

1 **High-resolution ultramicroscopy of the developing and**
2 **adult nervous system in optically cleared *Drosophila***
3 ***melanogaster***

4 Pende et al.

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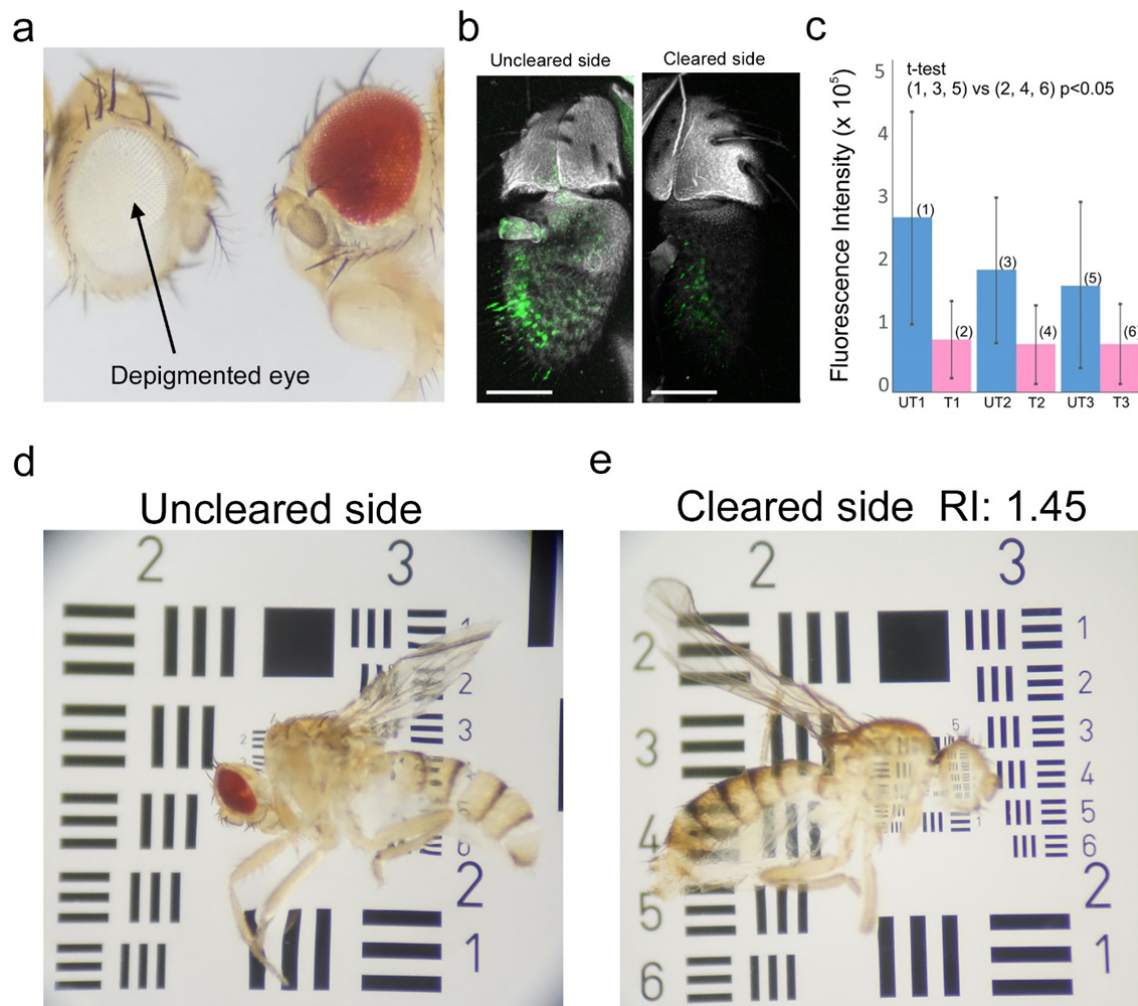
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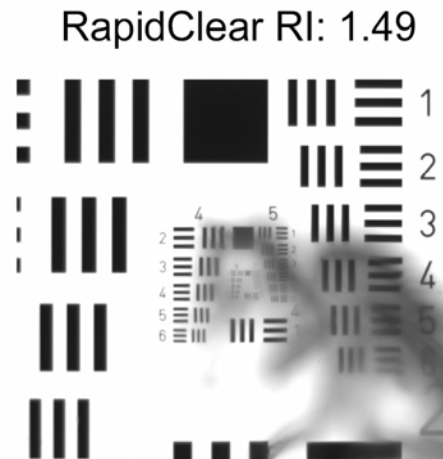
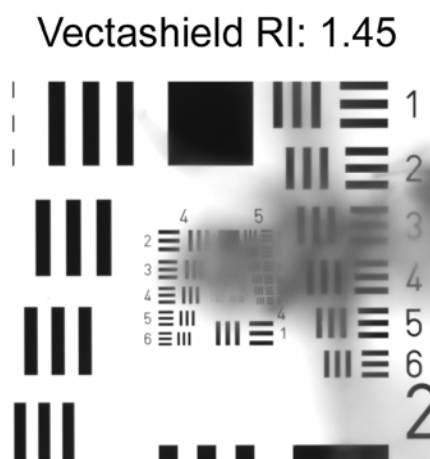
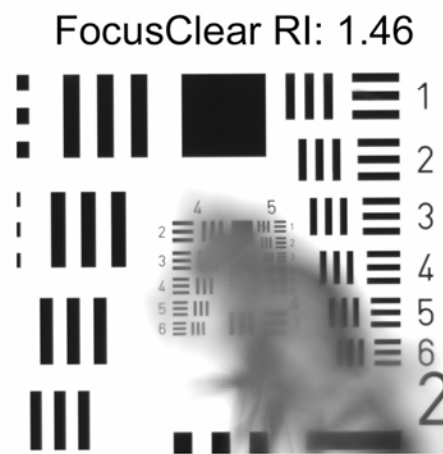
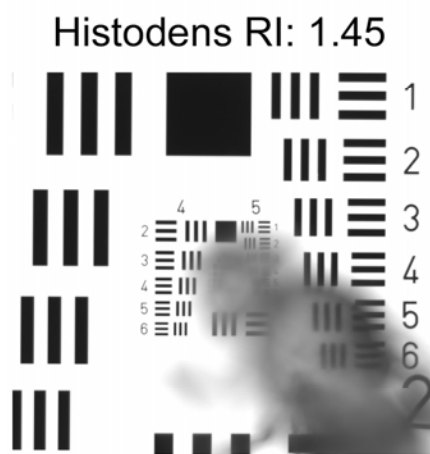
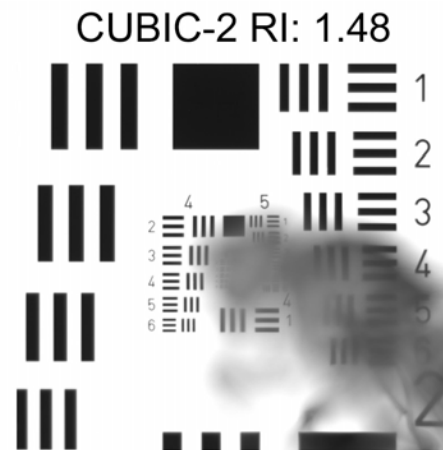
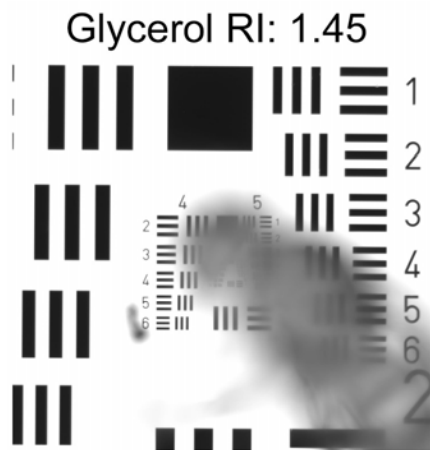
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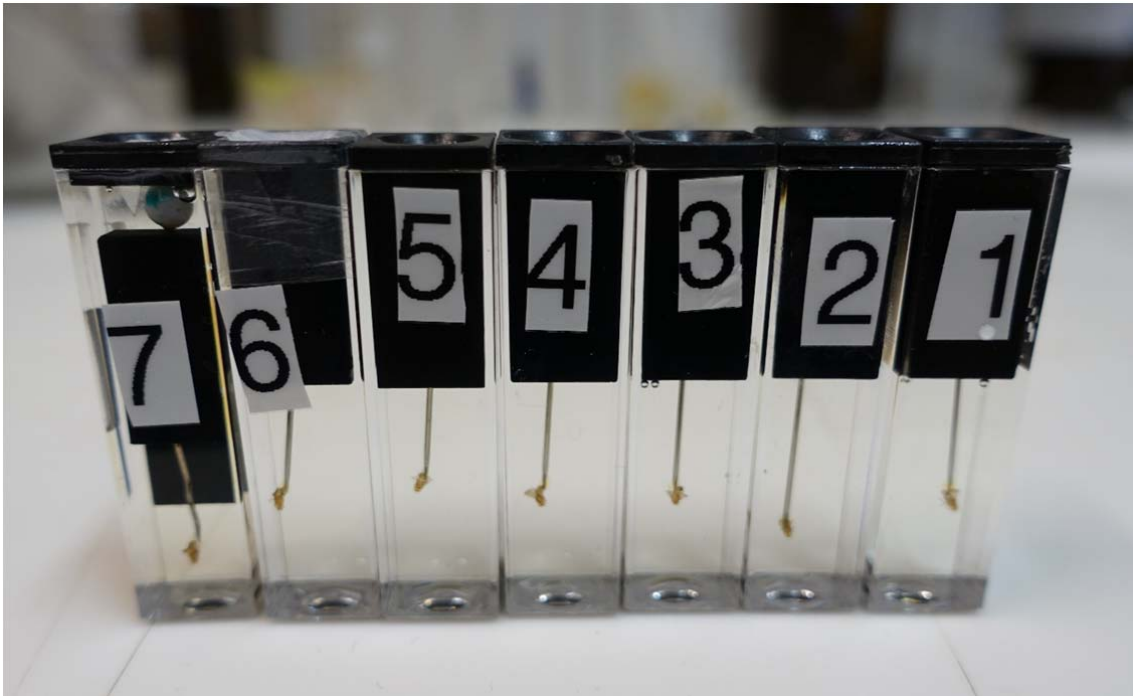
Supplementary Figure 1 | Comparison of uncleared and FlyClear treated adult flies. **a**, **d** and **e** Incident light image showing comparison of uncleared and cleared sides of the same fly. **b** Confocal image of weak GFP signal in the maxillary palp of uncleared and cleared sides of the same fly. **a** Comparison of the depigmentation properties of *Solution-1*. *Solution-1* treated side shows complete depigmentation of the adult eye (image was taken in PBS). **b** Comparison of GFP signal in Uncleared and four days in *Solution-1* cleared sample. Both sides of the same fly were mounted on one slide in Vectashield and recorded with the same laser intensity. **c** Diagram shows the results from three independent experiments comparing the intensities ($\pm$ s.d.) of untreated (UT) and *Solution-1* treated (T) sides of flies ($n=3$). Significance was assigned with unpaired t-test ($p<0.05$). **d** Uncleared right side of adult fly **e** FlyClear processed cleared left side of the same fly. Images in **a**, **d** and **e** were acquired with a stereomicroscope with a 1x objective (Leica, Plan APO 1.0X, WD 61,5mm). Images in **b** were acquired with a 20x immersion objective (Leica, HCX PL APO CS, 0.7 NA, 260 μ m WD). Genotype: ; *UAS-mCD8:GFP* ; *Or47b-Gal4*. Scale bars in **b** represent 50 μ m.



