Methodology for testing visual responses in the Anterior Optic Tubercle in the Honey Bee: Towards investigating Magnetoreception

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Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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Certificate

This is to certify that this dissertation entitled Full Title of Your Thesis Should Appear Here towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sagar Santwani at the University of Trento under the supervision of Albrecht Haase, Associate professor, Department of Physics, Center for Mind/Brain Sciences - CIMeC during the academic year 2025-2026.

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Declaration

I hereby declare that the matter embodied in the report entitled “ Methodology for establishing a visual response in the Anterior Optic Tubercle in the Honey Bee: Towards investigating magnetoreception ” are the results of the work carried out by me at the Center for Mind/Brain Sciences (CIMeC), University of Trento, Italy, under the supervision of Dr Albrecht Haase, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to clearly indicate this, with due reference to the literature and acknowledgement of collaborative research and discussions.

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Abstract

Honeybees (Apis mellifera) are known to possess magnetic sensitivity, yet the neural and biochemical mechanisms underlying magnetoreception remain poorly understood. The radical pair mechanism (RPM), a quantum-mechanical phenomenon, has been proposed as a basis for light-dependent magnetoreception, predicting that magnetic sensing is coupled to the visual system through cryptochrome-based photochemistry. To investigate this hypothesis, we developed an in vivo two-photon calcium imaging preparation targeting the Anterior Optic Tubercle (AOTu), a higher-order visual processing region of the honeybee brain, using FURA-2 dextran as a calcium indicator. A visual stimulus protocol was established using a 473 nm blue laser to characterise ON-OFF neural responses in the AOTu. An 80% day-two bee survival rate was achieved by the end of the study. Despite multiple experimental trials, a clear and reproducible visual response was not obtained. Contributing factors are discussed, including laser light leakage into the fluorescence recording, seasonal reduction in bee neuronal activity during winter months, and preparation quality. One recording provided tentative evidence of a visual response at stimulus onset and offset, partially obscured by light leakage. This work establishes a methodological framework for future experiments aimed at directly testing RPM predictions in honeybees, including light dependence, polarity independence, and disruption by radiofrequency fields.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Albrecht Haase, who, with patience and encouragement, guided me throughout this entire project. A special thanks to lab members Alan Oesterle, Sabrina Cighetti, and Maria Bortot for teaching me core lab skills and clearing all my doubts.

Finally, I sincerely thank my family, who have been immensely supportive in this journey.

Contributions

Contributor name	Contributor role
----	Conceptualization (Ideas)
Dr. Alan Oesterle, Dr. Albrecht Haase	Methodology
Dr. Alan Oesterle, Dr. Albrecht Haase	Software
—	Validation
—	Formal analysis
—	Investigation
Dr. Albrecht Haase	Resources
—	Data curation
—	Writing – original draft preparation
—	Writing – review and editing
—	Visualization
Dr. Albrecht Haase, Dr. Alan Oesterle	Supervision
Dr. Albrecht Haase	Project administration
—---	Funding acquisition

Generative AI usage

The abstract of this document and the contribution statements were generated, and references rearranged by the Claude Sonnet 4.6 model, a generative AI model developed by Claude. Beyond this, I declare that the use of generative AI did not extend beyond what is described in Section 4.6.1 of the ‘Guidelines for Generative AI usage at IISER Pune.

1. Introduction:

Honey bees (Apis mellifera) are highly social insects that exhibit complex behaviours such as navigation, communication, learning, and memory. Thanks to their relatively small brains, with about one million neurons, and their well-characterised neuronal architecture, they serve as an excellent model for studying the brain network function underlying these behaviours.

Studies have shown that honey bees use magnetic fields to forage and navigate around their hives (Walker & Bitterman, 1989). But how they sense the magnetic field is still unknown to us. However, one proposed theory for how bees may sense the geomagnetic field is the radical pair mechanism.

The radical pair mechanism (RPM) is a quantum-mechanical phenomenon proposed as a basis for light-dependent magnetoreception (see section 2.2). Our lab investigates how magnetic fields affect honey bees and the role of RPM in this process.

Since the RPM is initiated by photoexcitation, magnetoreception is expected to be light-dependent. And a primary candidate molecule for the RPM, the cryptochrome, is normally found in the retina of numerous insects and birds and has also been shown to be necessary for magnetoreception in several insects and birds (Möller et al., 2004; Gegear et al., 2008; Bazalova et al., 2016) (more in section 2.2). Both suggest that the magnetosensation and visual neural pathways are likely functionally coupled. This study investigates neural activity in the Anterior Optic Tubercle (AOTu), a higher-order visual processing region, in response to visual stimuli and eventually magnetic fields.

