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Supplemental Information

Neuronal Architecture of a Visual Center that Processes Optic Flow

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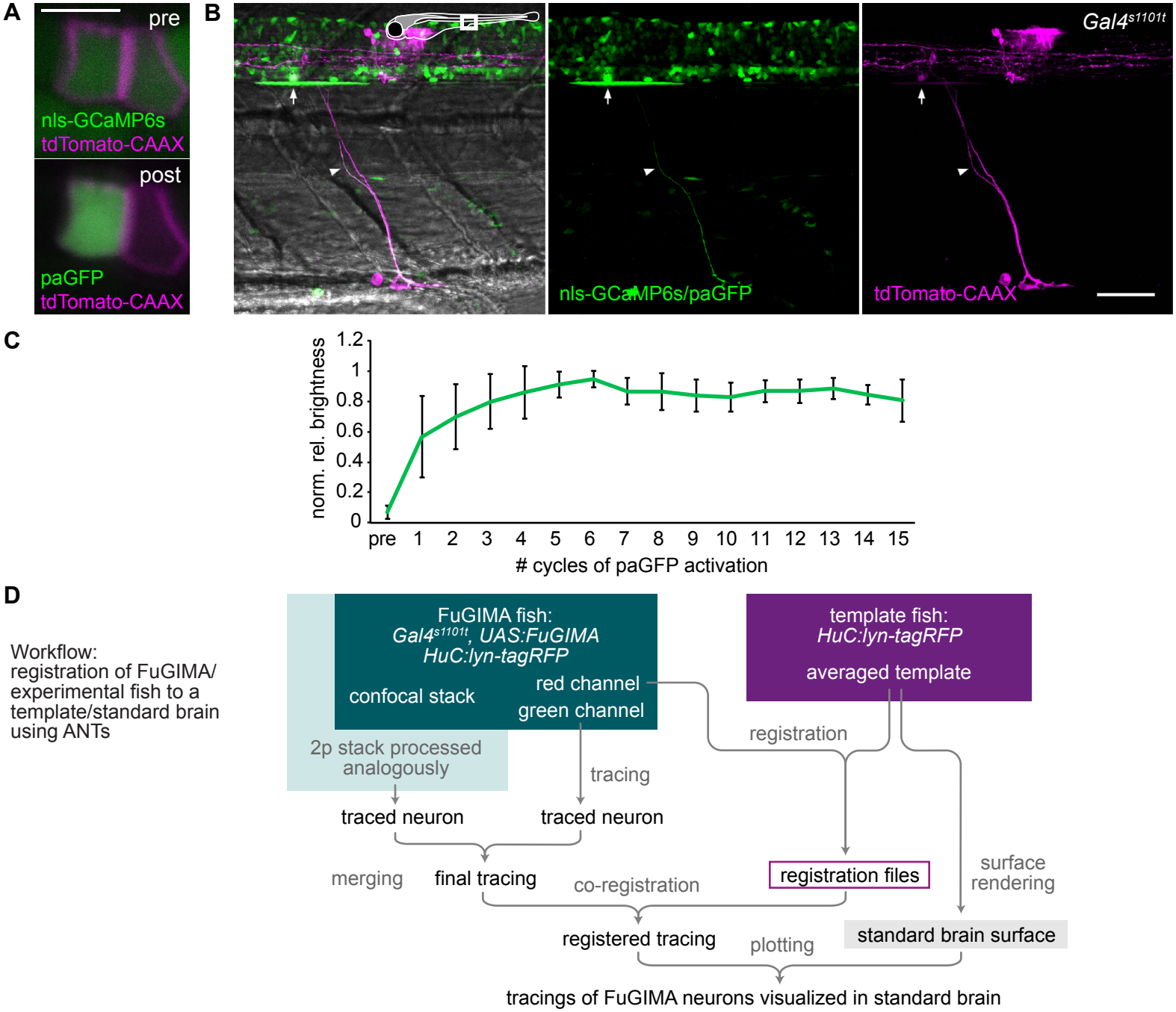
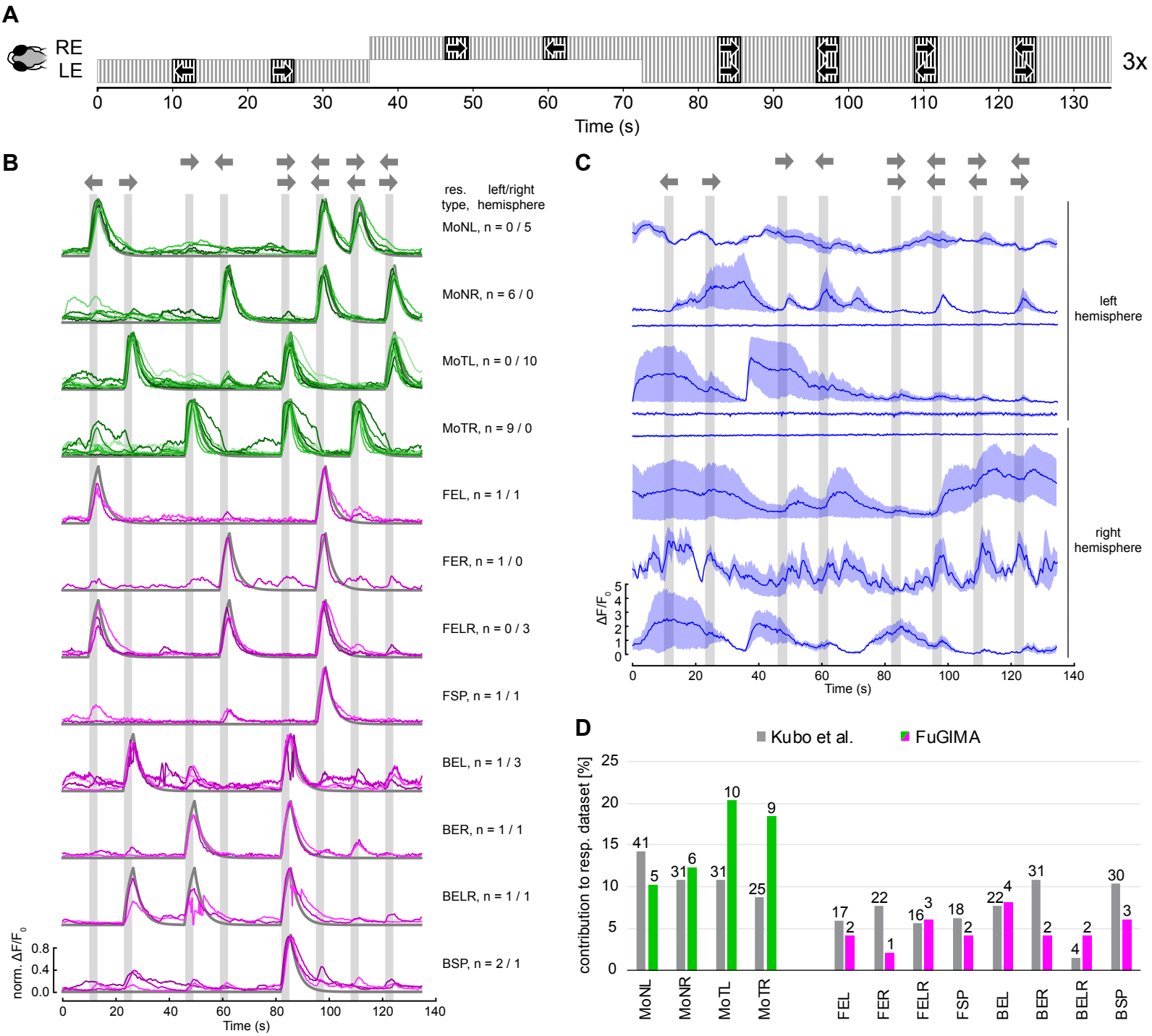