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34 **Supplementary Figure 2** | Comparison of transparency achieved after treatment
 35 with *Solution-1* and commercial refractive index (RI) matching media. Wide-field
 36 image of optically cleared specimens placed on top of a USAF1951-chart.



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40 **Supplementary Figure 3** | Mounting of optically cleared flies for signal
41 quantification. Optically cleared flies were mounted with a UV glue on a needle tip,
42 immersed in a cuvette filled with *Solution-2*, and sealed for imaging and storage.

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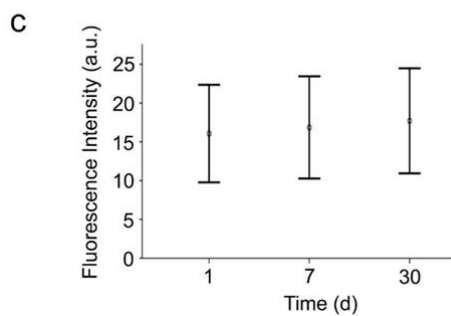
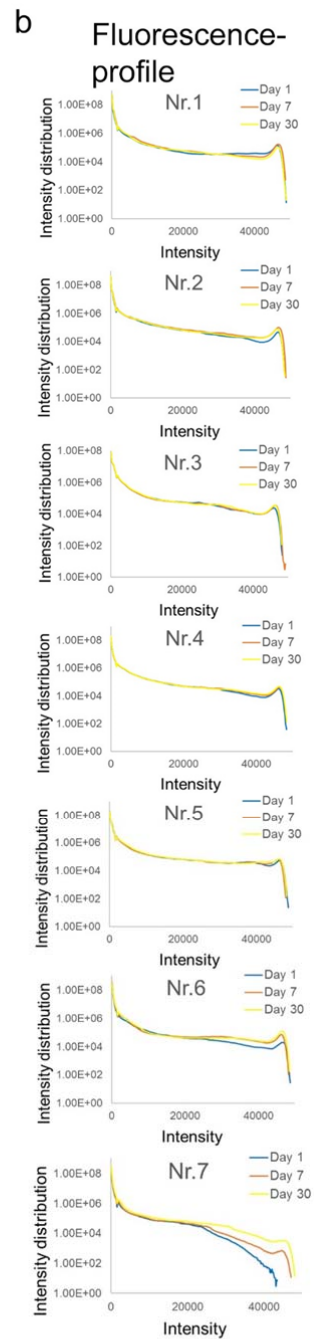
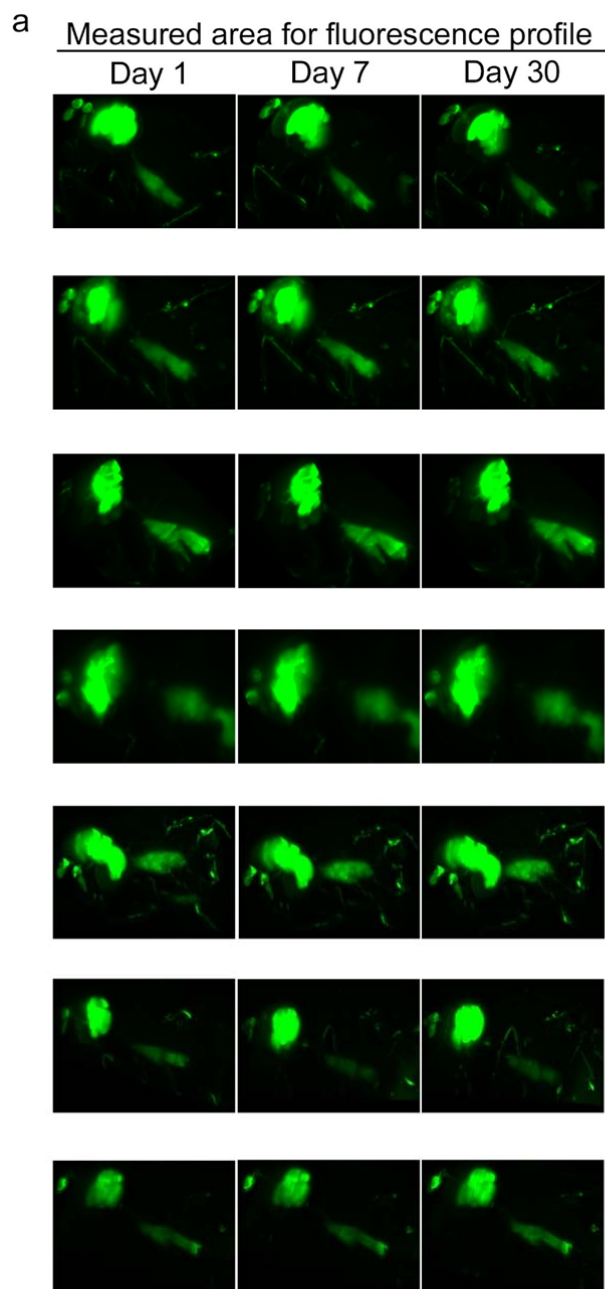
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53 **Supplementary Figure 4** | Stability of the GFP signal in adult fly after FlyClear
54 procedure. **a** Light-sheet stacks of 600 images show GFP signal in *Drosophila*
55 *melanogaster* measured with the same settings in the same areas at three different
56 time points (n=7). **b** Histogram data showing the comparison of fluorescence level
57 profiles within these areas over time. **c** Dotplot representing the average $\pm$ s.d. of the
58 intensity distribution from seven independent measurements after one day, one
59 week, and one month. For measuring significance, p-values were assessed with
60 one-way ANOVA ($P < 0.911$). All images were acquired with a 4x Objective (Olympus,
61 XLFluor4x/340, 0.28 NA, WD = 29,5mm) with custom-made correction of optics for a
62 refractive index of 1.45 (WD after correction 10mm). Genotype: *dscam-Gal4/CyO*;
63 *UAS-mCD8::GFP*

