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Previously undiscovered neurons of the Bogong moth brain: 3D-reconstruction and registration

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Abstract

The Australian Bogong moths are thought to have the ability to sense the earth's magnetic field. This ability has to be reflected in the animal's neural architecture. Hence reconstruction of three brain neurons was carried out, though these respond to visual stimulation instead of magnetic. These reconstructions were compared to that of other known specie's visual responsive neurons to provide a basis for future investigation into the Bogong moth's magnetic processing. The neurons were reconstructed with the Amira5.3 program to give 3D representations of their morphology. From this it was concluded that two of the neurons lack homologous ones in other species, these two being previously undiscovered. Implying a possible deviance in how the Bogong moth processes visual information.

Introduction

Seasonal migratory behavior is a common feature among flying animal species [1,2]. Species migrate from one region to another during specific seasonal periods, often distances of several 100 miles [1]. Some bird species display such migratory behavior [1], as do some members of the arthropod family (here focus is namely on insects). These include, but are not limited to, the desert locust (*Schistocerca gregaria*), north American Monarch butterfly (*Danaus plexippus*) and the Australian Bogong moth (*Agrotis infusa*) [3,4,5]. Both the Desert locust and the Monarch butterfly are known to be diurnal (day active) and have been shown to utilize atmospheric polarized light, originating from the sun, as well as other solar clues to determine the direction they are heading in, much like a solar compass [6]. These solar cues are integrated with information from the circadian clock in the central area of the brain; the time of day is hence accounted for. As a consequence they can know the relative position of the sun and thus their faced direction. With this mechanism both the Desert locust and the Monarch butterfly are thought to navigate during their respective migrations [6].

Unlike these two species the Bogong moth is known to be nocturnal, meaning it is mainly active at dimmer light levels [5]. Nevertheless it is an accomplished migrant. Each year the Bogong moths travel from southern Queensland (breeding grounds) to the alpine region of New South Wales. During the summer months (November to February) the Bogongs gather in caves in the Australian Alps, where they are in an aestivating state (a hibernation-like state) it is not until late summer or early autumn that the Bogongs migrate back to their breeding grounds. Breeding ensues once they arrive and the adult moths then die. Then the next generation of Bogongs takes up the migratory mantle, migrating to the alpine region for aestivation and later return to the breeding ground. Given that each moth only performs the migration once, this species sense of direction can not come from any previous experience of the migratory path nor from guidance by older individuals [6, Heinze, pers. comm.]. Hence, sense of direction must be mediated by the individual's sensory information.

As a nocturnal species the moth mainly navigates when there is a lack of solar cues. Therefore, it has to rely on other systems, than the one described above, in order to generate a sense of direction. This could be either the moon, polarized skylight from the moon, the Milky Way, or even the Earth's magnetic field [6]. Based on behavioral evidence the system that the Bogongs are believed to utilize is the ability to sense the Earth's magnetic field, most likely with the aid of a light-dependent chemical-based mechanism in their eyes [Heinze, pers. Comm., 6]. There is also some evidence of the Monarch butterfly being able to utilize the earth's magnetic field as a directional cue when its primary solar cues are lacking [7], with the close kinship between the Monarchs and the Bogongs the ferro-sensory system could be homologous. Also, preliminary electrophysiological results have demonstrated that neurons in the brain of the Bogong moth respond to visual compass cues like the atmospheric

polarized light [Heinze pers. comm.]. This suggests that the neurons underlying the visual compass of diurnal migratory insects might also be present in the Bogong moth and might additionally integrate with the magnetic field information. Therefore, the Bogong moth provides a unique access for understanding the neural basis of the magnetic compass sense.

Neural systems reflect the inherent ability of a species. Knowing the makeup of the brain is therefore essential for understanding how sensory information can be processed in order to give rise to a particular behavior [8]. The head of arthropods contain two ganglia both comprised of several different regions, or neuropils. Both visual processing and olfactory processing takes place in the supraesophageal ganglion of the insect head [9], where the concerned neuropils are located. Due to the overall fusion of the two ganglia they will throughout be referred to as the brain of the animal.

Neurons, the functional unit of the nervous system, are the cells that carry out the primary signal transduction in a nervous system. These cells receive input from other neurons or receptor cells in the form of electrical or chemical information, this information (if sufficiently strong) will affect the properties of the signaled to neuron, instigating or inhibiting further signal transduction. Neurons can often be characterized by their unique appearance, consisting of a soma where the nucleus is located, dendrites as the primary input region and an axon that serves as the primary output region. The dendrites, classified as the input processes of the cell, receive and react to the information from a presynaptic cell. Axons are responsible for the summation of intracellular electrical change caused by dendritic input, the initiation of action potentials (in the Axon hillock) as well as signaling other innervated neurons. The area between one cell's axons and the second cells dendrites is referred to as the synaptic cleft [8]. In insect species the soma is commonly located on the exterior surface of the neuropil and unlike vertebrate cells the dendrites and axons do not directly protrude from the soma; instead the soma is mainly excluded from the signal propagation step. Rather, the dendrites and axons form a continuous unit with the soma only connected via a side branch (the primary neurite) [10]. Dendritic branches (the input) and axonal branches (the output) can often be distinguished by their morphological appearance: dendrites are slender, smooth and possesses very fine spine like structures, while axonal branches are often thicker and characterized by ball-like swellings (varicosities). This allows deduction of the direction of information flow based solely on anatomical information [10]. Furthermore, it is within the neuropils that the synaptic connections of neurons are located [11]. Therefore, by knowing the morphology of its neurons it is possible to make an estimate of how information is transmitted in the brain. Combining several neurons into a network forms the basis for processing different inputs, which might lead to complex abilities such as perception of or reaction to external stimuli [8].

For the Monarch Butterfly a group of neuropils designated the central complex (CX) are believed to be the main processing center for polarized-light signals that ultimately affect the behavior of the insect, the motor planning [10,11]. The CX is targeted by neural pathways originating in the optic lobes and that travel through (also with processes in) the anterior optic tubercles (AOTU) and the lateral accessory lobes (LAL), terminating in specific CX subunits. Input is mainly mediated by tangential neurons to these CX-subunits, most prominently the upper or lower divisions of the central body (CBU and CBL respectively) and to a lesser extent also the protocerebral bridge (PB) and the paired noduli (NO) [10]. Because the central complex is believed to be the neuropil where the sense of direction is created within the Monarch butterfly [11] it is likely that these centers have a similar role in the Bogong brain. The sense of direction mediated by the ferro sensor is likely created here (the ferro-sensor itself is probably located in the eyes). As mentioned, the CX consists of four different types of neuropils CBU, CBL, PB and NO. These subunits are a major integration center for visual- and mechanosensory-information in the Monarch butterfly [12]. The AOTU and the LAL, are tightly linked to the CX, with the gal (GA) and bulb (BU) being closely

associated with the LAL in both function and spatial location [13]. Primary visual processing is carried out in the optic lobes, containing several distinct brain regions. Notably, the Lobula (LO) with its associated Lobula plate (LOP) and the medulla (ME) [14].

To understand how the Bogong moth function, it is essential to understand how these different brain regions are connected. Primarily the Bogong CX would have to be anatomically characterized on the level of neuropils as well as neurons. Hence, knowing how the neurons of its brain look, where they receive their input and where they have their output regions, essentially which brain regions they connect and what other neurons they are in contact with, is pivotal for the understanding of the Bogong. To that end this project is concerned with the three dimensional reconstruction of Bogong moth brain neurons.

Though the neurons that process the magnetic field information of the Bogong moths is the end goal, understanding how the visual system works provides an opportunity to compare it with the other insect species. Revealing to what extent Bogong's neural systems are similar to the Monarch butterfly's and to other specie's. With Bogongs and Monarchs being closely related system similarities should be evident. And with the Monarch likely having a ferro-sensor, Bogongs might have homologous visual processing systems. Hence, the stimuli that induces a response in these neurons was not the described magnetic one. Here only cells responding to visual stimuli were recorded from, that is only visually responsive neurons were reconstructed.

Experimental Procedures

The project was subdivided into three main parts: histology, confocal imaging and image processing; concerned with staining the cell for neuron visualization, capturing the images of the neuron in optical slices and actual 3D reconstruction of the neuron, respectively. These methods are based on Heinze et al. 2013 [11].

Before any of these steps could take place electrophysiological recordings were performed (by Andrea Adden and Stenley Heinze). Carrying out intracellular recordings is a complex and difficult procedure and was therefore beyond the scope of this project (refer to [10] for an overview of the electrophysiological method). The neuron's responses to different stimuli were first determined via these electrophysiological recordings in vivo. The recorded from neurons were injected with a substance that when put through histological preparations provided the basis for fluorescence detection via confocal microscopy. With the neurons captured in optical slices they were then processed in the Amira5.3 program and reconstructed in 3D. Their relative position could be assessed through the reconstruction of proximal neuropils and the registration into a reference brain. Eventually three whole neurons were reconstructed in this fashion.