A
pre
nls-GCaMP6s
tdTomato-CAAX
post
paGFP
tdTomato-CAAX
B
nls-GCaMP6s/paGFP
Gal4s1101t
tdTomato-CAAX
C
norm. rel. brightness
1.2
1
0.8
0.6
0.4
0.2
0
pre 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
cycles of paGFP activation
D
Workflow:
registration of FuGIMA/
experimental fish to a
template/standard brain
using ANTs
FuGIMA fish:
Gal4s1101t, UAS:FuGIMA
HuC:lyn-tagRFP
confocal stack
red channel
green channel
2p stack processed
analogously
template fish:
HuC:lyn-tagRFP
averaged template
registration
tracing
traced neuron
traced neuron
merging
final tracing
co-registration
registration files
surface
rendering
registered tracing
plotting
standard brain surface
tracings of FuGIMA neurons visualized in standard brain
E
distance LMstandard - LMexp [µm]
30
25
20
15
10
5
0
LM1 LM2 LM3 LM4 LM5 LM6 LM7 LM8 LM9 LM10 LM11
20 µm
L
20 µm
P
midline
dorsal view
LP
20 µm
V
oblique view
HuC:lyn-tagRFP
reference LM
LM1: posterior commissure
LM2: interpeduncular nucleus (IPN)
LM3: blood vessel near AF6
LM4: dorsal tip of AF7
LM5: neuropil rostral to nMLF
LM6: tecto-bulbar tract
LM7: optic tract ventral to OT
LM8: large blood vessel
LM9: AF9 to OT tract
LM10: tract in intermediate hypothalamus
LM11: commissure dorsal to IPN

Fig. S1

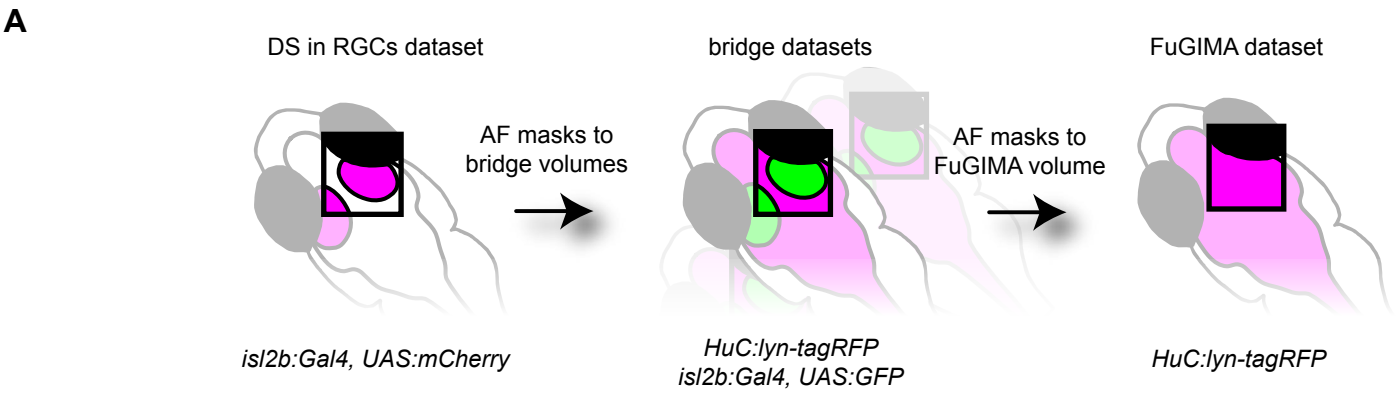
Supplementary Figure S1 (related to Figure 1). Characterization of paGFP activation in single cells, registration procedure of tracings and alignment precision of landmarks. **(A)** Application of the FuGIMA photoactivation protocol in a single spinal cord neuron co-expressing *UAS:FuGIMA* and *UAS:tdTomato-CAAX* (driver: *Gal4*^{s1101t}). Photoactivation in a single spinal cord neuron, pre- and post-photoactivation with a single activation cycle (brightness/contrast adapted separately). **(B)** Lateral views of the tail with the photoactivated neuron extending from the spinal cord after full photoactivation protocol of 15 cycles. (Inset: rectangle on fish schematic indicates the field of view. Green: nls-GCaMP6s/paGFP, magenta: tdTomato-CAAX, white arrow: soma of photoactivated neuron, arrowhead: filled neurite). **(C)** Time course of paGFP brightness in the soma over the course of 15 cycles of photoactivation (n = 5 pretectal neurons in 3 fish, mean +/- STD). **(D)** Workflow of image registration enabling visualization of FuGIMA neurons in the standard brain. Experimental z stacks are split into two separately processed channels. Neurons are traced in the nls-GCaMP6s/paGFP channel and tracings of neurons imaged at both the two-photon (2p) and the confocal microscope are merged. In parallel, the reference marker channel (*HuC:lyn-tagRFP*) is registered to the standard brain (averaged *HuC:lyn-tagRFP*). The resulting registration files are applied to tracings (co-registration), enabling their visualization within the volume of the standard brain. **(E)** Quantification of distances between the location of landmarks in the standard brain and in the registered experimental fish. Left: combined box plot and swarm plot (middle horizontal line: median, horizontal box outlines: first and third quartile, whiskers: last points included in 1.5 * interquartile range from the respective quartile), right: 3D rendering of landmarks in the standard brain (gray surface: reference marker *HuC:lyn-tagRFP*, dark gray: landmark position in standard brain, colors: registered landmarks from experimental fish, n = 8 z stacks from 6 fish for LM 1 – 9 and 11, n = 6 z stacks from 4 fish for LM 10, middle: dorsal view, black arrow: viewing direction for lateral view, shown on the right, LM, landmark). Scale bar: 5 µm in (A), 50 µm in (B).