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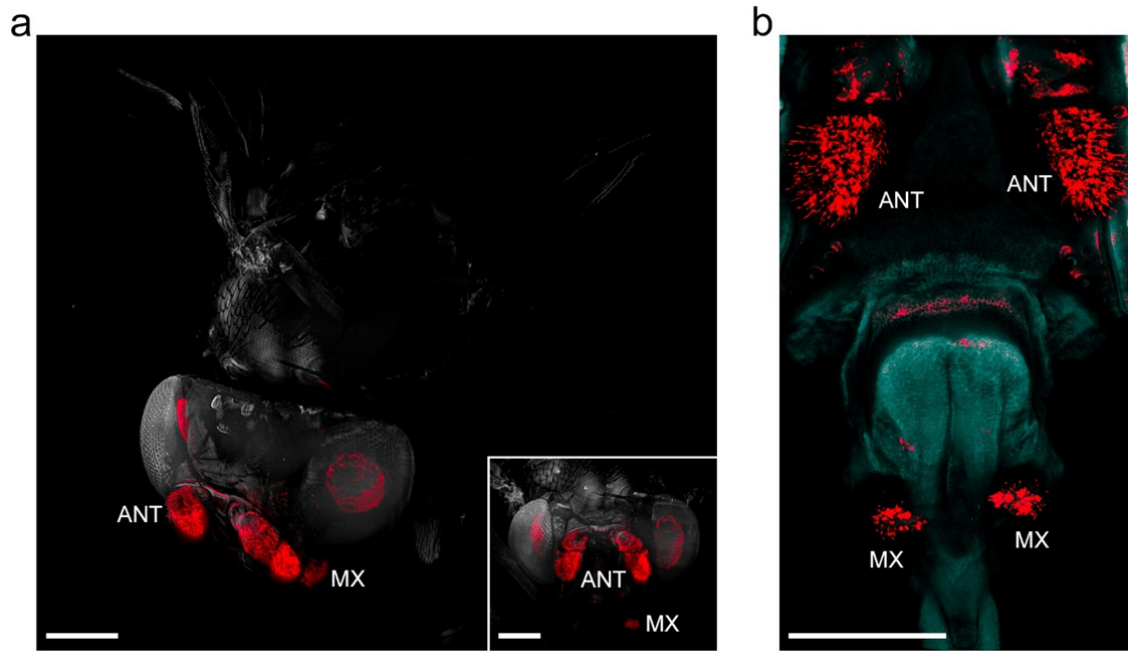
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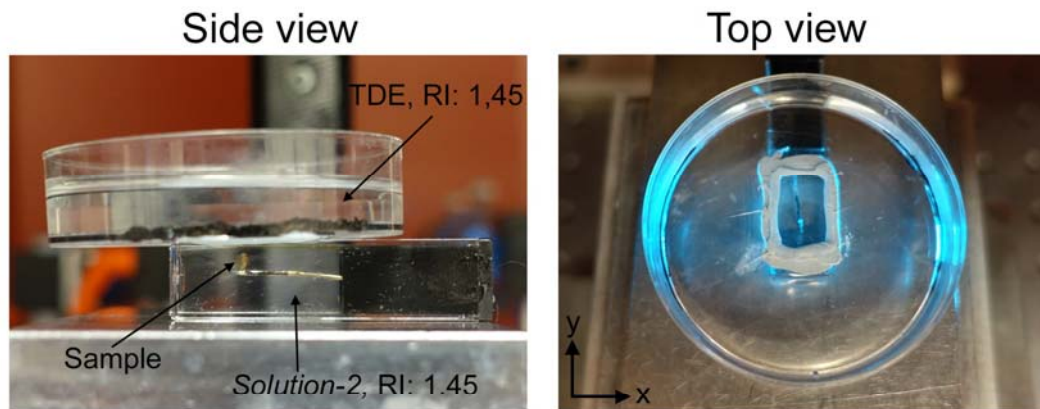
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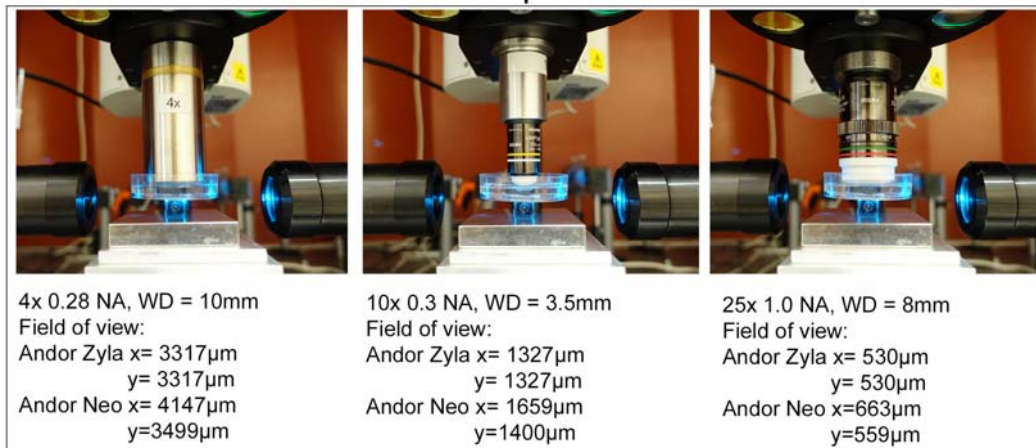
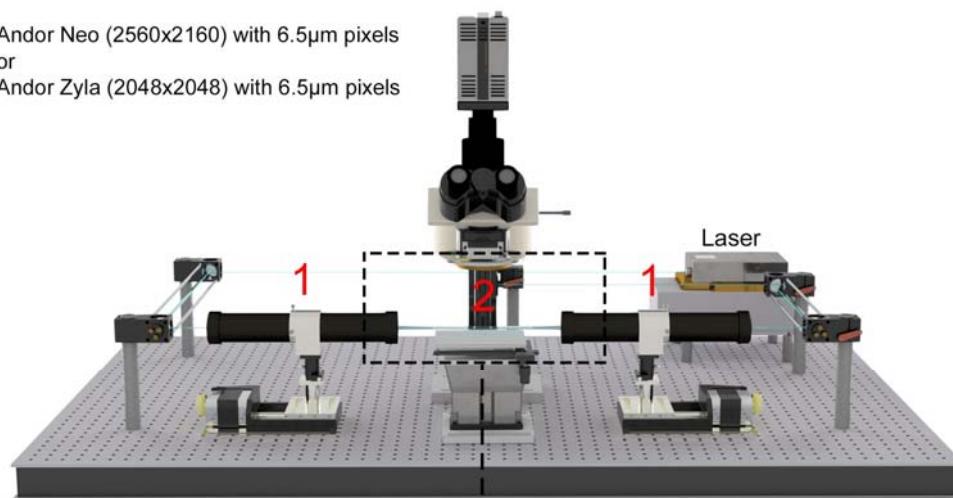
Supplementary Figure 5 | Visualisation of mCherry labeled adult *Drosophila* chemosensory system using FlyClear with confocal- and optimised ultramicroscope. **a** Light-sheet images and **b** confocal images of mCherry expression. **a** Olfactory neurons in antenna (ANT) and maxillary palp (MX). Inset shows coronal view of fly head. **b** Higher magnification of ANT and MX. Images in **a** were acquired with 0.5x post-magnification in combination with a 10x water-immersion objective (Olympus, UMPlanFLN, 0.3 NA, WD = 3.5mm) with custom-made correction of optics for a refractive index of 1.45 (WD = 3.5mm after correction). Image in **b** was acquired with a 20x immersion objective (Leica, HCX PL APO CS, 0.7 NA, 260µm WD). Genotype: *;;R88E12-Gal4 UAS-mCherry*. Scale bars represent 200µm.

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Andor Neo (2560x2160) with 6.5 μ m pixels
or
Andor Zyla (2048x2048) with 6.5 μ m pixels

