaged glomeruli future studies will
362 be able to determine how OB circuits transform peripheral input into neural representations of
363 odors at the OR level. Additional gene targeted mouse lines amenable to functional imaging
364 will also enable us to better understand interglomerular connectivity and how patterns of
365 inhibition are established between nearby glomeruli. The combination of our newly generated
366 OR-OB positional data with ligand-receptor deorphanization data will therefore enable large-
367 scale investigations into how the odor space relates to topographical features in the OB and
368 will facilitate the development of unified, systematic models for how odorants are processed,
369 interpreted, and transformed into behavior.

370

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390

391 **AUTHOR CONTRIBUTIONS:**

392 Conceptualization: KWZ, MW, HM

393 Methodology: KWZ, SDB, JDS, MW, HM

394 Investigation: KWZ, SDB, MHN, MW, HM

395 Visualization: KWZ, SDB, MHN, CAdM, MW, HM

396 Funding acquisition: KWZ, SDB, MW, HM

397 Project administration: MW, HM

398 Supervision: MW, HM

399 Writing – original draft: KWZ, SDB, MW, HM

400 Writing – review & editing: KWZ, SDB, MHN, JDS, CAdM, MW, HM

401

402 **COMPETING INTERESTS:**

403 HM has received royalties from Chemcom. HM has received research grants from Givaudan.

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406

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Fig. 1. Target capture sequencing consistently enriches OR transcripts. (A)

Methodological overview for targeted enrichment of OR and TAAR transcripts from OB sections. Briefly, RNA is extracted from OB tissue and used for cDNA synthesis and library preparation. Capture probes are designed against coding sequences (CDS) of interest, enabling enrichment of target genes following binding and washing steps. RNA-Seq of enriched libraries allows for high-throughput positional analyses when combined with systematic tissue sectioning. (B) Pre- and post-capture normalized abundances (transcripts per million; TPM) of intact OR and TAAR genes (red) and other intact genes (black) from a whole OB. (C) Frequency distribution of OR gene abundances pre- and post-capture from a whole OB. (D) Technical replicates demonstrating OR and TAAR gene abundances from independent capture enrichments of the same whole-OB RNA. (E) Biological replicates demonstrating OR and TAAR gene abundances from capture enrichment of whole-OB RNA

620 samples from different animals.

Fig. 2. Glomerular OR expression correlates with OE expression along the DV axis. (A)

623 Heatmaps for 980 ORs and TAARs across 22 DV sections sorted by mean position of
624 expression from three replicate mice. **(B)** Merged representation of DV replicates. Ordering of
625 genes (Y axis) is consistent across all heatmaps. **(C)** Distribution of normalized TPM
626 (maximum observed value = 1, minimum observed value = 0) for all 980 ORs and TAARs
627 from position of mean expression. **(D)** UMAP projection of 22 DV sections from all three
628 replicates. **(E)** Line is loess smoothed regression of OE DV index from Tan and Xie, *Chem.*
629 *Senses* 2018 across DV mean positions from our targeted spatial data. **(F)** Distribution of
630 ranked DV mean positions for the 980 ORs and TAARs by OE zone, OR class, and gene
631 family. Statistic is Mann-Whitney U-test.

Fig. 3. Glomerular OR expression is bimodally distributed along the anteroposterior

634 **axis. (A)** Heatmaps for 980 ORs and TAARs across 23 AP sections sorted by mean position
635 of expression from three replicate mice. **(B)** Merged representation from A. Ordering of genes
636 is consistent across all heatmaps. **(C)** Distribution of normalized TPM (maximum observed
637 value = 1, minimum observed value = 0) for all 980 ORs and TAARs from position of mean
638 expression. **(D)** UMAP projection of 23 AP sections across all six replicates. **(E)** Distribution of
639 ranked AP mean positions for the 980 ORs and TAARs by OR class and gene family. Statistic
640 is Mann-Whitney U-test. **(F)** Distribution of the ranked AP mean position for the set of ORs
641 cloned from the anterior and middle OB positions vs the posterior OB position from
642 Nakashima et al. *Cell*. 2013. Statistic is Mann-Whitney U-test.

643

Fig. 4. OR sequence similarity correlates with glomerular position more strongly

among ventral than dorsal Class II ORs. (A) Pairwise comparisons between mean position distance and OR protein sequence identity for ORs split by the family level homology cutoff ($\geq 40\%$ in orange, $< 40\%$ in black) for the AP (top), DV (middle), and ML dimensions (bottom). Statistic is Mann-Whitney U-test. **(B)** Pairwise comparisons between mean position distance and OR protein sequence identity for ORs split by the family, subfamily, and orthologous OR level homology cutoffs (40-60% in yellow, 60-80% in red, $\geq 80\%$ in magenta) for the AP (top), DV (middle), and ML dimensions (bottom). Statistic is Mann-Whitney U-test. **(C)** Phylogenetic