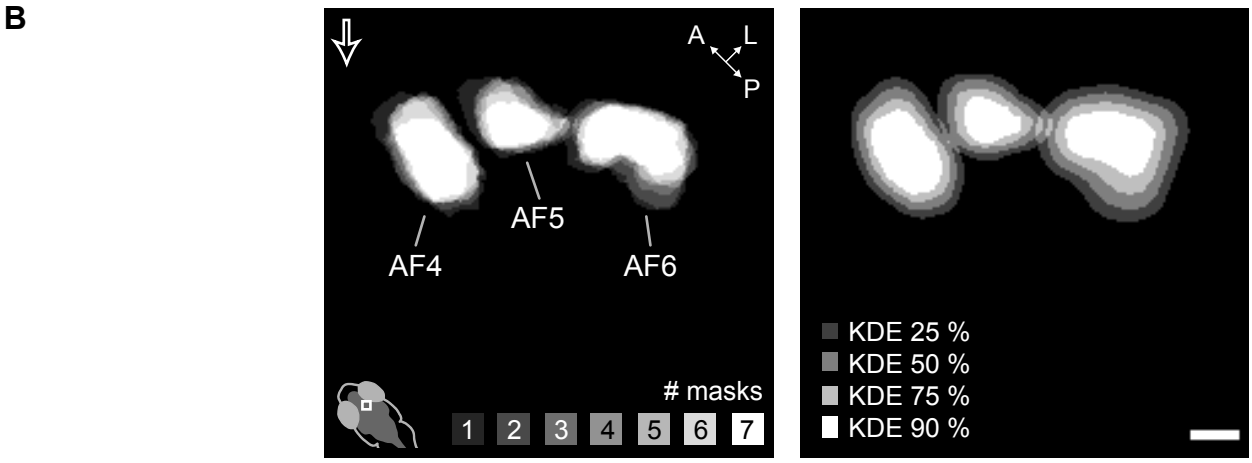


response type	mirrored equivalent	
MoNL	MoNR	
MoNR	MoNL	monocular nasalward ipsilateral - not in FuGIMA dataset
MoTL	MoTR	
MoTR	MoTL	monocular temporalward ipsilateral - not in FuGIMA dataset
FEL	FER	
FER	FEL	
FELR		
FSP		
BEL	BER	
BER	BEL	
BELR		
BSP		

Fig. S2

Supplementary Figure S2 (related to Figures 1 and 2). Visual stimulus protocol, functional imaging time series of all FuGIMA neurons and comparison of response type sampling with Kubo *et al.* (2014). (A) During functional imaging, fish are presented with the following whole-field motion stimulus, consisting of eight motion phases with three repetitions (same order): Horizontally moving gratings (3 s each, black arrows) are presented in four monocular phases (left nasalward, left temporalward, right temporalward, right nasalward), followed by four binocular phases (backward, forward, clockwise and counter-clockwise) and interspersed by the presentation of stationary gratings (gray, 10 s) (RE: right eye, LE: left eye). **(B)** Normalized fluorescence traces of motion-sensitive pretectal FuGIMA neurons grouped according to their response type, numbers indicate occurrence in FuGIMA dataset and hemisphere of origin. **(C)** Fluorescence traces of non-motion-sensitive pretectal FuGIMA neurons and hemisphere of origin (blue line: average over three repetitions, light blue: SEM). **(D)** Comparison of response type frequency between Kubo *et al.* (2014) (number of cells per fish) and this work (total number of cells in the dataset). Proportions of response type are normalized to the total number of neurons across the investigated motion-sensitive response types (four monocular DS and eight translation-selective), absolute number of neurons are indicated on top of each bars. **(E)** FuGIMA neurons were imaged in both left and right hemispheres. Mirroring leads to a change in response type name as indicated.


A
DS in RGCs dataset
bridge datasets
FuGIMA dataset
AF masks to bridge volumes
AF masks to FuGIMA volume
isl2b:Gal4, UAS:mCherry
HuC:lyn-tagRFP
isl2b:Gal4, UAS:GFP
HuC:lyn-tagRFP


B
A
L
P
AF4
AF5
AF6
masks
1 2 3 4 5 6 7
KDE 25 %
KDE 50 %
KDE 75 %
KDE 90 %

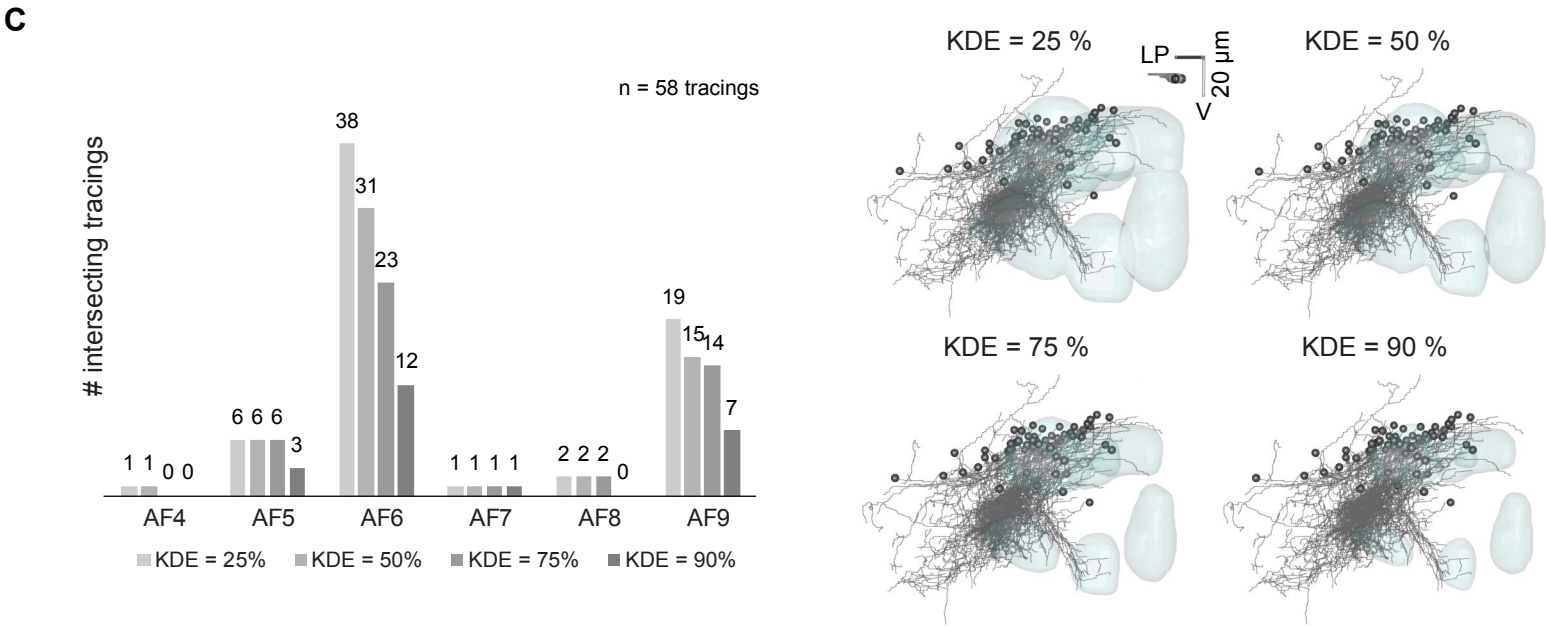

C
n = 58 tracings
# intersecting tracings
1 1 0 0
6 6 6 3
38 31 23 12
1 1 1 1
2 2 2 0
19 15 14 7
AF4
AF5
AF6
AF7
AF8
AF9
KDE = 25%
KDE = 50%
KDE = 75%
KDE = 90%
KDE = 25 %
KDE = 50 %
KDE = 75 %
KDE = 90 %
LP
V
20 µm

Fig. S3

Supplementary Figure S3 (related to Figure 2). Establishment of arborization field approximation boundaries and effect of boundary stringency on intersections with FuGIMA tracings. **(A)** Schematic illustrating registration of AF masks from the RGC standard volume via bridging z stacks (derived from multiple fish) to the FuGIMA reference brain. **(B)** Generation of approximation boundaries of AFs based on a kernel-density estimation (KDE) over registered AF masks (underlying Figure 2C). Left: Overlap of registered AF masks (n = 7 bridging z stacks, from 4 fish, open arrow: direction of oblique view), right: KDE of registered masks, thresholded to 25, 50, 75, and 90 %. **(C)** Quantification of intersections of FuGIMA tracings with AF boundaries of various stringency (KDE=25, 50, 75, and 90%). Right: 3D renderings of AF boundaries of various stringency and the full FuGIMA dataset (oblique view). Scale bar: 20 µm in (B).