Electrophysiology

Because this part of the method was beyond the scope of the project it will only be explained briefly, however it has some importance for the interpretation of the results. It aims to record the specific reaction a neuron has when the alive animal is exposed to a certain stimuli. The insect is first mounted in the examination chamber. For recordings the brain of the animal needs to be exposed. Once the head is fully secured a small hole is cut on the upper part of the head between the ocelli and towards the antennae, pronase is then added to the brain. The pronase enzyme digests the membrane that forms a protective cover around the brain. The membrane can then be removed with the help of fine forceps. Glass electrodes are then able to freely access the brain without risk of breaking. A reference electrode (silver wire) is inserted into the head near the proboscis muscle, while the measuring electrode is inserted into the brain. The electrode is lowered into the brain with the help of a micromanipulator until a neuron that responds to the investigated stimuli is found; preferably one adjacent to the central complex. Once a cell shows a response, either excitatory or inhibitory, the experiment

is conducted. The studied modality consisted of several visual stimuli, namely translational optic flow, wide-field rotation, as well as the extent of the neurons receptive field. (Results of electrophysiological recordings referred to in this report have been received from Andrea Adden)

Histology

Directly following electrophysiological recordings of the randomly chosen neuron, tracer particles known as Neurobiotin were injected into the studied neuron. With the help of their internal charge these small particles were forced out of the glass electrode and into the neuron, after which they rapidly diffused throughout the cell. The work in the context of this project began after the intracellular dye injections.

Dissection of the brain then followed. During dissection the brain was exposed by slowly removing the cuticle of the head as well as the cornea of the eyes, while the moth remained attached to the recording holder. Finally the head was separated from the body and transferred to a wax dish, in which the brain was removed from the remaining head capsule. In detail: With the underlying brain exposed, a droplet of moth ringer solution (150 mM NaCl, 3 mM KCl, 10 mM TES, 25 mM sucrose, 3 mM CaCl2; pH = 6.9) was placed over the insects head and covering the brain. The proboscis and its muscles as well as easily accessible trachea were removed, making the brain more accessible. Incisions around the rim of the eyes made the cornea removable, remaining cuticle between the above opening and this recent were also removed (including the part where the ocelli were located). A black ring just underneath the recently exposed optic lobes were then cut and carefully removed on each side, as to not damage the optic lobes. Now the head was severed and moved to a drop of ringer solution within the wax dish. By holding the back part of the head with one pair of forceps another pair could grab hold of trachea still attached to the brain and with the application of a small pulling force the brain was separated from the back of the head. The brain, now being completely freed from the head, was cleaned up (trachea were removed) and then placed in 0.5ml standardized cross-linking fixative, namely neurobiotin fixative (4% paraformaldehyde, 0.25% glutaraldehyde, 2% saturated picric acid in 0.1M phosphate buffer) overnight in the fridge (4°C) to prevent brain structures from deteriorating.

A wash series consisting of 4 times 15 minutes each in 0.5ml 0.1 M PBS (80 ml of stock solution 1 (1.4 g Na2HPO4 in 100 ml distilled water) and 20 ml of stock solution 2 (1.6 g NaH2PO4 x H2O in 100 ml distilled water) with 8.5 g NaCl in 900 ml distilled water, pH 7.2) was conducted the next morning. The brain was then incubated under dark conditions in a solution consisting of 500µl PBT and 0.5µl streptavidin-Cy3 complexes at 4°C for 3 days. The streptavidin molecule is linked to the fluorescent molecule Cy3; this complex readily penetrates the brain and binds with high affinity to neurobiotin, thus enabling the visualization of the injected neuron.

The following steps were all performed under dark conditions, as not to bleach the fluorescent molecule. Six subsequent washing steps each consisting of 20 minutes in 0.5ml PBT (PBS with 0.25% Trition-X, pH 7.2) were performed. Then 2 additional washing steps were also performed, this time consisting of 20 minutes in 0.5ml PBS. A series of incubations with increasing concentrations of ethanol concentrations (50%, 70%, 90%, 95%, 2x 99.8%), each of which spanning 15 minutes, was used to dehydrate the brain. For later visualization of deep brain structures the brain had to be made transparent with the use of methyl salicylate. First, incubation for 15 minutes in a 0.5ml mixture of methyl salicylate and 99.8% ethanol (1:1) was performed (this was carried out in a glass container because of methyl salicylate's inherent reactiveness with plastic). This was followed by a 1 hour incubation in pure (>99%) methyl salicylate.

The brain was then prepared for mounting, first the coverslips needed to be prepared. Eight staple reinforcing rings were stacked upon each other, with the two upmost

ones having a cut in them (so excess mounting medium could exit without difficulty), they were placed on a coverslip. Transfer of the brain ensued; the hole created by the staple reinforcing rings was filled with Permount mounting medium, by grabbing onto other attached tissue apart from neural tissue the brain was transferred into this hole. Another coverslip was then placed above, excess mounting media was pushed out using a small weight. The brain had to set in the mounting media for about 3 days before confocal imaging could be conducted.

Confocal imaging

The preparations were imaged with a confocal microscope equipped with a laser that excited the florescent Cy3 molecules at a wavelength of 561nm. The excited molecules, and hence the stained neuron, emits light at 570nm, which was detected. Also, some unspecific binding of streptavidin-Cy3 to surrounding brain structures had occurred, making it possible to visualize not only the recorded neuron but also the surrounding neuropil structures. The used objective's magnification was 25x, numerical aperture: 0.8.

Over a working distance of about 500µm the confocal microscope was able to create optical slices, i.e. different layers showing structures located at specific depth of the sample. Hence the image had, apart from having a height and width (x and y axis), also a thickness (z axis) covering about 1µm. Image series along the z-axis of many hundreds of images at voxel dimensions (volume pixels) of 0.5x0.5x0.9µm were thereby generated for further analysis. Several image stacks were taken that together cover the full extent of the neuron with all its arborizations.

Image processing

This part was subdivided into a neuron tracing part and a neuropil reconstruction part. The latter was needed for the neuron to be fitted into an already reconstructed reference brain, a Bogong moth's brain that was considered representative. The software Amira5.3 was used for all processing.

First the reconstruction of the stained neuron was completed. It began with the image stacks from the confocal microscopy, where 2-3 image stacks were often needed to cover the entire neuron. Image stacks were aligned to ensure that all parts of the neuron were located within a common frame of reference, allowing for seamless tracing of branches across image-stack boundaries. At first reconstruction involved the SkeletonTree function (skeletonize plugin). Here, neuronal branches were added at points of great light intensity, i.e. brightly stained parts, as compared to the surroundings (figure 1) [16].