653 tree of all Class II dorsal OB ORs (top left, n = 354), all Class II ventral OB ORs (top right, n =
654 464), the most anterior (35%, blue) and most posterior (35%, red) Class II ORs from the
655 dorsal (bottom left) and ventral OB (bottom right) DV zones. **(D)** Snakeplot of the Class II OR
656 consensus protein sequence; orange residues have significantly different physicochemical
657 properties for ventral, anterior or posterior, Class II ORs compared to all ventral Class II ORs.
658 Green residues indicate residues known to be involved in Class A GPCR activation through
659 contact with the G protein (* indicates the single residue which was identified as being both
660 associated with G protein contact and identified as significantly different for ventral, anterior,
661 Class II ORs). **(E)** Protein sequence logos for the four identified intracellular loop 3 residues
662 associated with ventral Class II anterior/posterior ORs depicting the conservation of specific
663 amino acid residues at each position. Red arrows indicate the specific residue within the
664 Class II OR consensus snakeplot (C) and the corresponding position in the sequence logo.
665 **(F)** Homology model of the mouse Class II consensus OR. Transmembrane helices (TM) are
666 numbered with residues associated with A/P positions (orange) and residues in contact with
667 the G protein (green) depicted in licorice with transparent regions indicating the residue
668 surface. Right, highlight of intracellular loop 3 (ICL3) where residues associated with A/P
669 positions are intermingled with the residues in contact with the G protein.

670

672 **Fig. 5. A three-dimensional model for OR glomerular positions from combined single-**
673 **dimension targeted sequencing data. (A)** UMAP projections of OR populations constructed
674 using the mean position from each individual replicate from the AP, DV, and ML dimensions.
675 DV mean position color reflects the calculated average mean position from all DV replicates.
676 OE Index (Zapiec 2020) refers to the OE index established by Zapiec and Mombaerts *Cell*
677 *Reports* 2020. FI Surface refers to the differential expression analysis calculated from dorsal
678 and ventral OB samples in this paper. **(B)** Three-dimensional predictions for the 709 ORs and
679 TAARs with DV mean positions (top) and with observed values in OE DV indices (middle and
680 bottom). **(C)** Three-dimensional predictions for the 709 ORs and TAARs with colors revealing
681 contrasting distributions of Class I vs Class II ORs, functional imaging surface enriched vs not
682 enriched ORs, and TAARs vs ORs. **(D)** Three-dimensional predictions for labeled ORs
683 generated in this study and from Zapiec and Mombaerts *PNAS* 2015. High probability
684 positions indicate the set of adjacent voxels containing highly ranked probabilities for that OR
685 with the best probability color indicates the voxel with the highest ranked probability within
686 that cluster of voxels. **(E)** Distance between predicted OR position and OR positions
687 determined from gene-targeted mouse lines compared to 50 random ORs from the same DV
688 zone (15 ORs used for unusual zone) and side of the OB. Statistic is Mann-Whitney U-test.
689 **(F)** Distribution of ranked mean DV model position for the best probability voxel in each
690 predicted glomerulus for all 709 ORs and TAARs for OE zone, OR Class, and gene family.
691 Statistic is Mann-Whitney U-test.

Fig. 6. Deorphanization of Olfr1377 and Olfr881. (A) Whole-mount confocal maximal intensity projection of compound heterozygous Olfr1377-IRES-mKate2; OMP-IRES-tTA; tetO-GCaMP6s and Olfr881-IRES-mKate2; OMP-IRES-tTA; tetO-GCaMP6s mice following two-photon functional imaging. (B) Baseline fluorescence and GCaMP6s $\Delta F/F$ responses to 2-s presentation of 4-methylacetophenone during two-photon functional imaging of the heterozygous lines in (A). Estimated concentration of delivered odorant (in nM) provided in parentheses here and elsewhere. Olfr1377 response map scaled from 0-80% $\Delta F/F$. Olfr881 response map scaled from 0-30% $\Delta F/F$. (C) Spectra of Olfr1377 and Olfr881 glomerular $\Delta F/F$ responses (sorted by Olfr1377 response magnitude) to a subset of ligands detected within a larger odorant panel. Odorants presented at an estimated concentration on the order of 10^0 nM, with the exception of 4-methoxyacetophenone (~ 3.5 nM for Olfr881; $\sim 3.5 \times 10^{-3}$ nM for Olfr1377). (D) In vivo concentration-response function of the Olfr1377 glomerulus to 4-

705 methoxyacetophenone. (**E**) In vitro concentration-response function of the Olfr1377 receptor
706 to an array of cyclic ketone odorants.

707

708 **SUPPLEMENTARY MATERIALS:**

709 **DATA AVAILABILITY**

710 All sequencing data will be made publicly available under NCBI BioProject
711 PRJNA773191 upon publication. Additional raw and processed data that support the findings
712 of this study are available from the corresponding authors upon request.

713

714 **CODE AVAILABILITY**

715 Key code for the project has been made publicly available at
716 <https://github.com/kanazian/obmap>.

717

718 **MATERIALS AND METHODS**

719 ***Statistics***

720 All statistical tests were performed in R. All Mann-Whitney U-tests were two-tailed and
721 performed with continuity corrections using the wilcox.test function from the stats package.
722 Dunn's Kruskal Wallis test performed with Benjamini Hochberg false discovery rate correction
723 using the dunn.test function from the dunn.test package. All box and whisker plots display the
724 median value as the middle line, the first and third quartile as the hinges, and the farthest
725 values within 1.5 inter-quartile range of the first or third quartile as the middle line as the
726 whiskers. * indicates $p \text{ value} \leq 0.05$, ** indicates $p \text{ value} \leq 0.01$, *** indicates $p \text{ value} \leq 0.001$.