This thesis starts by providing a more detailed background on two-photon microscopy, the RPM, and cryptochrome. It then moves on to the preparation of bees for imaging, imaging trials, and the results obtained. While the original goal was to obtain magnetic stimulus responses, this could not be attempted, as proving a firm correlation between visual stimuli and a neural activity spike proved harder than expected.

2. Theoretical Background:

2.1 Radical pair mechanism

The radical pair mechanism (RPM) is a quantum-mechanical phenomenon that has been experimentally demonstrated under laboratory conditions (Hore & Mouritsen, 2016).

A pair of bound molecules (the radical pair) undergoes a photochemical transfer of one electron between them, giving rise to a radical molecule with two unpaired quantumly entangled electrons. These electrons initially exist in a singlet state configuration, i.e., their spins are antiparallel (total spin is 0), but then rapidly oscillate between singlet and triplet states (both have total spin 1).

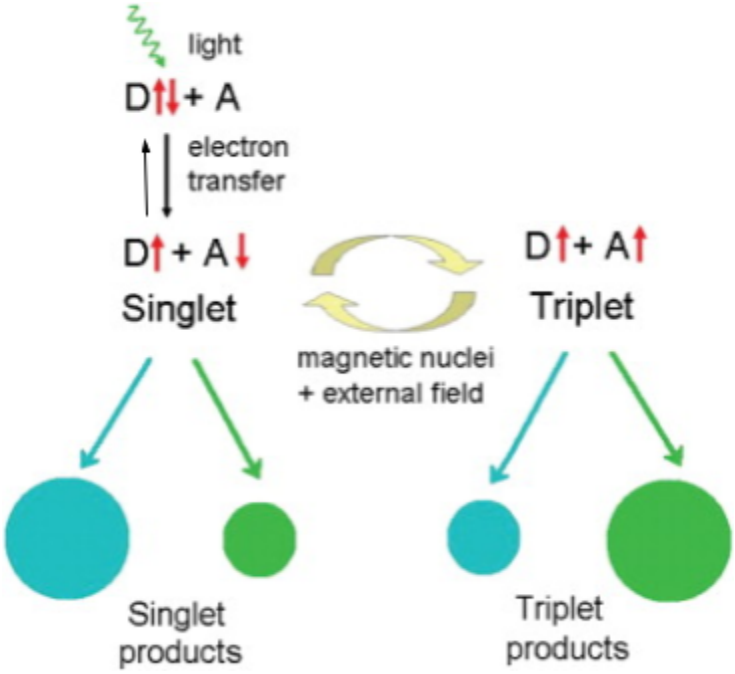


Figure 2.1 A proposed mechanism of how RPM can bring about biochemical changes due to Earth's magnetic fields (Reproduced from Biophysical Journal, Ritz et al., 2009, with permission from Elsevier).

But the probability of which state they occupy most of the time is determined by the magnetic field they experience (Lambert et al., 2012). The state they are in, in turn, influences various reactions and, in theory, helps the bee navigate the geomagnetic map and markers.

On the other hand, one of the biggest disadvantages of this theory is the decoherence of the electron spin states. The magnetic fields influencing the singlet-triplet oscillations require that the unpaired electrons are entangled and coherent. But environmental interactions lead this coherent state to rapidly decay and fall into decoherence. And so for magnetic fields to interact with these radicals, there would need to be sufficiently long coherent electronic states (~100 microseconds). This is, however, a much longer lifespan than has been shown in experiments. Hence, if it is shown to be a coherent mechanism underlying magnetoreception, RPM can demonstrate that studying biological systems is a powerful way to extend the lifetimes of quantum-coherent and entangled systems and apply them to many useful applications. (Oesterle, 2026; Lambert et al., 2012; Gauger et al., 2011)

2.2 Cryptochrome

A very promising candidate for the site of the radical pair mechanism is the cryptochrome protein (CRY). This protein is found in the retina of numerous animals (Sancar, 2000).

Cryptochromes are a class of flavoproteins and photoreceptors that have been known to be responsible for maintaining circadian clocks and circadian rhythms in animals and regulating a cascade of events in plants (Liedvogel & Mouritsen, 2009). They have also been reported in studies to form radical pairs upon photoexcitation in birds and in computer simulations with millisecond lifetimes. (Liedvogel et al., 2007; Biskup et al., 2008). This is why the cryptochrome protein is widely considered as a highly favourable candidate for the radical-pair mechanism in the field of magnetoreception. However, whether and how these cryptochromes are responsible for magnetoreception, and whether the radical-pair mechanism is the cause, are widely discussed.

