# Materials and Methods

## Flies

Experimental animals were one to three week old adult female *Drosophila melanogaster*. They were kept on a standard cornmeal medium (100ml contained: 91.8 ml water, 11.8g sugar beet syrup, 1.08g yeast, 0.406g agar, 9.4g cornmeal, 0.24ml propionic acid) at a constant temperature of 25°C in an incubator and subjected to a 12:12h light-dark cycle. All flies carried the binary Gal4-UAS system (Figure 1) (Brand and Perrimon, 1993) where the yeast transcription activator Gal4 was expressed under the control of an olfactory receptor gene (OR) and the Upstream-Activating-Sequence (UAS) was upstream of a reporter gene. Three different OR-Gal4 lines were used: Or22a-Gal4, Or47b-Gal4 (Vosshall et al., 2000) and Or83b-Gal4 (flies provided by Dr. Leslie Vosshall, Rockefeller University, NY, USA). Or22a-Gal4 flies were crossed to UAS-Cameleon2.1 flies (Fiala et al., 2002) for AL and antennal imaging experiments and for electroantennogram recordings (see Appendix); they were crossed to UAS-CD8-GFP flies for confocal microscopy of antenna and AL (flies provided by Dr. Leslie Vosshall, Rockefeller University, NY, USA). Or47b-Gal4 flies were crossed to UAS-Cameleon2.1 flies for AL imaging experiments and to UAS-CD8-GFP; UAS-GFP flies for confocal microscopy of antenna and AL (flies provided by Dr. Marien de Bruyne, Free University, Berlin, Germany). Or83b flies Or22a-Gal4 flies were crossed to UAS-Cameleon2.1 flies for AL imaging experiments.

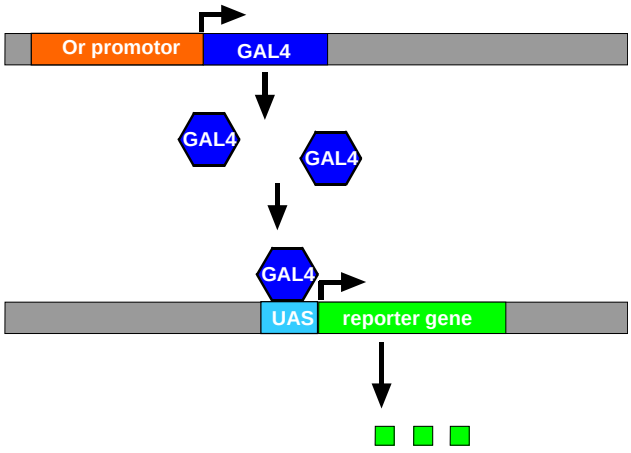


**Figure 1 The binary Gal4-UAS system.** Gal4 is a yeast transcription factor which binds to the Upstream Activating Sequence (UAS). If Gal4 is placed downstream of an olfactory receptor gene's promoter it is only expressed within cells which also express the respective OR gene. In these cells Gal4 binds to the UAS and the downstream reporter gene is transcribed. Through this system it is possible to label genetically identified cells.

## Calcium sensor

Cameleon2.1 is a ratiometric FRET-based calcium indicator dye (Miyawaki et al., 1999). As can be seen in Figure 2, it consists of two green fluorescent-protein-based moieties: enhanced cyan fluorescence protein (ECFP) and enhanced yellow fluorescent protein (EYFP) which are linked to each other via calmodulin and the calmodulin binding peptide M13. Upon binding of four calcium ions, calmodulin binds to M13. This in turn leads to a conformational change bringing ECFP and EYFP in closer proximity. This enables Fluorescence Resonance Energy Transfer (FRET) from the donor, ECFP, to the acceptor, EYFP, leading to a decrease in ECFP fluorescence and an increase in EYFP fluorescence. The overall signal strength elicited by a change in calcium concentration is given by the ratio between the acceptor and donor fluorescence change.