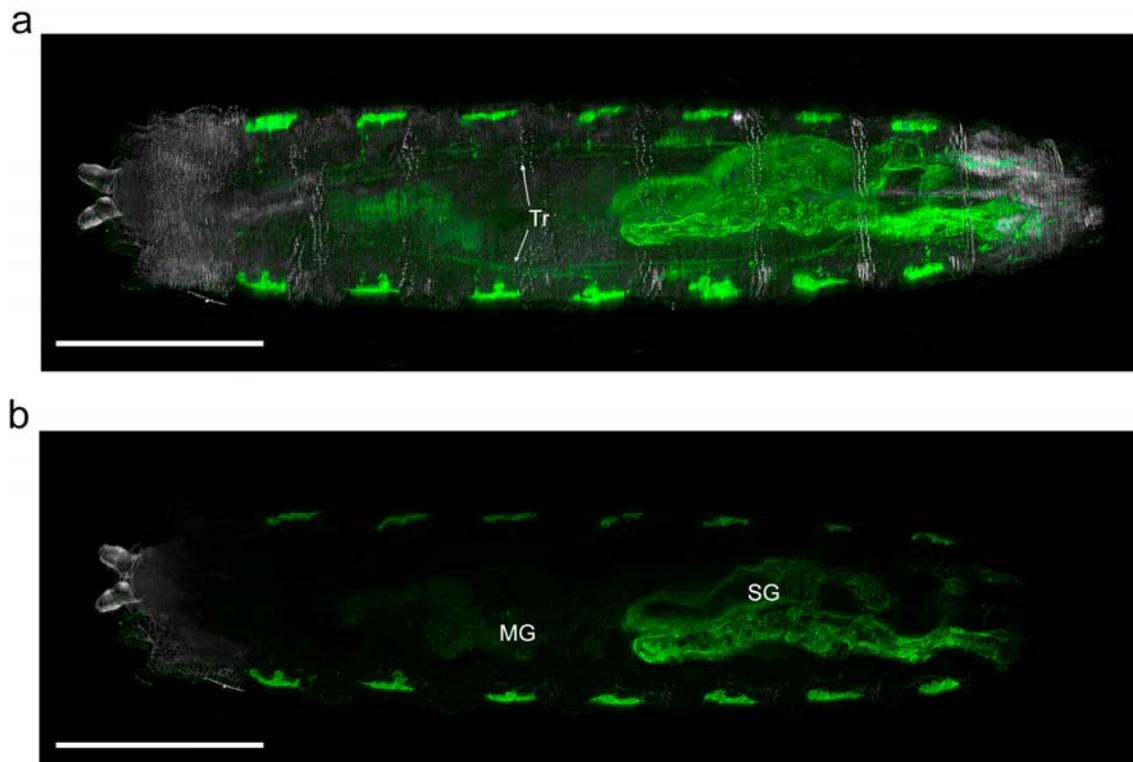


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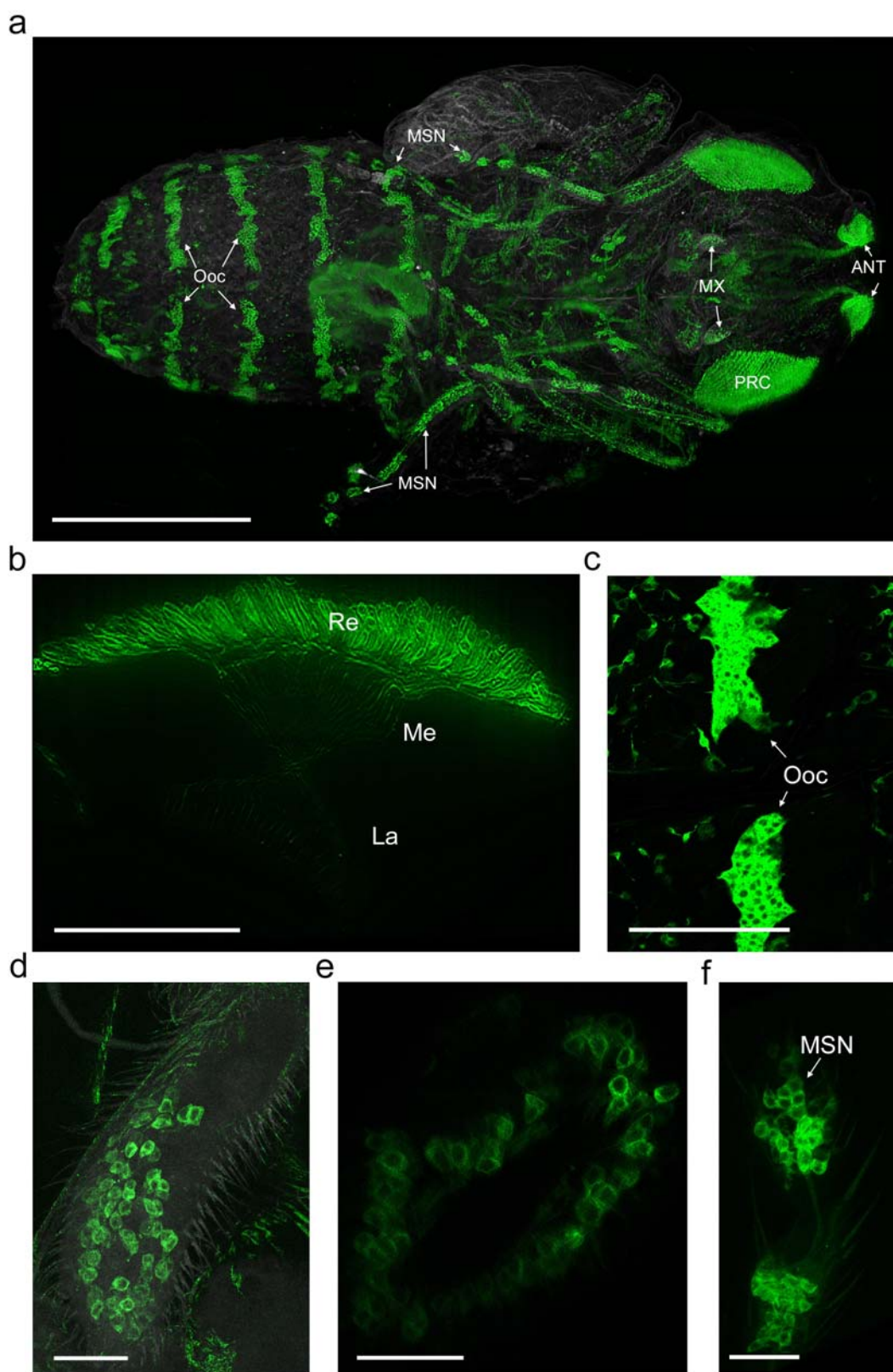
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Supplementary Figure 6 | Ultramicroscope system and imaging chamber (a) Side and top view of the imaging chamber. The sample containing chamber is filled with *Solution-2* (RI: 1,45) the objective immersion chamber is filled with aqueous Thiodiethanol solution (RI: 1,45). (b) Scheme of the ultramicroscope system. (1) Light-sheet generator (illumination lens), (2) detection lenses. Boxed area indicates different detection lenses used for imaging. (From left to right) 4x Objective (Olympus, XLFluor4x/340, 0.28 NA, WD = 29,5mm) with custom-made correction of optics for a refractive index of 1.45 (WD after correction 10mm), 10x water-immersion objective (Olympus, UMPlanFLN, 0.3 NA, WD = 3.5mm) with custom-made correction of optics for a refractive index of 1.45 (WD = 3.5mm after correction), 25x objective (Olympus, XLPlanN, 1.0 NA, WD = 8mm)