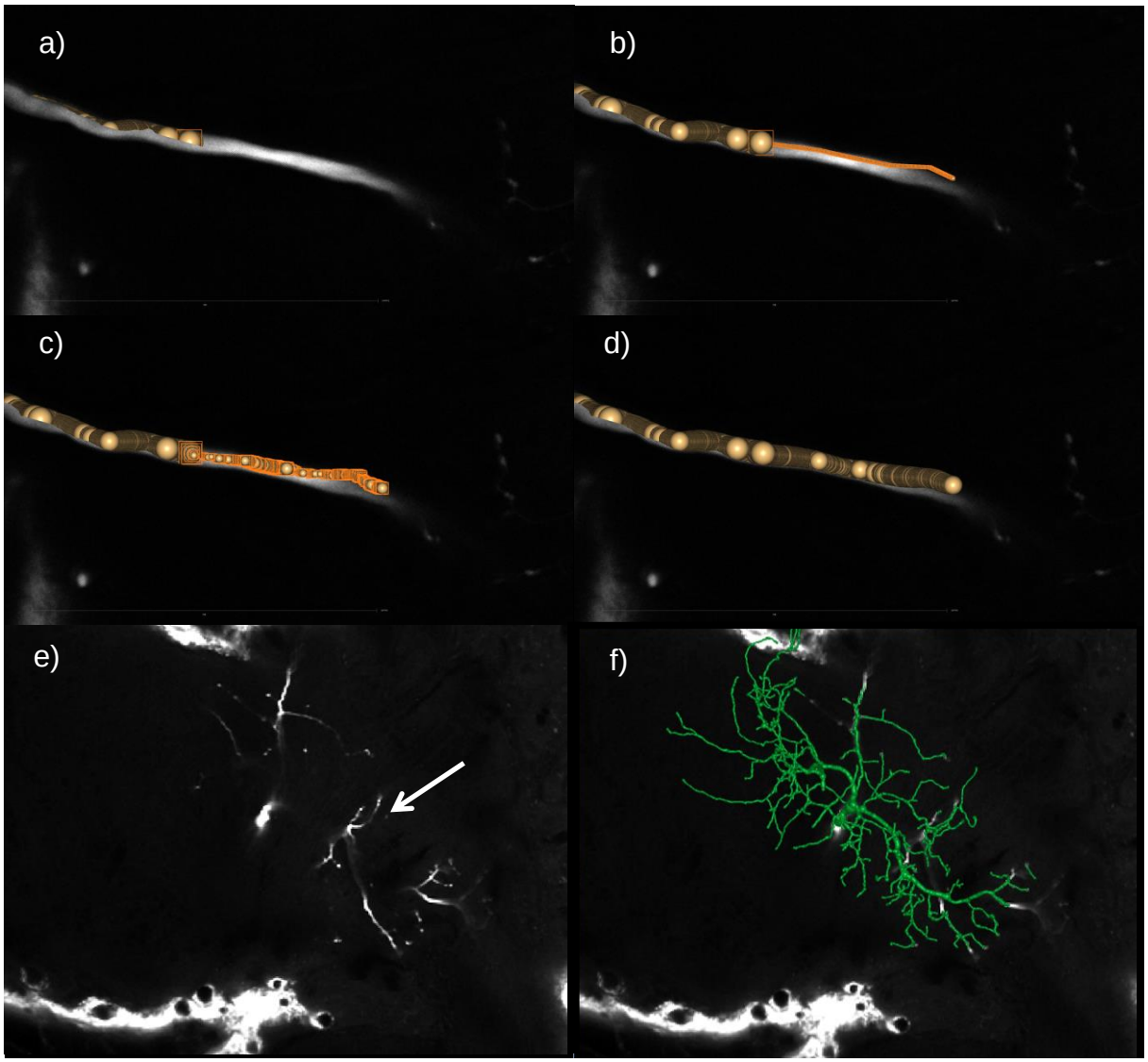


Figure 1. Reconstruction steps of a neuron branch as well as neuronal reconstruction in a larger region. **a)** Image data of an optical slice with some reconstructed neuron present (orange 3D structure). The brighter part of the picture is the stained neuron. **b)** The bright parts have been manually tracked and neuron parts added, however the added branch is thinner than the brightness suggests. **c)** With the aid of the software the added branch have now been fitted to match the center of the stained neuron on image. **d)** The diameter of the added branch now matches the stained neuron image. This is accomplice with the aid of the program. (Scale bar is 100µm long in a-d). **e**) Pure image data with the stained neuron shown as the more bright parts (not counting the outer layer of the brain). These bright parts, indicated with an arrow, are all targeted with the reconstruction tool. **f**) Reconstructed neuronal parts of the region are visible, they cover the brightly stained region in the displayed slice (as well as having some reconstructed branches above this section). (Scale bar is 200µm long in e-f.)

Second, a neuronal aborization extends in all three dimensions, hence it proved important to not only track it in the x and y plane but also the z plane. Once a branch had been manually added (represented in the form of spheres, figure 1 a-b) the computer corrected, with the help of the brightness information from the image stack, the center of the aborization as well as the thickness of the branches was made to match the image information more closely (figure 1 c-d).

In order to gain information about the position of the neuron within the insect brain the surrounding landmark neuropils had to be reconstructed. This was also performed in Amira. To do this the previously aligned image stacks had to be resampled to 1x1x1µm and merged with one another, yielding one entire image of the captured areas. The outlines of the neuropils were traced in selected sections in each image plane. The outline was determined to be where the intensity of grey shifted (figure 2a), i.e. where a difference could be detected between two compartments. Reconstruction took place in all three dimensions yielding flat surfaces along all three axes (figure 2b). Utilizing the Wrap function in Amira the computer then approximated a volume out of this scaffold information (figure 2c). The resulting label-field was then used to compute a 3D surface representation of each neuropil, with which the neuron was displayed (figure 2d). For the name of concerned neuropils and their abbreviations see table 1.

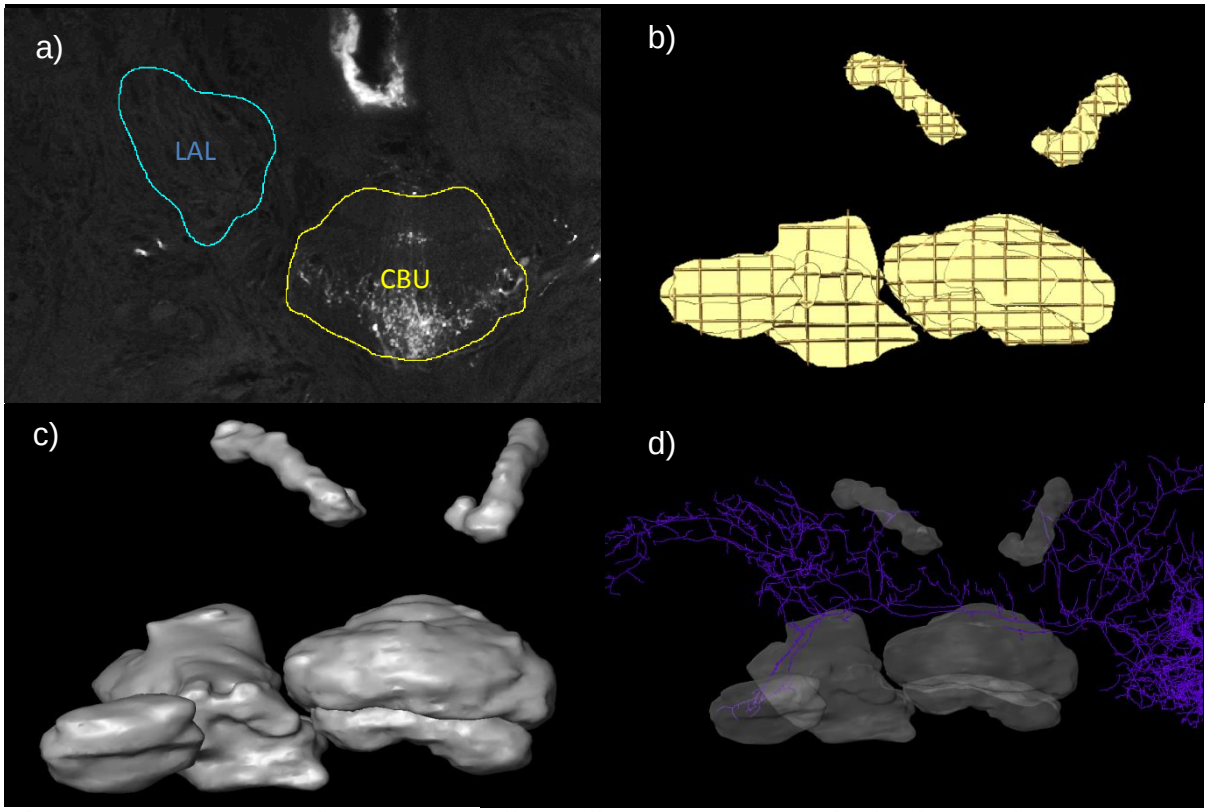


Figure 2. Reconstruction of neuropils at different stages. **a)** The image information, encircled area showing where the neuropils are located, approximated with grey value difference to determine its border. **b)** Reconstructed neuropil scaffold with labelled section in all three planes. **c)** The volume approximation of these neuropils as estimated by the software. **d)** Parts of a neuron displayed in relation to these neuropils.

The volumetric neuropil information was then registered with a representative Bogong moth brain, i.e. the neuropils reconstructed in this project were aligned with the neuropils from a representative brain. It was done both manually and then with the aid of the program's AffineRegistration tool to get the most representative overlap for the two brains. Consequently, giving the position of all neurons within the same representative brain, enable all neurons to be displayed in the same frame of reference. Manual fitting relied on positional movement in all three dimensions, rotation around all three axes as well as overall shrinkage or enlargement of reconstructed brain. Computerized fitting did positional movement, rotation as above and was able to enlarge or shrink individual dimensions through shearing (df. = 9).

Once fitted into the reference brain other neuropils, reconstructed in the reference brain, were displayed in relation to the neuron. Specific morphological features of the neuron were then analyzed.

Table 1. The name and abbreviations of the different neuropils mentioned.

