A 4-dimensional representation of antennal lobe output based on an ensemble of characterized projection neurons

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ABSTRACT

A central problem facing studies of neural encoding in sensory systems is how to accurately quantify the extent of spatial and temporal responses. In this study, we take advantage of the relatively simple and stereotypic neural architecture found in invertebrates. We combine standard electrophysiological techniques, recently developed population analysis techniques, and novel anatomical methods to form an innovative 4-dimensional view of odor output representations in the antennal lobe of the moth *Manduca sexta*. This novel approach allows quantification of olfactory responses of characterized neurons with spike time resolution. Additionally, arbitrary integration windows can be used for comparisons with other methods such as imaging. By assigning statistical significance to changes in neuronal firing, this method can visualize activity across the entire antennal lobe. The resulting 4-dimensional representation of antennal lobe output complements imaging and multi-unit experiments yet provides a more comprehensive and accurate view of glomerular activation patterns in spike time resolution.

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1. Introduction

How much neural activity is necessary to encode sensory or motor information? This seemingly simple question has not been satisfactorily answered in any sensory or motor system. So far, we know that single neurons play major roles only in rare cases (Eaton and Bombardieri, 1978; Hedwig, 1996; Hedwig and Heinrich, 1997) and even then, not under all circumstances (Hedwig, 2000). In most cases, neural populations underlie sensory or motor encoding (Ruiz et al., 1995; Georgopoulos, 1996), but the necessary population size, in other words the amount of influence a single neuron exerts, is still under debate (Groh et al., 1997; Houweling and Brecht, 2008; Huber et al., 2008).

In the last decade, multi-unit recording and imaging techniques, which allow recording of ensemble activity, became widely available. Both approaches have greatly expanded our knowledge about and understanding of odor processing in the vertebrate olfactory bulb and the insect antennal lobe. Imaging studies have allowed us to understand spatial aspects of odor processing. They show, that an odor consistently excites roughly the same subset of glomeruli in a species-specific manner (Galizia et al., 1999b; Rubin and Katz, 1999). These activation patterns differ for different odors (Friedrich

and Korsching, 1997; Joerges et al., 1997; Uchida et al., 2000; Meister and Bonhoeffer, 2001; Ng et al., 2002; Wachowiak et al., 2002; Hansson et al., 2003). In most animals, both the glomerular pattern and the degree of activation change with odor concentration (Rubin and Katz, 1999; Sachse and Galizia, 2003). The only exception seems to be the turtle, where the pattern of activated glomeruli is concentration invariant (Wachowiak et al., 2002). Usually, the percentage of glomeruli, which are activated in response to an odor stimulus, is not quantified in imaging studies, but it seems to be in the range of approximately 13–30% (Joerges et al., 1997; Uchida et al., 2000; Sachse and Galizia, 2002; Silbering and Galizia, 2007).

Recently, it has been shown for mammals and insects that olfactory receptor neurons, which express the same receptor protein (Buck and Axel, 1991; Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999), project into the same glomerulus in the primary olfactory neuropils (Mombaerts et al., 1996; Wang et al., 1998; Vosshall et al., 2000). For some insects, the glomeruli of the antennal lobe were identified early on (Pinto et al., 1988; Flanagan and Mercer, 1989; Stocker et al., 1990; Rospars and Hildebrand, 1992; Laissue et al., 1999; Rospars and Hildebrand, 2000). The finding that olfactory receptor neurons with the same receptor proteins project to the same glomeruli provides an anatomical basis for imaging studies. Furthermore, recently novel tools for the anatomically correct reconstruction of brains and their neuropils have been developed. Together, they lead to the development of new

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reference atlases of the antennal lobe for a number of insects (Galizia et al., 1999a; Laissue et al., 1999; Berg et al., 2002; Huetteroth and Schachtner, 2005).

Multi-unit recording studies on the other hand have enhanced our understanding of the temporal aspects of odor processing. Current estimates from *Manduca sexta* antennal lobes suggest that in response to brief stimulation (100 ms) with undiluted odors about 40% of recorded units in an ensemble are excited, about 30% are inhibited and 30% are not affected by an odor stimulus (Daly et al., 2004b). This suggests that odor-driven responses are more distributed than imaging studies imply. Ensemble responses evolve in an odor-dependent manner over time. That is, they are dynamic in that different units fire at different times and with different temporal patterns during an odor response (Laurent et al., 1996; Wehr and Laurent, 1999; Friedrich and Laurent, 2001; Stopfer et al., 2003; Daly et al., 2004b; Lei et al., 2004).

Both, imaging and multi-unit methods have, thus, added much more meaning to the conceptual term “across fiber” pattern (Pffaffmann, 1955). However, because of the inherent limitations of these methods, we still have no clear understanding of how little or how much activity is necessary to represent an odor at behaviorally defined detection thresholds and/or behaviorally relevant concentrations. Optical recording methods for example are restricted to a single 2-dimensional optical section and, thus, activity in the third dimension is not accounted for. Furthermore, the temporal resolution of optical recordings in imaging studies is usually about 3–5 Hz, which is too slow to resolve neural dynamics. Multi-unit recordings, although revolutionizing our view of neural dynamics, do not permit a morphological identification of the recorded units and provide no, or at best limited (Lei et al., 2004) evidence about their spatial distribution. Moreover, because of physical constraints, the recorded units can only stem from a restricted area near the electrode site and, thus, typically only 10–25 units (up to 43; Daly et al., 2004b) of the approximately 1200 antennal lobe interneurons (Homberg et al., 1989) are recorded.

Therefore, we sought to complement these approaches by combining a number of established and more recent methods in a novel way. We take advantage of the very successful “identified neuron” approach in invertebrate neurobiology (e.g. Nolen and Hoy, 1984; Jacobs and Miller, 1985; Brodfuehrer and Friesen, 1986; Boehm and Schildberger, 1992; Borst and Egelhaaf, 1994; Poulet and Hedwig, 2006) by recording intracellularly from single antennal lobe projection neurons and staining each of them (e.g. Matsumoto and Hildebrand, 1981; Kanzaki et al., 1989; Anton and Hansson, 1994; Christensen et al., 1998; Kloppenburg et al., 1999; Reisenman et al., 2005). We combine this with recently advanced anatomical reconstruction methods (AMIRA; e.g. Lin et al., 2007; Rø et al., 2007) and created a 3-dimensional reference antennal lobe of *M. sexta* based

on previously standardized glomeruli (Huetteroth and Schachtner, 2005). The physiological data of all the sequentially recorded, stained, and characterized projection neurons are combined to create a population of projection neurons (Georgopoulos et al., 1988), which we call a “virtual” ensemble (Skaggs and McNoughton, 1998). Subsequently, this can be analyzed in a similar way as neural ensembles from multi-unit recordings (Stopfer et al., 2003; Daly et al., 2004b; Brown et al., 2005). The final combination of the anatomical and physiological data results in a 4-dimensional representation of odor responses, which allows quantification of both the spatial and the temporal aspects of odor output representations across the antennal lobe. Previous accounts of this work have been published as abstracts (Staudacher et al., 2007a,b).