Cryptochromes in animals can be broadly classified into three types: Drosophila-like type 1 CRY(CRY1), mammalian-like type 2 CRY(CRY2) and bird-like type 4 CRY (CRY4). Different animals and insects show different types; some even express more than one type of CRY.

Gegear et al. (2008) were among the first to show that magnetoreception is both light and CRY1 dependent in Drosophila. They noticed that removing either led to a loss of responses to the magnetic field in the flies. However, transgenic flies lacking CRY1 and expressing CRY2 recovered their magnetosensation. Studies in P. americana also show that directional magnetosensation is CRY2 dependent. These results suggest that CRY2 might be directly responsible for magnetoreception in bees. However, CRY2 is light-insensitive (Kutta et al., 2017), suggesting that another mechanism for electron transfer and subsequent radical-pair formation may exist.

CRY1/Drosophila-like type 1 CRY	**CRY2/mammalian-like type 2 CRY**
Light sensitive (Wan et al., 2021)	Not sensitive to light. (Kutta et al., 2017) RPM needs photoexcitation.
Expressed in several insects like mosquitoes, butterflies and cockroaches.	Found in all insects except Drosophila. Only CRY2 is present in honey bees.
Magnetoreception is lost upon making CRY1 deficient in drosophila (Gegear et al., 2008,)	CRY2 regulates directional magnetoreception in P. americana cockroaches, which only express CRY2 (Bazalova et al., 2016)

2.3 Two-photon microscope:

In recent times, two-photon microscopy has become very popular in neuroscience imaging studies. Its penetration depth is about 500 μm to 1 mm, which falls within the range for the bee brain and makes two-photon microscopy ideal for our experiments.
As the name suggests, two-photon microscopy uses two photons of the same wavelength to simultaneously excite an electron in the fluorophore, which then releases a low-wavelength, high-energy photon emission. (Figure 2.2) This has many advantages, such as:

- It drastically increases the spatial resolution of the scans compared to confocal microscopy. This is because the two-photon absorption, excitation and resultant emission (from de-excitation) only occur at around the intended focal point. This makes it easier to distinguish low-energy emissions from other parts and planes of the brain and eliminates the need for a pinhole to exclude out-of-focus emissions.