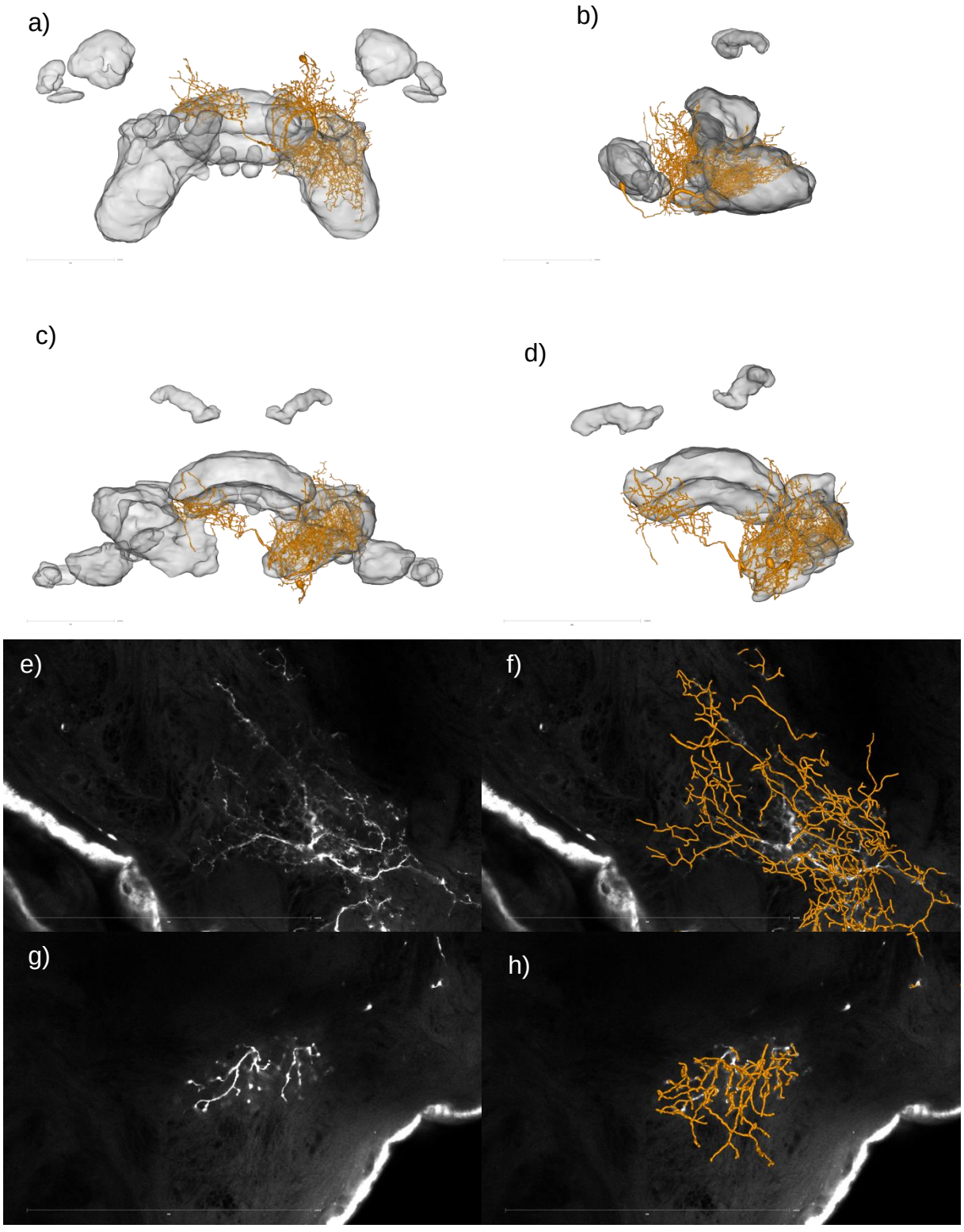
Abbreviation	Name
CBU	upper division of central body
CBL	lower division of central body
PB	protocerebral bridge
NO	Noduli
LAL	lateral accessory lobe
AUTO	anterior optic tubercle
BU	bulb
MB	mushroom body
LO	lobula
LOP	lobula plate
ME	medulla
AL	antennae lobe

Results

Reconstruction was carried out on three different neurons named 140312, 150225 and 150429 individually. Bellow follows an in-depth description of the individual neurons characteristics.

Individual morphologies of reconstructed neurons

Neuron designated 140312. The input tree of the cell is located on the left side of the moth brain in the LAL (figure 3a-c). This is further evident when the image slice data for this particular aborization tree is looked at (figure 3e-f). The density of this tree is high, individual fibers are located in close proximity to one another, indicating the many input locations for information throughout the LAL. The cell possesses three major output trees, two located in corresponding regions on either side of the midline, in un-reconstructed regions of the brain. They appear dorsal of the central complex and posterior of the mushroom body lobes, likely in the inferior protocerebrum (figure 3g-j). Another output tree is also present on the left side of the brain and located posterior to the input tree (see figure 3k-l). The aborization density in output fibers is lower than that of the input fibers and they do appear to remain in a small localized region of space, possibly within single neuropils. The soma of the neuron is located close to the dorsal surface of the brain. The individual's neuropils in reconstructed brain were distorted (figure 3d).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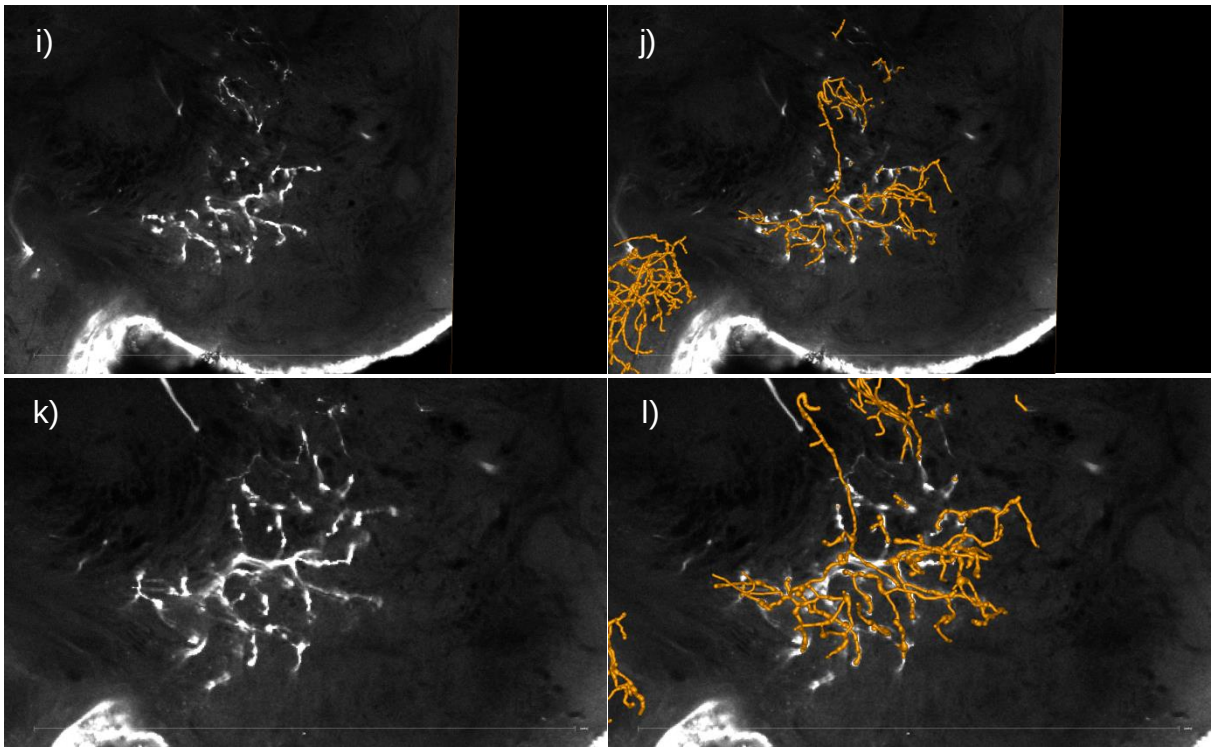
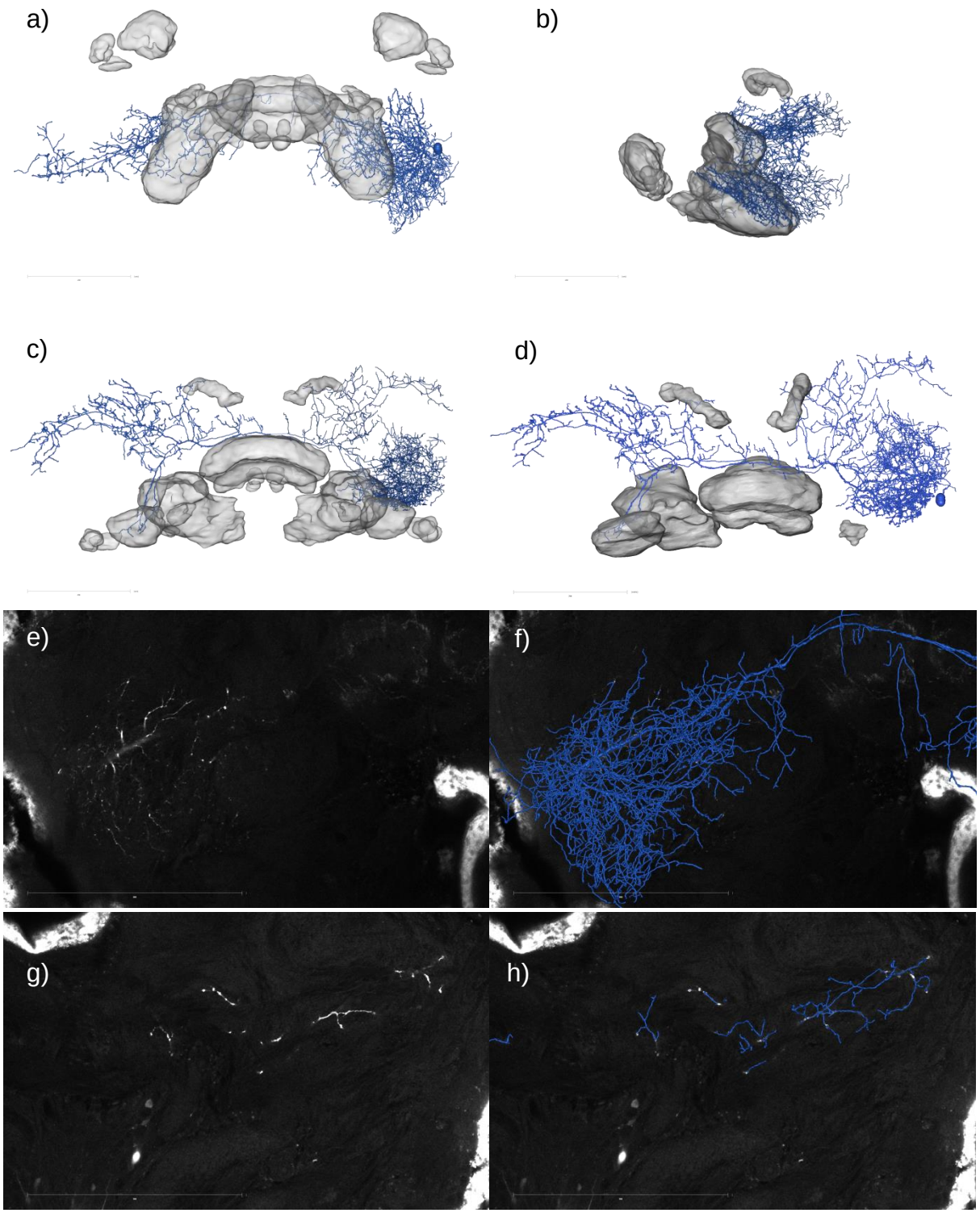