2. Materials and methods

2.1. Animals

Each of the *M. sexta* L. pupae received from the Arizona Research Laboratories Division of Neurobiology was placed in a separate paper bag. They were kept in an incubator (Percival Scientific, Inc.; I66VLC8) on a reversed light:dark cycle of 16:8 h. Ambient temperature and relative humidity were kept constant at 25 °C and 75%, respectively. Every day, bags with newly eclosed moths were dated and only 3–10 day old males were used (median: 6; 1st quartile: 5; 3rd quartile: 7; $N = 78$). To account for the nocturnal activity pattern of the moths, all experiments were performed within the first 4 h of their subjective night.

2.2. Electrophysiology

The dissection followed an established protocol (Christensen and Hildebrand, 1987). In short, parts of the head capsule, all mouthparts and musculature inside the head and part of the tentorium were removed to expose the brain. Both antennal joints and antennae were left intact. The brain was superfused with *Manduca* saline throughout dissection and experiment (Heinbockel et al., 1998). A small area of the left antennal lobe was desheathed to enhance electrode penetration.

Borosilicate glass tubing (Sutter Instruments, Inc.; BF100-50-10; OD 1.0 mm, ID 0.5 mm) was pulled to form microelectrodes with a Brown-Flaming type puller (Sutter Instruments, Inc.; P-2000). Electrode tips were filled with 5% Neurobiotin in distilled water (Vector Laboratories, Inc.; SP-1120), while shoulders and part of the stems were filled with 2 M potassium acetate. Connection to the amplifier was made with a silver/silver chloride wire, the tip of which was immersed in the potassium acetate solution. Electrode resistances were about $238 \pm 48 \text{ M}\Omega$ (mean $\pm$ standard deviation,

Fig. 1. (A) The photograph of the preparation shows brain with the antennal lobe, the glass tube with the left flagellum inserted and other details of the recording situation. (B) The original recording of an odor-driven response of a projection neuron shows the low spontaneous activity, a strong, high frequency response, and both I1 and I2 inhibition. Abbreviations: I1 and I2, inhibition No. 1 and 2.

$N=78$). Another silver/silver chloride wire served as reference electrode (Fig. 1A). Neural signals were amplified in bridge mode with an Axoclamp 2B amplifier (Molecular Devices, Inc.; Fig. 1B). Intracellular recordings from neurites lasted up to 137 min with an average duration of 39 ± 29 min (mean $\pm$ standard deviation, $N=78$). Recording times longer than 7 min were usually sufficient to passively stain the recorded neuron.

All data were recorded onto the hard disk of a personal computer with 10 kHz sampling rate (16 bit; Molecular Devices; Digidata 1440A) using Clampex (version 10.1; Molecular Devices) as software interface.

2.3. Odor stimulation

Compressed air was dried and cleaned by passing it through Drierite (W. A. Hammond Drierite, Ltd.) and activated charcoal (Sigma; C3014). The output of this filter array was then split into three lines, each of which passed through a separate three-way valve (The Lee Company; LFAA1200118H). They joined again via a four-port stainless steel manifold, the fourth port of which was used as a common output into a glass tube. Two of the lines had custom-made odor cartridges (inner diameter: 6 mm; length: 70 mm; volume: 1.6 ml) with Luer fittings inserted between valve and glass tube. These lines were used for odor stimulation, while the third line provided a constant, clean air stream. The left flagellum was inserted into the glass tube, which had a diameter of 2.5 mm and was 64 mm long (Fig. 1A). Airflow through the three lines could be regulated (Cole Parmer; PMR1-01293) and was calibrated to 250 ml/min with a flow meter (Agilent Technologies; ADM100).

Odor cartridges were loaded with a strip of filter paper (Whatman International, Ltd.; No. 1), which was impregnated with 3 μ l of odorant. When no stimulus was presented, the valve, which had no odor cartridge in-line was open to provide a constant airflow across the flagellum. For odor stimulation, this valve was closed, while one of the other two valves was opened simultaneously under software control (Clampex, version 10.1; Molecular Devices). Each stimulus presentation consisted of five repeats of a 100 ms pulse of either a blank (clean air only) or a neat odor separated by 10 s of clean air. Each of the five repeats was defined as peri-stimulus time from 0.8 s before to 9.2 s after stimulus onset. To develop the method described here, we used undiluted 2-hexanone (Aldrich; 103004), 1-hexanol (Sigma; 471402), 2-octanone (Sigma; O4709), 1-octanol (Sigma; O4500), 2-decanone (Aldrich; 196207) and 1-decanol (Aldrich; 150584). These odors were selected, because they have been used previously in behavioral studies with *M. sexta*, are known to elicit a conditioned feeding response, and can be discriminated between (Daly and Smith, 2000; Daly et al., 2001a,b). Neat concentration was chosen to compare the data to an earlier multi-unit study (Daly et al., 2004b).

2.4. Histology

After the experiments, the brains were fixed in 4% formaldehyde in 0.1 M Millonig's buffer (pH 7.4) for 2 h at room temperature, washed with and then stored in Millonig's buffer (pH 7.4) for up to 4 weeks at 4 °C. Then, brains were embedded in 7.5% Agarose (Low EEO; Fisher Scientific; BP160-500) and sectioned with a vibratome (Leica; VT1000S). Free-floating sections (70 μ m or 240 μ m) were washed before they were incubated at 4 °C either for 20 h with Avidin-Texas Red conjugate (Molecular Probes; A820; 70 μ m) or for 40 h with Streptavidin-CY3 conjugate (Jackson ImmunoResearch; 016-160-084; 240 μ m). After washing and dehydration, the mounted sections were coverslipped with Permount (Fisher Scientific; SP15-500).

A compound microscope (Olympus; BX61) equipped with a confocal laser-scanning unit (Olympus; FV1000) and controlled by the appropriate software (Olympus; FV10-ASW, version 01.06b) was used to evaluate and scan the specimens. Sections were either scanned with a 10 $\times$ or 20 $\times$ objective (Olympus; UplanSApo 10 $\times$ /0.40 or 20 $\times$ /0.75, respectively) with XY pixel sizes of 1.242 or 0.621 μ m/pixel and Z steps of 0.66 or 0.35 μ m, respectively. Each section was simultaneously scanned at λ 488 nm and λ 543 nm to record the green auto-fluorescence of the brain tissue, especially the glomeruli of the antennal lobe, and the morphology of the cells in red. All data were exported as tagged image file stacks for further analysis in AMIRA (version 4.1; Visage Imaging, Inc.). All anatomical descriptions are based on the head axis, not the embryonal neuroaxis.

