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A digital three-dimensional atlas of the honeybee antennal lobe based on optical sections acquired by confocal microscopy

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Abstract We present a digital atlas of the glomeruli in the antennal lobe of the honeybee, *Apis mellifera*, accessible to the scientific community via the Internet. The atlas allows the identification of glomeruli in preparations in which the glomeruli can be recognized, be it in sections or in whole-mounts. The high resolution of the anatomical data upon which the atlas is based and its electronic form should prove to be an important tool for anyone involved in the study of the honeybee antennal lobe. Its accessibility via the Internet is a step towards interactive and freely accessible databases of animal brains.

Key words Olfactory glomeruli · Olfaction · Anatomical reconstruction · Fluorescent tracer · Internet · Honey bee, *Apis mellifera* (Insecta)

Introduction

The most striking similarity between the olfactory systems of mammals and insects is the glomerular organization of their primary processing centres in the brain. It is widely believed that glomeruli form the elementary units of an olfactory code. To understand their role, glomeruli have to be studied individually. In mice, individual glomeruli have been labelled with molecular tools (Mombaerts et al. 1996; Vassar et al. 1994). However, complete mapping of the 2000 (approximately) glomeruli in each olfactory bulb appears as yet out of reach.

In insects, many researchers have focused on the pheromone system, because of the clear identifiability of its glomeruli on morphological grounds (Hildebrand 1996). These glomeruli form the macroglomerular complex in male Lepidoptera (Hansson 1995) or the macroglomerulus in *Periplaneta americana* (Boeckh and Tolbert 1993). Male honeybees (drones) also have macroglomeruli (Arnold et al. 1985).

The spatial arrangement of non-pheromonal glomeruli appears to be highly stereotyped in each insect species. *Drosophila* has 43 glomeruli, all of which have been identified (Stocker et al. 1990; Laissue et al., in press). The glomeruli in *Manduca sexta* have also been counted and mapped (Rospars and Hildebrand 1992).

The worker honeybee possesses between 156 and 166 glomeruli (Flanagan and Mercer 1989a; Arnold et al. 1985). The branching pattern of the honeybee antennal nerve (AN) into the olfactory glomeruli has been described (Suzuki 1975). Where it enters the antennal lobe (AL), it splits into six tracts (T1 to T6), of which four (T1 to T4) innervate the glomeruli of the AL. T1 and T2 pass through the coarse neuropil, with T1 innervating about 70 glomeruli in the dorso-rostral part of the AL, and T2 innervating exactly seven medial glomeruli. T3 and T4 run ventro-caudally outside of the AL neuropil, together with T5 and T6 (see Tb in Fig. 2). T3 innervates about 70 ventro-caudally positioned glomeruli and T4 exactly seven posterior glomeruli. Glomeruli innervated by T4 are unique, as the receptor cells terminate within the entire glomerular neuropil. All other glomeruli are divisible into cortex and core, with receptor cells almost exclusively innervating the cortex (Fig. 1). Tracts T5 and T6 bypass the AL to enter the dorsal lobe, which is the "antennal mechanosensory and motor centre" (Homberg et al. 1989). The glomeruli are arranged stereotypically and are invariant enough in position to make it possible to create an atlas of the glomerular arrangement in the AL. Indeed, an atlas of the worker honeybee AL has already been published (Flanagan and Mercer 1989a).

The availability and easy accessibility of such a reference work is of paramount importance for functional and comparative investigations of the glomerular role in olfactory development and functional coding. Therefore, we have created a new computerized atlas, building upon the previously published identification of glomeruli by Flanagan and Mercer (1989a). Part of this work has been presented in abstract form (McIlwrath et al. 1997). This new atlas

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is based on optical sections obtained by using a confocal microscope, is completely digitized and is available on the Internet. Because of the high resolution of the primary data and the software that is now available for image processing, sections in any plane and surface views from any angle can be created from the image stacks. This should allow researchers to identify glomeruli in their experiments, irrespective of whether they have whole-mounts or sections at their disposal.

Materials and methods

Staining procedures

In order to visualize the glomeruli in the confocal microscope, mass fills from the ANs were performed. Bees (*Apis mellifera carnica*) were collected from the hive, fixed in metal tubes and fed to satiation. All bees were foraging adult worker bees. In winter, bees were kept in a flight chamber under an artificial 12 h light/12 h dark rhythm. The antennae were immobilized by fixing them to the head capsule with wax. The scapus of one antenna was then cut and a small mould was formed around the antennal stump with vaseline. The mould was filled with neurobiotin solution (2.5% in H_2O, Vector Laboratories). The bees were subsequently kept overnight in a humid chamber at room temperature (20°C) to allow complete tracer transport. The head capsule was then opened, the tracheae and glands were removed and 4% formalin was injected into the head capsule. This procedure was chosen in order to reduce any tissue distortion that could occur if the brain had been removed from the cuticule before fixation. Following a 15-min prefixation period, the brain was removed from the capsule. After an additional fixation of 1.5 h in 4% formalin on ice, the brains were rinsed in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and in a series of alcohol solutions (50%, 70%, 90%, 99%, 100% ethanol, 10 min each; xylol, 5 min; 100%, 99%, 90%, 70%, 50% ethanol, 10 min each). This was followed by rinsing the preparations in Triton solution (0.1% Triton X-100, Serva, in PBS) at 37°C, incubation in digestive solution for 30 min (1 mg/ml collagenase, 1 mg/ml hyaluronidase, both from Sigma, 0.1% Triton solution in PBS), and saturation in NGS solution (10% normal goat serum, Vector Laboratories, in 0.1% Triton PBS) for 30 min. The brains were then rinsed in Triton-PBS and incubated overnight in the chromophore solution (1.5% Cy3 conjugated to Streptavidin, Jackson Immuno Research Laboratories, in PBS). The following day, the brains were rinsed in PBS, dehydrated in increasing ethanol solutions (50%, 70%, 90%, 99%, 100%, 10 min each) and mounted as whole-mounts on a coverslip in methyl salicylate.

Confocal data acquisition and processing

The whole-mounts were imaged with a confocal microscope (Leica TCS 4D with a DM RBE microscope) by using an LD 16× oil immersion objective (NA=0.5). Cy3 was excited with the 568-nm line of an ArKr 150-mW laser and detected with an LP 590 filter; an 8× line average was used to improve the signal to noise ratio. The entire AL was imaged into one stack. The resolution differed in the various preparations but was generally about 0.8 µm/voxel in the image plane (512×512 pixels) and about 2.0 µm/voxel in axial dimension (80–100 planes). This axial resolution was chosen in order to have each glomerulus in at least 10 sections, since the diameter of glomeruli varies between 30 and 50 µm. The raw data was stored on CD-ROM.

Confocal images were processed by using Imaris (Software Package by Bitplane, Zürich, Switzerland) on a Silicon Graphics Workstation. Tools used to improve image quality were background noise subtraction, emission attenuation correction and contrast enhancement.