Figure 3. The 140312 neuron reconstructed in 3D as well as optical slices for more intricate analysis. **a-c)** Neuron included in the reference brain shown from the anterior, lateral and dorsal view respectively. As seen the input region innervates the left LAL, while output regions likely located in the left inferior protocerebrum and right inferior protocerebrum as well as one located posterior to the input tree). **d)** Neuron included with neuropils in the reconstructed from brain. Distortions of the neuropils as compared to the representative are present, the angle is similar to that of picture c (scale bar is 150µm long in a-d). **e-f)** optical slices of the LAL input tree without and with neuron included. **g-h)** optical slices of one of the left output trees, likely located in the left inferior protocerebrum, without and with neuron included. **i-j)** optical slices of the right output tree, likely located in the right inferior protocerebrum, without and with neuron included. **k-l)** optical slices of the other left output tree, located posterior to the input tree, without and with neuron included (scale bar is 200µm long in all section pictures). Brain regions that are shown: CBU, CBL, PB, LAL, AUTO and BU.

Neuron designated 150225. The 150225 cell show four major aborization regions, two on the ipsilateral side of the brain and two on the contralateral, with the cell crossing the midline just above the CBU (figure 4a-c). No innervation of reconstructed neuropils is observable except for some side branches in the LAL. However, using the optical slice it is possible to conclude that the dense input tree is mainly located within the anterior part of the venrolateral protocerebrum (figure 4e-f). One input tree innervates a dorsal unreconstructed structure (figure 4g-h). Two output regions appear to innervate similar structures on their respective side of the brain, close to the posterior surface of the brain, likely in posterior parts of the inferior protocerebrum (figure 4i-l). The soma appears to be located on the left lateral side of the insect's brain. Note that in the 3D reconstruction (figure 4a-c) there is a part of the neuron, just above the CBU, that appear as a straight line. Because of severe distortions of this individual's brain, the registration process only led to a satisfactory result when stretching this

part of the neuron. The length of this neurite is therefore overestimated. Despite this stretching artifact, the overall position of aborization trees in the final reconstructed neuron closely resembles their positions in the original brain (figure 4d). There is one notable exception to this, one part of the right ventral aborization tree appear to stretch into the optical lobe, in the representative brain. However, when the imaged sections are looked at it appears to not extend into the optic lobe (figure 5).



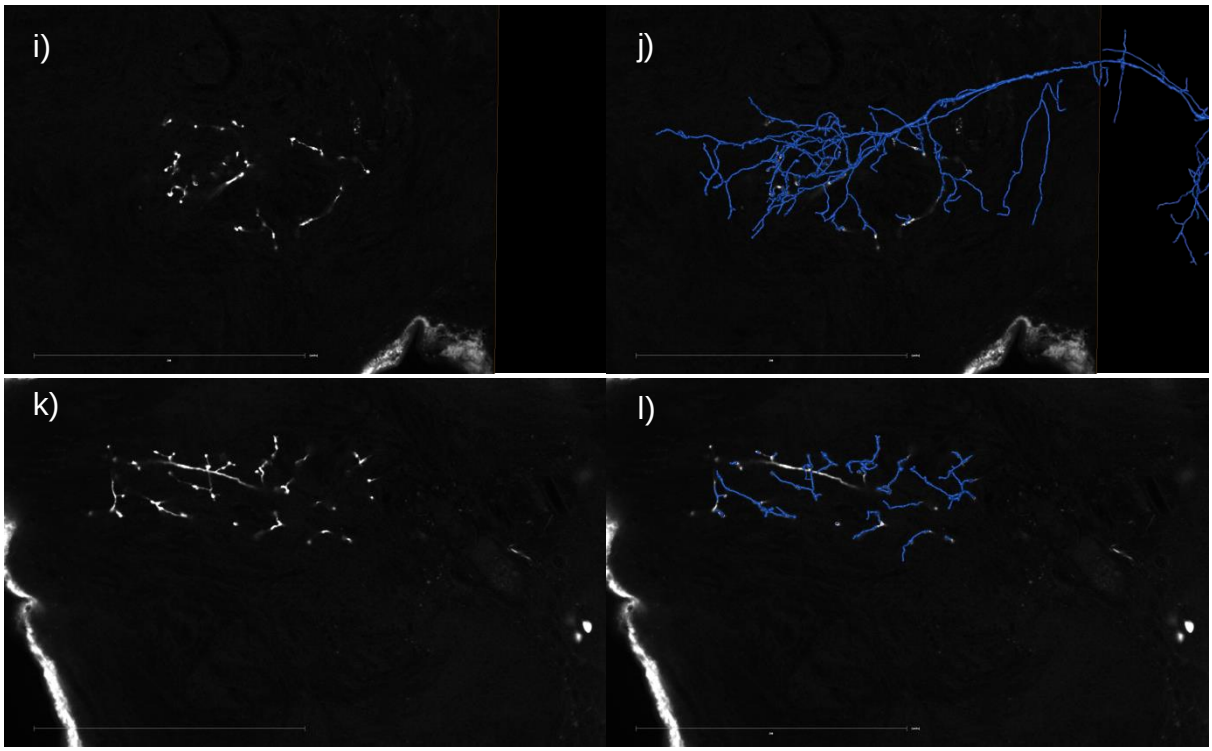


Figure 4. The 150225 neuron reconstructed in 3D as well as optical slices for more intricate analysis. **a-c)** Neuron included in the reference brain shown from the anterior, lateral and dorsal view respectively. As seen the input region innervates a region lateral to the LAL, while output regions innervate unreconstructed structures two to the left and one on the right side. **d)** Neuron included with neuropils in the reconstructed from brain. Distortions of the neuropils as compared to the representative are present. The angle is similar to that of picture c. **e-f)** optical slices of the input tree without and with neuron included. **g-h)** optical slices of the left anterior output tree without and with neuron included. **i-j)** optical slices of one of the right output trees without and with neuron included. **k-l)** optical slices of right inferior output trees without and with neuron included. (Scale bar 200µm throughout.) Brain regions that are shown: CBU, CBL, PB, LAL, AUTO and BU.