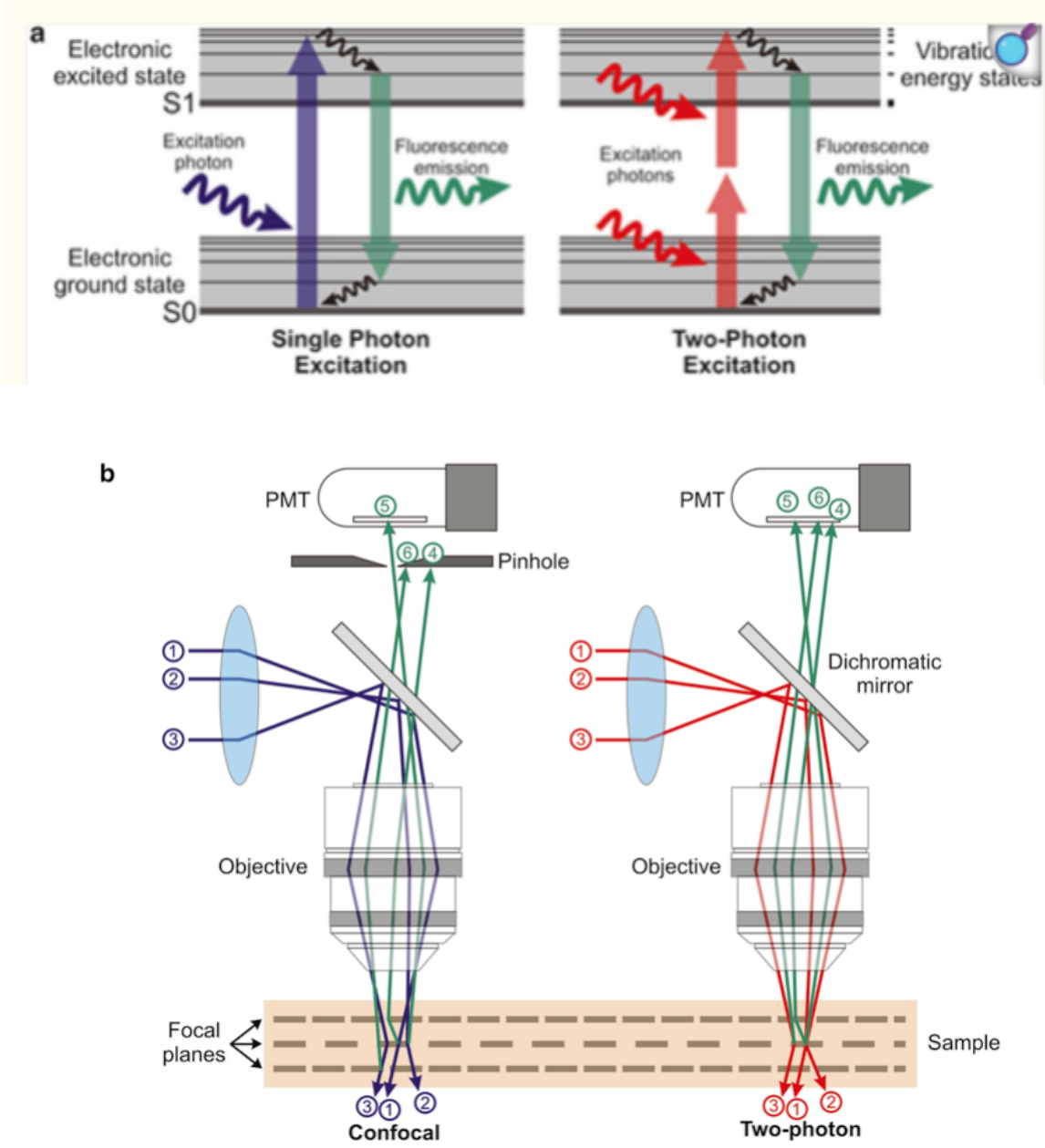


Figure 2.2 (A)Jablonski diagram for one photon excitation vs two photon excitation working, two photons contribute to the excitation of the fluorescence dye in the two-photon microscope, resulting in a higher wavelength emission spectrum and reduced photodamage outside the focal planes, due to lower energy photons hitting the sample and (B)Different microscope setups for two-photon and confocal microscopy. A pinhole is not needed for a two-photon microscope, thus enabling the collection of the full emission spectrum. Different ray diagrams (Mostany et al., 2014)

- Higher-wavelength excitation light in the infrared spectrum can be used to achieve deeper penetration (Figure 2.3) and lower scattering (Rayleigh Scattering).

- A high-wavelength (low-energy) excitation light reduces photodamage and photobleaching where the beam is not concentrated.
- There is an easier separation of the emission and excitation spectra due to a large difference in their wavelengths.