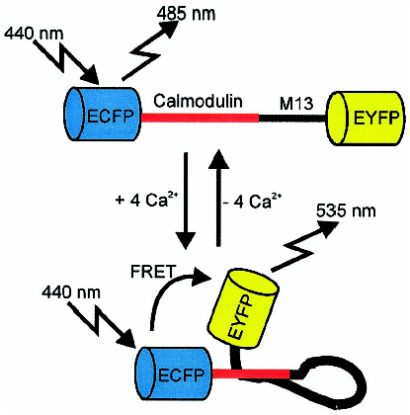


**Figure 2 Cameleon2.1 is a FRET based ratiometric dye.** ECFP stands for Enhanced Cyan Fluorescence Protein and EYFP stands for Enhanced Yellow Fluorescence Protein. M13 is a calmodulin binding peptide. Binding of four $Ca^{2+}$ ions to calmodulin enables it to bind M13 which leads to a conformational change of Cameleon2.1. This conformational change brings ECFP and EYFP in closer proximity enabling Fluorescence Resonance Energy Transfer (FRET). Excitation wavelength of Cameleon is 440nm which is absorbed by ECFP having an emission wavelength of 485nm. During FRET the energy absorbed by ECFP is transmitted to EYFP which has an emission wavelength of 535nm. Figure adapted from (Diegelmann et al., 2002)

## *In Vivo* preparation of flies

*Antennal lobe experiments*

Prior to fixing in a perspex stand (Figure 3), flies were cooled for 30 minutes in a plastic vial on ice in order to reduce movements. Flies were attached to the stand at the back and front of their neck. The antennae were pulled forward with a fine wire (Rediohm-800, HP Reid Inc, Palm Coast, FL, USA) so that the antennal lobes (AL) were visible after opening of the head capsule. A polyethylene foil was sealed to the head with two component silicon (KwikSil, WPI, Germany). A hole was cut into the foil. The hole was filled with ringer (130mM NaCl, 5mM KCl, 2mM $MgCl_2$(*$6H_2O$),

2mM($CaCl_2$(*$2H_2O$), 36mM Saccharose, 5mM Hepes, pH 7,3 (Estes et al., 1996)) and the head capsule was opened. After removal of gland tissue, air sacks and tracheae, flies were immediately placed underneath the microscope for subsequent $Ca^{2+}$ imaging. The same procedure was applied for confocal images of ALs.