Out of a total of 53 scanned ALs, 8 were selected for further processing by applying the following criteria: glomerular outlines were detectable throughout the AL, contrast was high in the entire stack and no damage to the tissue was visible. Optical sections in these ALs were then compared with the sections published by Flanagan and Mercer (1989a). A single left AL was chosen in which the orientation of the optical sections appeared closely to match the published sections and in which the identity of the glomeruli could thus be reconstructed. The outlines of the glomeruli could be seen in every individual optical section. Polygons were drawn by hand around the glomeruli with a computer mouse, tracing each glomerulus through all optical sections in which it appeared. This procedure yielded the raw data of the atlas as a stack of bitmap files spanning the AL.

Glomerular identification and labelling

This raw atlas was compared with the published atlas (Flanagan and Mercer 1989a) and the individual glomeruli were identified. Where there was uncertainty concerning the identity of a glomerulus, we compared the image with that of the other seven selected ALs.

In bitmap files, each pixel can be attributed a particular value, ranging from 0 to 255 for the 8-bit resolution. Since the total number of glomeruli was about 160, we assigned a different value to each glomerulus; this allowed us to assign each glomerulus a unique value (see Table 1 for the appropriate list).

We tried to follow the labelling proposed by Flanagan and Mercer (1989a) as closely as possible. Pixel values were given according to the glomerulus name, with an offset of 0 for T1, offset of 80 for T2, offset of 90 for T3 and offset of 170 for T4, in the following way. Glomeruli of tract 1, labelled T1(1) to T1(71), were given pixel values from 1 to 71. Glomeruli from tract 2 (T2–1(1) to T2–1(6)) were given values from 81 to 87. Glomeruli from tract 3, labelled T3(1) to T3(71) were given values from 91 to 161, i.e. the glomerulus number plus 90. Glomeruli from tract 4 (T4–1(1) to T4–7(1)) were given values from 171 to 177, i.e. glomerulus plus 170. For purposes of brevity, labels that had to be printed into the slices (e.g. Fig. 2) were shortened in the following way: instead of the tract being put into parentheses after the glomerulus number, a single letter was apposed to it, with A denoting T1, B denoting T2 and so on, e.g. A45 was used for 45 (T1) and C60 for 60 (T3). In order not to create confusion among the glomeruli innervated by branches of tract 2, T2 glomerulus T2–1(1) was labelled B01(1) and T2–2(1) was labelled B01(2), whereas all other T2–1(*n*) were labelled B*n*. T4-*n*(1) was labelled D*n*. Full details are given in Table 1.

With NIH Image, a simplified version of the AL atlas was created, with different colours for the four tracts and their glomeruli, which were directly labelled for identification. This version allows for fast browsing of the frontal sections but reslicing will yield unsatisfactory results.

Viewing tools

Browsing through the atlas is easy with any three-dimensional-capable graphics package. We have used Imaris (Bitplane, Zurich, Switzerland) on a Silicon Graphics Workstation, IDL (Research Systems, Boulder, Colo.) on a Macintosh or IBM-compatible computer, for which we have included a sample procedure in the website, and NIH Image (a public domain Macintosh program developed at the U.S. National Institute of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/; basic procedures are described in the Appendix) on a Macintosh.

With any program capable of displaying the grey-level value, the identity of the glomerulus can be read from the screen. For example, utilizing the computer program NIH Image, the cursor can be moved into any place within the atlas and the value of the pixel can be read in the information window. Comparison of the pixel value with Table 1 leads to the identification of the structure. This program allows the novel reslicing of the entire atlas in any orientation and the creation of projections from any angle.

Table 1 Comparison of LUT values (voxel values) in the digital value atlas, short name-tags in the digital named atlas and glomerulus identity according to Flanagan and Mercer (1989). Glomeruli not explicitly mentioned in Flanagan and Mercer (1989) are labelled *nn*

LUT value	Short label	**Flanagan & Mercer**	LUT value	Short label	**Flanagan & Mercer**	LUT value	Short label	**Flanagan & Mercer**
01	A01	01 (T1)	56	A56	56 (T1)	121	C31	31 (T3)
02	A02	02 (T1)	57	A57	57 (T1)	122	C32	32 (T3)
03	A03	03 (T1)	58	A58	58 (T1)	123	C33	**nn**
04	A04	04 (T1)	59	A59	59 (T1)	124	C34	34 (T3)
05	A05	05 (T1)	60	A60	60 (T1)	125	C35	35 (T3)
06	A06	06 (T1)	61	A61	61 (T1)	126	C36	36 (T3)
07	A07	07 (T1)	62	A62	62 (T1)	127	C37	37 (T3)
08	A08	08 (T1)	63	A63	63 (T1)	128	C38	38 (T3)
09	A09	09 (T1)	64	A64	64 (T1)	129	C39	**nn**
10	A10	10 (T1)	65	A65	**nn**	130	C40	40 (T3)
11	A11	11 (T1)	66	A66	**nn**	131	C41	**nn**
12	A12	12 (T1)	67	A67	**nn**	132	C42	42 (T3)
13	A13	13 (T1)	68	A68	**nn**	133	C43	43 (T3)
14	A14	14 (T1)	69	A69	**nn**	134	C44	44 (T3)
15	A15	15 (T1)	70	A70	**nn**	135	C45	45 (T3)
nn	nn	16 (T1)	71	A71	**nn**	136	C46	46 (T3)
17	A17	17 (T1)				137	C47	**nn**
18	A18	18 (T1)	81	B01 (1)	T2-1(1)	138	C48	48 (T3)
19	A19	19 (T1)	82	B01 (2)	T2-2(1)	139	C49	49 (T3)
20	A20	20 (T1)	83	B02	T2-1(2)	140	C50	50 (T3)
21	A21	21 (T1)	84	B03	T2-1(3)	141	C51	51 (T3)
22	A22	**nn**	85	B04	T2-1(4)	142	C52	52 (T3)
23	A23	23 (T1)	86	B05	T2-1(5)	143	C53	53 (T3)
24	A24	24 (T1)	87	B06	T2-1(6)	144	C54	54 (T3)
25	A25	25 (T1)				145	C55	nn
26	A26	26 (T1)	91	C01	01 (T3)	146	C56	56 (T3)
27	A27	**nn**	92	C02	02 (T3)	147	C57	57 (T3)
28	A28	28 (T1)	93	C03	**nn**	148	C58	58 (T3)
29	A29	29 (T1)	94	C04	04 (T3)	149	C59	59 (T3)
30	A30	30 (T1)	95	C05	05 (T3)	150	C60	60 (T3)
31	A31	**nn**	96	C06	06 (T3)	151	C61	61 (T3)
32	A32	32 (T1)	97	C07	07 (T3)	152	C62	62 (T3)
33	A33	33 (T1)	98	C08	08 (T3)	153	C63	63 (T3)
34	A34	34 (T1)	99	C09	09 (T3)	154	C64	**nn**
35	A35	35 (T1)	100	C10	10 (T3)	155	C65	**nn**
36	A36	36 (T1)	101	C11	11 (T3)	156	C66	**nn**
37	A37	37 (T1)	102	C12	12 (T3)	157	C67	**nn**
38	A38	38 (T1)	103	C13	13 (T3)	158	C68	**nn**
39	A39	39 (T1)	104	C14	14 (T3)	159	C69	**nn**
40	A40	40 (T1)	105	C15	15 (T3)	160	C70	**nn**
41	A41	41 (T1)	106	C16	16 (T3)	161	C71	**nn**
42	A42	42 (T1)	107	C17	**nn**			
43	A43	43 (T1)	108	C18	18 (T3)	171	D01	T4-1(1)
44	A44	44 (T1)	109	C19	19 (T3)	172	D02	T4-2(1)
45	A45	45 (T1)	110	C20	20 (T3)	173	D03	T4-3(1)
46	A46	46 (T1)	111	C21	21 (T3)	174	D04	T4-4(1)
47	A47	47 (T1)	112	C22	22 (T3)	175	D05	T4-5(1)
48	A48	48 (T1)	113	C23	23 (T3)	176	D06	T4-6(1)
49	A49	49 (T1)	114	C24	24 (T3)	177	D07	T4-7(1)
50	A50	50 (T1)	115	C25	25 (T3)			
51	A51	51 (T1)	116	C26	26 (T3)	200	AN	
52	A52	52 (T1)	117	C27	27 (T3)	201	T1	
53	A53	53 (T1)	118	C28	28 (T3)	202	T2-1	
54	A54	54 (T1)	119	C29	29 (T3)	203	T2-2	
55	A55	55 (T1)	120	C30	30 (T3)	204	T3	
						206	TB	