2.5. Identification of glomeruli

A 3-dimensional male antennal lobe reference atlas was created according to the protocol described in el Jundi et al. (in press). The resulting 63 glomeruli (60 ordinary and 3 sex specific glomeruli) were named and numbered according to Rospars and Hildebrand (2000). The reference atlas was subsequently used to determine (1) in which glomerulus a projection neuron arborized, (2) in which tract the axon was located, and (3) where the soma was located. For this, the tagged image file stacks of all the sections of each brain were loaded into AMIRA and aligned in all dimensions. By using the "merge"-module of AMIRA, they were combined to one single 3-dimensional stack. Comparing this stack with the reference antennal lobe and the reconstructed antennal lobes of Rospars and Hildebrand (1992, 2000) allowed identification of the glomeruli, in which individual projection neurons arborized. Antenna-cerebral tract identity and cell group of the recorded projection neuron were also determined on the basis of this aligned 3-dimensional stack. The labels of identified glomeruli were false-color coded according to anatomical or physiological data. Pictures of different views were taken with the snapshot tool of AMIRA.

2.6. Ensemble analysis

Physiological data were imported from Clampex (version 10.1; Molecular Devices) to Matlab (R2007a; The Mathworks, Inc.) for all analysis. As a first step, the time of occurrence of each action potential was extracted by thresholding the spike train. Ensemble raster plots are based on aligning the responses of all neurons to the same odor/concentration by stimulus onset. These aligned data sets were the basis for calculating peri-stimulus time histograms for each repeat of the seven stimuli (blank and six odors). For every projection neuron, 10 s of each of the five repeats, i.e. peri-stimulus time from -0.8 s to +9.2 s, were divided into 500 separate 20 ms bins and the number of action potentials per bin was counted. These action potential count based data were saved for later analysis. These counts were also transformed into z-score based data sets in the following way. For every bin, the mean action potential count across the repeat was subtracted from the action potential count of the current bin and then divided by the standard deviation of the action potential count across the repeat. The resulting z-score value expresses how many standard deviations the action potential count of a given bin is above or below the mean action potential count across this repeat.

Based on the ensemble responses, Euclidean distances were calculated (Stopfer et al., 2003; Daly et al., 2004b; Brown et al., 2005) separately, but in the same way for action potential counts and z-score transformed data. For both data sets, the bin-width was 20 ms, which resulted in 500 bins for each repeat. Like the raster plots, the Euclidean distance plots show data from peri-stimulus time -500 ms to +1480 ms, which is represented in bins

16–115; stimulus start, bin 41, is defined as time zero. Fig. 2 schematizes how Euclidean distances were calculated. The following was repeated for every bin of every repeat of every stimulus. At each bin, the action potential count/z-score value of each of the projection neurons represented the value of one coordinate of a single point in a multi-dimensional space with the size of the ensemble (columns in Fig. 2A). The resulting points were represented in a multi-dimensional coordinate system, which is shown as 3-dimensional for clarity (Fig. 2B). Euclidean distance was always calculated for two points of the same time bin but from different repeats and/or odor stimuli (e.g. green vs. blue in Fig. 2B); it is the length of the shortest connection between the two points (red lines in Fig. 2B) and was calculated according to the Pythagorean theorem (Fig. 2C). Euclidean distance was calculated for all time bins in pair-wise comparisons across all five repeats and the seven stimuli. Depending on the comparison, mean Euclidean distance values and standard errors were calculated based on the appropriate subsets of these data and then plotted.

To create activity maps of antennal lobe responses to each stimulus, the z-score data of each neuron were translated into a normalized false-color code. The color information representing the neuronal activity was then transferred to AMIRA and the respective glomeruli of the 3-dimensional antennal lobe representation were false-color coded accordingly. In principle, the final 4-dimensional representation of antennal lobe responses to the seven stimuli was

prepared in the same way. For every 20 ms bin, the color code was applied to the identified glomeruli of the 3-dimensional antennal lobe representation in AMIRA and with the snapshot tool a separate picture was saved as a tagged image file. Finally, these images were sequentially loaded into AMIRA and, with the aid of the “Time Series Control” and “Movie Maker” tools, a QuickTime® movie was created and saved for each of the seven stimuli (Animations). Supplementary Fig. 2 shows the same posterior and anterior views presented in the animations, but with the glomeruli labeled.

3. Results

Of the 78 recorded cells one descending neuron, 35 local interneurons and 29 projection neurons were stained successfully. Three of the 29 projection neuron recordings were discarded because only two odors could be presented. A fourth recording was not used because a second neuron was visible in the histology and, therefore, the anatomical data could not be unambiguously matched with the physiological recording. Therefore, 25 of the 29 successfully recorded and stained projection neurons were included in this analysis. The steps towards a 4-dimensional representation of their activity across the antennal lobe were as follows: (1) each neuron was characterized and the relevant morphological parameters extracted, (2) information on the glomerulus each cell arborized in was inserted into the antennal lobe map, (3)

Fig. 2. (A) At each peri-stimulus time bin for all repeats of all stimuli, the action potential count/z-score value of each neuron of the ensemble represents one coordinate value for a single point in a space, the dimensionality of which is equal to the size of the ensemble. (B) Red lines represent the Euclidean distances between two points of the same peri-stimulus time bin for two fictive responses (green and blue points). To illustrate the principle, Euclidean distances between corresponding points are exemplified in a 3-dimensional space. (C) Euclidean distances between corresponding points were calculated according to the Pythagorean theorem. This calculation was performed on every odor and repeat comparison to provide both within odor and between odor distance values at each peri-stimulus time bin. Abbreviations: ED, Euclidean distance; R1(X)Od1(2,Y,Z), repeat one (X) for odor one (two, Y, Z).

Fig. 3. Two examples (A and B) of recorded and filled projection neurons. Maximum projections show the dendritic trees of two projection neurons (red) and the outlines of antennal lobe glomeruli (green). Yellow dotted lines indicate the borders of two arbitrarily chosen individual glomeruli. Abbreviations: AC/LC, anterior/lateral soma cluster; IACT/OACT, inner/outer antenno-cerebral tract; G52 and G27, identity of innervated glomerulus.

the physiological data were analyzed, and (4) the antennal lobe map was color coded based on the analysis of the physiological data.

3.1. Morphological parameter extraction

All sections containing antennal lobe structures and/or parts of the stained projection neuron were scanned. In all cases, the recorded tissue auto-fluorescence showed the outlines of dense neuropils, e.g. glomeruli (Fig. 3A and B). The red fluorescence clearly showed the morphology and in all projection neurons allowed a complete reconstruction of their antennal lobe ramifications. Axon terminals in the mushroom body calyces were stained well in 14 cells, weakly in five neurons, but were not

stained in 6 cells. Projection images of the relevant tagged image file stacks were usually sufficient to determine, in which cluster the soma of a projection neuron was located and through which tract the axon projected to the protocerebrum. The somata of the 25 projection neurons were located in all three clusters (anterior cluster: 9; lateral cluster: 5; medial cluster: 11). Their axons ran in three tracts (dorso-medial antenno-cerebral tract: 1; inner antenno-cerebral tract: 22; outer antenno-cerebral tract: 2).