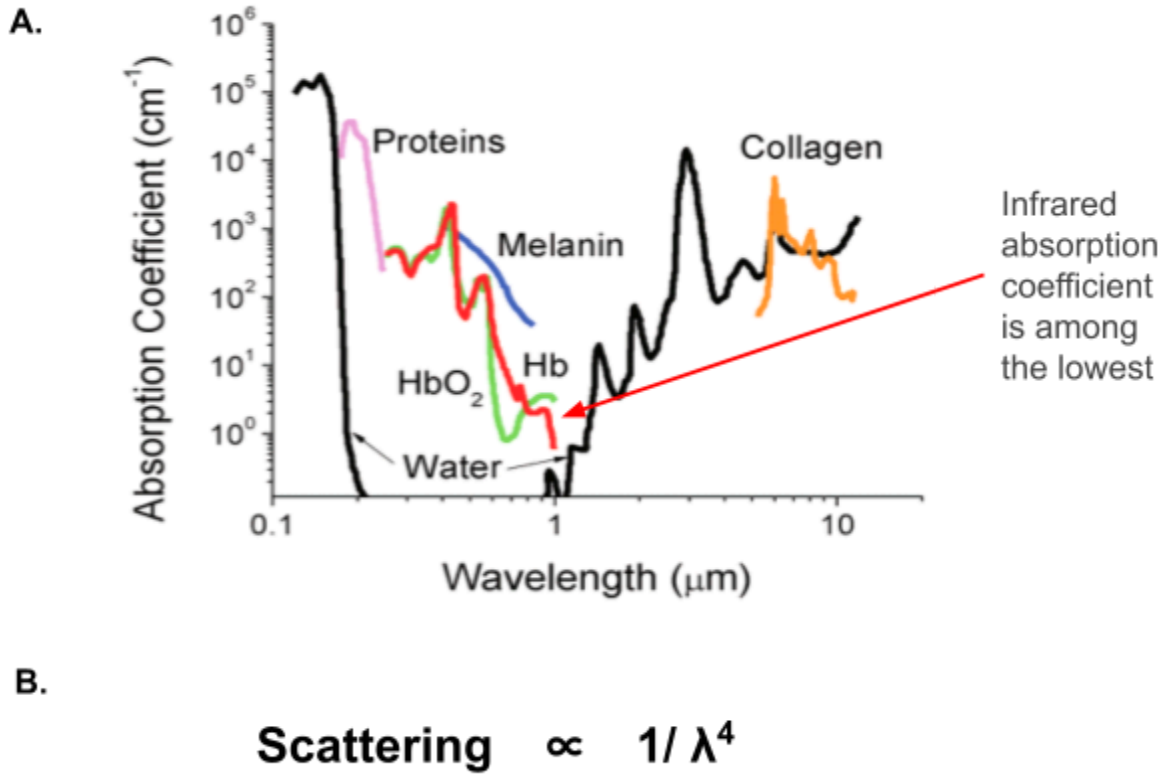


Figure 2.3 (A). Absorbance of various tissues and blood components from 200 nm to 10 μm. The lowest overall tissue absorption occurs in the infrared region. (Adapted with permission from Pansare et al., Chemistry of Materials, 2012. Copyright © 2012, American Chemical Society. (B). The Rayleigh scattering formula. With a higher wavelength, we get a much sharper decrease in scattering

A microscope lens with a 20x magnification is used in the two-photon apparatus with a 1.0 Numerical Aperture. The absorption peak wavelength of free fura-2 dextran is 380 nm, which shifts to 340 nm upon binding to calcium. The dextran component makes the dye impermeable to the cell membrane, preventing it from leaving the neuron.

780 nm infrared light is used to obtain high-quality fluorescence scans at different depths, primarily exciting free fura-2 dextran (780 nm corresponds to an absorption peak at 390 nm in a two-photon imaging apparatus, much closer to that of free fura-2). So when neural

activity increases, there's a calcium influx, decreasing the concentration of free fura-2, and hence reducing the fluorescence. This is why neural activity is expected to show an inverted response in the temporal fluorescence imaging setup. A 525 nm ± 35 nm chroma filter and a photomultiplier tube (PMT) are used to capture the fura-2 fluorescence.

While imaging, a 256 x 256 pixel resolution is used, with a pixel size of 4.17μm x 4.17μm, at a frame rate of 4.3 Hz. However, during visual response trials later, the resolution was changed to 128 x 128 pixels and zoomed in to approximately 1.65 μm x 1.65 μm, resulting in a subsequently faster frame rate of 6.06 Hz.

We use two-photon imaging techniques because they offer far greater three-dimensional resolution and much lower photobleaching and photodamage.

3. Methods:

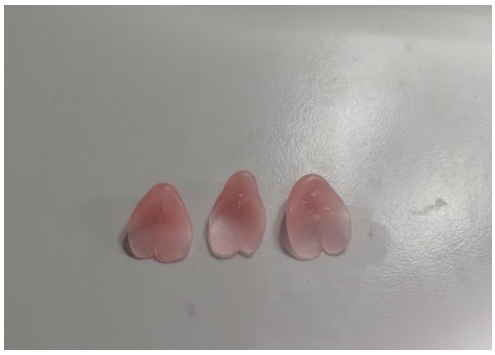
In this section, we detail an exhaustive, in-depth in vivo preparation process for imaging the Anterior Optic Tubercle (AOTu) while keeping the bees alive. We first collect the bees and mount them in a bee holder, followed by dissection and cleaning to make the brain clearly visible for injection and imaging. This is a laborious, precise process that requires some time to master.

(A) **(B)**

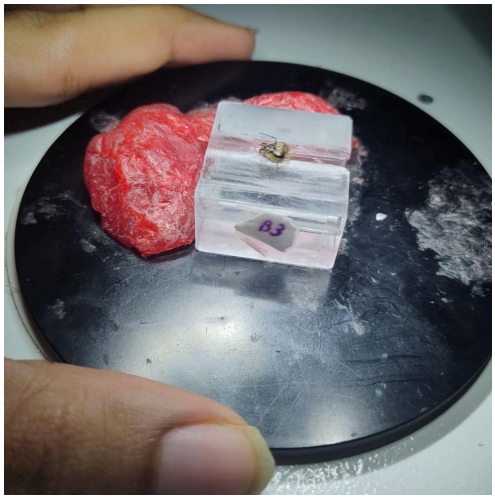
Figure 3.1 (A) A protective body suit and gloves worn for bee collection, and (B) Bee hives.