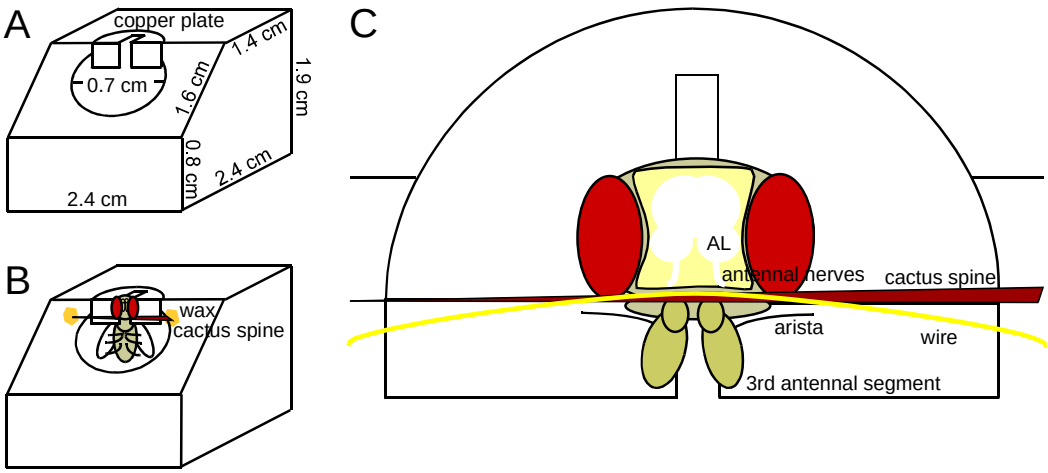


**Figure 3 Preparation of flies for antennal lobe imaging experiments. A** Perspex stand. The stand had a width and depth of 2.4cm, and a height of 1.9cm, the diameter of the whole was 0.7cm. A copper plate designed for electron microscopy (Athene Grids, Plano GmbH, Wetzlar, Germany) with a diameter of 3.05mm a thickness of 10 – 12µm, a slit of 2mm length, 125µm width was cut such that one end of the slit was open and attached to the stand with super glue (Blitz Kleber gel by Pattex, Henkel KGaA, Düsseldorf, Germany). **B** Fly attached to the Perspex stand. After flies were immobilized by cooling them on ice, they were placed in the Perspex stand by sliding their neck through the slit in the copper plate. The back of the fly's head was attached to the stand with grated colophony dissolved in 70% alcohol. By attaching a cactus spine with the aid of wax (Deiberit, Dr. Böhme and Schöps Dental GmbH, Goslar, Germany) on either side in front of the fly's neck the fly was prevented from slipping out of the copper plate. **C** The antennae were pulled forward with a fine wire (Rediohm-800, HP Reid Inc, Palm Coast, FL, USA) in order to make the antennal lobes visible after opening of the head capsule. The head capsule was cut along both eyes. These cuts were connected by another cut through the ocelli and a cut through the fold behind the antennae. After removal of glands, airsacks and trachea, the antennal lobes and the remainder of the brain were visible.

*Antennal experiments*

Flies were fixed in a 10µl pipette tip such that the head just emerged from the opening (Figure 4). The back of the head was fixed to the tube with n-eicosan (Sigma, Germany). One of the fly's antennae was pulled backwards with a fine wire (Rediohm-800, HP Reid Inc, Palm Coast, FL, USA) such that the dorso-medial position where Or22a positive cells are located was facing upwards. A coverslip was

mounted onto the flies head such that the antennal surface was touching it. A drop of aqua dest. was placed on top of the coverslip and the fly was placed under the microscope for imaging with a water immersion lense. Note that this preparation leaves the animal surgically intact.

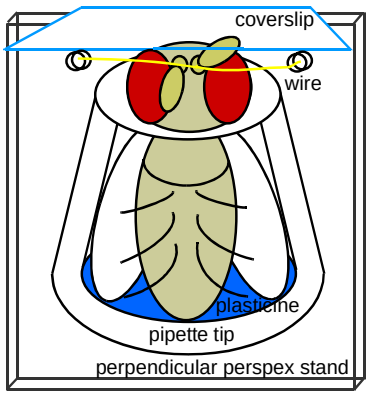


**Figure 4 Preparation of flies for antennal imaging experiments.** Flies were blown into a 10µl pipette tip with an aspirator. The pipette tip was cut such that the fly's head with the antennae just emerged. The lower end of the pipette tip was sealed by pressing it into plasticine. The back of the head was glued to the pipette with n-eicosan (Sigma, Gräfelfing, Germany). The pipette tip was attached to a perpendicular Perspex plate using utility wax (Kerr) with the fly's head slightly above the upper end of the plate. A fine gold wire (Rediohm-800, HP Reid Inc, Palm Coast, FL, USA) was placed between the 2$^{nd}$ and 3$^{rd}$ antennal segment and threaded through holes in the Perspex plate in order to bend the antenna backwards such that the dorsomedial area opposite to the arista where ORNs expressing Or22a are situated was facing upwards. Then a coverslip was placed on top of the antenna slightly touching it. A drop of aqua dest. was placed on top of the coverslip in order to be able to use a water immersion lense for imaging.

## Confocal pictures

All confocal pictures were acquired on a Leica DMRXE confocal microscope with a 40x water immersion objective for AL pictures (NA 0.8, HCX APO Leica) and with a 20x air objective for antennal pictures (NA 0.7). Z-stacks of 1µm were projected into a single plane employing WCIF ImageJ (http://www.uhnresearch.ca/facilities/wcif/). For antennal picture of ORNs expressing Or22a or Or47b whole antennae were pulled from the fly's head and immediately placed in a solution of 60% glycerin dissolved in DMSO (Sigma, Germany). For AL pictures of ORNs expressing Or22a or Or47b flies were prepared and dissected as described above. Excitation laser light was 488nm with an argon laser.

**Imaging set-up**

*Antennal lobe experiments*

All experiments were done on a modified Polychrome II imaging setup described by Fiala et al (2002) (TillPhotonics, Gräfelfing, Germany; Figure 5, left) with a xenon lamp and a monochromator as light source. The fluorescence microscope (Olympus BX51WI) was equipped with a 20 x water immersion objective (NA 0.95, XLUM Plan FI, Japan). Excitation wavelength was 440nm and the exposure time was 65-100 ms. The primary dichroic mirror was 470 DCLP. The emitted light was split by a 520 dichroic and filtered with BP 530-565 (EYFP, acceptor) and BP 473-494 (ECFP, donor). The light was captured by two CCD cameras (TILL imago, Till Photonics GmbH, Gräfelfing, Germany), one for each emission wavelength of the FRET-based ratiometric $Ca^{2+}$-sensitive dye Cameleon2.1 (Miyawaki et al., 1999). Binning on chip was 1.6µm/pixel (image size 153 x 106 pixels corresponding to 244.8µm x 169.6µm). Images were taken at a rate of 3 Hz.