However, the identification of the glomerulus, in which a given projection neuron arborized, was only possible by using a species-specific 3-dimensional antennal lobe map (Fig. 4). For this, the tagged image file stacks of the relevant sections were combined. The green auto-fluorescent channel of the stacks was essential to

Fig. 4. Colored and numbered glomeruli in these four views of the 3-dimensional reference antennal lobe represent the locations of the dendritic trees of the uniglomerular projection neurons recorded and stained in this study. The three colors represent the output tracts through which the axons of these neurons leave the antennal lobe and project to the protocerebrum. Abbreviations: AC/LC/MC, anterior/lateral/median soma cluster; Cu, Cumulus; DMACT/IACT/OACT, dorso-medial/inner/outer antenno-cerebral tract; To, Toroid.

Fig. 5. Raster plots of odor-dependent changes in the responses of single projection neurons and, consequently, of the virtual ensemble. Stimulus marker: grey vertical bar. Tables on the left side of the plots provide details on the glomerulus, in which the corresponding projection neuron ramified in, soma cluster and output tract. Horizontal grey bars indicate specific stimuli that were not tested in a given projection neuron. *Abbreviations:* AC/LC/MC, anterior/lateral/median soma cluster; DMACT/IAC/OACT, dorso-medial/inner/outer antenno-cerebral tract.

fit these data to the reference antennal lobe and to identify, in which glomerulus each projection neuron ramified. The 25 projection neurons shown had dendrites in 19 of the 63 glomeruli of the reference male antennal lobe. Two cells each arborized in glomeruli 15, 16, 36, 37, 52, and the toroid. Based on these single cell data, we created a 3-dimensional representation of these glomeruli (Fig. 4). Additionally, they were color-coded according to

the tract in which the axon of a projection neuron runs towards the protocerebrum. Fig. 4 shows that the glomeruli we recorded from were not located in one plane, but rather distributed across the entire antennal lobe. Furthermore, this representation can be viewed from all angles without losing details. That any desired color-coding scheme can be applied easily and efficiently is important for linking physiological data to this anatomical map.

Fig. 6. Plots of Euclidean distance based on binned (20 ms) action potential data (A and B) and z-score transformed data (C and D) show that response dynamics is well preserved in z-score data and that this virtual ensemble could discriminate different odors very well. Stimulus marker: grey vertical bar. *Abbreviations:* AP, action potential; sem, standard error of the mean.

3.2. Analysis of physiological data

The raster plots in Fig. 5 show that the spiking responses of single projection neurons and, consequently, the activity patterns of the ensemble constructed with 25 projection neurons changed in an odor-dependent manner. In all projection neurons that responded, an initial interruption of firing was observed, and in 12 of the 25 recordings, this could be directly attributed to inhibitory postsynaptic potentials causing the so-called I1 inhibition (cf. Fig. 1B and Supplementary Fig. 1). This gap in firing varied in an odor-dependent manner in terms of both onset latency and duration, but usually occurred in the same time window as I1 inhibition, which started around 126 ± 20 ms (mean and standard deviation, $n = 180$ in 12 neurons) and had a duration of 44 ± 31 ms (mean and standard deviation, $n = 180$ in 12 neurons). Relative to the command voltage initiating the odor valve opening, most stimulus-response latencies of the projection neurons were about 160–220 ms (191 ± 60 ms; mean and standard deviation, $n = 192$ in 12 neurons), but they could be as short as 100 ms (cf. Fig. 5 and Supplementary Fig. 1A, 2-hexanone; cell 4: 7, anterior cluster, inner antenno-cerebral tract). Spike frequency during the excitatory phase of the response was both odor and cell dependent (Fig. 5). However, not all projection neurons responded with excitation or at all. Three of the 25 cells never responded to odor (cell 6: 15 (+25), anterior cluster; cell 19: 63, lateral cluster; cell 23: Toroid, lateral cluster; all inner antenno-cerebral tract), 2 cells always ceased firing (cell 21: labial pit organ glomerulus (LPOG), lateral cluster, inner antenno-cerebral tract; cell 25: 55 (+60), lateral cluster, outer antenno-cerebral tract), and 5 were excited by every odor (cell 7: 16, anterior cluster; cell 12: 37, anterior cluster; cell 15: 44, medial cluster; cell 18: 53, anterior cluster; cell 20: Discbase, medial cluster; all inner antenno-cerebral tract). Thus, about 50% of the 25 projection neurons were excited, 25% stopped firing and 25% remained inactive in response to an odor stimulus (11.43 ± 2.64 , 5.86 ± 2.04 , and 6.29 ± 1.38 , respectively).

The peri-stimulus raster plots show, that excitatory responses were terminated by a second distinct gap in firing (Fig. 5), which could be directly attributed to the so-called I2 inhibition in 7 of the 25 recordings (cf. Fig. 1B). This phase of the response, which was more variable than I1, started about 375 ± 152 ms (mean and standard deviation, $n = 39$ in 7 neuron) after stimulus onset and lasted for about 415 ± 168 ms (mean and standard deviation, $n = 39$ in 7 neurons). The details of these epochs of interrupted firing were also cell and odor dependent. Despite the fact that each cell was recorded in a different animal, all of these findings are consistent with results of multi-unit experiments (Daly et al., 2004b; Brown et al., 2005). Thus, constructing a virtual ensemble from single, characterized neurons does not seem to introduce obvious artifacts.

Raw spike trains are, however, not suited for use in a 4-dimensional representation of antennal lobe activity, because neuronal activity has to be comparable across projection neurons with very different baseline and response firing rates. Thus, the raw spike train data have to be transformed. This transformation should not change the dynamics of the neural activity patterns and should not lead to distortions of the results of subsequent analyses.