3.1 Collecting and Mounting Bees Protocol

1. Bee collection:
    - Bees were collected around 12-1 pm, preferably when the sunlight was falling directly on the hives.
    - In November and December, bees were collected by opening the hives' tops. This is done quickly and promptly, so that cold air is kept out as much as possible. The bee containers are then kept inside insulation for transport.
    - Preferably, bees that have pollen on their legs were collected. This shows that they are active foragers and neurologically mature, making them ideal for brain imaging experiments.
    - This process is very laborious, so only 6 bees are collected at once.



(A)



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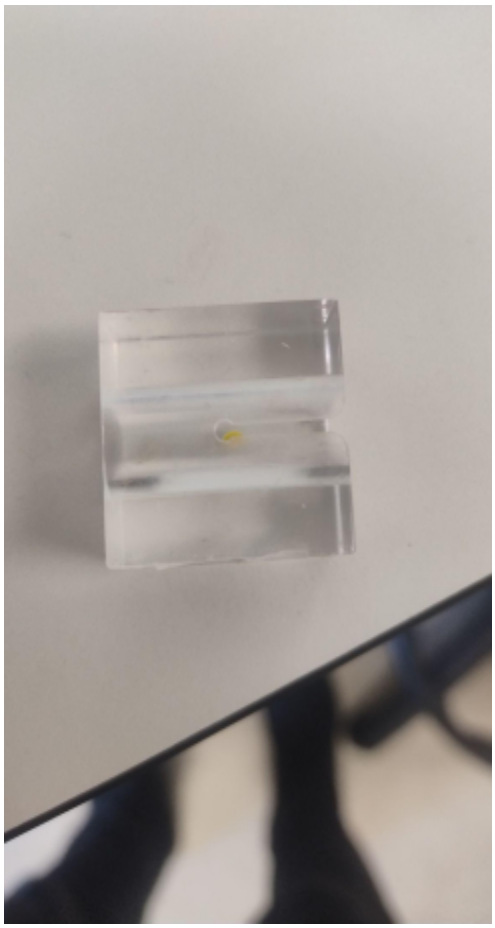


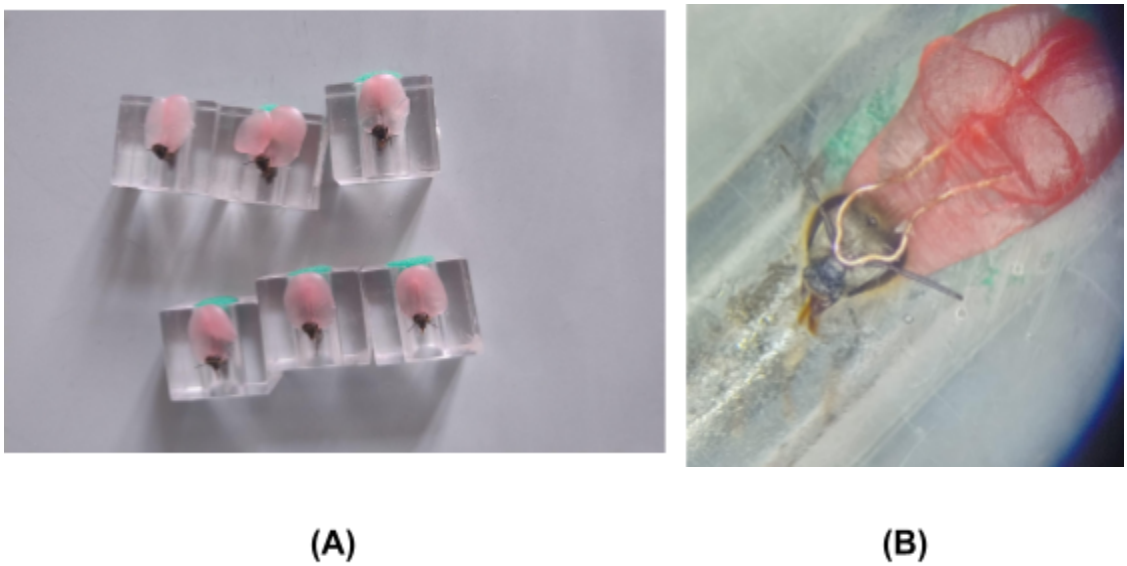
(C)

Figure 3.2 (A) Dental wax, (B) A mounted bee and (C) A bee holder with a narrow slit for the bee neck.