*Antennal experiments*

The antennal recordings were done on two different experimental setups. One was the same as for the AL data with an alteration of the binning on chip (3.2 µm/pixel, image size 76*53 pixel corresponding to an area of 243.2 × 190.8 µm) and an exposure time of 70ms. The other setup consisted of a fluorescence microscope (Olympus BX50WI) equipped with a 20x water immersion objective (NA 0.95, XLUM Plan FI, Japan), and a TILLVision imaging system (TILLPhotonics, Germany). The two emission wavelengths were split by a beamsplitter (DualView, Optical Insights, LCC, Tuscon, USA) recorded on the same CCD camera (VisiCam, Visitron Systems GmbH, Puchheim, Germany) and separated during data analysis. The primary dichroic mirror was 455 DCLP followed by a LP 470. The emitted light was split by a 505 dichroic and passed through a LP 515 for EYFP and BP 465-495 for ECFP. Images were taken at 3 Hz. Excitation wavelength was 435nm, exposure time was 25ms.

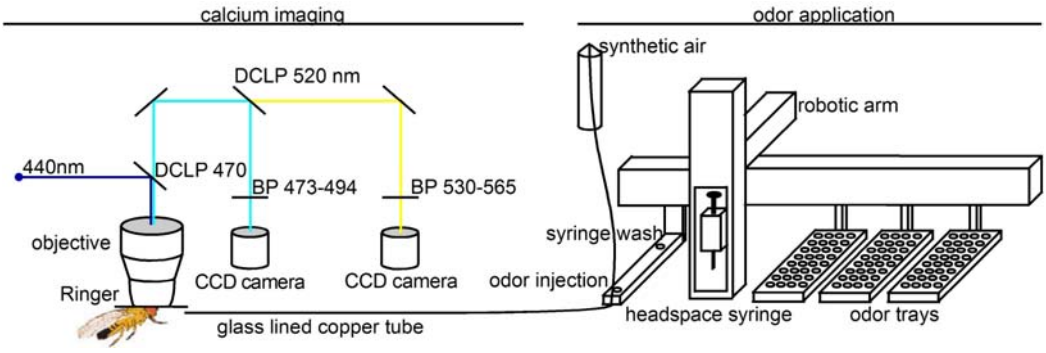


**Figure 5 Imaging set-up and automated stimulus application system. Left** A xenon lamp and a monochromator provided 440nm excitation wavelength focused via a 20x water immersion objective onto the brain. Emission wavelengths passing a 470nm dichroic long pass mirror (DCLP 470nm) were split by a second dichroic long pass mirror (DCLP 520nm), and the two beams were guided through band pass filters (BP 473-494nm for the ECFP emission, and BP 530-565nm for the EYFP emission). The two emissions were simultaneously recorded by two independent CCD cameras. **Right** The olfactometer employed in the study was originally designed as an autosampler for head-space gas chromatography. It was adopted for usage as a stimulus application system by injecting the head space samples into a constant airstream directed at the fly's antennae rather than into a gas chromatograph. Odors were diluted in 5ml mineral oil in 20ml vials which were placed on one of three trays. Each tray could hold up to 32 vials. The robotic arm of the autosampler could be programmed to go to each of the different vials. The vials were sealed with an aluminum cup with a 1cm diameter hole which was closed by a piece of Teflon. The robotic arm could suck up a given amount of headspace from the vials via a syringe which perforated the Teflon piece and inject it into the airstream directed at the fly's antennae. The hole within the Teflon resealed after removal of the syringe. After each odor injection the syringe was rinsed by blowing synthetic air for 100 seconds trough it while simultaneously sucking the air from below before the next odor injection.