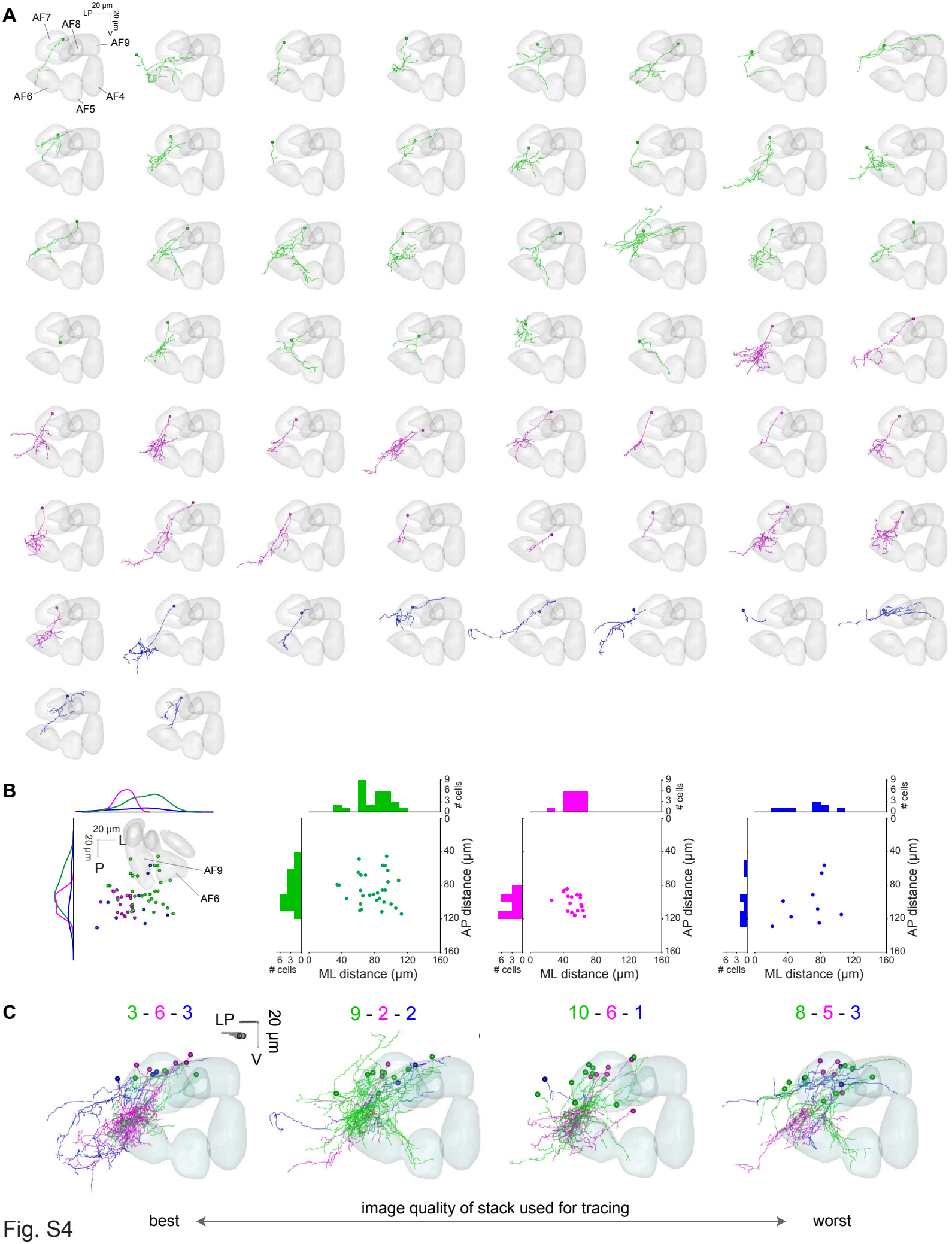
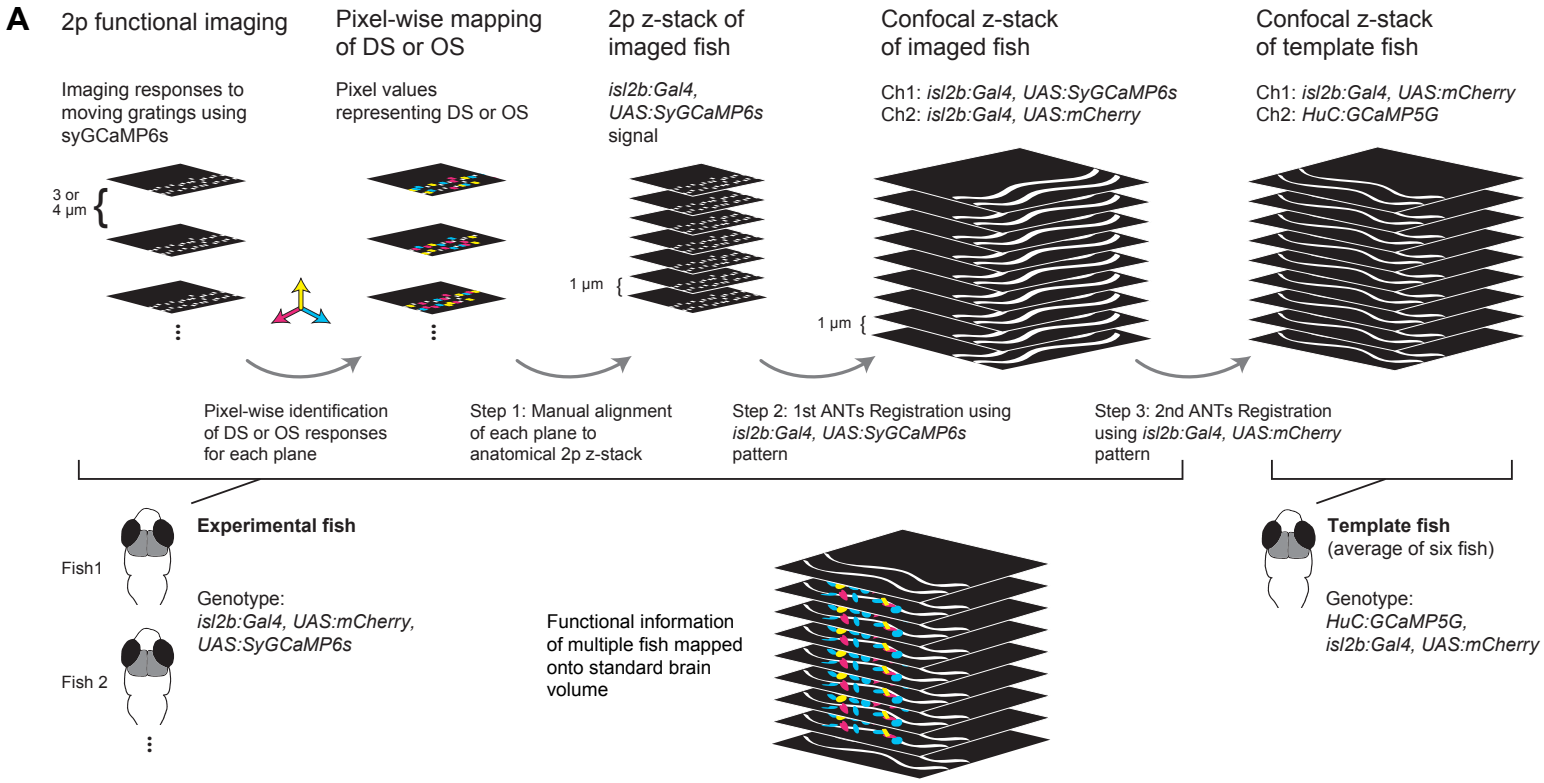
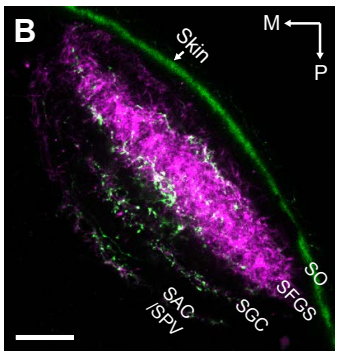
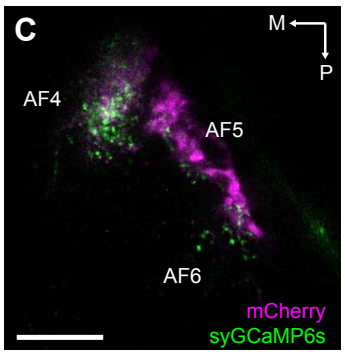