2. Once the bees are collected in the containers, we mount them individually:
   - A container is placed in a box of ice for 1-2 minutes, until the bee is immobilised. (anaesthetising the bees)
   - A piece of dental wax is molded into an appropriate shape for restraining the bee head (Figure 3.2 A). This dental wax has a narrow slit that allows the bee neck to fit.
   - The bees are mounted in the bee holder by placing the head in the narrow slit.
   - The proboscis needs to be extended out before the next step. This is done to ensure that the proboscis doesn't get stuck under the bee holder and the bee is able to feed

- The prepared dental wax is applied around their neck and head to prevent them from moving their head too much. This is a tricky step because the wax needs to be tight so as to severely limit their movement, but not so tight that they are unable to consume the sugar solution.
- A sponge is also added to prevent them from moving their body too much.
- A drop of sugar water is placed in front of their heads where their proboscis can reach and they are able to feed(33% sucrose weight by weight solution)
- If bees don't or are unable to drink the sugar solution on the first day, their chances of surviving are greatly decreased.

3.2 Protocol for Dissection



(A) (B)

Figure 3.3 (A) Six bees are usually mounted and prepared for each experiment. (B) A copper wire is used to temporarily block the movement of the antennae

1. Antennal blocking: Bees' antennae are one of the primary ways bees use to sense their environment. These antennas, however, can interfere with the dissection and injection processes. So we generally block their movement using n-eicosan wax;
   - The antenna is first fixed in place using a copper wire. (Figure 3.3 B)
   - N-eicosan is then taken on a low-temperature soldering iron. (There is a metal coil attached to the solder, as seen in the picture. This is done because the tip of the

solder is too wide relative to the bee's head. The tip of this coil is small enough to produce a smaller n-eicosan drop, which can then be used quite comfortably.)
    - The solder is heated to 50 degrees Celsius to melt the n-eicosan.
    - This wax is then applied to the antennas to glue them in place.
    - The copper wires are then removed.

2. Dissecting: We then move to the dissection of the head. The head capsule is cut into a rectangular shape by the edge of a razor blade. Figure 3.4 (A) below illustrates the cuts made in the order of red, orange, green and blue. The last cut, shown in blue, is made only halfway, as it becomes very difficult to do the dissection all the way through. The head capsule is then torn carefully, using forceps. This head capsule is carefully kept aside on a piece of tissue paper.

3. Cleaning: The salivary glands and the trachea on one side (usually the left) are removed or pushed aside to expose the desired injection site. Very careful precision and months of practice are needed to produce very calibrated and fine movements, so that the brain is not scratched or damaged. Once this step is refined, the bee's chances of surviving on the second day drastically improve. On the first day, only minimal cleaning (to expose the injection site) is performed to maximise bee survival and to preserve optimal brain function. Care must also be taken to avoid damaging the forceps tips, as they are quite small and delicate.

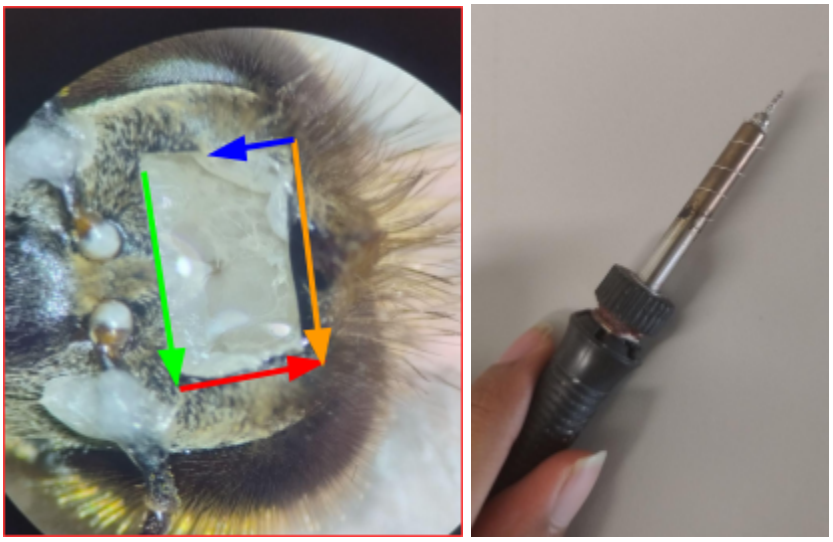


Figure 3.4 (A)Above illustrate the incisions made to remove the head (B) Iron solder used to apply n-eicosan