Results

Histological preparation

Application of neurobiotin to the antennal stump and its subsequent labelling with Cy3 yielded strong and reliable staining in all glomeruli of the AL (Fig. 2) and strong staining in the dorsal lobe neuropile and some motoneurone somata (data not shown). The AN was always brightly labelled.

Dehydration and subsequent immersion in methyl salicylate resulted in preparations of good transparency, thus allowing for optical confocal sectioning through the entire AL. This treatment, however, led to tissue shrinkage of between 10% and 20% (approximate value determined by comparison with living ALs not yet removed from the head capsule and with histological preparations that had not been dehydrated). As the size of ALs varies between individuals (Winnington et al. 1996) and is strongly affected by season, with bees in the early spring having much smaller brains (and heads) than their summer sisters (unpublished observations), the overall size is not a relevant parameter for a standardized atlas. The size of the atlas lies in the range of the sizes of ALs found in unfixed living bees.

Identification of glomeruli

Glomeruli could be individually recognized as units. For the glomeruli in T1, T2 and T3, the innervation pattern was taken as the main aid (Fig. 1): whereas the centres of the glomeruli were unstained, their cortex was marked with dense branching of stained neurons, with neurites growing in all directions. This was opposed to fibres that passed between glomeruli and that were often oriented along the glomerular border (arrows in Fig. 1).

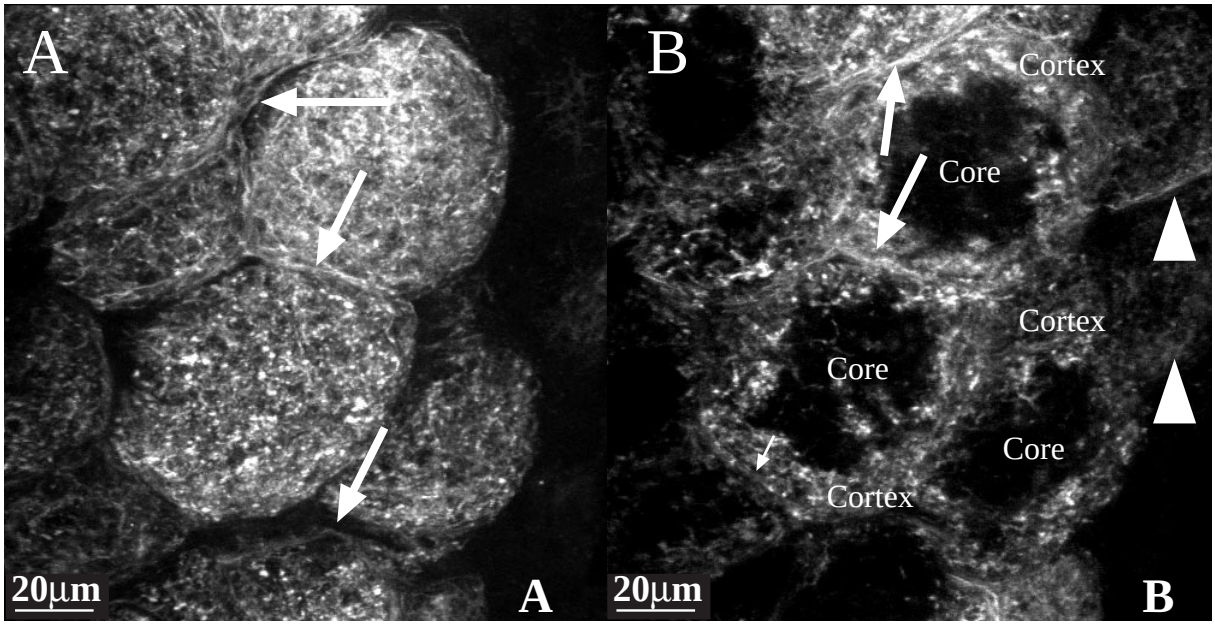
Following the approach described by Rospars and Hildebrand (1992), "primary" glomeruli were identified first in each section. "Primary" glomeruli were defined as glomeruli that could be identified solely on the basis of their form, size and relative position to prominent landmarks, such as major tracts or, in the case of the honeybee, the lateral passage. Then, "secondary" glomeruli were identified by their relative position to the primary glomeruli (for a thorough description of glomerular shape, reciprocal position and identification, see Flanagan and Mercer 1989a).