A
AF7
AF8
AF9
AF6
AF4
AF5
20 µm
LP
V
B
20 µm
L
P
AF9
AF6
cells
AP distance (µm)
ML distance (µm)
C
3 - 6 - 3
9 - 2 - 2
10 - 6 - 1
8 - 5 - 3
LP
V
20 µm
best
image quality of stack used for tracing
worst

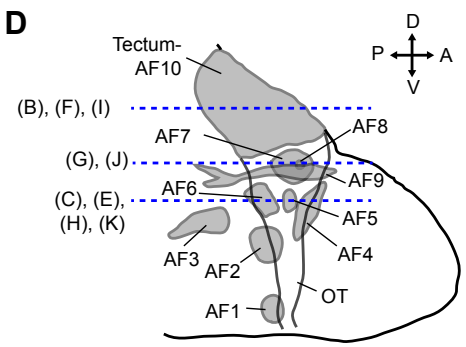
Fig. S4

Supplementary Figure S4 (related to Figure 2). Morphology of all FuGIMA neurons, soma locations regarding response class and split of dataset according to z stack quality. **(A)** Individual FuGIMA neurons plotted together with AF masks (KDE=50%), tracings color-coded according to response class (green: monocular DS, magenta: translation-selective, blue: non-motion-sensitive, color-code as in Figure 1C, oblique view). **(B)** Soma location of FuGIMA neurons color-coded according to their response class. (Left) Montage of 3D rendering of FuGIMA somata with surfaces of AFs 4-9 and KDE for ML and AP distributions. (Right) Plot of soma location with histogram of ML and AP distribution (separated by response class). ML: medial-lateral, AP: anterior-posterior, distance measured from the origin as defined in Kubo et al., 2014. **(C)** Split of FuGIMA dataset into four categories according to image quality of the z stack (“best” to “worst”, manual annotation). For each category, the tracings of each class are color-coded as in (A), and the number of each class is stated on top. All four categories contain tracing of all response classes, with relatively more translation and non-motion-sensitive tracings emanating from the best quality z stacks.


A
2p functional imaging
Imaging responses to moving gratings using syGCaMP6s
3 or 4 µm
Pixel-wise mapping of DS or OS
Pixel values representing DS or OS
2p z-stack of imaged fish
isl2b:Gal4, UAS:SyGCaMP6s signal
1 µm
Confocal z-stack of imaged fish
Ch1: isl2b:Gal4, UAS:SyGCaMP6s
Ch2: isl2b:Gal4, UAS:mCherry
1 µm
Confocal z-stack of template fish
Ch1: isl2b:Gal4, UAS:mCherry
Ch2: HuC:GCaMP5G
Pixel-wise identification of DS or OS responses for each plane
Step 1: Manual alignment of each plane to anatomical 2p z-stack
Step 2: 1st ANTs Registration using isl2b:Gal4, UAS:SyGCaMP6s pattern
Step 3: 2nd ANTs Registration using isl2b:Gal4, UAS:mCherry pattern
Fish1
Fish 2
Experimental fish
Genotype:
isl2b:Gal4, UAS:mCherry, UAS:SyGCaMP6s
Functional information of multiple fish mapped onto standard brain volume
Template fish
(average of six fish)
Genotype:
HuC:GCaMP5G, isl2b:Gal4, UAS:mCherry