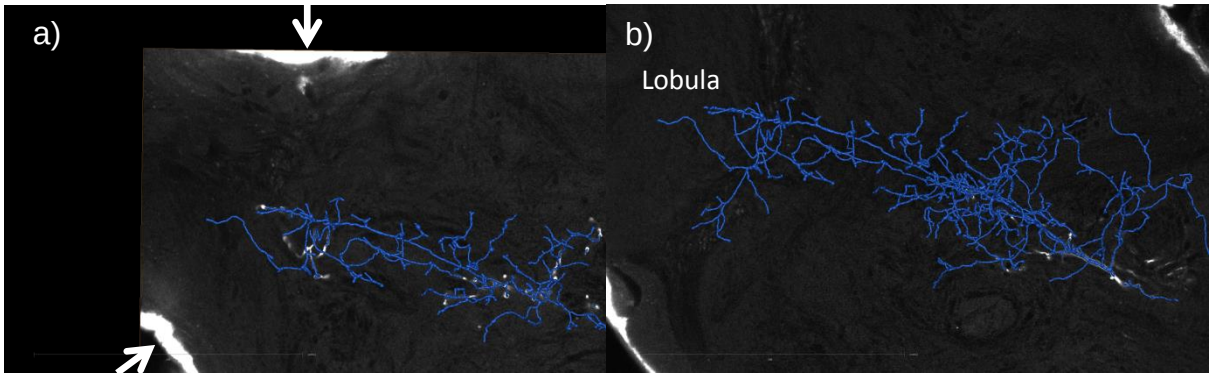
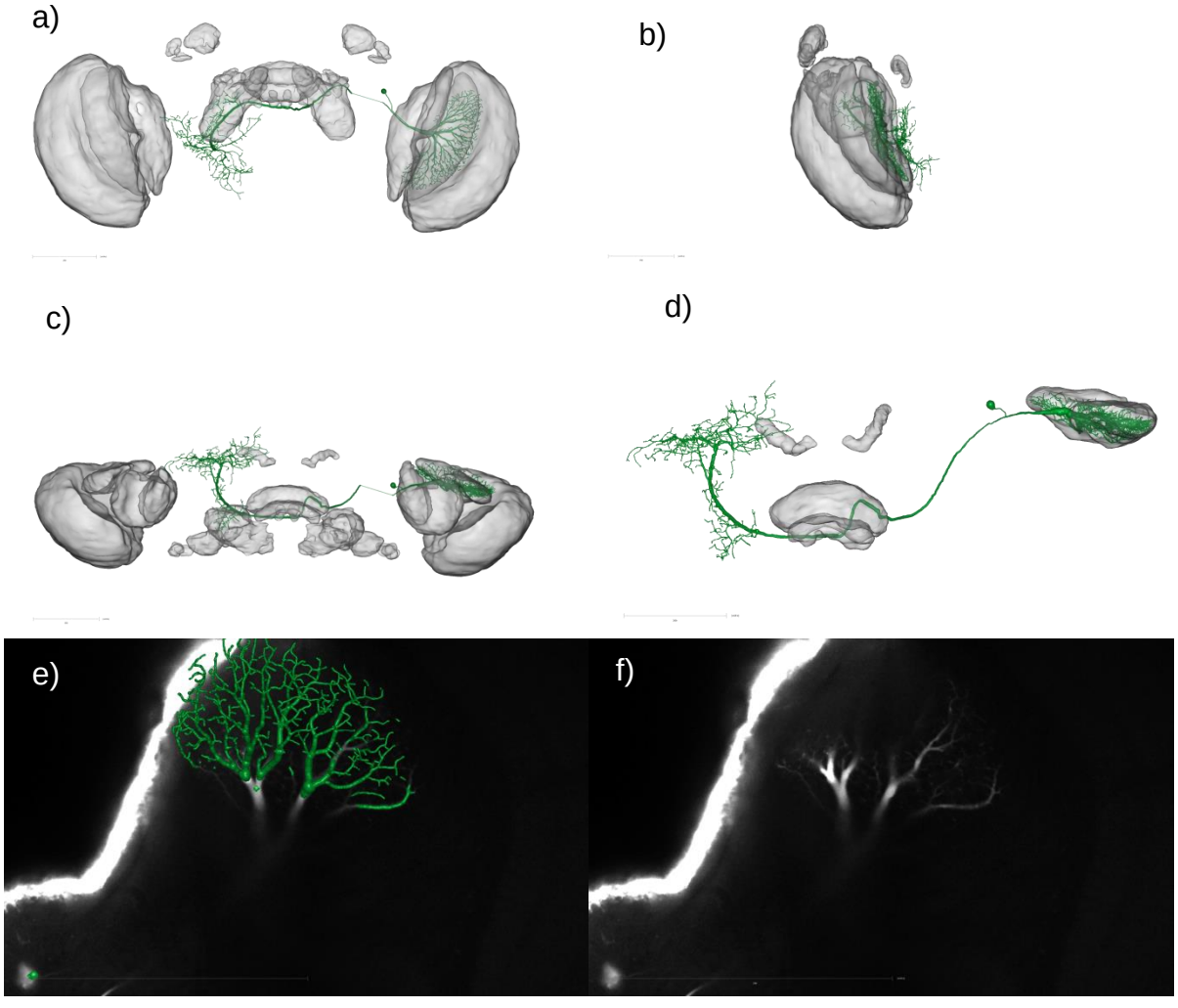


Figure 5. The output tree of the 150225 neuron shown with image data showcasing that this part of the tree is not within the optic lobe. **a)** Image with arrows that mark of where the optic lobe starts and expands into the left most part. **b)** Showing the neuron and the lobula, which the neuron is not in contact with.

Neuron 150429. Neuron 150429 possesses three aborization regions: input is present in the lobula plate, while output regions are present in a small part of the right LAL and major output in posterior brain, likely in the posterior lateral protocerebrum (figure 6a-j). The main neurite passes over to the contralateral side, traveling close to the CBU, with the soma located on the left side of the brain, at the root of the left optical lobe. Note that the registration process resulted in a long stretched neuron segment on the left side of the brain, where the neurite approaches the lobula plate (figure 6a-c). This stretching was necessary, as the mounting of this individual brain and the reference brain resulted in differing positions of the optic lobes. This individual brain's optic lobes were tilted upwards when compared to the reference brain, their three-dimensional positions varied in respect to the central neuropils. Thus, a single registration step could not obtain a satisfactory compromise for neuropil overlap. Instead, two separate registrations were made: one reflecting just the optic lobes and one focusing on the central brain neuropils. The neuronal overlap for the two registrations was poor. Hence, a large stretching of the neurite was required, resulting in this large uncertainty of the neurite's position close to the stretched part (compare figure 6a-c with 6d).