## Odorant application

*General parameters*

A constant air stream (1ml/s) coming from a synthetic air gas bottle (Messer-Griesheim, Berlin, Germany) was guided through a glass-lined copper tube with an inner diameter of 1mm and a length of 38cm, the tip placed at a distance of 1 cm from the fly's antennae. The pure odor substances were diluted in 5ml mineral oil (Sigma-Aldrich, Taufkirchen, Germany) in 20ml headspace vials (CleanPack, Germany) to their final concentration (ranging from $10^{-2}$ to $10^{-10}$ [vol/vol]). During stimulation 2ml of headspace were injected by a computer controlled autosampler (Figure 6, right; CombiPAL, CTC analytics, Switzerland) in 2 seconds into the constant air stream, which was interrupted with a computer controlled solenoid valve (Stimulus Controller, Siemens, Germany) during stimulus application. Interstimulus interval was 2 minutes.

*Or22a Experiments*

Unless otherwise stated experiments were done according to the following sequence: Each stimulus protocol started with three 'control' measurements, i.e. a presentation of the diluent mineral oil, room air and a reference odor. The reference odor (ethyl propionate at a concentration of $10^{-2}$ [vol/vol]) was used to monitor the fly's responsive state. The 'control' measurements were followed by eight odor presentations which either differed in their chemistry or in their concentration. After eight of these, the control block was presented again. Odor presentations were pseudo-randomized for different flies in order to avoid sequence effects. Individual flies were recorded for up to 2 hours with an interstimulus interval of 2 minutes during which the AL preparation was superfused with fresh Ringer's solution. Odors used were of the highest purity available and purchased from Sigma, Fluka or Aldrich. For list of odors see Tables 1 and 2.

*Or47b Experiments*

In these experiments E2-hexenal was used to monitor the fly's responsive state, in two flies at a concentration of $10^{-2}$ [vol/vol], in three flies at a concentration of $10^{-1}$ [vol/vol]. Between presentations of E2-hexenal four to eight different odors were presented. Odors were of the highest purity available and purchased from Fluka (hexanal, 2-hexanone) and Aldrich (benzaldehyde, E2-hexen-1-ol, E2-hexenal, E2-hexyl acetate, 4-methylcyclohexanol, 1-hexanol, hexyl acetate, Z3-hexen-1-ol). All enantiomeric odors were racemic mixtures, CAS-number were identical to those in the Or22a experiments (see Tables 1 and 2).

**Gaschromatography**

Gaschromatographic measurements were used to confirm that decadic odor dilutions resulted in decadic headspace concentrations for a subset of the odors. To this end 2ml headspace of decadic odor dilutions of ethyl acetate ($10^{-5}$, $10^{-4}$, $10^{-3}$, $10^{-2}$ [vol/vol]) and methyl 3-hydroxy hexanoate ($10^{-4}$, $10^{-3}$, $10^{-2}$ [vol/vol]) were splitlessly injected with an autosampler (CombiPAL, CTC analytics, Switzerland) into a gaschromatograph (GC; Trace GC Ultra, Thermo, Electron Corporation, Waltham,

MA, USA). The GC was equipped with a fused-silica capillary column with an intermediate polarity (10m, ID 0.18mm, 0.2µm film thickness (14% cyanopopylphenyl, 86% dimethyl polysiloxane; RTX-1701, Restek, Bellefonte, PA, USA). Intitially, the temperature program started at 30°C for one minute, then the temperature was raised at a rate of 10°C/minute to 280°C where it was held for another minute. For methyl 3-hydroxy hexanoate this proved to result in sufficient resolution of the GC's Flame Ionization Detector (FID) signal. For ethyl acetate, however, split injections (ratio 1:10) were used to avoid column overloading at high concentrations and the program was changed to a first temperature ramp of 2°C per minute up to 50°C followed by a second ramp of 20°C per minute to 280°. For both odors, peaks eluted at the same respective retention time for all concentrations (ethyl acetate: 1 min 7 sec; methyl 3-hydroxy-hexanoate 7 min 19 sec). For ethyl acetate two sets of dilutions were prepared and measured in order to test the reproducibility of data. Differences between data sets were negligible, so that only a single measurement was done for methyl 3-hydroxy hexanoate. As shown in Figure 6 for both odors the peak area, a measure for the amount of injected substance, increased linearly with increasing amounts of odor diluted in mineral oil when plotted double-logarythmically (ethyl hexanoate $\log Area = 1.0051 \times x + 8.776$, $R^2 = 1$; methyl 3-hydroxy-hexanoate $\log Area = 1.0043 \times x + 7.0534$, $R^2 = 0.9997$).