727

728 ***Animals***

729 Wild-type C56BL6 mice were purchased from the Jackson Laboratory and used for
730 breeding. Olfr1377-IRES-mKate2 and Olfr881-IRES-mKate2 mice were generated by
731 inserting *IRES-mKate2* cassettes downstream of the CDS of each OR using *Easi-CRISPR*⁶⁶.
732 OMP-IRES-tTA mice⁶⁷ were provided by C. Ron Yu (Stowers Institute, Univ. of Kansas). tetO-

733 GCaMP6s mice⁶⁸ were purchased from the Jackson Laboratory (Stock No. 024742). Mice
734 were group housed with food and water available ad libitum and kept on a 12-h-light/dark
735 cycle. All procedures for animal handling and tissue collection were approved by the
736 Institutional Animal Care and Use Committees of Duke University and the University of Utah.

737

738 ***Sample acquisition and sequencing library preparation***

739 Pups were sacrificed between postnatal day 20 and 22. Whole brains with olfactory
740 bulbs intact were dissected and placed in a solution of 3% Low Melting Point Agarose
741 (American Bioanalytical) within an embedding mold (Polysciences Inc, Peel-A-Way R30)
742 before placing on ice. Once solidified, the mold was removed and the agarose-embedded
743 brain was prepared for vibratome sectioning by cutting away agarose to leave a triangular
744 shape with the tip forming at the bulb on the surface that will be cut first for that specific
745 direction. The triangular block was glued (Loctite 404) onto the vibratome stand (Leica
746 VT1000S with Feather carbon blades), submersed in cold 1X HBSS (Gibco) and 100 µm
747 sections serially cut. Sections were placed into 1.5mL tubes and stored at -80C. 400 µL of
748 Buffer RLT (Qiagen) with 10% BME was added to each frozen tissue section before
749 homogenization at 30,000RPM for 4 seconds using a mounted Biogen PRO200 with 5mm flat
750 tip generator probe. The homogenizer probe was rinsed three times with DI water between
751 samples. RNA was extracted using a Qiagen RNeasy kit according to the manufacturer's
752 instructions. cDNA synthesis was performed using a SMART-Seq v4 (Takara) kit with 10 ng
753 total RNA used for input in half-sized reactions. 10 ng cDNA was used in the KAPA Hyperplus
754 library construction kit following the manufacturer's instructions. All quantifications were
755 performed using Qubit.

756

757 ***Target capture sequencing and alignment***

758   We selected a set of ORs, TAARs, V1Rs, and V2Rs for inclusion in our assay based
759 on the mouse genome annotation available at the time (GRCm38.p4 release M10). This set of
760 transcripts was submitted to Roche Nimblegen for inclusion in a targeted enrichment probe
761 panel. All sections from a single OB were uniquely indexed and combined in equal amounts
762 to create a 1000 ng pool which was processed for target capture according to the
763 manufacturer's protocol using a 20 hour hybridization period. Target capture library pools
764 were sequenced on an Illumina NextSeq 500 Sequencing System in the 75SR or the
765 HiSeq2500 in 50SR mode at the Duke Center for Genome and Biology shared core facility.
766 Snakemake v3.5.5⁸⁰ was used to process read files through alignment and quantification.
767 STAR v2.7.0d⁸¹ was used to generate a genome index using the primary assembly and
768 comprehensive gene annotation file of the mouse genome (Gencode GRCm38.p6 release
769 M25). Reads were aligned to this genome index using STAR with default options except for –
770 quantMode TranscriptomeSAM which maps genome alignments to transcript coordinates.
771 STAR output transcriptome SAM files were quantified using RSEM v1.3.1⁸² using default
772 options to generate gene level counts for differential expression.

773

774 ***Sample normalization***

775   TPM values for each sample from each replicate OB were weighted by a factor that
776 accounts for the proportion of OMP found in each sample and the approximate proportion of
777 the OB glomerular layer collected in the sample (see below: Three-dimensional model of OR
778 glomeruli positions). Weighted TPM was then normalized between 0 and 1 using the
779 minimum and maximum values for each OR. Merged heatmaps were generated using the
780 median normalized values at each position of the expression array for DV and ML heatmaps
781 (n = 3). For AP heatmaps, the merged representations was generated from the median of four
782 individual replicates along with the mean value of the four replicates at each position. Matrix

783 of median values were then renormalized for each OR across samples. The following ORs
784 were excluded from all analyses due to abnormally high expression values that potentially
785 indicate ectopic expression of the OR within OB cell types: Olfr287, Olfr32, Olfr361, Olfr1033.