Therefore, we binned the action potential train data of each cell (bin-width: 20 ms) and afterwards transformed these binned data to z-score values. A comparison of the histograms of raw and z-score data showed that the dynamics of the neural responses were preserved by this transformation (data not shown). In fact, because negative z-scores are possible, gaps in firing are more easily detected. To test whether population analysis following this transformation was distorted, we implemented a Euclidean distance analysis. This analysis also allowed us to establish how our serially recorded virtual ensemble compares to similar ensembles from multi-unit recordings (cf. Brown et al., 2005). A comparison of the action potential-based (Fig. 6A and B) and z-score Euclidean dis-

tance (Fig. 6C and D) plots shows that the peaks and troughs were preserved in a one-to-one fashion throughout. The Pearson's correlation coefficients between the action potential-based and z-score based Euclidean distance curves were between 0.91 and 0.99 (all $p \ll 0.0001$), indicating highly significant linear correlations. The slopes of the action potential and z-score based Euclidean distance

plots appear different because of the different ordinates. However, this scaling effect did not change the time course of the Euclidean distance dynamics of the z-score transformed Euclidean distance (cf. below). In other words, z-score based Euclidean distance measures were not distorted as compared to action potential-based Euclidean distances. Furthermore, using standard deviations as the

Fig. 7. Z-score transformed data were used to quantify how many neurons increased, stopped or did not change their firing at each bin of the peri-stimulus time. For this, the normalized z-score colorbar was broken into three regions: red, increased firing, ≥ 2 ; blue, pause in firing, ≤ -0.5 ; black, no change, > -0.5 and < 2.0 standard deviations. Neuron counts within sliding windows of 20 ms (A) and 180 ms (B) averaged across the seven stimuli (blank and the six odors: $n = 7$; 15–19 glomeruli). Data are plotted as means $\pm$ standard error of the mean (broken lines). (C and D) The same sliding windows (C: 20 ms; D: 180 ms) were used for the separate counts for each stimulus. *Abbreviation:* gl, number of glomeruli included in each of these counts.

Fig. 7. (Continued).

measurement unit makes it possible to discern statistically significant from non-significant changes of neuronal activity, which cannot be done with action potential count based arbitrary units (Stopfer et al., 2003).

As in ensembles based on multi-unit recordings, responses to the same odor ('within odors') showed low distance values. This indicates that odor-driven responses are highly consistent (Fig. 6A and B). For the various comparisons between different odors (Fig. 6), the Euclidean distance values increased by at least two times. There

was always a brief decrease in distance between 100 and 140 ms followed by a rapid increase in Euclidean distance, which started about 120–140 ms after stimulus onset. This drop in Euclidean distance reflects the initial pause in firing across the artificial population, whereas the increasing Euclidean distance is consistent in response time with the odor-dependent sequence of activations (cf. Fig. 5). In all cases, the Euclidean distance values peaked about 220–260 ms after stimulus onset, marking the end of the activation phase of the projection neuron responses, and they declined over the sub-

Fig. 8. Posteromedian views of the 3-dimensional reference antennal lobe show representations of stimulus driven projection neuron activity for each condition, i.e. blank and six odors. Glomeruli are colored according to the normalized z-score colorbar. Their colors indicate the maximum z-score value for each glomerulus within the time window between 100 and 400 ms after stimulus onset. *Abbreviation:* LC/MC, lateral/median soma cluster.

sequent 500–550 ms. These values are consistent with what has previously been reported for multi-unit data (Daly et al., 2004b).

3.3. Activity over time

The data have a sub-millisecond temporal resolution, which provides an opportunity to – post hoc – compare glomerular activation patterns at different temporal resolutions. Thus, the bin-width of

the analysis can be chosen to emphasize the temporal or the spatial aspect of odor coding or any compromise between these extremes. A normalized color code makes it possible to display and to easily discern the number of positive and negative deviations from the mean action potential count (Figs. 7 and 8). This color code has been further reduced in Fig. 7 to discriminate between increased firing rate (red: z-score ≥ 2 standard deviations), decreased firing (blue: z-score ≤ -0.5 standard deviations) and non-significant vari-

ations in firing (black: $-0.5 < z\text{-score} < 2$ standard deviations). The threshold for decreased firing maximizes the overlap of I1 inhibition, as observed in original recording traces (Supplementary Fig. 1), with low $z\text{-score}$ values, but similarly low $z\text{-score}$ values can occur at any other peri-stimulus time too (cf. Section 4). The responses are shown as averages across the seven stimuli (Fig. 7A and B) and separately for each of these stimuli (Fig. 7C and D). Fig. 7A, which emphasizes the temporal aspect (20 ms sliding window), shows that up to 4 of the 19 glomeruli (21%) were excited per 20 ms time bin and this maximum activation was reached about 260 ms after stimulus onset (Fig. 7A red line). Because of their different response properties, not all glomeruli were simultaneously active. This means that the number of glomeruli, which were active during the whole odor response, was higher than at any one moment in response time (cf. Movies). The blue curve shows, that 160 ms after stimulus onset about 9 of the 19 glomeruli (47%) fired less, which can be explained by the occurrence of the first pause of firing in a typical projection neuron. In approximately the same number of projection neurons/bin a second pause in firing occurred by about 450 ms. The black curve shows that in parallel with the first pause in firing, about seven projection neurons (37%) did not change their firing levels. At the peak of the response, 9 of the 19 glomeruli (47%) were not activated at all. As expected, a longer integration window of 180 ms (Fig. 7B) leads to higher counts per window and condition; consequently, the sum of three categories has to be higher than the number of neurons in the ensemble. Here, 8 of the 19 glomeruli (42%) are excited about 260 ms post stimulus onset (Fig. 7B red curve). The effects of the first and second pause in firing can still be observed (Fig. 7B blue curve). This shows that despite some smoothing by the sliding window the general dynamics of glomerular activation/inactivation were preserved.

In Fig. 7C and D, the data are plotted in the same way, but separately for the blank and each of the six odors. Although not identical, these plots show the basic features of the responses, which were described above. The initial drop in firing (black and blue curves), the excitatory phase (red curves), second drop in firing (black and

blue curves) and the return to baseline activity (black and blue curves) were very similar across the blank and the different odors. However, these activity counts do not provide any spatial or temporal information.

In the next to the last step, we, therefore, combined the 3-dimensional antennal lobe representation of the recorded projection neurons (cf. Fig. 4) with the $z\text{-score}$ transformed spike train data. In contrast to the raster plots (Fig. 5), we can apply an objective criterion (≥ 2 standard deviations) for significant activation of a glomerulus in these plots, because activity is represented by $z\text{-scores}$. In the normalized color code very low action potential counts/bin ($z\text{-score} \leq -0.5$ standard deviations) are marked by cool colors, excitation ($z\text{-score} \geq 2$ standard deviations) by warm colors, while green represents changes around average counts/bin ($-0.5 < z\text{-score} < 2$ standard deviations). In Fig. 8, activity in a window of 100–400 ms after stimulus onset is shown; the use of a 300 ms integration window provides a good basis for a comparison to imaging data. Glomeruli, of which two recordings are available, are represented by the activity of the projection neuron with the more complete physiology and/or morphology data. The long time window reveals how much of the antennal lobe is actively encoding for an odor. Fig. 8 shows the pattern of glomeruli across the antennal lobe that increased, stopped or did not change firing in response to odor stimulation. Currently, there is no obvious focal point of neural activity for any of the odors used. Active glomeruli were not located along single surfaces of the antennal lobe, but were distributed across the entire surface of the antennal lobe. Furthermore, the spatial patterns of glomerular activation were odor specific. This is obvious from a comparison of the activation patterns of some glomeruli, which are located in the posteromedian plain. 2-Hexanone elicited significant excitation in glomeruli 2, 27, 37, 43, 44, 53 and four more out-of-view glomeruli, while glomeruli 52, 55, 64 and one out-of-view glomerulus stopped firing. Stimulation with 1-hexanol led to a different pattern. Now, glomeruli 27, 37, 43, 44 and four more out-of-view glomeruli were excited, while glomeruli 2, 42, 52, 53, 55, 64, and one out-of-view glomeru-