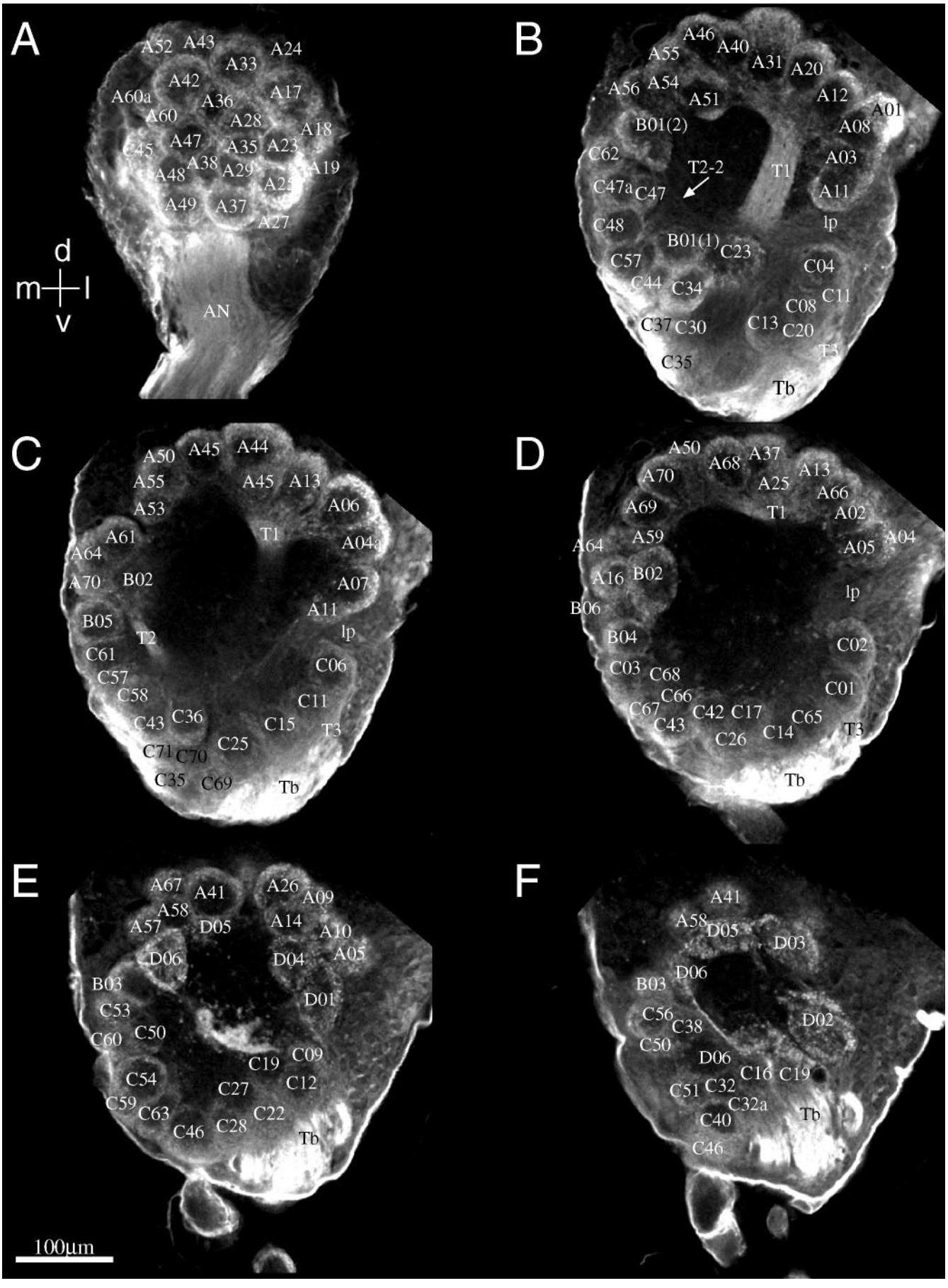
Database for the digital atlas

The digital atlas was based on the optical slices of one AL. An exemplary slice of each of the two datasets that we created is shown in Fig. 3. Each glomerulus was manually

Fig. 1A,B Example of a close-up view of three glomeruli innervated by T1, seen at two different depths. **A** Optical section through the cortex of the glomeruli. Note the dense and apparently random branching of the afferent axons. Also notice that the density of labelled fibres is lower in between the glomeruli and that fibres appear to project in one direction (*arrows*). **B** Optical section through the middle region of the same three glomeruli as in **A**. Note that the core of the glomeruli is almost void of afferent innervation and that the cortex is only between 5 and 10 μm thick (compare with the *scale bar*). Axons that bypass the glomeruli can be seen between them (*arrows*). The cortex of two additional glomeruli can be seen *right* (*arrowheads*)

Fig. 2A–F A series of six confocal optical horizontal sections (**A** 26 μm, **B** 78 μm, **C** 104 μm, **D** 130 μm, **E** 169 μm, **F** 195 μm) of the left AL of *Apis mellifera* in which receptor cell axons were fluorescently stained. Individual glomeruli are labelled (see Table 1 for full names). Note that, in most cases, the perimeter of each glomerulus can be clearly seen. Also notice the unrestricted innervation of glomeruli innervated by T4 (*D01–D07*) compared with the exclusively peripheral innervation of the other glomeruli. Glomeruli *A60* and *A60a*, and *C47* and *C47a* are anomalous (*lp* lateral passage, *T1-T4* tracts of the antennal nerve, *Tb* tracts T3-T6, *AN* antennal nerve, *d* dorsal, *v* ventral, *m* medial, *l* lateral). *Bar* 100 μm

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B01(2) A08
C62 A03
T2-2 T1
C47a C47 A11
C48 lp
B01(1) C23
C57 C04
C44 C34 C11
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C37 C30 C13 C20
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A55 A45 A13
A53 A06
A61 T1 A04a
A64
A70 B02 A07
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C59 C63 C22
C46 C28 Tb
100μm
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A41
A58 D05 D03
D06
B03
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C50
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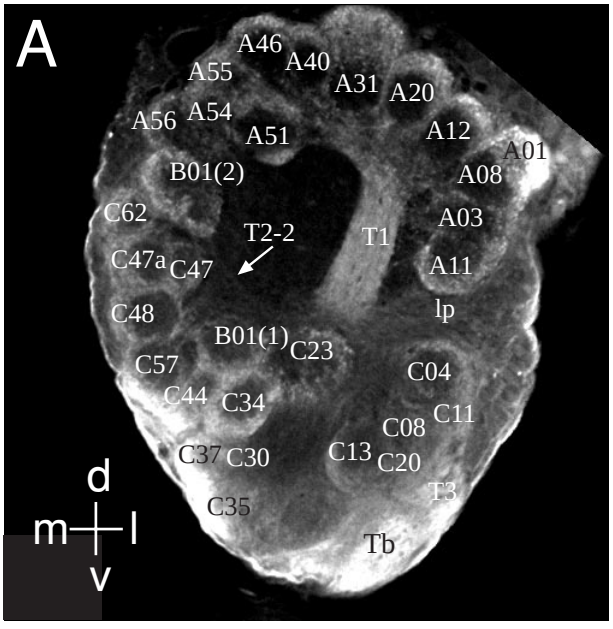
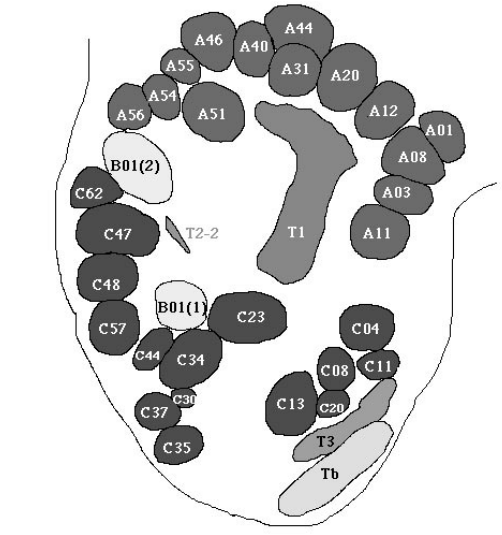
C51 C32
C32a Tb
C40
C46