B
M
P
Skin
SO
SFGS
SGC
SAC
/SPV


C
M
P
AF4
AF5
AF6
mCherry
syGCaMP6s


D
D
P
A
V
Tectum-AF10
(B), (F), (I)
(G), (J)
(C), (E), (H), (K)
AF7
AF8
AF9
AF6
AF5
AF4
AF3
AF2
AF1
OT

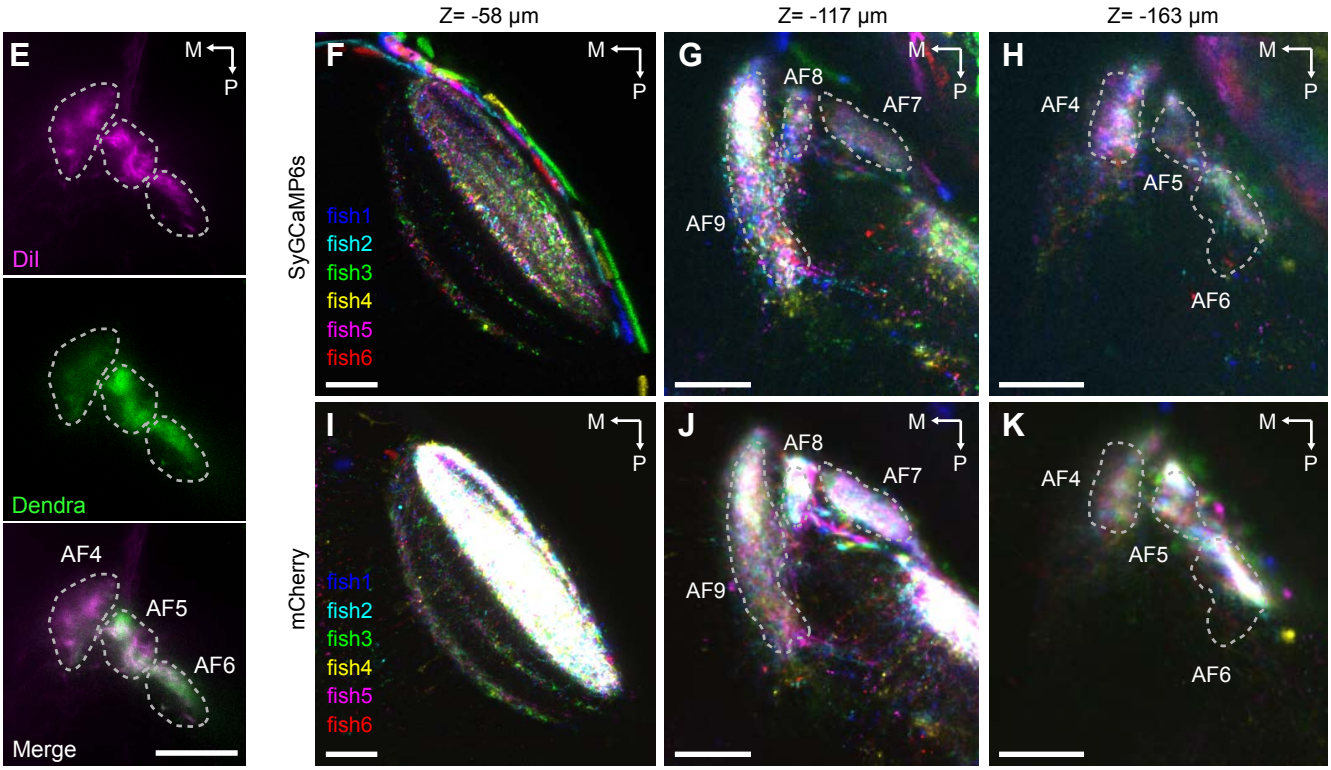

Z= -58 µm
Z= -117 µm
Z= -163 µm
E
M
P
DiI
Dendra
AF4
AF5
AF6
Merge
F
G
H
I
J
K
SyGCaMP6s
mCherry
fish1
fish2
fish3
fish4
fish5
fish6
AF8
AF7
AF9

Fig. S5

Supplementary Figure S5 (related to Figure 3). Image registration work flow to generate a 3D map of direction-selectivity, characterization of *isl2b:Gal4, UAS:syGCaMP* expression and overlay from image registration. (A) Schematic workflow for registering functional responses with anatomical structures. For clarity, only 3 preferred directions are represented here. See STAR Methods for details. **(B, C)** Subcellular localization of syGCaMP6s in the tectum/AF10 (B) and AF4, AF5 and AF6 (C) in *isl2b:Gal4, UAS:syGCaMP6s, UAS:mCherry* fish. Note that syGCaMP6s expression exhibits punctate signals in RGC terminals, in contrast to uniform mCherry signals in *en passant* RGC axon bundles. SO, *stratum opticum*; SFGS, *stratum fibrosum et griseum superficiale*; SGC, *stratum griseum centrale*; SAC, *stratum album centrale*. **(D)** Schematic illustration of AFs (modified from Burrill and Easter (1994)). Blue dotted lines indicate approximate z-planes shown in other panels of this figure. OT, optic tract. **(E)** Lipophilic dye DiI injection of the RGC axons in *isl2b:Gal4, UAS:Dendra-kras* fish. Note that the *isl2b:Gal4* line labels most of RGCs projecting to AF4, AF5 and AF6. **(F-K)** Overlay of 6 different transgenic fish (*isl2b:Gal4, UAS:syGCaMP6s*, *UAS:mCherry*) that have been registered into a reference system (RGC standard brain based on *isl2b:Gal4, UAS:mCherry*). Z-position indicates the distance from the dorsal most surface of AF10. Note that both syGCaMP6s (F-H) and mCherry (I-K) patterns from 6 fish occupy conserved space in the registered volume. A, anterior; P, posterior; D, dorsal; V, ventral, M, medial. Scale bars represent 30 μm.