Fig. 9. Pictures of four frames from the animations illustrate how activity patterns change in odor (A and C vs. B and D) and time (A vs. C and B vs. D) dependent ways.

lus stopped firing. In other words, there is an odor specific spatial code, which is distributed widely across the entire antennal lobe. Based on this time window, which encompasses all the significant parts of the odor responses, about 47% of the 19 glomeruli were excited, 26% stopped firing and 27% did no change their activity in response to an odor. In other words, on average, about 73% of the antennal lobe actively contributes to the output code for any of the odors used.

3.4. 4-Dimensional representation

In the last step, we created 4-dimensional representations of the neural responses to each of the seven stimuli (Movies). It may be helpful to use [Supplementary Fig. 2](#), which shows the glomerulus numbers, as legend while reading the following descriptions and watching the animations. Furthermore, if each animation is opened in a separate window, the responses can be directly compared at each point in time. These animations are based on the z-score transformed and false-color coded data used in [Figs. 7 and 8](#), but they provide a more comprehensive view of the spatial aspects of the responses to the six odors and the blank stimulus.

The animations show that fluctuations around the mean counts/bin were widespread across the entire antennal lobe. Response maxima were reached at about 240–260 ms after stimulus onset and then activity decreased slowly over about 500–540 ms (cf. [Fig. 6](#)). Furthermore, not all responsive glomeruli were active/inactive at the same time, but they became active/inactive in odor-dependent sequences and combinations. Thus, the odor-dependent sets of active/inactive glomeruli show time variant degrees of differences and overlap. The movies indicate that projection neurons, which were excited by a stimulus, responded with an initial pause in firing, which was followed by excitation and then by a second pause in firing (cf. [Fig. 1B](#)); the responses of glomeruli 7 and 43 to 2-hexanone stimulation illustrate this (cf. also 2-hexanone and 1-hexanol: glomeruli 37 and 44). Latencies and durations of the initial phase of interrupted firing were different in different glomeruli responding to the same odor (e.g. 2-hexanone: glomerulus 7 vs. glomerulus 43), and a comparison between the movies shows that they were also odor dependent (e.g. glomerulus 43: 2-hexanone vs. 1-hexanol). The excitatory phase of the stimulus driven response also shows a stimulus specific pattern of onset latencies. Furthermore, glomeruli were excited in different combinations across the whole antennal lobe. For example, 260 ms after the start of the 2-hexanone stimulus ([Fig. 9A](#)), anterior glomeruli 2 and 4, lateral glomeruli 7 and 15, medial glomerulus 43 and posterior glomerulus 44 were excited, while 20 ms later ([Fig. 9C](#)) this pattern changed to anterior glomeruli 4 and 16, lateral glomeruli 7 and 15, medial glomerulus 43 and posterior glomeruli 37 and 44. A comparison with the 1-hexanol response shows that these patterns were odor dependent. In response to 1-hexanol, anterior glomerulus 16, lateral glomeruli 7 and 15, medial glomerulus 27 and posterior glomerulus 44 responded at 260 ms ([Fig. 9B](#)), while lateral glomeruli 7 and 15 and posterior glomeruli 37 and 44 responded at 280 ms ([Fig. 9D](#)).

Moreover, the animations show that glomeruli with decreased or no counts/bin were distributed across the entire antennal lobe and that the temporal sequences of their decreases in firing were also odor dependent. For example, 240 ms after the beginning of the 2-hexanone presentation glomeruli 27, 36, 52, 55 and 64 had decreased or no counts/bin, while 240 ms after 1-hexanol stimulation on-set glomeruli 2, 42, 52, 53, 55 and 64 fired less or not at all. Yet, a different set, glomeruli 43, 55 and 64, did not fire 240 ms after stimulation with 2-octanone. Odor-dependent decreases in counts/bin also included phases, which could be attributed to the rather variable second gap in firing, which followed excitatory responses (e.g. glomerulus 44: 2-hexanone vs. 1-decanol). These

phases with pauses in firing may be an important feature for odor representations. Thus it may be necessary to consider both the numbers of glomeruli, which were excited and stopped firing, to completely describe any odor-driven response.

4. Discussion

There is a gap between imaging based and multi-unit neural ensemble methods. On the one hand, imaging methods provide an indicator of the spatial distribution of neural population activity, but with limited temporal resolution. On the other hand, multi-unit methods reveal response dynamics across neural populations with high temporal precision, but provide only very limited spatial information. The novel approach described here, complements imaging and multi-unit techniques. It takes advantage of the known species-specific pattern of glomeruli. Sequentially recorded, stained and characterized individual projection neurons are used to assign neural spiking activity to the specific glomeruli, they arborize in. Combining these serial recordings results in an output representation for each stimulus, which is visualized in the anatomical context of a newly created reference antennal lobe. Z-score transformed data preserve response dynamics and, thus, provide a good basis for further population analysis. Stimulus–response dynamics of this virtual ensemble are consistent with results from multi-unit data. Furthermore, the approach shown here adds spatial information for a more direct comparison with imaging methods. Therefore, this 4-dimensional representation of antennal lobe responses provides a meaningful tool to analyze odor output coding in primary olfactory neuropil.

4.1. Comparison to other techniques

Extracellular multi-site and multi-unit recording techniques have been used in a variety of vertebrate and invertebrate systems with great success (e.g. [Wilson and McNaughton, 1993](#); [Henze et al., 2000](#); [Wessberg et al., 2000](#); [Chapin, 2004](#); [Daly et al., 2004a](#); [Brown et al., 2005](#); [Mazor and Laurent, 2005](#)). Spike sorting allows classification of events based on a multitude of criteria and to form so-called units. However, the error rates of spike sorting depend on many physical, physiological, and other factors and can be rather high ([Harris et al., 2000](#)). Furthermore, except in locusts and cockroaches ([Laurent and Davidowitz, 1994](#); [Husch et al., 2009](#)), most local interneurons in insect antennal lobes produce sodium spikes ([Christensen et al., 1993](#); [Anton and Homberg, 1999](#); [Wilson et al., 2004](#); [Wilson and Laurent, 2005](#)). Moreover, like local interneurons ([Christensen et al., 1993](#)), projection neurons can have short stimulus–response latencies (e.g. [Fig. 5](#), cell 4: 7, anterior cluster, inner antenno-cerebral tract). These factors increase the difficulty of discriminating between projection neurons and local interneurons or subpopulations of these two major classes ([Matsumoto and Hildebrand, 1981](#); [Christensen et al., 1993](#); [Anton and Homberg, 1999](#)), because the cells recorded from are not stained in multi-unit experiments. However, only projection neuron activity is read-out by and, thus, significant for higher brain centers. Thus, because multi-unit recordings represent both local processing (local interneuron) and output activity (projection neuron) in most insects except locusts, interpreting the significance and meaning of output by using extracellular methods is challenging. The new approach reported here, overcomes these limitations of extracellular recording, because each neuron is precisely classified and there is no issue of contamination or missing action potentials.