Fig. 3A–C Example and description of the coding used for individual glomeruli in the digital atlas. **A** Optical section through the antennal lobe. Individual glomeruli are labelled with the shortened names (see Table 1 for name conventions). This is the same section as that in Fig. 2B. **B** Corresponding section from the labelled atlas (LabelAtl.tif). Here, each glomerulus is *outlined* and labelled. This form of the atlas is ready to use irrespective of the software employed. **C** Corresponding section from the value-coded atlas (ValueAtl.tif). The LUT attributes *grey* levels to the pixel values, starting from 0 (*white*, background), to *1* (glomerulus A01 or 1 (T1)), *81* (first glomerulus innervated by T2), *94* (91 would be the first in the list of glomeruli innervated by T3), *201* (values above 200 are tracts) and *255* (*black*, for the perimeter of the AL), to name just a few examples. The *grey* values for each glomerulus are read out and written to the side (*arrows*). The corresponding glomerular identity is given *right or left*, first with the shortened format and, in the *external columns*, with the nomenclature of Flanagan and Mercer (1989a). The corresponding glomerular identity of the grey level values can be found in Table 1

traced and the pixel grey scale values within the glomerulus were set to a level unique to that glomerulus (Fig. 3C). Therefore, in the volumetric dataset, voxels (volume elements) with a grey value of, say, 113, uniquely denote a position in space belonging to glomerulus C23. The important information is not the colour but the pixel value: with different colour lookup tables (LUT), the attribution of voxel value to colour can be changed depending on the needs of

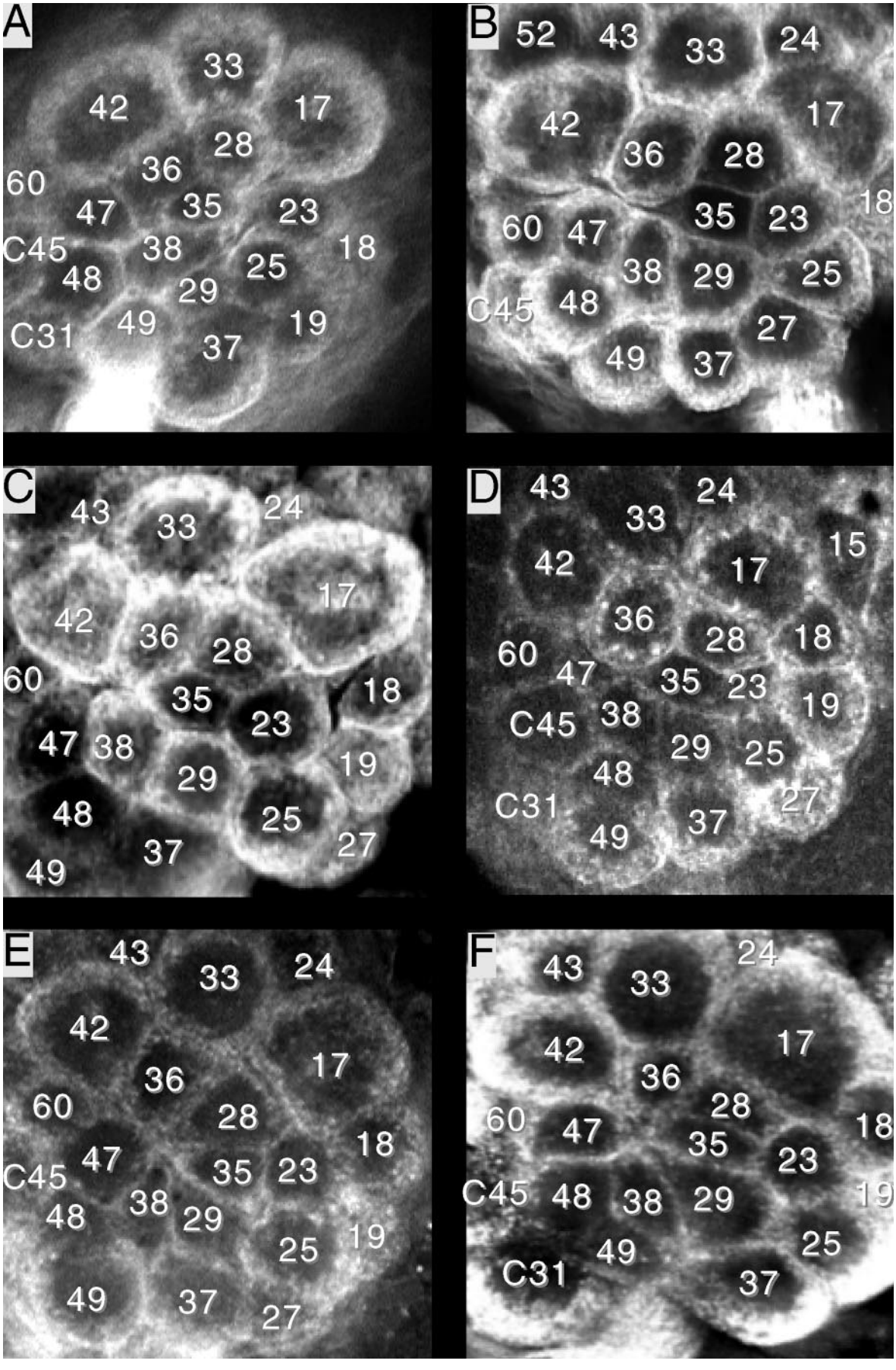
Fig. 4A–F Comparison of confocal sections in different individuals. Each panel (**A–F**) shows an optical section through the AL, with the glomerular identity being marked. All glomeruli are innervated by T1, with exception of *C31* and *C45*, which are innervated by T3. Note the variability of the position of some glomeruli, such as *47* (T1), which fails to touch *36* (T1) in **C** and almost touches *28* (T1) in **E**. The AL in **E** is the AL upon which the atlas is based ▶