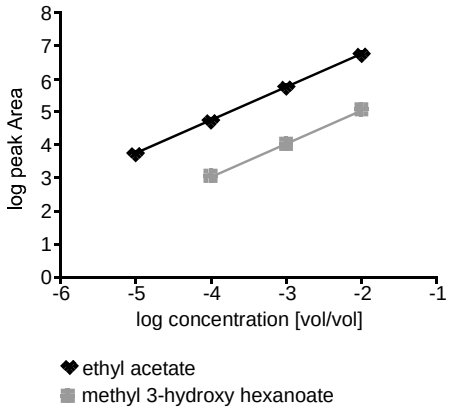


**Figure 6 Correlation between [vol/vol] dilutions and amount of headspace.** Headspace injections of increasing decadic dilutions of ethyl acetate (black diamonds, n = 2 for each concentration) and methyl 3-hydroxy hexanoate (grey squares, n = 1 for each concentration) resulted in decadically increasing FID signal strength (indicated as log peak area).

**Pharmacological experiments**

Picrotoxin (PTX) (Sigma, Germany) was first dissolved in DMSO (Sigma, Germany) to a concentration of 100mM. Further dilutions to a final concentration of 5µM were done in Ringer's solution. Muscimol (Sigma, Germany) was dissolved in aqua dest. to a concentration of 100mM and further diluted in Ringer to a final concentration of 500µM. The odors used for this set of experiments were ethyl hexanoate at a concentration of $10^{-7}$ [vol/vol] and 1-butanol at a concentration of $10^{-3}$ [vol/vol]. All flies were measured with a standardized protocol. For each treatment the odors were presented twice and responses were averaged during data analysis. First odors were presented in the presence of Ringer, then PTX or muscimol were applied by manually exchanging the Ringer for 100µl of the pharmacon. Approximately four minutes after application of the pharmacon the odors were presented again. Then the pharmacon was washed by exchanging the Ringer five times.

**Data analysis**

*General*

All data analysis was done with custom written IDL software (Research Systems, USA). For each fly, measurements were chosen for further analysis if they showed stable responses to the reference odor at the beginning and the end of one control block. Prior to signal calculation the data was semi-automatically corrected for movement artifacts first within measurements and then between measurements. Images from the CCD camera(s) were median filtered in space to reduce noise (filter size: 3 pixels). Then, the EYFP image was divided by the ECFP image. For AL traces, averages of a 7 × 7 pixel box (11.2 × 11.2 $\mu m^2$) were calculated as a function of time. For antennal traces the box was 11 × 11 pixels (35.2 × 35.2 $\mu m^2$). We calculated the ratio EYFP/ECFP multiplied by factor 100. Background fluorescence was determined by an average of 6 frames before stimulus onset and was subtracted from each frame of the measurement, thereby shifting traces to zero before stimulus onset. The resulting values are labeled $\Delta$ Ratio [%]. Odors which evoked an increase in fluorescence intensity were called activating odors; odors which evoked a decrease in fluorescence intensity were called inactivating odors; odors which did not evoke a

change in fluorescence intensity were called non-activating odors. False color coded pictures were calculated by averaging three frames around the time point of the response maximum within 5 seconds after stimulus onset. A threshold was chosen above which fluorescence intensities were color coded and below which the raw fluorescence picture was shown. Pictures were median filtered in space (filter size: 3 pixels).

*Or22a experiments*

A total of 100 flies was measured for the AL, and 121 flies for the antennae. AL and antennal data are based on 2982 and 4244 analyzed odor presentations, respectively. For quantification of odor-evoked response magnitude, an average of three frames around the maximum of the response within 5 seconds after stimulus onset was taken. This time interval was chosen to include the maxima of slow responses which occurred for AL responses to low odor concentrations and for antennal responses in general.