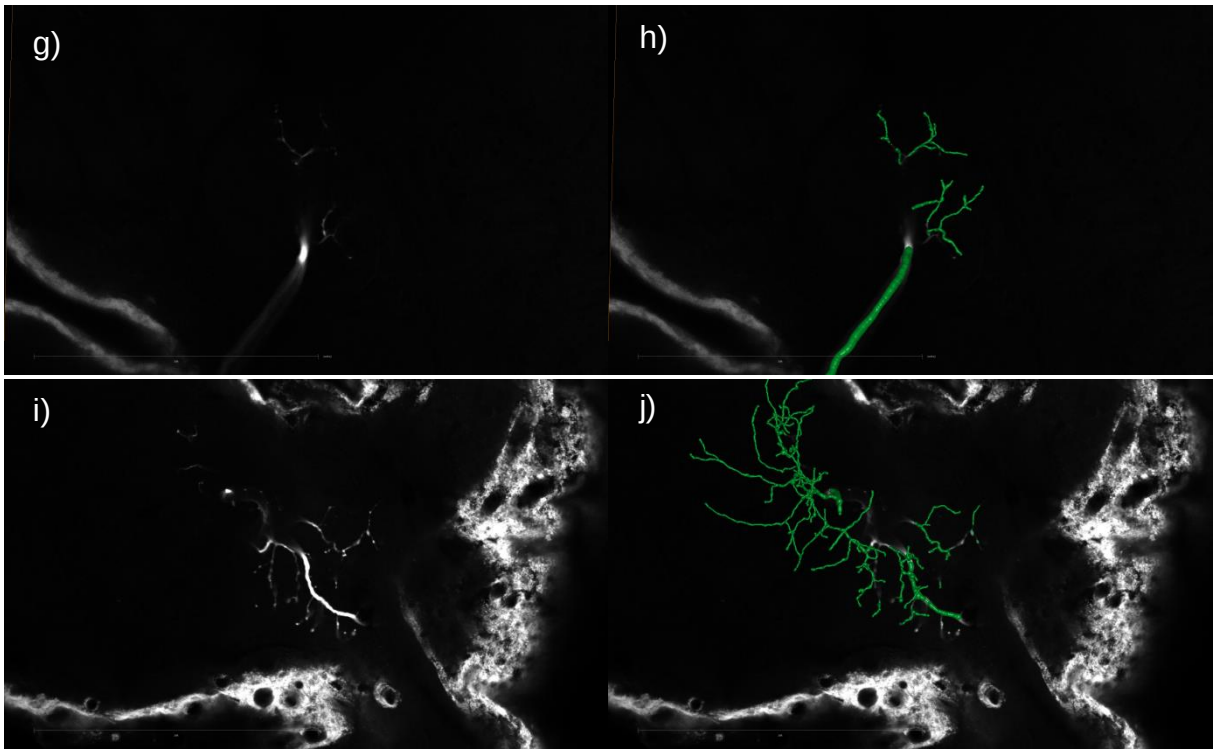


Figure 6. The 150429 neuron reconstructed in 3D as well as optical slices for more intricate analysis. **a-c)** Neuron included in the reference brain shown from the anterior, lateral and dorsal view respectively. As seen the output region innervates a region lateral to the LAL with minimal fibers in the LAL, while input regions innervate the lobula plate. **d)** Neuron included with neuropils in the reconstructed from brain. Distortions of the neuropils as compared to the representative are present but few except for the position of the optic lobes. The angle is similar to that of picture c. **e-f)** Optical slices of the input tree without and with neuron included. **g-h)** optical slices of the minor output tree in the LAL without and with neuron included. **i-j)** optical slices of the major output tree within the LAL without and with neuron included. (Scale bar 200µm throughout.) Brain regions that are shown: CBU, CBL, PB, LAL, AUTO and BU.

Combined neurons.

The three cells show little resemblance in what areas they innervate, all having their major input and output regions in different spatial locations (figure 7). However, all do appear to have some affiliation with the LAL: the 140312 cell having its major input tree in the left LAL, the 150225 cell having a few input branches in the left LAL and the 150429 cell having a few output branches in the right LAL. Furthermore, except for the 140312's input tree in the left LAL and the 150429's input tree in the left optic lobe all other aborization trees are located in un-reconstructed neuropils. The input trees for all neurons are located on the left side of the brain. Also, it is only the 150429 neuron that lack a bilateral output trees, both 140312 and 150225 display this feature.

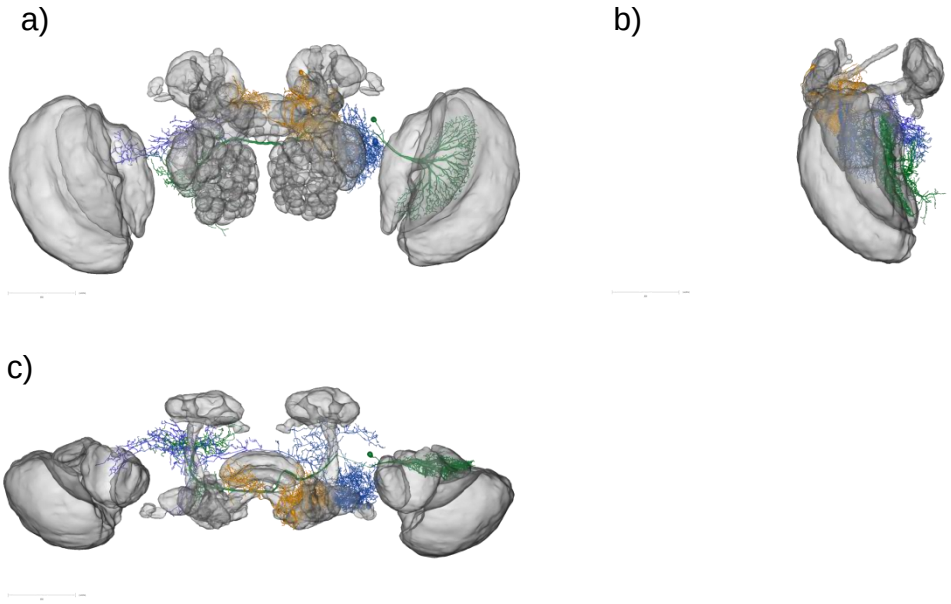
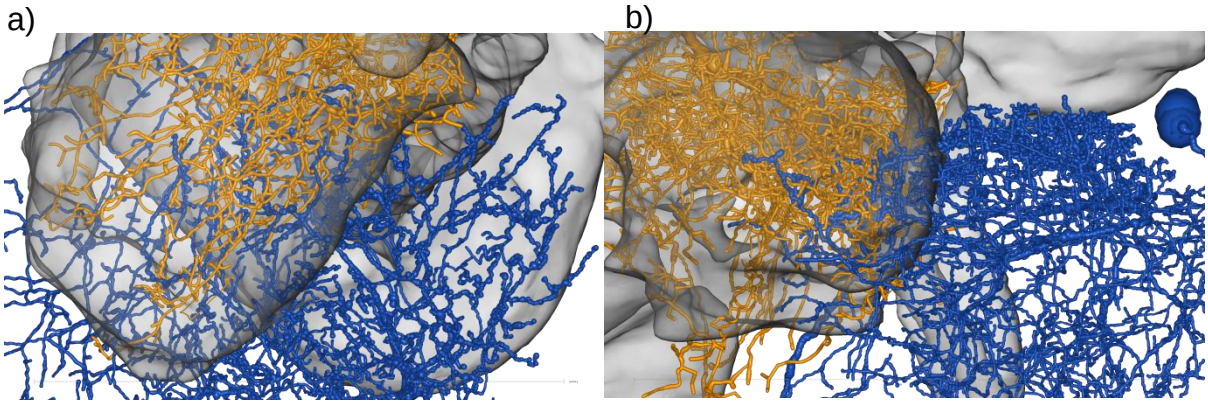


Figure 7. The three different neurons included in the reference brain showing major aborizations in different regions when compared to one another. The neurons are all displayed in a different color: 140312 in orange, 150225 in blue, 150429 in green. **a)** frontal view. **b)** lateral view. **c)** dorsal view. (Scale bar 200µm throughout). Brain regions that are shown: CBU, CBL, PB, LAL, AUTO, BU, AL, MB, LO, LOP and ME.

Possible interactions between the reconstructed neurons

As seen above only one noticeable overlap between the three neurons aborization trees were detected. This is a potential overlap between the 140312 and the 150225 cells. The left LAL is the main input neuropil for the 140312 neuron and, as mentioned, a few input fibers from the 150225 neuron also innervate this neuropil. An overlap appears possible in the reference brain (figure 8a-b), but the overlap appear to be less pronounced within the optical slice pictures (figure 8c-f). Here, the amount of branches entering the LAL is low for the 150225 neuron and the 140312 neuron have most of its branches contained within the LAL.