C

46(T1)	A46	46	44	A44	44(T1)
55(T1)	A55	55	40	A40	40(T1)
54(T1)	A54	54	31	A31	31(T1)
56(T1)	A56	56	20	A20	20(T1)
51(T1)	A51	51	12	A12	12(T1)
T2-1(1)	B01(2)	82	01	A01	01(T1)
62(T3)	C62	152	08	A08	08(T1)
47(T3)	C47	137	03	A03	03(T1)
48(T3)	C48	138	11	A11	11(T1)
T2-2(1)	B01(2)	81	201	T1	
57(T3)	C57	147	113	C23	23(T3)
44(T3)	C44	134	94	C04	04(T3)
34(T3)	C34	124	98	C08	08(T3)
30(T3)	C30	120	101	C11	11(T3)
37(T3)	C37	127	103	C13	13(T3)
35(T3)	C35	125	110	C20	20(T3)
AL Perimeter		255	204	T3	
			206	TB	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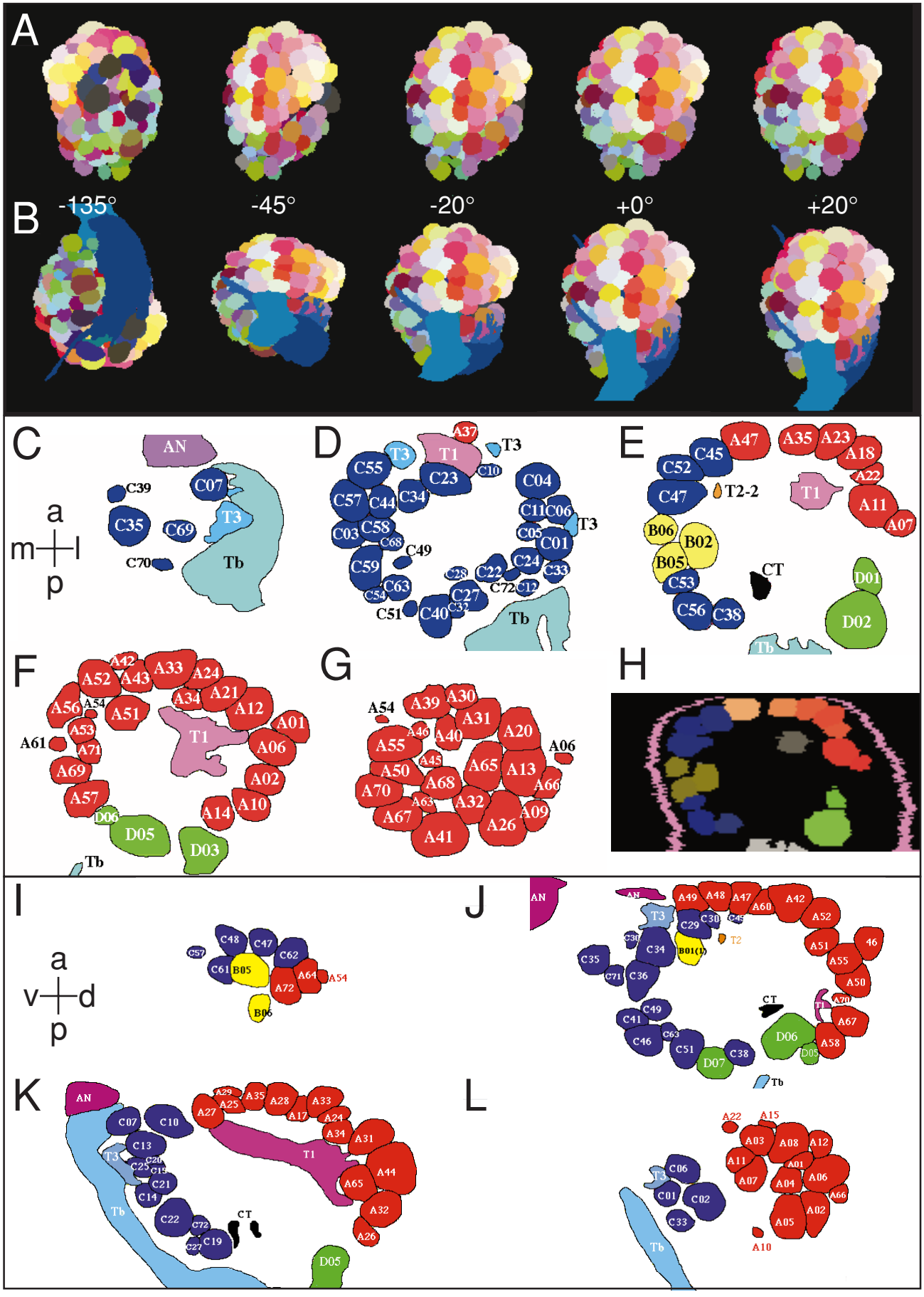
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the researcher. For example, on assigning grey levels to the values (as in Fig. 3C), glomerulus A1, which has the value of 1, is thereby (almost) invisible. The colours of the glomeruli in Fig. 5 are an example of another attribution of colours to voxel values.

This volumetric standard atlas of the bee brain is named "ValueAtl.tif". It is 2.6 MB in size, has a resolution of 2.4 μm in all three dimensions and is accessible on the Internet (see Appendix). A version consisting of all layers as single TIFF, JPEG or GIF files is also available in subfolders.

At positions at which there is only one glomerulus in most ALs, we sometimes found two. An example is given in Fig. 3A with glomeruli C47a and C47. In these cases, we included just one glomerulus in the atlas (see Fig. 3B). The following glomeruli where included as single glomeruli in the atlas but where double in one of the investigated specimens: 30(T1), 35(T1), 37(T1), 46(T1), 60 (T1), 62 (T1), 64(T1), 32(T3), 42(T3), 43(T3), 47(T3) and 59(T3). We never saw more than five doubled glomeruli in one AL. We failed to find glomerulus 16(T1), which is described in the published atlas and which should lie ventral to T4–1(1); this glomerulus is therefore missing from the digital atlas.

The variability between six individuals in a comparable optical section is shown in Fig. 4: the three largest glomeruli, 42, 33 and 17, are always apparent. The typical neighbourhood relationships should also be noted, as should the band of glomeruli 60, 47, 38, 29 and 25, and the way in which their shape differs between individuals.

Visualizing the glomerular layout of the AL

By using the digital atlas, it is possible to project and cut the AL at any angle. In the electronic files on the Internet, examples are given for rotations in space around the vertical and the horizontal axes. In Fig. 5A, B, some exemplary frames of these sequences are shown, for which the colour LUT "system" of the Macintosh was applied. This LUT is convenient, as it attributes different colours to voxel values that lie close together. The animations on the Internet give a better impression of the three-dimensional structure.

Projections and reslicing of the AL can be made by using various standard software packages. The pictures in Fig. 5 were obtained by using NIH Image. The procedure is briefly described in the Appendix.

An exemplary section from the volumetric atlas is shown in Fig. 5H, in which the different colours denote their different pixel values. Graphically reworked digital sections of labelled glomeruli can be seen in Fig. 5C-G, I-L. These sections give an impression of the way in which the digital atlas of the AL can be applied.