Neural activity can be optically recorded with voltage-sensitive dyes, Ca^{2+} sensitive dyes, by utilizing intrinsic signals or when synaptopHluorin is expressed by the targeted cells. These methods

are used to study single cells (e.g. [Single and Borst, 1998](#)) and populations of neurons (e.g. [Bonhoeffer and Grinvald, 1993](#); [Shang et al., 2007](#)). Ca^{2+} -imaging has been most prevalent in olfaction research (e.g. [Friedrich and Korsching, 1997](#); [Joerges et al., 1997](#); [Wachowiak et al., 2002](#); [Hansson et al., 2003](#); [Spors et al., 2006](#); [Silbering and Galizia, 2007](#)). For this, the tissue is usually superfused with dye, which is taken up into cells over a preparation-specific staining period. It is, however, not known, which cells take the dye up and if this uptake occurs evenly across the preparation and the different cell types. Furthermore, when imaging large regions of neuropil, it is not possible to resolve individual unit activity patterns; this is true even when dye loading is restricted to a specific subpopulation (e.g. [Sachse and Galizia, 2003](#)). In all imaging systems, the light sensor, mostly a CCD camera, samples light from a relatively thin section within the tissue, i.e. the focal plane plus light from the depth of field and some scattered light. This works well in planar neuropils like parts of the olfactory bulb in vertebrates, but has limits in 3-dimensional structures like the insect antennal lobe. [Fig. 8](#) and the animations of the 4-dimensional response representation show, that a significant number of glomeruli responding to odor would be missed, if only a single plane of the antennal lobe were considered.

Fluorescent signals are usually very small, which means that light has to be sampled over relatively long periods of time. Therefore, with only few exceptions ([Wachowiak et al., 2002](#); [Spors et al., 2006](#); [Wesson et al., 2008](#)), the temporal resolution of Ca^{2+} -imaging experiments is usually not higher than 3–5 Hz when large neuropil areas are imaged. In other words, the temporal resolution of imaging techniques is two orders of magnitude slower than an action potential. As a corollary, odor-driven glomerular activation patterns may seem more similar than they actually are based on the integration by the imaging technique. Furthermore, if the I1 and I2 phases are integrated with a brief excitatory response when using Ca^{2+} -imaging, it may be possible to miss meaningful excitatory responses. In some circumstances, it may be possible that brief excitatory responses, which are flanked by I1 and I2 phases, may actually appear as suppression using these methods. Thus, because temporal dynamics of the neural response are not resolved well, differences in the sequence of glomerular activation may be invisible and, thus, missed in an interpretation of the results. In contrast to Ca^{2+} -imaging, the approach introduced here, works on a sub-millisecond temporal resolution, is not limited to a small volume around one focal plane, and is based on characterizing each cell of a virtual ensemble.

The new approach shown here is complementary to imaging and multi-unit techniques and, like other methods, has a number of limitations and is based on some assumptions. One limitation is that data from a serially recorded virtual ensemble cannot be used for studies on synchronous firing of populations of neurons, because there is no synchronous neural activity across animals. For the same reason, this method does not provide a means to quantify the relationship between population spiking and local field potentials. In addition, we make assumptions that should be clarified. One of these is that stimulus delivery is precise and consistent between animals. To address this issue, we made certain that airflow velocity and distance from the odor cartridges to the antennae were identical across preparations. Nevertheless variability in delivery between preparations could introduce a small amount of variability in response latencies. But given that the delay from odor valve opening to the odor reaching the base of the antenna is on the order of 10 ms we estimate this contribution to response variability to be nominal. One indicator of stimulus consistency is that we observe little variability across the five repeats in the responses of single projection neurons. Another assumption is that the response latencies of cells, which arborize in the same glomerulus, but are recorded in different animals, are the same. The ultimate test of

this assumption would require to record the same identified neuron in different animals, which is fairly difficult. However, when we consider the time course of odor-driven population responses from multi-unit studies we find no difference, suggesting that between animal variability is minimal. Finally, from a practical standpoint, a serial reconstruction based on single cell recordings is not feasible in olfactory systems with a large number of glomeruli like in mammals.

4.2. Inhibition and statistical significance

Depending on the location of the recording site relative to the spike initiation zone, intracellular recordings can contain both, action potentials and postsynaptic potentials. The latter provide direct evidence regarding the inhibitory and excitatory events, which shape the spiking activity of a given neuron. In about half of our recordings, graded potentials were represented well enough to statistically describe I1, which is based on GABA_A mediated lateral inhibition ([Waldrop et al., 1987](#); [Christensen et al., 1998](#)) and I2 inhibition, the mechanism of which is unknown. Both, I1 and I2 are hallmarks of antennal lobe projection neuron responses to odor. In recordings where subthreshold currents were not observed, the possible causes underlying pauses in firing cannot be determined. Therefore, we discuss these times as “pauses in firing” and not as inhibition.

Our analysis is based on action potentials, because they are the output of the antennal lobe that reaches and is used by neurons in higher brain centers, like the mushroom bodies or lateral horn. To make meaningful statements about input codes to higher brain centers, their analyses must be based on the signals arriving there, trains of action potentials. The added benefit is that analysis tools, established for multi-unit data, can be used with the spike train data from virtual ensembles.

A projection neuron fires up to four, maybe five action potentials per 20 ms bin during the excitatory phase of an odor response; other times it fires no action potentials during one or more 20 ms bins. Because no neuron can fire less than zero action potentials, there is an asymmetry between increased and decreased action potential counts per time bin, which is carried over into the z-score data (range: –1.2 to 5.5 standard deviations) and any linear transformation of the spike data. We compared a variety of analysis methods, but no currently available method overcomes this floor effect.

With regard to increased action potential counts/bin, we could use a value of ≥ 2 as significance criterion, because the maximum z-score value was 5.5 standard deviations. The lower limit for decreased action potential counts/bin was –1.2 standard deviations. Therefore, we chose ≤ -0.5 as limiting criterion, because it maximized the temporal overlap of small z-score values with phases when I1 inhibition was actually observed in recordings (cf. above). Thus, this criterion increases our ability to pinpoint times when a pause in the firing of a neuron was probably based on I1. Nevertheless, because the relationship between statistical and neurobiological significance is unknown, we do not know if a statistically insignificant increase or decrease in action potential counts/bin really is insignificant for a postsynaptic neuron. Moreover, we do not know if biological significance is symmetrical or asymmetrical with regard to increased/decreased action potential counts/bin. Thus, we do not speak of statistically significant low action potential counts/bin.