In order to summarize data across animals with varying overall response strengths, responses within an animal were scaled to the mean maximum of the response to the reference odor (ethyl propionate $10^{-2}$[vol/vol]). The resulting values are labeled 'normalized response strength'. Dose-response curves were established by first measuring the responses to a panel of 104 odors at a concentration of $10^{-2}$ [vol/vol]. Subsequently, only odors which elicited responses clearly higher than those to the diluent mineral oil and air were tested at a concentration one log unit lower ($10^{-3}$ [vol/vol]). This procedure was repeated until no response was measurable to any odor. Effective Concentrations eliciting a halfmaximal (50%) response (EC50) were calculated by fitting the Hill equation with a weighted (weights equal variance of each data point) non-linear least squares algorithm to the normalized data which were averaged across animals for each odor and its respective concentration.

*Or47b experiments*

For Or47b experiments a total of five flies were measured and 63 odor presentations were analyzed. For quantification of the odor evoked responses three

frames around the maximal amplitude, either positive or negative, within five seconds after stimulus onset were averaged. This value was labeled ‘mean response’.

**Estimation of ppm**

I estimated the ppm of the odors at EC50 on the basis of the work by Cometto-Muniz and co-workers (2003). The authors describe the relationship between liquid- and vapour-phase concentrations for 60 volatile organic compounds diluted in mineral oil based on numerous gas chromatographic measurements of the respective headspaces. They deduced an equation by which the odor concentration in the vapor phase can be calculated from the odor concentration in the liquid phase, i.e. mineral oil:

$$[Cst]_{vap} = a \times [Cst]_{liq}^{\beta}$$

where $[Cst]_{vap}$ equals the concentration of the solute in vapor phase, $[Cst]_{liq}$ equals concentration of the solute in liquid phase. ‘a’ and ‘β’ are experimentally deduced constants and dependent on the solute, i.e. the odor. The authors present the values of ‘a’ and ‘β’ for 60 different organic compounds as well as the respective vapor pressures. Since the relationship between log ‘a’ and log vapor pressure is linear ($\log a = 1.359 \times \log vp + 2.0404$, where vp represents Vapor Pressure, $R^2$ = 0.9123 [acetic and formic acid were excluded as they represented the only strong outliers]), I could calculate factor ‘a’ for each of the odors with known vapor pressure around 23°C, the temperature at which our experiments were conducted. As I was not able to find a relationship between β and either factor ‘a’ or vapor pressure, I set β to 1 for each odor, which was the mean of β for all odors. For the 60 odors investigated by Cometto-Muñiz and co-workers (2003) we could calculate the ratio between the estimated ppm and the experimentally deduced ppm at a given concentration (I chose $10^{-2}$ [vol/vol]). These ratios enabled us to calculate 95% confidence limits for each odor (Supplemental Table 1).

**Superposition of 3D molecular structures of Or22a activating odors**

The molecules included in the MRR of Or22a were aligned based on their three-dimensional structures employing a multiple semi-flexible algorithm developed for super positioning small molecules (Baum, 2005). Semi-flexible algorithms use precomputed conformers to take into account the flexibility of the ligands. This is necessary, as generally the active forms of the ligands are not known. First, the three-dimensional structures of the odor molecules were generated using CORINA (Sadowski and Gasteiger, 1993) or downloaded from the internet. These structures were then used as input to the program ZIBMol (Fischer et al., 2002) which generated between 1 and 71 metastable conformations for each odor molecule (median = 8; for number of conformers generated for each odor see Table 1). From these conformations the lowest energy conformers were chosen for further analysis. The conformers were parameterized using the Merck Molecular Force Field (MMFF). The parameterization was needed for assigning atom types such as donor, acceptor, hydrophobic, and aromatic.

The algorithm required the preselection of a reference molecule. Each reference conformer was separately compared to all query molecules. This comparison resulted in a large number of diverse pairwise matchings which have been locally optimized starting from some initial transformation. These pairwise matchings were then combined to multiple matchings according to the common substructures with the reference conformation. Several parameters were computed for each multiple matching, such as the number of query molecules contained in the matching, the size of the common substructure and the average score value of the matchings (favoring atom pairs such as donors or acceptors, and penalizing atom pairs consisting of, e.g., a donor and a hydrophobic atom). According to these parameters, the multiple matchings were sorted into different sets, each set containing a number of ‘equally’ good matchings. These multiple matchings with their respective multiple superpositions could then be interactively looked at.