786

787 ***Sequence and position analysis and identification of anterior and posterior OR***
788 ***associated residues***

789 Mouse OR protein sequences were downloaded from HORDE⁸³ and OR pairwise
790 alignments were computed using the pairwiseAlignment and pid functions from the R package
791 Biostrings using the BLOSUM62 substitution matrix, a gapOpening penalty of 11 and a
792 gapExtension penalty of 1. 1090 mouse OR protein sequences were aligned using Clustalx
793 with manual adjustments as previously published⁶⁵. To identify residues that were more
794 conserved by the 139 (30% set) most anterior, ventral, Class II ORs than by chance, we
795 simulated distributions of Grantham distances for random subsets of 70 (50% of size of set)
796 ventral Class II ORs for all pairwise combinations for each residue in the alignment. Random
797 sampling was performed 1000 times. The per-residue mean Grantham distances were
798 computed for all combinations of the 139 most anterior, ventral, Class II ORs and used to find
799 p-values under the null distribution after FDR correction. Alignment positions with gaps in
800 more than 10% of OR sequences were excluded from the analysis. Analysis was repeated for
801 all sets (20%, 27.5%, 35%) for both anterior and posterior Class II ORs. Visualization of the
802 structural sequence for Olfr539 were generated using GPCRsnakeplotter
803 (<https://github.com/Yue-Jiang/snakeplotter>). Sequence logos were created using WebLogo
804 (<http://weblogo.threeplusone.com/>) using default settings.

805

806 ***Homology model building***

807   The protocol follows a previously published method⁵⁷. Aligned protein sequences of
808 1092 mouse ORs were manually aligned to pre-aligned protein sequences of 11 GPCRS
809 including bovine rhodopsin (PDB ID 1U19), human chemokine receptors CXCR4 (PDB ID
810 3ODU) and CXCR1 (PDB ID 2LNL), and human adenosine a2A receptor (PDB ID 2YDV)
811 using Jalview⁸⁴. Four experimental GPCR structures (1U19, 3ODU, 2YDV, and 2LNL) were
812 used as templates to build the Class II mouse consensus OR by homology modeling using
813 Modeller. Five models were obtained and the one fulfilling several constraints (sufficiently
814 large binding cavity, no large folded structure in extracellular loops, all TMs folded as α -
815 helices, and a small α -helix structure between TM3 and TM4) was kept for further
816 visualization.

817

818 ***Differential expression analysis***

819   The R package, edgeR (v3.34.0), was applied to the sample count table for all genes
820 in the reference⁸⁵. Common, trended, and tagwise dispersions were estimated separately and
821 in the sequence listed. For identification of ORs in the functional imaging surface and ORs
822 responding to 4-methylacetophenone, results were subset to olfactory receptor genes
823 (pseudogenes included) and FDR values were recalculated from the p values obtained by
824 performing differential expression using all genes.

825

826 ***Three-dimensional model of OR glomeruli positions***

827   A DTI model of the mouse brain⁶¹ was converted from .nii to .stl filetype and loaded in
828 Blender. An array of cubes with the number of cubes per dimension matching the number of
829 sections collected from each dimension was stretched in each dimension to encompass the
830 entire OB in order to account for difference in age of mice. OB and cube structures were
831 exported as separate .ply files and imported into R where a custom script was used to

determine the position of cubes which contain OB surface polygons. Additional OB surface-containing cubes were included to remove gaps, model the medial surface, and account for a 100 μm wide glomerular layer within the outer surface of the OB. The final set of OB surface-containing cubes was exported as three-dimensional coordinates for use as a scaffold for the shape of the OB glomerular layer.

The composition of glomeruli in each voxel along the OB surface was calculated as the weighted average of the composition obtained from sequencing each OB section that intersected that voxel weighted by the total number of surface voxels in each section. To account for sequencing noise, the composition of glomeruli in each sequence was estimated using a Bayesian multinomial-Dirichlet model with a Dirichlet prior parameter of 0.65 reflecting the weak prior knowledge that each section likely contained only a subset of all possible glomeruli in the OB. For each sequenced section, 1000 posterior samples from this model were obtained and used in subsequent calculations of the glomerular composition in each surface voxel. Custom functions were applied to determine positional clusters of high probability voxels for each gene based on the Bayesian posterior probability and the expected DV location of the OR based on class and Tan and Xie *Chem. Senses*. 2018 index identity. Briefly, each gene was designated as dorsal, ventral, unusual zone, or non-OR based on gene name, OR class, OE position, and OB mean position. Based on this DV designation, the highest probability voxel cluster (minimum 3 adjacent voxels) from the corresponding DV hemisphere on each side of the line of symmetry was picked. The probability of the medial and lateral clusters was then compared and used to define the region of mirror symmetry for the cluster with the lower probability with the lower probability cluster being repicked from a mirror symmetry field across the line of symmetry. If the medial cluster had a higher probability, the lateral cluster was repicked from a field that was from the lateral half and ranging from 6 sections anterior to 1 section posterior of the medial cluster. If the lateral

cluster had a higher probability, the medial cluster was repicked from a field that was from the medial half and ranging from 6 sections posterior to 1 section anterior of the lateral cluster. Clusters that were found within 6 sections of the front or back of the model were repicked from fields between the end of the model and 6 sections inward. The probability of these two clusters was then compared to that of a single larger (5 adjacent voxels) cluster picked from a band of voxels running adjacent to the line of symmetry in order to process ORs whose proximity to the symmetry line has led to a single glomerulus or closely positioned glomeruli. If the probability of this symmetry line voxel cluster was greater than that of the best probability of the medial or lateral glomeruli, the symmetry line voxel cluster was chosen as the predicted position. If not, the split glomeruli were selected as the predicted position. ORs classified as being in the unusual zone in the OE were predicted as a single large cluster within the anterior, ventral, central face of the OB. Non-OR genes were predicted using the same method as dorsal ORs with only the top probability voxel cluster being returned.