The computer files

The computer files include the following datasets: one atlas in which each glomerulus can be identified by its individual voxel value (as a single file and also as folders with each section being in a separate file, in the formats TIFF, GIF and JPEG), one atlas with each glomerulus being individually labelled (as a single file and a folder with each section being given as an individual file) and a folder containing several files with examples of rotations of the atlas at different angles and with various settings (see the Appendix for a list of files).

Discussion

Methodological aspects: staining and demarcation of the glomeruli

Tracing the axons with the neuronal tracer marker neurobiotin and its subsequent visualisation with Cy3 resulted in clear fluorescent staining of the glomeruli in the AL. Use of the confocal microscope allowed the entire AL to be scanned as a whole-mount, with no need to slice the preparation. Thus, the resulting optical slices were perfectly aligned and could be used directly to construct the atlas by manual tracing of the glomerular borders.

How clearly can the individual glomeruli be delimited with this method? Sensory afferents innervate only the cortex of the glomeruli, with the exception of the glomeruli innervated by T4. Therefore, in the confocal reconstruction, these glomeruli appear as clearly identifiable empty spheres. Delimitations between glomeruli are also clear, as they are surrounded by a glial layer that is not innervated by afferents and are therefore dark in confocal sections. Moreover, some axons run parallel to the glomerular borders (see Fig. 1). Delimitations of the glomeruli towards the input side of the glomerulus are often arbitrary as the labelled axonal innervations pass directly from the tract into the glomeruli. This uncertainty affects T1 glomeruli on the side facing the coarse neuropil and the T3 glomeruli facing the bun-

◀ **Fig. 5A–L** Examples of projections onto the digital atlas. **A** Projected views of the antennal lobe from different angles (20°, 0°, –20°, –45°, –135°). The LUT system was chosen and the projection parameters set to no-transparency, first hit. Therefore, only the superficial glomeruli that can be seen from that particular angle appear (the rotated movie gives a much more impressive image, so please download it from the Internet). Rotation was around the dorso-ventral axis (*y-axis*) and was achieved by utilizing the computer program NIH Image. Tracts and AL borders were removed by excluding pixel values above 199 from the projection (see the Appendix for settings). **B** Projected views as in **A** but rotated around the *x-axis* (medio-lateral). Tracts were included (pixel values between 200 and 206), whereas the border of the AL was not (pixel value 255). **C–G** Series of sections taken at a distance of 70 μm from each other, from dorsal (**C**) to ventral (**G**). Anterior is *up* and posterior *down*, as shown in the inset (*a* anterior, *p* posterior, *m* medial, *l* lateral). The glomeruli in these images are individually labelled. Labelling and perimeters were reworked. Glomeruli innervated by T1 are *red*, by T2 are *yellow*, by T3 are *blue* and by T4 are *green*. **H** Example of the corresponding section of **E** as created by sectioning the digital atlas with the IDL program available on the Internet. The LUT has been chosen to give a different colour for each glomerulus but approximately keeping the *red/yellow/blue/green* code for the glomeruli innervated by T1/T2/T3/T4. **I–L** Series of sagittal sections taken at a distance of 80 μm from each other, from medial (**I**) to lateral (**L**). These sections have been colour-coded and labelled as in **C–G** (*d* dorsal, *v* ventral, *a* anterior, *p* posterior)

dle of tracts. It does not affect T2 and T4 glomeruli, each of which is innervated by narrow sub-branches. The hand-tracing of all boundaries is the main reason for the "jitter" in the glomerular borders from one optical section to the next, as can be seen in Fig. 4H.

Sometimes, two glomeruli were so closely adjacent to each other that they were difficult to tell apart. Here, a comparison between different preparations helped to distinguish between them and a hand-drawn compromise delimitation was included in the atlas. This concerns the following glomeruli: 60 (T1), 62 (T1), 64(T1), 32(T3), 47(T3) and 59(T3). Researchers who identify their glomeruli as one of these should be aware of the possibility that there may be two at that location. This uncertainty reflects a variability in the number of glomeruli between individuals. Flanagan and Mercer (1989a) found between 72 and 77 glomeruli in T3 and between 66 and 69 in T1. Arnold et al. (1985) always found 71 in T1 (as we did) and that 82–90 were innervated by T3. Part of this variability may be attributable to experimental shortcomings and may be overcome by new methods. On the other hand, glomerular shape and the reciprocal positions obviously vary between individuals, as can be seen in Fig. 4. Indeed, the size of individual glomeruli has been shown to be experience-dependent (Sigg et al. 1997).

The mentioned borderline difficulties do not affect the relative positions of the glomeruli and, therefore, do not compromise the main function of the atlas, which is to provide a clear three-dimensional grid for the glomerular positions. Nevertheless, the application of other staining techniques, including a combination of various tracers and chromophores, would certainly improve such an atlas. Moreover, the use of automated procedures for glomerular recognition is necessary to tackle the question of variability amongst a larger number of specimens and are presently being developed (Otto et al. 1998).

Why have a digital atlas?

The glomerular activity pattern in the worker honeybee has been shown to be specific for a particular odour as a stimulus (Joerges et al. 1997; Galizia et al. 1997) and to be bilaterally symmetrical (Galizia et al. 1998). It is as yet unclear, however, whether the pattern is conserved between animals. In order to address this question, glomeruli have to be identified individually and this can only be done with the help of an atlas. We have started this investigation by using the atlas presented in this paper. Local interneurones and projection neurones often branch in just one or in only a few glomeruli (Sun et al. 1993; Abel 1995). The odour-induced responses of these cells as recorded with an intracellular electrode (Flanagan and Mercer 1989b; Fonta et al. 1993) cannot yet be interpreted in terms of glomerular function. In order to tackle the olfactory code, the identification of individual glomeruli in optical recording studies and in electrophysiological studies with subsequent staining of the recorded cells is of paramount importance. Furthermore, glomeruli have been shown to vary in size depending on age and behavioural status (Sigg et al. 1997; Winnington et al. 1996). This variation in size is glomerulus-specific and makes the individual identification of glomeruli necessary.

The advantage of this new atlas compared with its predecessor (Flanagan and Mercer 1989a) is two-fold. First, because of the confocal technique, a higher resolution has been obtained based on whole-mount preparations. Adjacent slices lie 2.4 μm apart and there is no loss of information between them, so that no glomeruli are missing. Second, because of its digital form, the atlas can be resliced at at any angle. This is important, for example, when a glomerulus has to be identified in a histological preparation that has not been frontally sectioned. The digital atlas can be rotated and resliced until it "fits" the section to be investigated and can then be used to identify the glomeruli.