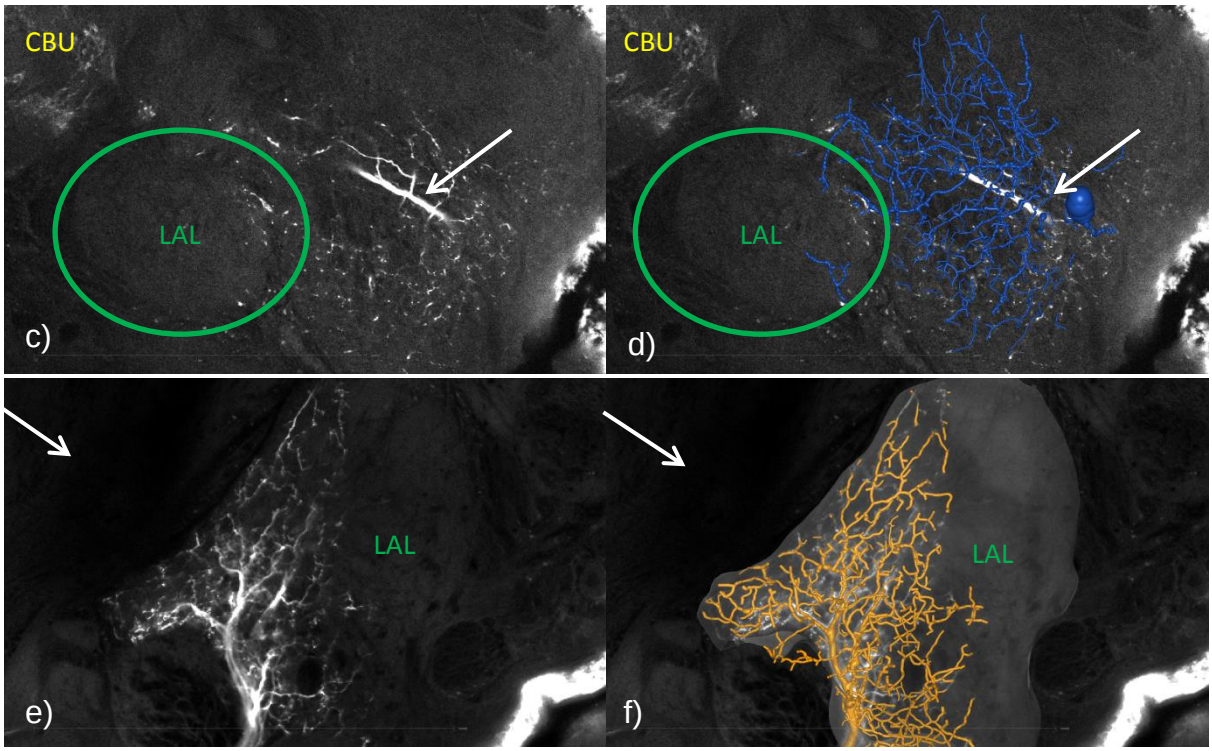


Figure 8. The possible overlapping region of the 140312's (orange) and 150225's (blue) respective input trees within the left LAL. **a-b)** Represented in the reference brain, here there appear to be overlap between quite a few fibers. **c-d)** Optical slice for the 150225 neuron in the concerned region without and with the reconstructed neuron respectively. The area marked with a green ring is the LAL. As seen there is little innervation of the LAL. **e-f)** Optical slices for the 140312 neuron in the concerned region without and with the reconstructed neuron respectively. The LAL is marked with a grey surface in f. It is heavily innervated along its lateral side, and few neurons protrude beyond this. The arrow approximately marks the same region in c-f. Note that when c to f are compared the extent to which the cells cover the same area appears lower than in a and b. (Scale bar 200µm throughout.)

Discussion

Certain limitations in the execution of the experimental procedure decrease the accuracy that information is obtained with. The Neurobiotin staining is specific to the recorded from neuron, with little leakage and with the streptavidin-Cy3 binding being specific enough to not extensively bind non-injected cells. If the preparations weren't thoroughly cleaned some aggregates in the brain could prove to be binding sites for the complex, causing brightly stained dots in the optical slices. The brains appeared to have been stained evenly. Also, when the brains were to be fixed between the cover glasses the optic lobes could be arranged differently in relation to the center brain, as is evident in the 150429 neuron. This is responsible for the neuron's large stretched region.

The resolution with the confocal microscope is not high enough to detect individual branches when they are very fine and located spatially close. Consequently, the reconstructed neurons only show the general features of the actual neurons, not the absolute aborizations locations, especially in finer branches. The more tissue the laser has to penetrate before the concerned slice, the worse the resolution gets. This in relation to the working

distance meant that the brains were often scanned from both sides, creating several image stacks.

The Amira program had limitations. The fitting of finer branches proved difficult, the brightness levels were often just higher than the background stain. Resulting in some unrealistic fitting which had to be manually checked and corrected. Furthermore, if two branches were spatially close, with one being thick, the other could often be fitted into the thicker branch. Two different stacks detailing the same region would sometimes interpret the same tissue part as being of a different brightness level, resulting in abrupt diameter change of some branches, evident in the lower part of the 150225 neuron. Two different stacks often had a discrepancy of a few µm where some branches were located in relation to one another. Hence, reconstructing a neuron over two image stacks often induced some artifacts of stretching and thickness diameter. These factors further strengthen the fact that the reconstructed neurons only show the general features of the actual neurons, not the absolute aborization locations.

Neuropil reconstruction relied on the background streptavidin-Cy3 staining of brain structures. Hence, the quality was low, often difficult to discern where a neuropil ended, resulting in some guess work. Therefore, the neuropil reconstruction was less reliable than if an anti-synapsin antibody had been stained with. It is often practical to avoid anti-synapsin staining because this process adds two extra weeks of waiting time before the neuron can be visualized. The background staining often provides enough information for reliable reconstruction. Future rehydration and anti-synapsin staining of brains is possible, but only carried out if a cell is deemed interesting and the background staining is poor. The studied brains are candidates for future anti-synapsin staining due to a less then desirable background staining making their reconstructed neuropils likely lack or have extra parts excluded/included.

With this in mind and the fact that individual brains have slight morphological differences, as well as 140312 and 150225 being deformed during dissection, the registration into the representative brain proved difficult. The match between them was often lower than desired, resulting in some neurons appearing in a different spatial position than is real. Mainly 140312 and 150225 are affected by this, due to their distortions. This is apparent in the 150225 neuron that does appear to be inside the optic lobe when in the reference brain, but not in the optical slices. This is also the likely reason that the 140312 and the 150225 cells appear to have greater interactions with one another in the reference brain than in the optical slices. Future anti-synapsin stainings might make neuropil reconstruction and as a result fitting into reference brain better, but difficulties due to deformations can not be rectified post their induction. Also, the representative brain is only an individual insect’s brain not accounting for individual morphological differences as an average would. An average brain would cancel out individual differences, making the neuronal position more accurate. However, an average brain was not available at the reconstruction opportunity. Furthermore, many innervated neuropils are unreconstructed, further neuropil reconstruction in the reference brain would add more information to the neurons innervation sites.

Due to the above information being lost when the neuron is fitted into the representative brain, neuronal positions will display distortions. Artifacts such as stretching can also be induced, evident in 140312 and 150429. However, there is a gain of information

considered worthwhile. Neurons can be displayed together with one another, as well as displayed together with neuropils that could not be reconstructed in their individual brains, providing desirable information about neuronal innervation sites and interactions respectively.

All of the cells showed responses to certain types of visual information when under electrophysiological examination. Neuron 140312 likely receives input from one or more cells in the LAL and then propagates this to two homologous bilateral brain regions as well as a third ipsilateral region. It gave a strong inhibitory response when exposed to large-field motion (independent of movement direction) in the receptive field between 0° (straight ahead) and +90° (perpendicular to the animal on its right side). Neuron 150225 probably receives input from the anterior part of the ventrolateral protocerebrum which is then transmitted to two homologous bilateral brain regions and a third region contralateral to the input tree. It responded to the individual motion of bars moved in a certain direction, with leftward motion giving a more pronounced response than rightward. However, the receptive field extended over only 20° in the animal's left visual field. Lastly 150429 likely gets its input from the lobula plate and then transmits out to the contralateral LAL and to an adjacent unreconstructed region. This cell gave strong responses to the leftward motion of both individual bars and to wide field motion. Similar stimuli in rightward motion gave a complete inhibitory response. The cells receptive field was on the left side of the animal (-30° to -90°). Though the 140312 and 150225 cells appear to have some overlapping input branches they appear to process slightly different kinds of information. Despite their close spatial positions they might not be in contact with the same presynaptic cells.

None of the cells appear to innervate the important central complex, where the generation of the compass sense is thought to be mediated. This might be because the CX is not the center of the compass sense in Bogongs, that it is not the center where visual clues integrate with the compass sense or, and which is most likely, that the information processed by these cells might only have a secondary effect on the compass sense. All these cells appear to process information that might not be crucial for the generation of this sense, instead information that might be used later in the processing for the compass sense. It might be that this information needs to be processed by other neurons before it is intergraded into the CX. In either case greater characterization of the Bogong's neural architecture is needed.

The 140312 and the 150225 neuron both lack previously discovered homologous neurons, making them never before seen cells. This suggests a possible difference in the Bogong moth's brain structure in relation to other animals, that the Bogong might process visual information in a different way to that of other species. It is also possible that these neurons do in fact exist in other species but are yet to been detected in these. In either case these neurons are brand new to the field of neuron reconstruction, making them a good addition to the neuronal database for insects. The 150429 neuron do have homologous neurons in the Blow fly (*Calliphora vicina*) brain [17], indicating that the visual processing of this specific stimulus (as described above) might be similar in the two species. With this processing possibly being similar it might be that the processing of other stimuli are also similar between the two species. However, with the Bogong moth and the Monarch butterfly sharing more general features of their existence, like displaying migratory behavior, further study would likely show a similarity between these specie's neural architecture.

The reconstructed neurons only show similarities to already known neurons in one of three cases. If this is due to them not being found but still present in other species, or if they are special for the Bogong moth, reflecting a unique characteristic of this species, is hard to discern. One thing remains certain, the Bogong moth's brain and neurons warrants further study.

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