Comparison against ORs with known glomeruli positions was performed by superimposing images of mapped positions from publications on a blank OB 3D model and recording AP, DV, ML coordinates for the boundaries of the depicted glomerulus. For each glomerulus of each OR with known information, we calculated all pairwise three-dimensional distances between the voxels within the predicted position and the depicted positions. Reported value was the minimum distance observed with any overlap between predicted and depicted position resulting in a value of 0.

pS6-IP RNA-Seq

Three P21 mice of either sex were used per odorant or control condition. Animals were individually habituated in sealed containers for 1 h, then transferred to a new container containing a piece of filter paper enclosed in a cassette (Tissue-Tek) spotted with 10 μ l of 4-

882 methylacetophenone diluted at 1% (v/v) in distilled water or with 10 µl of distilled water (no
883 odor control). After 1 hr of exposure, the animals were euthanized, then had their olfactory
884 epithelium dissected in cold 25 ml of dissection buffer (1 × HBSS +Ca²⁺, +Mg²⁺ [Gibco], 2.5
885 mM HEPES pH 7.4, 35 mM glucose, 100 µg/ml cycloheximide, 5 mM sodium fluoride, 1 mM
886 sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM beta-glycerophosphate). The
887 dissected tissues were transferred to 1.35 ml of cold homogenization buffer (150 mM KCl, 5
888 mM MgCl₂, 10 mM HEPES pH 7.4, 100 nM Calyculin A, 2 mM DTT, 100 U/ml RNasin
889 [Promega], 100 µg/ml cycloheximide, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1
890 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1x protease inhibitor [Roche]).
891 Bones were removed, and the tissue was mechanically dispersed three times at 250 rpm and
892 nine times at 750 rpm using a homogenizer (Glas-Col). The homogenates were centrifuged at
893 4600 rpm for 10 min at 4°C in a 1.5 ml Lobind tube (Eppendorf), then the supernatants were
894 collected into a new 1.5 ml Lobind tube and combined with a mixture of 90 µl 10% NP-40 and
895 90 µl 300 mM DHPC (Avanti Polar Lipids). The samples were centrifuged at 13000 rpm for 10
896 min at 4°C, then the supernatants were collected into a new 1.5 ml Lobind tube and incubated
897 with 20 µl pS6 antibody (Cell Signaling #5364) for 1.5 hr at 4°C under constant rotation.
898 During the last 30 minutes of incubation, 100 µl Protein A Dynabeads (Invitrogen) were
899 prepared by washing three times with 900 µl of wash buffer 1 (150 mM KCl, 5mM MgCl₂, 10
900 mM HEPES pH 7.4, 0.05% BSA, 1% NP-40). The samples were added to the beads and
901 incubated under constant rotation for 1 hr at 4°C, followed by four washes with 700µl of wash
902 buffer 2 (350 mM KCl, 5 mM MgCl₂, 10 mM HEPES pH 7.4, 1% NP-40, 2 mM DTT, 100 U/ml
903 recombinant RNasin [Promega], 100 µg/ml cycloheximide, 5 mM sodium fluoride, 1 mM
904 sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM beta-glycerophosphate). The
905 samples were moved to room temperature at the final wash, and the elution was performed

906 with 350 µl Buffer RLT (Qiagen). The RNA present in the eluate was purified with the RNeasy
907 Micro kit (Qiagen).

908

909 ***Heterologous cell expression***

910 The Dual-Glo luciferase assay was performed as described previously⁸⁴. Briefly,
911 Hana3A cells^{86,87} were plated on 96-well plates in MEM supplemented with 10% FBS. After
912 18-24 hr incubation at 37 °C and 5% CO₂, cells were transfected with 100 ng/µL plasmids
913 coding for Olfr1377 (5 µL/plate), muscarinic acetylcholine receptor M3R (2.5 µL/plate),
914 RTP1S (5 µL/plate), pRL-SV40 (5 µL/plate) and CRE-luciferase (10 µL/plate) using
915 Lipofectamine 2000 (Invitrogen). pCI vector (5 µL/plate) was used instead of Olfr1377 plasmid
916 for the transfection of control cells. 18-24 h after the transfection, the culture medium was
917 replaced with serial dilutions of the odorants in CD293 medium (GIBCO), then the plates were
918 incubated for 3.5 hours at 37°C and 5% CO₂ without the lid. Luminescence was measured
919 using a Polarstar Optima plate reader (BMG). Luminescence values (LV) were determined by
920 subtracting basal luminescence (of an empty 96-well plate), then dividing firefly luminescence
921 by renilla luminescence (to control for transfection efficiency and cell viability) as in the
922 formula: $LV = (\text{firefly luminescence} - 400) / (\text{Renilla} - 400)$. The LV values were normalized and
923 plotted using GraphPad Prism 9 by establishing the minimum LV = 0 and the maximum LV =
924 1 and using the analysis log(agonist) vs. response (three parameters). CAS numbers for
925 tested odorants are as follows: acetophenone - 98-86-2, 2-hydroxyacetophenone - 118-93-4,
926 4-methylacetophenone - 122-00-9, 4-methoxyacetophenone - 100-06-1.