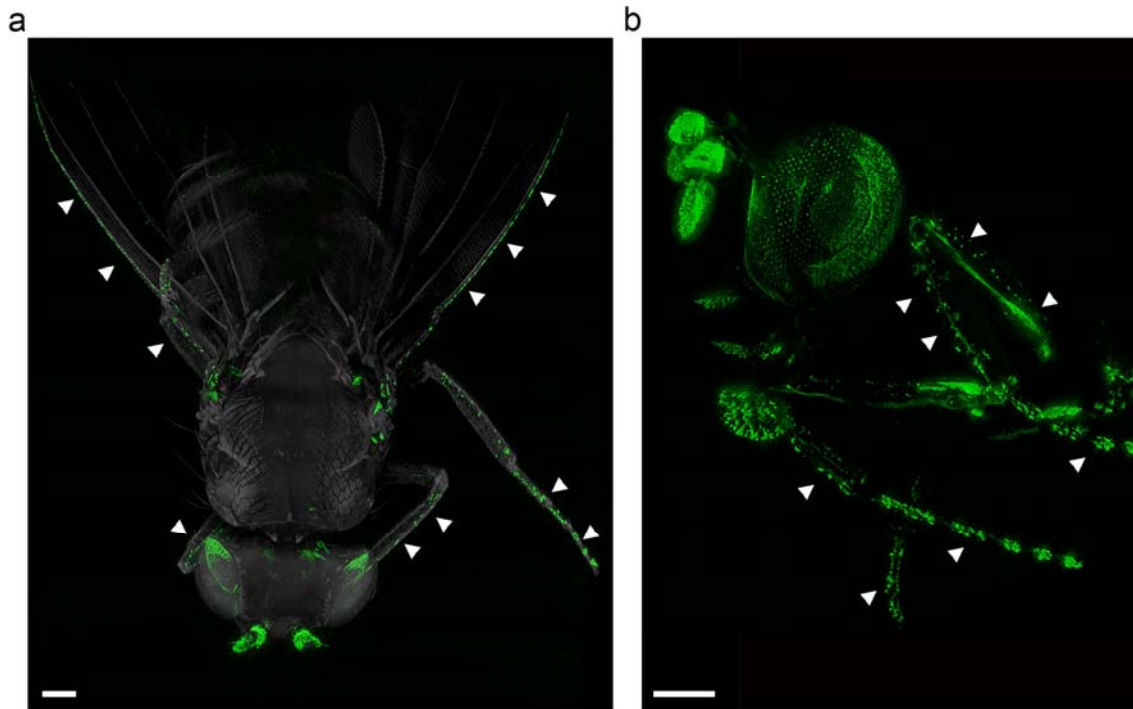
Supplementary Figure 7 | Imaging of FlyClear treated second instar larva using optimised ultramicroscope system. **a, b** Light-sheet images of GFP expression in larval organs. **a** Imaging of whole larva showing the trachea (Tr). **b** Dorsal view of a clipping plane in undiseased larva showing mid gut (MG) and salivary glands (SG). All images were acquired with a 10x water-immersion objective (Olympus, UMPlanFLN, 0.3 NA, WD = 3.5mm) with custom-made correction of optics for a refractive index of 1.45 (WD = 3.5mm after correction). Genotype: *Peb-Gal4 UAS-mCD8::GFP*. Scale bars represent 500µm.



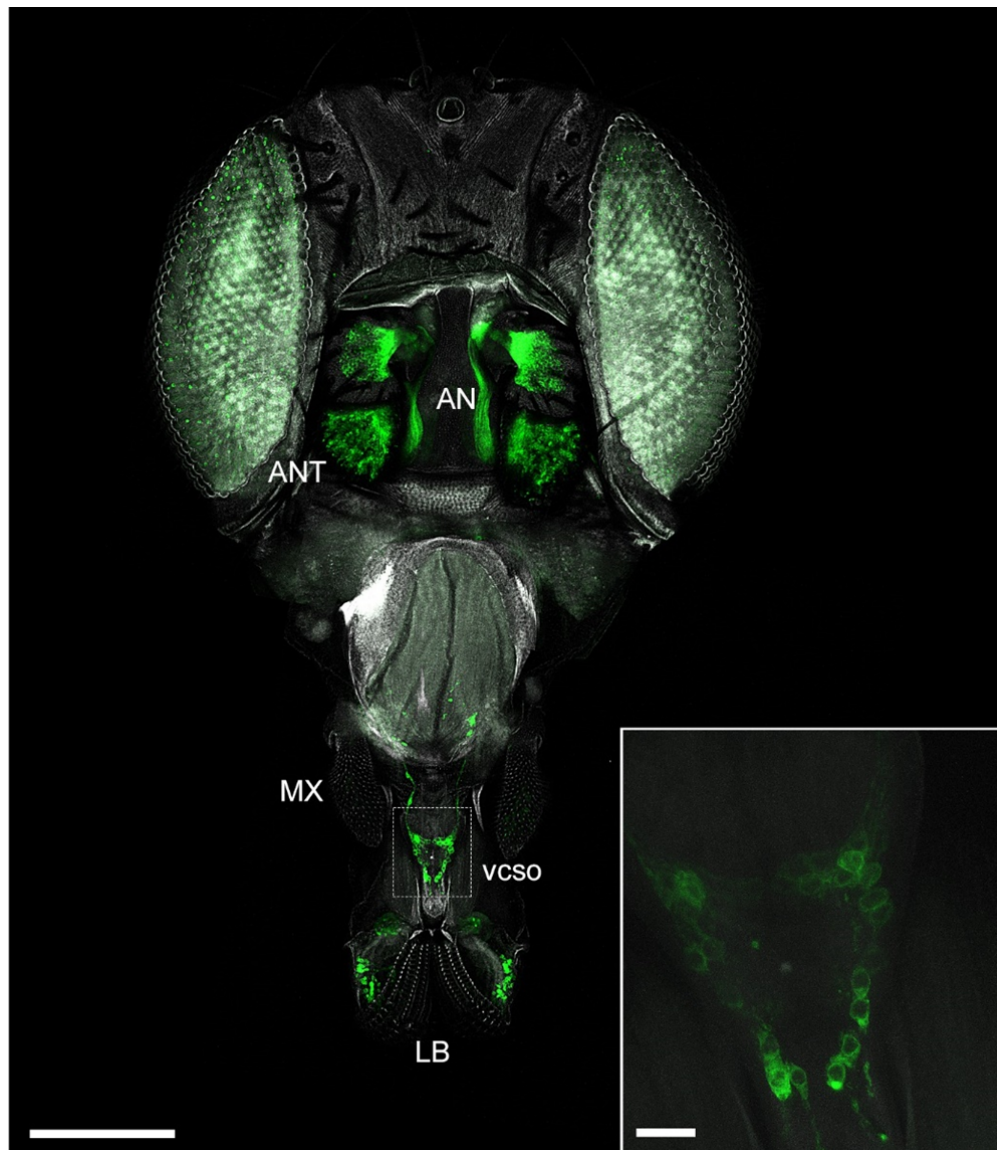
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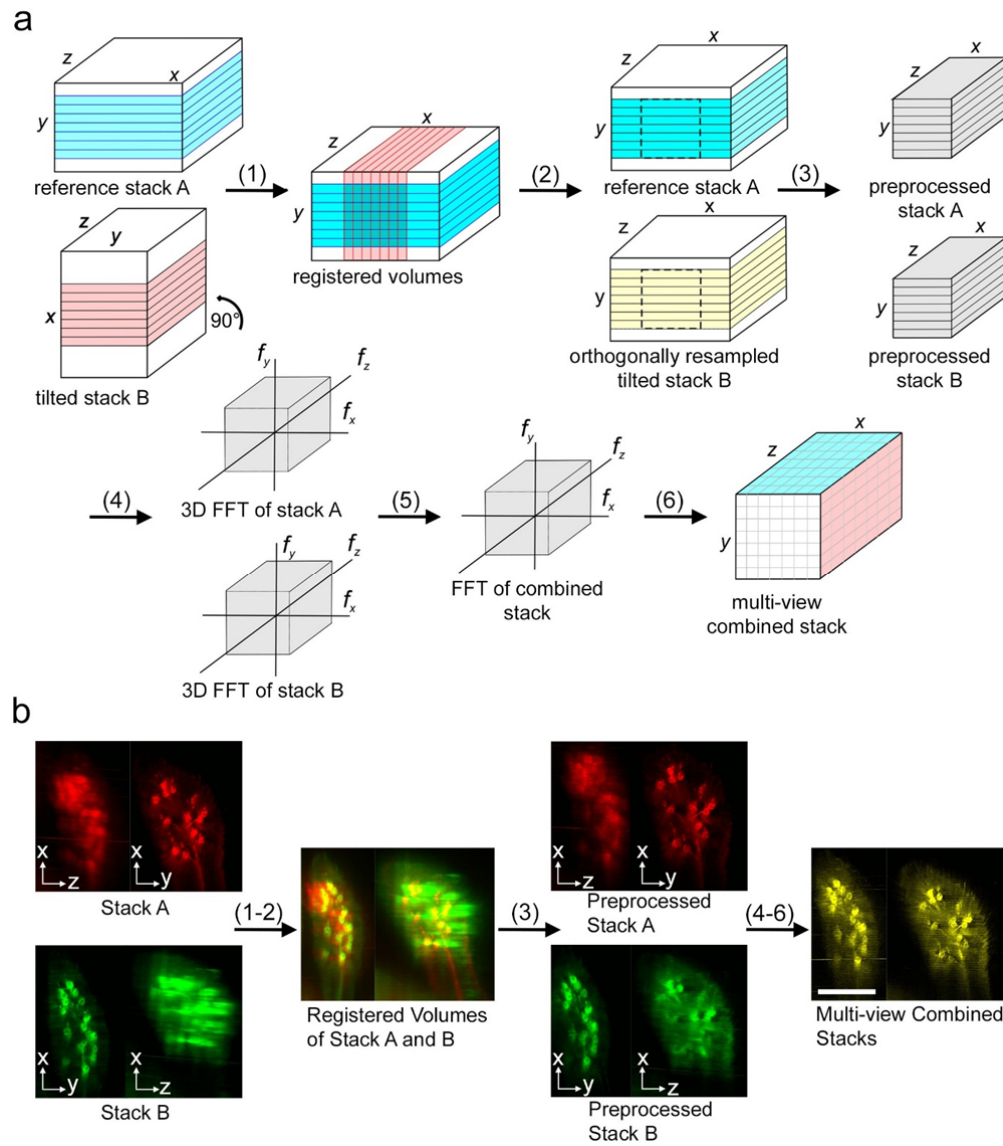
Supplementary Figure 8 | Imaging of reproductive, visual and sensory system of an intact pupa using confocal- and optimised aspheric ultramicroscope. **a, b** Light-sheet images and **c-f** confocal images of GFP expression. **a** Ventral view of Pebbled expression in whole pupa. Arrows indicate oocytes (Ooc) in abdomen, mechanosensory neurons (MSN) in the legs, photoreceptor cells (PCR) in the eyes and olfactory neurons in the maxillary palps (MX) and antenna (ANT). **b** Photoreceptor projecting from the retina (Re) through the lamina (La) into the medulla (Me). **c** oocytes, olfactory neurons in **d** maxillary palp and **e** antenna and **f** mechanosensory neurons in leg tip. Image **a** was acquired with 0.5x post-magnification in combination with a 10x water-immersion objective (Olympus, UMPlanFLN, 0.3 NA, WD = 3.5mm) with custom-made correction of optics for a refractive index of 1.45 (WD = 3.5mm after correction). Image in **b** was acquired with a 2x post-magnification in combination with a 25x objective (Olympus, XLPlanN, 1.0 NA, WD = 8mm). Confocal images in **c, d** were acquired with a 40x Oil-immersion objective (Leica, HCX OL APO CS, 1.25 NA, 100µm WD) and in **e, f** with a 63x Glycerol-immersion objective (Leica, HC PL APO, 1.3 NA, WD 280µm). Genotype: *Peb-Gal4 UAS-mCD8::GFP*. Scale bars represent 500µm in **a**, 100µm in **b, c** and 25µm in **d-f**.