Future developments: the biological information of the atlas

Digital cartography of the brain will soon become an important tool in all branches of neuroscience (Roland and Zilles 1994). Projects, such as Flybrain (Armstrong et al. 1995; http://www.flybrain.uni-freiburg.de/), the human brain atlas (http://www.med.uci.edu/), the Whole Brain Atlas (http://www.med.harvard.edu/AANLIB/) and the MuriTech Internet atlas of mouse development (http://www.muritech.com/atlas/), have initiated this development. The use of confocal microscopy to collect the raw data from whole-mounts has many advantages but also some limitations. For example, because of the limited transparency of brain tissue, structures investigated should not be too thick. Insect brains, such as those of *Drosophila melanogaster* or *Apis mellifera*, are certainly advantageous, because the entire brain can be imaged as a whole-mount. However, the amount of collected data is so vast that computer scientists are required to develop new methods for handling the large data files.

On the other hand, the biologist is requested to reduce the aquired raw data to a level where most profit can be drawn from the information. In the case of the AL, the glomerular borders and their identity appear to be the appropriate structures for the atlas. Resolution beyond the glomeruli is not useful for inclusion in an atlas of the entire AL.

The digital atlas is based on confocal sections from one individual. It therefore cannot be considered as a "standard" atlas. At present, there are no tools to create a complex volumetric reference work from several individuals: their development, however, is of great importance, if digital atlases are to incorporate both positional identity and information about intraspecific variability.

The rapid development in computer technology should lead to digital atlases becoming widespread tools in biological research. They will have two separate tasks. First, a digital anatomical atlas is a reference work that is superior to its non-digital predecessors, because of its potential for higher resolution and its flexibility in creating projections and slices at arbitrary angles. Second, digital atlases will

soon become the basis for biological databases, because cross-references to additional information can be integrated as hyperlinks to the pertinent structures of the atlas. This additional information may include representations of single-cell staining, databases with citation lists or any other relevant information concerning a particular structure.

Future developments: the digital form of the atlas

At present, the digital atlas is available on the Internet as a stack of successive pixilated slices through the AL. The different stacks vary in the way that the information of the glomerular identity is coded. In one file (see Appendix), borderlines are given and labels are printed within each glomerulus. In another file, no names are printed within the glomeruli but the voxel value within each glomerulus is unique and can be mapped according to its identity with the aid of Table 1, which is also present on the Internet. The format of the atlas is therefore extremely flexible. It can be read with almost any software capable of pixilated graphics processing, such as Adobe Photoshop, NIH Image, IPLab-Spectrum and IDL. The atlas can therefore be easily transported to any computer, with or without access to the Internet.

The disadvantage of this setting is that each researcher has to download the data to be able to work with it. Protocols suitable for the dissemination of three-dimensional information across the Internet, such as VRML (Virtual Reality Modeling Language), are available. In the future, we plan, in co-operation with other groups, to develop interactive tools that will expand the possibilities of the digital atlas. This will then allow access to the information for each glomerulus, resection of the atlas, and rotation of its three-dimensional model directly on the Internet. The Internet site will be updated accordingly.

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Appendix

List of files in the Internet

A list of relevant files that is available in the Internet can be found in Table 2; “read.me” and sample program files can also be downloaded.

Source for the IDL program

The source of an IDL program ("PlayAtla.pro") that loads the data is given on the Internet site. It allows reslicing in any of the three orthogonal directions and the color LUT can be changed. It is meant as an example and should aid the reader who wishes to write a similar program, including custom procedures.

IDL is distributed with a demonstration program, "slicer.pro". After "PlayAtla.pro" has been run, "slicer.pro" has access to the atlas information (on the command "COMMON VOLUME_DATA, AtlasArray" in "PlayAtla.pro"). Projections, oblique cuts and three-dimensional representations can be carried out with the slicer.

Description of NIH loading, rotation and reslicing

Open the program NIH Image (this program for Macintosh computers is available free of charge from http://rsb.info.nih.gov/nih-image/).With "File – Open", load the file "ValueAtl.tif". The atlas is now loaded. Use the magnifying glass to increase viewing size, and the period and comma to browse the slices. Place the cursor on the glomerulus of your choice and read the value in the Info Window. Compare the value with the glomerulus name given in Table 1.

If you want to reslice the dataset, select a line (Tools Window) and then "Tools – Reslice". Again, identify glomeruli by reading their values in the Info Window. For oblique slices, you have either to rotate the stack first and then slice it or to download the appropriate extension from the NIH Internet site.

If you want to see surface views from any angle, select the menu "Stacks", submenu "Project", and make your choices: "Slice Spacing" should be 1 but "initial angle", "total rotation" and "rotation angle increment" are up to you. Use "lower transparency bound" and "upper transparency bound" to select the area to be projected, e.g. 1–199 for all glomeruli but no tracts, 1–254 for all glomeruli and tracts but not the AL perimeter, 1–80 for T1 exclusively, etc., (see Table 1). "Surface opacity", "surface depth-cuing" and "interior depth-cuing" should all be set to 0 and "projection method" set to "nearest point", if you want to use the projection for glomerular identification purposes with the voxel values. Click "ok" and the projection is performed. By first selecting regions of the Atlas with the Tools Box, you can project the selection only.

Internet addresses from which the atlas can be downloaded

http://flybrain.neurobio.arizona.edu/
http://flybrain.uni-freiburg.de/
http://flybrain.nibb.ac.jp/
http.//www.neurobiologie.fn-berlin.de/honeybeeALatlas

Table 2 Relevant files in the Internet

Name	Size/type	Details
NamesAtl.tif	24.2 MB	One file consisting of a stack of 87 TIFF files with glomeruli individually labelled with name-tags. NIH Image format
ValueAtl.tif	2.6 MB	One file consisting of a stack of 87 TIFF files with glomeruli coded via their pixel value. NIH Image format
NamesAtl.dir	Directory	Containing the same data as the file NamesAtl.tif but as single files for each of the 87 layers. JPEG format
ValueAtl.dir	Directory	Containing the same data as the file ValueAtl.tif, but as single files for each of the 87 layers. Arranged in three separate subdirectories in the formats TIFF (each file 35 kB), GIF (each file 13 kB) or JPEG (each file 14 kB)
PrHoGlo.qt	2.2 MB	3-D view of the antennal lobe as surface projections rotated around the x-axis, tracts excluded. QuickTime movie
PrHoGlo.tif	2.4 MB	Same as PrHoGlo.qt. TIFF file collection
PrHoTra.qt	2.2 MB	Same as PrHoGlo.qt, tracts included. QuickTime movie
PrHoTra.tif	2.4 MB	Same as PrHoTra.qt. TIFF file col lection
PrVeGlo.qt	2.2 MB	3-D view of the antennal lobe as surface projections rotated around the y-axis, tracts excluded. QuickTime movie
PrVeGlo.tif	2.4 MB	Same as PrVeGlo.qt. TIFF file collection
PrVeTra.qt	2.2 MB	Same as PrVeGlo.qt, tracts included. QuickTime movie
PrVeTra.tif	2.4 MB	Same as PrVeTra.qt, TIFF file collection