927

928 ***Functional imaging***

929 Adult mice (2-3 months old) of both sexes were anesthetized and prepared for
930 functional imaging with 3 Hz artificial inhalation via a double-tracheotomy procedure as

931 previously described⁸⁸. Imaging was conducted using a resonant-scanning two-photon
932 microscope (Sutter Instruments) coupled to a pulsed Ti:Sapphire laser (Mai Tai HP, Spectra
933 Physics) tuned to 920 nm and a Fidelity-2 1070 nm laser (Coherent) and equipped with a 16X
934 0.8 N.A. water-immersion objective (Nikon), with emission separated by green (520/65 nm)
935 and red (641/75 nm) filters and collected by GaAsP photomultipliers (Hamamatsu H10770B).
936 Glomeruli were imaged at 15.5 Hz through thinned-bone windows. Odorants were presented
937 using a custom olfactometer⁸⁸ in pseudorandom order (except for 4-methoxyacetophenone
938 concentration series), with 2 s delivery duration and variable inter-delivery intervals. Odorants
939 were diluted in caprylic/capric medium chain triglycerides (C3465, Spectrum Chemical Mfg.
940 Corp.).

941 For response maps and spectra (Fig. 6B and C), $\Delta F/F$ responses were calculated as
942 the difference in mean fluorescence within 4 s following odorant onset from the 4 s preceding
943 odorant onset (the baseline fluorescence), divided by the baseline fluorescence. For display,
944 $\Delta F/F$ response maps were bilinearly interpolated by a factor of 2 and low-pass filtered
945 (Gaussian, standard deviation = 1 pixel) to improve map resolution and reduce map noise,
946 respectively. For the concentration-response function of the Olfr1377 glomerulus to 4-
947 methoxyacetophenone (Fig. 6D), ΔF responses were calculated as the difference in mean
948 fluorescence within 2.5 s following odorant onset from the baseline fluorescence. For the
949 lowest three odorant concentrations (in which responses fully decayed before subsequent
950 odorant presentations), baseline fluorescence was calculated as the mean fluorescence
951 within the 1 s preceding odorant onset. For the highest three odorant concentrations (in which
952 responses failed to fully decay before subsequent odorant presentation), ΔF responses were
953 calculated using the baseline fluorescence observed during the final 3.5×10^{-13} M odorant
954 presentation. All $\Delta F/F$ and ΔF responses are averaged across three odorant presentations.
955 All analysis was performed using custom code written in MATLAB (MathWorks).

956

957 ***Whole-mount visualization***

958   Adult mice of both sexes were transcardially-perfused with PBS followed by 4%
959 paraformaldehyde and postfixed overnight before brains were extracted. For whole-mount
960 confocal imaging, the ventral surface of the brain was adhered to a 35 mm culture dish to limit
961 movement, covered in PBS to maintain tissue hydration, and 10 μ m step z-stacks collected
962 using a FluoView FV1000 (Olympus) confocal microscope equipped with 5X air objective
963 (Olympus). Image analysis and maximal intensity projections were performed in ImageJ. For
964 mapping glomerular position from whole-mount preparations, OBs from postfixed brains were
965 removed intact and epifluorescence images of the dorsal and, subsequently, medial surfaces
966 of each OB captured at 5x magnification, with each surface positioned roughly perpendicular
967 to the imaging axis. Images were stitched manually in Photoshop before registration of OBs
968 and glomerular position mapping.

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Fig. S1. Targeted capture sequencing is consistent for enrichment of OR genes. (A)

Pre- and post-capture normalized abundances of intact probed genes (red) and intact non-

probed genes (black) from a whole OB. **(B)** Pre- and post-capture normalized abundances of

intact vomeronasal (Vmnr) genes (red) and intact non-Vmnr genes (black) from a whole OB.

(C) Pre- and post-capture normalized abundances of target gene families. **(D)** Technical

978 replicates of OR and TAAR gene abundances from independent capture enrichments using
979 two different whole-OB RNA samples (OB4, OB5) combined with different ERCC spike-in
980 mixtures (A, B). **(E)** Biological replicates of OR and TAAR gene abundances from
981 independent capture enrichments of three different whole-OB samples (OB1, OB2, OB3).

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Fig. S2. Dorsoventral OB spatial sections correlate with known OE positions. (A) Loess smoothed regression of OE DV index from Zapiec and Mombaerts, *Cell. Reps.* 2020 across DV mean positions from our targeted spatial data.

986

987 **Fig. S3. ORs display bimodal distributions along the anteroposterior axis. (A)** Heatmaps
988 for 980 ORs and TAARs across 23 AP sections sorted by mean position of expression from
989 three additional replicate mice.

Fig. S4. Mapped AP positions are consistent with published AP data. (A) Scatter plot of 31 ORs present in our AP dataset examining the relationship between ranked mean AP position and in vitro activity rank from Nakashima et al. *Cell*. 2013. Color indicates the reported OB position from which the OR was cloned from. (B) Data from B separated by OE DV zone. (C) Data from B, grouped. Statistic is Mann-Whitney U-test.