Supplementary Figure 9 | Distribution of mechanosensory neurons in legs and wings visualised using optimised aspheric ultramicroscope system. **a, b** Light-sheet images of GFP expression. **a** Dorsal view of sensory neurons in legs and wings marked with arrowheads. **b** Lateral view of sensory neurons in legs and wings marked with arrowheads. Images in **a** was acquired with 0.5x post-magnification in combination with a 10x water- immersion objective (Olympus, UMPlanFLN, 0.3 NA, WD = 3.5mm) with custom made correction of optics for a refractive index of 1.45 (WD after correction 3.5mm). Image in **b** was acquired with 2x post-magnification in combination with a 4x Objective (Olympus, XLFluor4x/340, 0.28 NA, WD = 29,5mm) with custom-made correction of optics for a refractive index of 1.45 (WD after correction 10mm). In **a** a post magnification of 0.5x was used. Genotype: *Peb-Gal4 UAS-mCD8::GFP*. Scale bars represent 200µm.



Supplementary Figure 10 | Visualisation of the connections in adult fly olfactory receptor system using confocal microscopy. Coronal view of olfactory receptor neurons in antenna (ANT), maxillary palp (MX), labellum (LB), and part of the antennal nerve (AN) projecting from the ANT. Inset shows a higher magnification image of ventral cibarial sense organ (vcso). Images were acquired with a 20x immersion objective (Leica, HCX PL APO CS, 0.7 NA, 260µm WD). Genotype: *Peb-Gal4 UAS-mCD8::GFP*. Scale bars represent 200µm and 10µm in the main figure and inset respectively.



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181 **Supplementary Figure 11** | Principle of multi-view combining of ultramicroscope-
 182 recordings from 90°-tilted directions. **a** Scheme of the workflow of multi-view
 183 combining. **b** GFP-labelled neurons in maxillary palp processed with multi-view
 184 combining. **a, b** (1) Two image stacks, A (reference stack) and B (tilted stack) were
 185 recorded from orthogonally tilted directions and spatially registered using Amira
 186 software. (2) After registration, stack B was virtually resliced to generate computed
 187 section planes that are coplanar with respect to stack A. (3) Non-overlapping regions
 188 were removed from stacks A and B using a binary masking operation. The remaining
 189 parts were scaled to approximately the same average brightness by scaling to the
 190 95% percentile. (4) A 3D Fast Fourier Transformation was applied to both stacks and
 191 the calculated magnitudes and phases were recombined according to eq. (3) and (4)
 192 in the methods section. (5) The multi-view combined stack comprising the sharper
 193 components of A and B was obtained by applying the inverse Fourier transformation.