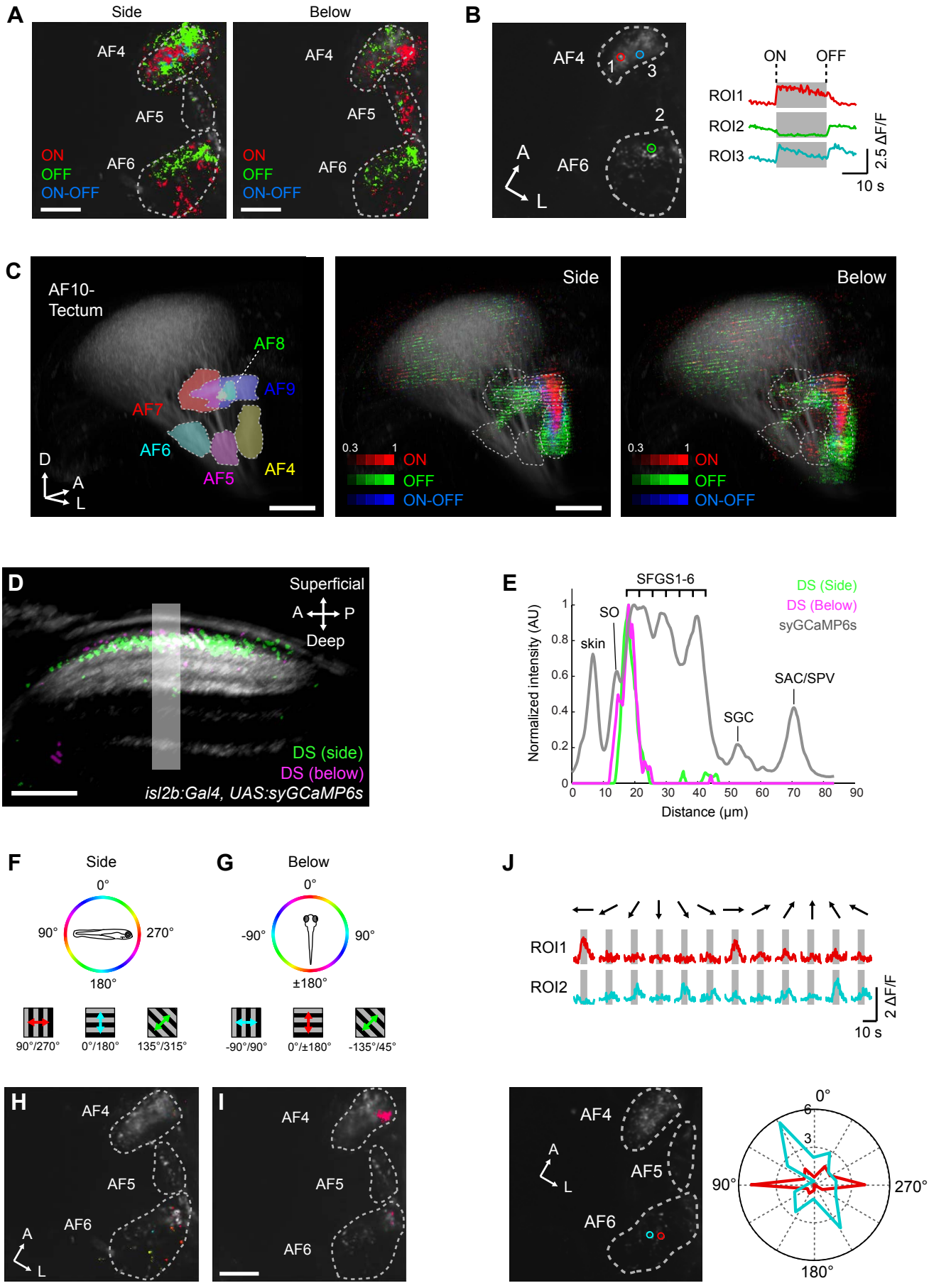
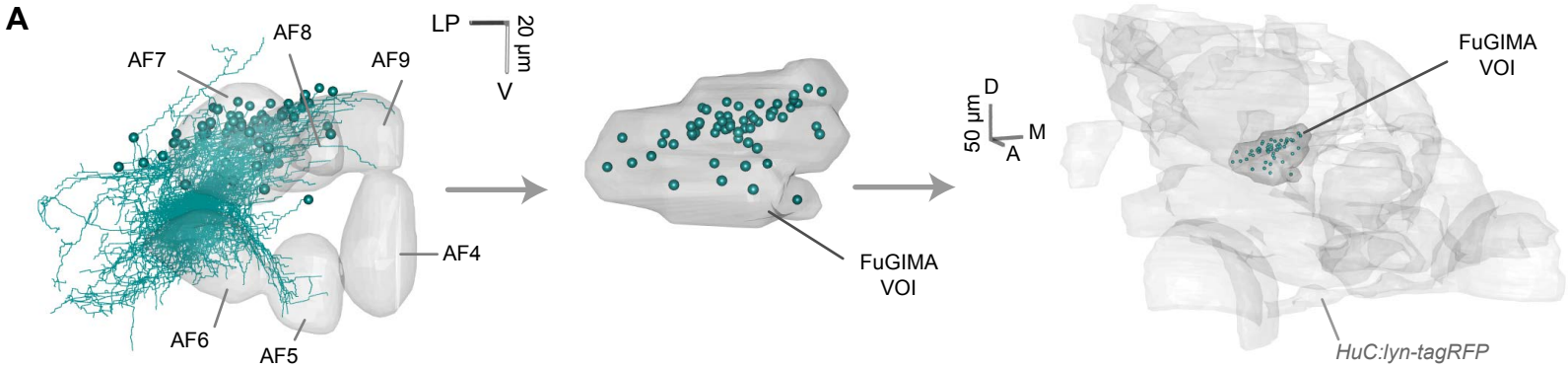

A
Side
Below
AF4
AF5
AF6
ON
OFF
ON-OFF
B
1
2
3
A
L
ON
OFF
ROI1
ROI2
ROI3
2.5 ΔF/F
10 s
C
AF10-
Tectum
AF8
AF9
AF7
AF6
AF5
AF4
D
A
L
Side
Below
0.3
1
ON
OFF
ON-OFF
D
Superficial
A
P
Deep
DS (side)
DS (below)
isl2b:Gal4, UAS:syGCaMP6s
E
SFGS1-6
DS (Side)
DS (Below)
syGCaMP6s
SO
skin
SGC
SAC/SPV
Normalized intensity (AU)
Distance (μm)
F
Side
0°
90°
270°
180°
90°/270°
0°/180°
135°/315°
G
Below
0°
-90°
90°
±180°
-90°/90°
0°/±180°
-135°/45°
J
ROI1
ROI2
2 ΔF/F
10 s
H
AF4
AF5
AF6
A
L
I
AF4
AF5
AF6
AF4
AF5
AF6
A
L
0°
6
3
90°
270°
180°

Fig. S6

Supplementary Figure S6 (related to Figure 3). Mapping of orientation-selectivity, direction-selectivity, and luminance responses in RGC terminals. (A) Response profile to luminance changes in AF 4, 5, and 6 presented from the side or below. Pixels are color coded according to the mutually exclusive luminance response types: pixels responsive to increase in luminance (ON), decrease in luminance, and both increase and decrease in luminance (ON-OFF). **(B)** Representative luminance response in AF4 and 6. Visual stimuli were presented from the side. ROIs correspond to synaptic puncta marked in the right panel. Note that AF5 is not contained in this optical plane. In (A) and (B), functional pixels are plotted on top of the mean image of syGCaMP6s (gray). **(C)** 3D representation of luminance response in RGC terminals. (Left) 3D model of AFs (as Figure 2B). For side presented 3D map, both AF10 and AF 4, 5, and 6 volumes are pooled from 6 functionally imaged volumes. For underneath presented 3D map, both AF10 and AF 4, 5, and 6 volumes are pooled from 7 functionally imaged volumes. The intensity of pixels corresponds to the frequency of a particular pixel to be luminance responsive across all imaged fish. Note that AF4 and AF9 contain highly luminance responsive RGC terminals and AF5 is weakly ON responsive (right panel). **(D)** Localization of DS pixels in tectal sublaminae. After registration into the RGC reference brain, DS pixels identified by the side (green) and below (magenta) presentations of visual stimuli were overlaid to the average image of *isl2b:Gal4, UAS:syGCaMP6s* signals of the same registered volume. DS pixels tuned to forward motion are plotted here for both side and below stimulus presentations. The volume was sliced obliquely to reveal laminar structure along the superficial-deep axis within the tectal neuropil. **(E)** Intensity plot along region indicated by a box shown in (D). Note that DS pixels for both stimulus positions (side, below) occupy SFGS1. **(F, G)** Color scheme of orientation space for motion presented from the side (F) and below (G). **(H, I)** Orientation selectivity (OS) in AF4, AF5 and AF6. The motion was presented from the side (H) and below (I). The color code is shown in (F, G). OS pixels are plotted on top of the mean image of syGCaMP6s (gray). **(J)** Representative responses of OS-RGC terminals in AF6. Visual stimuli were presented from the side. ROIs correspond to synaptic puncta