Fig. S5. Mapping of mediolateral positions. (A) Heatmaps for 980 ORs and TAARs across 22 ML sections sorted by mean position of expression from three replicate mice. (B) Merged

999 representation of A. Order of Y-axis genes is consistent across all heatmaps. **(C)** Distribution
1000 of normalized TPM (maximum observed value = 1, minimum observed value = 0) for all 980
1001 ORs and TAARs from position of mean expression. **(D)** UMAP projection of 66 ML samples
1002 from all three replicates. **(E)** Distribution of ranked ML mean positions for the 980 ORs and
1003 TAARs by OR class and gene family. Statistic is Mann-Whitney U-test.

Fig. S6. Relationship of OR protein sequence and OB position. (A) Scatter plot of mean position distance and OR protein sequence identity for ORs. Dashed lines indicate family (40% in yellow), subfamily (60% in red), and highly similar (80% in magenta) OR level

homology cutoffs for the AP (top), DV (middle), and ML dimensions (bottom).

Fig. S7. Anterior and posterior ORs along the dorsal and ventral OB. (A) Phylogenetic trees for anterior sets of Class II dorsal OB ORs (blue, left), posterior sets of Class II dorsal OB ORs (red, middle), and combined sets of dorsal anterior and posterior Class II OB ORs (right). Sets of different sizes (top row = 20% most anterior/posterior ORs, $n = 70$; middle row = 27.5% most anterior/posterior ORs, $n = 97$; bottom row = 35% most anterior/posterior ORs, $n = 123$) used as conditions for identifying significantly different residues as compared to

1016 Class II dorsal OB ORs. **(B)** Phylogenetic trees for anterior sets of Class II ventral OB ORs
1017 (blue, left), posterior sets of Class II ventral OB ORs (red, middle), and combined sets of
1018 ventral anterior and posterior Class II OB ORs (right). Sets of different sizes (top row = 20%
1019 most anterior/posterior ORs, n = 92; middle row = 27.5% most anterior/posterior ORs, n =
1020 127; bottom row = 35% most anterior/posterior ORs, n = 162) used as conditions for
1021 identifying significantly different residues as compared to Class II dorsal OB ORs. **(C)**
1022 Distributions of dorsal and ventral anterior and posterior ORs by OR family as classified in
1023 Olender et al. *BMC Evol. Bio.* 2020 for dorsal OB ORs (left) and ventral OB ORs (middle).
1024 Distribution of all Class II dorsal and ventral ORs by OR family is depicted in the top right.

Fig. S8. Position of anterior and posterior associated residues within ventral Class II ORs. (A) Snakeplot of the Class II OR consensus protein sequence with residues color coded as being associated with glomerular AP position (orange), involved in G protein contact (green), involved in the OR activation mechanism (yellow), and located in the odorant binding cavity (blue). Residues filled with one color and bordered in a different color were associated with both categories. (B) Homology model of the mouse Class II consensus OR. Left, residues associated with AP positions (orange) are depicted in licorice with transparent regions indicating residue surface. Right, transparent regions represent the surface of

residues located in the extracellular (EC) loops (gray), located in the odorant binding cavity (blue), associated with the activation mechanism (yellow) and associated with G protein binding (green). (C) Snakeplot of the Class II OR consensus protein sequence with blue shaded residues were identified as having significant different physicochemical properties for ventral, anterior, Class II ORs compared to all ventral Class II ORs. Red shaded residues were identified as having significantly different physicochemical properties for ventral, posterior, Class II ORs compared to all ventral Class II ORs. Residues highlighted in green indicate mammalian OR residues known to be involved in Class A GPCR activation through contact with the G protein (* indicates the single residue which was identified as being both associated with G protein contact and identified as significantly different for ventral, anterior, Class II ORs).

A

Fig. S9. Sequence logos for selected ventral Class II anterior and posterior residues.

Protein sequence logos for positions associated with ventral Class II anterior OR residues (left six columns) and ventral Class II posterior OR residues (right four columns) depicting the conservation of specific amino acid residues within different sets of sequences. Red arrows indicate the specific residue within the Class II OR consensus snakeplot (fig. S8, A and C) and the corresponding position in the sequence logo.

Fig. S10. Positional features are conserved when only mean position data is used. (A)

UMAP projections of OR populations constructed using the mean position from each replicate from all dimensions (top row), AP replicates only (second row), DV replicates only (third row), and ML replicates only (bottom row). AP, DV, and ML mean position reflects the calculated average mean position from each dimension. OE Index (Zapiec 2020) refers to the OE index established by Zapiec and Mombaerts *Cell Reports* 2020. OE Index (Tan 2018) refers to the OE index established by Tan and Xie *Chem. Senses* 2018 with values less than 2 labeled as Dorsal and equal or greater than 2 labeled as Ventral. FI Surface refers to the differential

expression analysis calculated from dorsal and ventral OB samples in this paper. **(B)** UMAP
projection of captured OB spatial samples constructed using transcript abundances for the
980 ORs and TAARs from all dimensions (left), separated by dimension (middle), and for the
order of section number by replicate from each dimension (right).