194 (6) Finished multi-view combined stack. Scale bar represents 50µm and is
195 representative for all images in **b**.

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199 **Supplementary Table 1** | Resolution of the USAF1951-chart in µm

Element	Group Number						
	1	2	3	4	5	6	7
1	250	125	62.5	31.25	15.63	7.81	3.91
2	222.72	111.36	55.68	27.84	13.92	6.96	3.48
3	198.43	99.21	49.61	24.8	12.4	6.2	3.1
4	176.78	88.39	44.19	22.1	11.05	5.52	2.76
5	157.49	78.75	39.37	19.69	9.84	4.92	2.46
6	140.31	70.15	35.08	17.54	8.77	4.38	2.19

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220 **Supplementary Table 2 | Imaging specifications**

Figure	Animal	System	Objective	Postmagnification	Image pixel size	z-step	Number of channels	Imaging depth	Number of merged stacks	Data volume	Imaging Time
3a	Prepupa	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	936µm	2	19GB	2h
3b	Prepupa	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	Clipped plane of 3a		n.a	n.a
3c	Prepupa	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	Clipped plane of 3a	2	n.a	n.a
3d	Prepupa	Confocal	Leica 20x Gly		0.757µm x 0.757µm	2.01µm	2	247µm		190MB	11min
3e	Prepupa	Confocal	Leica 20x Gly		0.757µm x 0.757µm	1.3µm	2	119µm		260MB	8min
4a	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	563µm		4GB	40min
4b	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	546µm		4GB	40min
4c	adult <i>Drosophila</i>	UM	Olympus 25x RI:1.45		0.259µm x 0.259µm	0.259µm	2	226µm		12GB	45min
4d	adult <i>Drosophila</i>	UM	Olympus 25x RI:1.45		0.259µm x 0.259µm	0.259µm	2	196µm		10GB	40min
4e	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	459µm		4GB	35min
4f	adult <i>Drosophila</i>	UM	Olympus 25x RI:1.45		0.259µm x 0.259µm	0.259µm	2	248µm		16GB	45min
5a	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45	2x	0.324µm x 0.324µm	1.539µm	2	672µm	2	4GB	40min
5b	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45	2x	0.324µm x 0.324µm	1.539µm	1	672µm	2	2GB	20min
5c	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	601µm		4GB	40min
5d	adult <i>Drosophila</i>	UM	Olympus 25x RI:1.45	0.5x	0.54µm x 0.54µm	1.08µm	2	844µm		7GB	55min
6c	adult <i>Drosophila</i>	UM	corr. 4x RI: 1.45	2x	0.81µm x 0.81µm	1.62µm	2	1313µm		3GB	30min
6d	adult <i>Drosophila</i>	UM	corr. 4x RI: 1.45	2x	0.81µm x 0.81µm	1.62µm	2	2198µm	2	10GB	55min
6e	adult <i>Drosophila</i>	UM	corr. 4x RI: 1.45	2x	0.81µm x 0.81µm	1.62µm	2	Dorsal 1313µm; Sag. 2198µm	2 (multiview combined)	25GB	1h 15min
Supp. Figure	Animal	System	Objective	Postmagnification	Image pixel size	z-step	Number of channels	Imaging depth	Number of merged stacks	Data volume	Imaging Time
1b	adult <i>Drosophila</i>	Confocal	Leica 20x Gly		1.250µm x 1.250µm	1µm	2	74µm		100MB	11min
4a	adult <i>Drosophila</i>	UM	corr. 4x RI: 1.45		1.642µm x 1.642µm	1.642µm	1	500µm-800µm		n.a.	n.a.
5a	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45	0.5x	1.302µm x 1.302µm	2.432µm	2	1884µm		6GB	45min
5b	adult <i>Drosophila</i>	Confocal	Leica 20x Gly		0.757µm x 0.757µm	2.01µm	2	267µm		280MB	11min
7a	Larva	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	895µm	2	16GB	1h20min
7b	Larva	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	2	Clipped plane of 7a	2	n.a	n.a
8a	Pupa	UM	corr. 10x RI: 1.45	0.5x	1.302µm x 1.302µm	0.651µm	2	801µm	2	12GB	60min
8b	Pupa	UM	Olympus 25x RI:1.45	2x	0.129µm x 0.129µm	0.4µm	1	clipping plane of stack of 422µm		6GB	30min
8c	Pupa	Confocal	Leica 40x Oil		0.378µm x 0.378µm	1.09µm	2	one image		1MB	<1min
8d	Pupa	Confocal	Leica 40x Oil	1.8x Zoom	0.215µm x 0.215µm	0.59µm	2	44µm		160MB	6min
8e	Pupa	Confocal	Leica 63x Gly	1.8x Zoom	0.135µm x 0.135µm	0.04µm	1	one image		1MB	<1min
8f	Pupa	Confocal	Leica 63x Gly	1.8x Zoom	0.137µm x 0.137µm	0.71µm	1	52µm		80MB	6min
9a	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45	0.5x	1.302µm x 1.302µm	2.916µm	2	2145µm		6GB	50min
9b	adult <i>Drosophila</i>	UM	corr. 4x RI: 1.45	2x	0.81µm x 0.81µm	1.62µm	1	1061µm		800MB	12min

10a	adult <i>Drosophila</i>	Confocal	Leica 20x Gly		0.757µm x 0.757µm	2µm	2	136µm	2	288MB	25min

221 UM = ultramicroscope, corr. =corrected

222 **Supplementary Table 3 | Video specifications**

Supp. Video	Animal	System	Objective	Postmagnification	Image pixel size	z-step	Imaging direction	Imaging depth
1	Larva	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	Dorsal	895µm
2	Prepupa	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	Dorsal	936µm
3	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45	0.5x	1.302µm x 1.302µm	2.916µm	Dorsal	2145µm
4 (Multiview)	adult <i>Drosophila</i>	UM	Olympus 25x RI:1.45		0.259µm x 0.259µm	0.259µm	Coronal	196µm
	adult <i>Drosophila</i>	UM	Olympus 25x RI:1.45		0.259µm x 0.259µm	0.259µm	Sagittal	318µm
5	Pupa	UM	Olympus 25x RI:1.45		0.259µm x 0.259µm	0.259µm	Dorsal	512µm
6	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45	2x	0.324µm x 0.324µm	1.539µm	Sagittal	672µm
7	adult <i>Drosophila</i>	UM	corr. 10x RI: 1.45		0.651µm x 0.651µm	0.651µm	Sagittal	601µm
8 (Multiview)	adult <i>Drosophila</i>	UM	corr. 4x RI: 1.45	2x	0.81µm x 0.81µm	1.62µm	Dorsal	1313µm
	adult <i>Drosophila</i>	UM	corr. 4x RI: 1.45	2x	0.81µm x 0.81µm	1.62µm	Sagittal	2198µm

223 UM = ultramicroscope, corr. = corrected

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