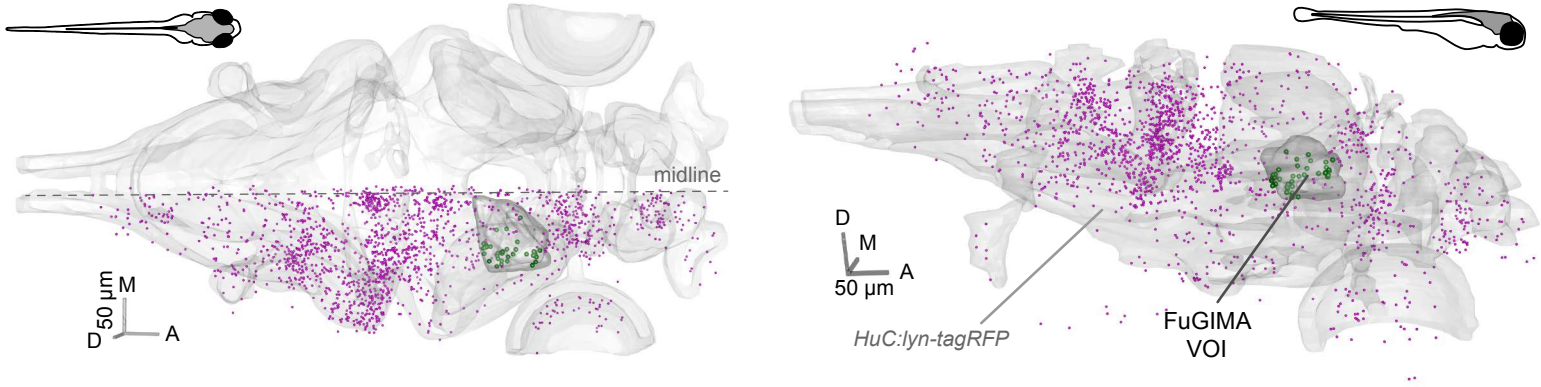
marked by the two circular ROIs in the bottom left image. Polar plot (bottom right) is derived from the ΔF/F traces shown above. A, anterior; P, posterior; D, dorsal, L, lateral. SO, *stratum opticum*; SFGS, *stratum fibrosum et griseum superficiale*; SGC, *stratum griseum centrale*; SAC, *stratum album centrale*. Scale bars: 10 μm (A, I), 30 μm (D) and 50 μm (C).



FuGIMA VOI: surface encompassing somata of FuGIMA neurons (n = 58)

B search in single-neuron atlas:

• somata in FuGIMA VOI (n = 38), • somata outside of FuGIMA VOI (n = 1705)



C

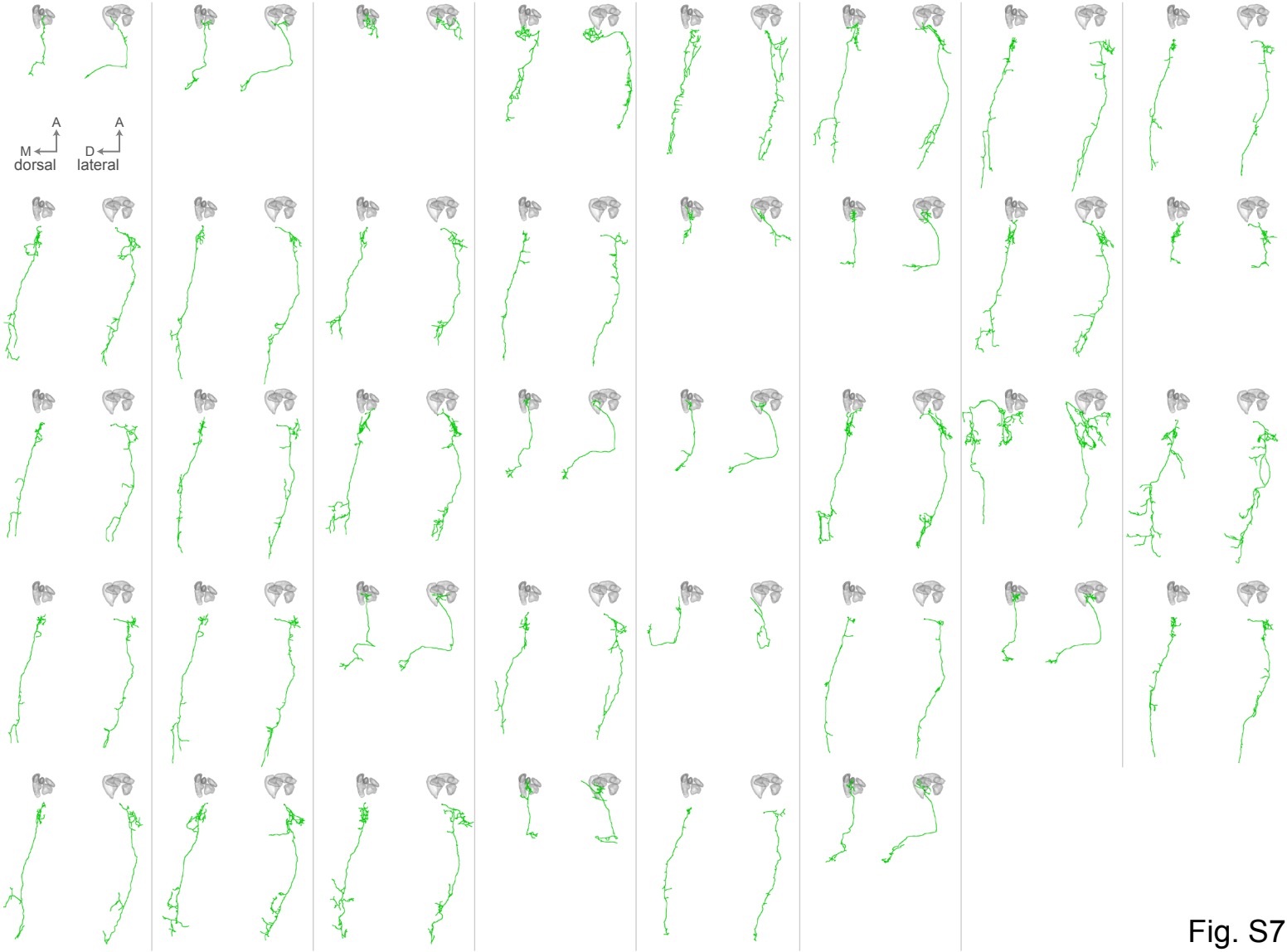


Fig. S7

Supplementary Figure S7 (related to Figure 6). Search strategy to complement FuGIMA tracings with a single-neuron atlas and results. (A) Definition of the FuGIMA "volume-of-interest" (FuGIMA VOI), the search area to find neurons complementing FuGIMA neurons (pretectal projection neuron (PPNs)) in a single-neuron atlas. The surface encompasses the somata of all FuGIMA neurons (n = 58 neurons) and is registered to the volume of a single-neuron atlas (Kunst et al. (2019), this issue of *Neuron*). **(B)** 3D rendering of the single-neuron atlas standard brain (*HuC:lyn-tagRFP,* Kunst et al. (2019), this issue of *Neuron*) and all somata from this single-neuron atlas, color-coded according to position within (green, n = 38, pretectal projection neurons, PPNs) or outside of the FuGIMA VOI (magenta, n = 1705, out of 1743 tracings in the atlas) (left: dorsal view, right: lateral view). **(C)** Individual tracings of PPNs, plotted with AFs 4 – 9; two plots per neuron: dorsal view (left) and lateral view (right), respectively.