Fig. S11. A three-dimensional model for OR glomeruli positions from single-dimension targeted sequencing data. (A) Three-dimensional predictions for all 709 ORs and TAARs colored by AP mean position (left) and ML mean position (right). **(B)** Three-dimensional

l069 predictions for the 709 ORs and TAARs colored to show the distribution of Class I, functional
l070 imaging surface enriched ORs, and TAARs without opposing features in order to better
l071 demarcate positions. **(C)** Distribution of ranked mean model position (AP axis for top row and
l072 bottom left and DV axis for bottom right) for the best probability voxel in each predicted
l073 glomerulus for all 709 ORs and TAARs for OE zone, OR Class, gene family, and enrichment
l074 in the functional imaging surface. Statistic is Mann-Whitney U-test. **(D)** Heatmaps for labeled
l075 ORs from individual replicates along the AP (left), ML (top right), and DV (bottom right)
l076 dimensions.

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l079 **Fig. S12. Differential expression analysis for functional imaging surface samples. (A)**
l080 OB schematic showing approximation of functional imaging (FI) surface (red) and OB
l081 remainder (blue) dissected for differential expression analysis. **(B)** Volcano plot comparing
l082 expression of ORs in functional imaging surface and OB remainder samples. The FI surface
l083 enriched ORs, Olfr1377 and Olfr881 are labeled in green and cyan, respectively. **(C)** Volcano
l084 plot comparing expression of ORs in pS6-IP RNA-Seq stimulation with 1% 4-
l085 methylacetophenone. The highly ranked responding ORs, Olfr1377 and Olfr881 are labeled in
l086 green and cyan, respectively. **(D)** Coronal sections from an Olfr881-IRES-mKate2 mouse
l087 indicating a dorsal-central location of the mKate2 labeled ORs **(E)** Volcano plots for
l088 differential expression from functional imaging samples and OB remainder samples for all
l089 genes, ORs labeled in red. **(F)** Volcano plot for only ORs, FDR readjusted, Class I ORs
l090 labeled in red. **(G)** Volcano plot for only TAARs, FDR readjusted. **(H)** Volcano plot for only
l091 vomeronasal receptors (VMNRs), FDR readjusted.

Fig. S13. Whole-mount confocal microscopy and glomerular phenotypes of Olfr1377-IRES-mKate2 and Olfr881-IRES-mKate2 OBs. (A) Whole-mount maximum intensity projections of the dorsal surface of Olfr881-IRES-mKate2 OBs from male and female mice with inlays showing the mKate2-positive glomeruli. (B) Whole-mount maximum intensity projections of the dorsal surface of Olfr1377-IRES-mKate2 OBs from male and female mice with inlays showing the mKate2-positive glomeruli.

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Fig. S14. Position of Olfr1377 and Olfr881 glomeruli. (A) Whole-mount stitched epifluorescence images (together with whole-mount confocal maximal intensity projections

l102 from fig. S13) reconstructing the medial (left) and dorsal (right) faces of Olfr1377-IRES-
l103 mKate2 mice OBs. Red arrows indicate glomerulus. **(B)** Same as (A) for Olfr881-IRES-
l104 mKate2 mice. **(C)** Positions of Olfr1377 fluorescent glomeruli as recorded from the whole-
l105 mount OB images. Red bordered circles indicate the mean position of the individual
l106 replicates. **(D)** Same as (C) for Olfr881.

Fig. S15. In vivo two-photon GCaMP6s response kinetics and concentration-dependence of Olfr1377 and Olfr881 glomeruli. (A) Raw two-photon GCaMP6s

fluorescence of a mKate2-labeled Olfr1377 glomerulus during sequential presentation of odorants in pseudorandom order to a compound heterozygous Olfr1377-IRES-mKate2; OMP-IRES-tTA; tetO-GCaMP6s mouse. * marks long-term activation by 4-methoxyacetophenone. Red text highlights response to 4-methylacetophenone. Odorants presented at an estimated concentration on the order of 10^{-1} nM. **(B)** Similar to (A) for a mKate2-labeled Olfr881 glomerulus imaged in a compound heterozygous Olfr881-IRES-mKate2; OMP-IRES-tTA; tetO-GCaMP6s mouse. Note the lack of long-term activation by 4-methoxyacetophenone. Odorants presented at an estimated concentration on the order of 10^0 nM. **(C)** Chemical structures of a subset of ligands identified by functional imaging for Olfr1377 (solid box) and Olfr881 (dashed box), including multiple overlapping cyclic ketone ligands. **(D)** Raw two-photon GCaMP6s fluorescence of a mKate2-labeled Olfr1377 glomerulus during sequential presentation of increasing concentrations of 4-methoxyacetophenone, revealing long-term activation with 3.5×10^{-2} nM and higher concentrations.

123 **SUPPLEMENTARY TABLES**

124 **Table S1:** Mice used for sequencing

125 **Table S2:** Sequencing read depth for each sample

126 **Table S3:** Probe coverage and enrichment statistics

127 **Table S4:** Target gene expression and normalization weights

128 **Table S5:** Dimensional mean positions for target genes

129 **Table S6:** Summary statistics for percent identity score-based groups of Class I, Class II
130 Dorsal OB ORs, and Class II Ventral OB ORs

131 **Table S7:** Residue identities of Class II OR consensus sequence and significance for all
132 tested sets of anterior/posterior ORs

133 **Table S8:** Three-dimensional model posterior medians

134 **Table S9:** Differential expression analysis for functional imaging surface and pS6-IP 4-
135 methylacetophenone studies

136 **Table S10:** Heterologous cell assay results

Supplementary Files

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- [obmapsuptables.zip](#)