4.3. Neural activity across the antennal lobe

The virtual ensemble shown here has a response dynamic that is essentially identical to what has been reported for multi-unit data. Odor responses evolve in time; that is, different projection neurons, which can be equated to identified glomeruli in this study,

respond with different onset latencies and fire (or not) with different odor-dependent patterns (e.g. [Daly et al., 2004b](#); [Brown et al., 2005](#)). Euclidean distance analysis ([Fig. 6](#)) shows that odor representations evolve dramatically over time, producing unique output codes, which presumably contribute to unique odor representations and, hence, discrimination. About 100 ms after stimulus onset, Euclidean distance briefly dips, then rapidly increases, reaching a peak within about an additional 140 ms, and then declines over about 500 ms. This time course is very similar to what has been reported for extracellular multi-unit data ([Daly et al., 2004b](#); [Brown et al., 2005](#)). A recent report, using the same approach described here, obtained similar results in the moth *Bombyx mori* ([Namiki and Kanzaki, 2008](#)). The large increase in Euclidean distance by 4 or more standard deviations is significant and indicates that this virtual ensemble could discriminate very well between classes of odorants (ketones vs. alcohols) and single odors of the same moiety that differ only in carbon chain length.

Our new approach allows the correlation of temporal dynamics produced by action potentials with a 3-dimensional spatial perspective. We find that per response, about 47% of the glomeruli are excited, about 26% stop firing, and about 27% do not change activity. These figures roughly match with previously reported distributions of excited, inhibited and unchanged units in multi-unit recordings ([Daly et al., 2004b](#)). Now, we can, however, relate these figures to the activity distribution across the antennal lobe. If one extrapolates this finding, it would mean that actually about 46 glomeruli of the 63 of an antennal lobe are actively involved in coding for a particular odor, 30 of them excited and 16 not firing. This would contrast strongly with the results of imaging studies, which indicate that only about 13–30% of the glomeruli are actively involved in odor coding ([Joerges et al., 1997](#); [Uchida et al., 2000](#); [Sachse and Galizia, 2002](#); [Silbering and Galizia, 2007](#)).

Will the percentages we present here change when recordings from more glomeruli are added? The current data set comprises approximately 30% of the antennal lobe glomeruli, which were sampled randomly and are distributed across all surfaces of the antennal lobe (cf. [Fig. 4](#)). The animations (e.g. 2-hexanone) clearly show that during every odor response glomeruli from different areas of the antennal lobe are active (e.g. 2-hexanone). The sample size of our data is in the same range as in multi-unit recording experiments and spans a significant portion of the antennal lobe ([Daly et al., 2004b](#)). Based on these facts, we expect no major changes in the percentages of glomeruli, which are excited, stop firing or do not change their activity.

Why is the identity of the glomerulus important with regard to projection neurons? It was indicated by [Buck and Axel \(1991\)](#) and later shown ([Mombaerts et al., 1996](#); [Wang et al., 1998](#); [Vosshall et al., 2000](#)) that olfactory receptor neurons expressing the same receptor protein project to the same glomerulus. This seems to indicate that each glomerulus is not just an anatomically, but, based on ORN identity, a functionally distinct entity. Recently, it was shown that projection neurons have a broader odor tuning than the olfactory receptor neurons they are connected to ([Wilson et al., 2004](#)). This finding suggested the existence of excitatory connections between glomeruli by local excitatory interneurons, which were recently described ([Olsen et al., 2007](#); [Root et al., 2007](#); [Shang et al., 2007](#)). Odor responses are, furthermore, shaped by interglomerular presynaptic inhibition ([Olsen and Wilson, 2008](#)). Because of the lateral interactions between different glomeruli of the antennal lobe, it follows that the more precise descriptor for any projection neuron is the glomerulus it arborizes in and not the ORN it is connected with. The approach shown here accounts for this, because it takes advantage of the 3-dimensional antennal lobe reference atlas and uses characterized projection neurons as a basis for identifying their respective glomeruli.

Along these lines, should odor responses of the approximately 14 projection neurons arborizing in the same glomerulus (63 glomeruli, 900 projection neurons; [Schachtner et al., 2005](#)) not be identical? For six of the glomeruli, two projection neurons were recorded in this study. Within these projection neuron pairs, the responses to the blank and the six odors differ in most but not all cases. Differences in odor responses of projection neurons from the same glomerulus have been described in earlier studies too ([Roche King et al., 2000](#); [Sadek et al., 2002](#); [Guerenstein et al., 2004](#); [Masante-Roca et al., 2005](#)). On the other hand, it was reported that projection neurons from the same glomerulus have very similar response properties ([Reisenman et al., 2005](#)). Moreover, paired recordings show an increased probability for synchronous firing if two antennal lobe/olfactory bulb neurons arborize in the same glomerulus ([Schoppa and Westbrook, 2001](#); [Lei et al., 2002](#)).

Like others, we face questions about the variability of the responses of projection neurons and glomeruli (cf. [Galizia et al., 1999b](#)). But in no study have primary olfactory interneurons been morphologically and physiologically identified like, e.g. in the cricket auditory ([Wohlers and Huber, 1982](#)) and cercal system ([Jacobs and Murphey, 1987](#); [Miller et al., 1991](#)). Without such rigorous identification, it is not possible to discriminate between the variability of identified neurons from different animals and the variability between cells of the same type, which arborize in the same glomerulus. Because there are no quantitative data, it is difficult to assess the impact of these two types of variability on the 4-dimensional representation presented here. However, it is difficult to conceive that either source of variability would not have some impact on the virtual ensemble presented here or any other data set collected with a different method.

In conclusion, the new approach shown here will also contribute to clarifying this issue and, thus, contribute to a further understanding of odor encoding. As our database of recorded and stained neurons grows, it will allow exploration of whole antennal lobe responses to different odors as a function of various stimulus parameters, such as concentration and duration. This database will also provide unique opportunities to perform more focused population analyses of specific subpopulations of projection neurons based on morphological characteristics, such as differences between distinct cell clusters and output tracks. This will lead to a deeper understanding of the functional role of multiple output cell types and pathways, which are found in both vertebrates and invertebrates. Finally, future studies will match psychophysical experiments, which characterize detection and discrimination thresholds in this species. Thus, the dynamic response pattern of a virtual ensemble of characterized neurons will be correlated with biologically relevant concentrations and sensory limits of the model system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jneumeth.2009.03.019](https://doi.org/10.1016/j.jneumeth.2009.03.019).

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