3.3 Protocol for dye loading and injection

1. Preparing FURA-2 dextran dye needles (Dye loading):
   - A glass needle with an extremely thin tip (~10 microns) is prepared using a machine (Figure 3.5 A)
   - The dye is photosensitive, so one needs to be careful to expose it to as little light as possible. 20 ml of tiny fura-2 crystals with forceps are added until the solution turns yellow.
   - A pipette is used to place a drop of dye on a clean slide. Then, under the microscope, the dye is carefully placed on the glass needle. This is a delicate process that requires the dye to be of a particular consistency. If too dry, the tip may break, and if too watery, the dye will not stick to the needle. So a little deionised water drop is added, and we wait for the dye to dry and reach a suitable consistency. If it becomes too dry, another drop of water can be added. The ideal consistency is when the dye droplet appears to form a film. Care needs to be taken that the needles don't break and act as little capillaries to absorb the dye. This is because the dye can be quite expensive and should not be wasted.

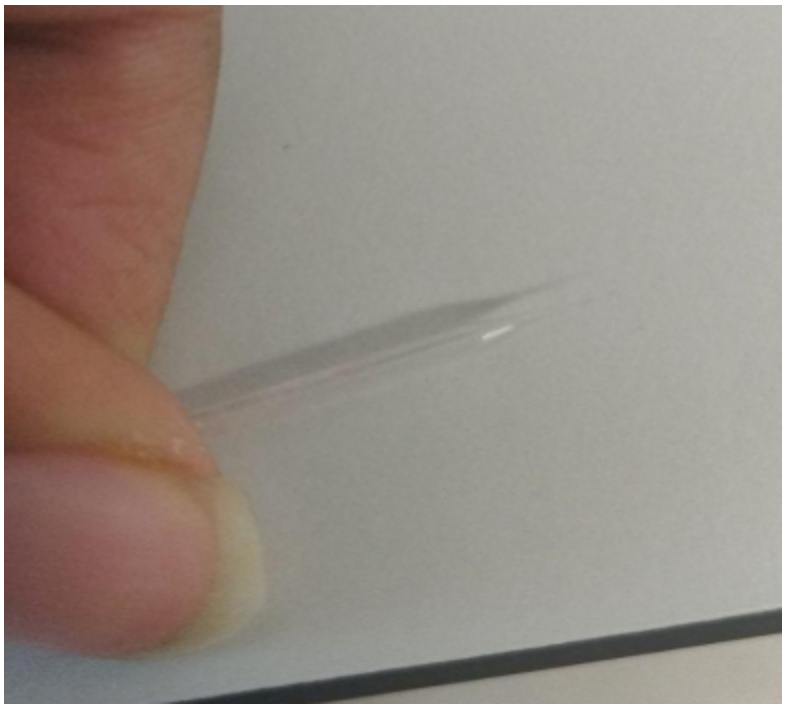


Figure 3.5 A glass needle with an extremely thin tip (~10 microns).

   -

- After enough dye needles are prepared (this can vary depending on how many bees are prepared in a day and how many injections are used per bee), another deionised water droplet is added to the dye droplet and mixed. Then, using a pipette, the dye droplet is transferred back to the original dye container for reuse.

In the paper Oesterle (2026), three dye injections were used for each bee. However, in my setup, I observed that when more than two dye injections are used, the bees' survival rate is severely reduced. But this is a preliminary observation and might improve with a better and improved technique.
FURA-2 dextran is a calcium-binding fluorescent dye that, in neuronal populations, shows an inverted fluorescence response to increased neuronal activity. That is, the fluorescence observed under the microscope dips whenever neuronal activity increases.

2. Dye Injection:
   - The injection is made into the tubercle region (usually done on the left side of the brain).
   - The removed head capsule is then placed back on the head. This capsule naturally seals on its own and prevents the hemolymph from drying out.
   - The bee is then left overnight in a box with a wet tissue placed underneath it (for sufficient humidity). We leave it overnight to allow the dye to diffuse to the tubercle on the right side of the brain. The reason is that the injection often damages the tubercle region on the left side of the brain. This is not always the case, as we try to inject into the ventral inter-tubercle tract (vITT), but it might nevertheless directly damage the AOTu (if I miss) or indirectly compromise its functioning by damaging the neuron.

3. Protocol for cleaning the right side of the brain:
   - The next day, about 80% of the bees survive. The survival rate depends on how developed the technique is. I achieved an 80% survival rate by the end of my thesis.
   - For those that survived, we again remove the head capsule and clean the other side of the brain (i.e., remove or push aside the salivary glands and the trachea to expose the tubercle region for imaging). Again, it is recommended to push the salivary glands and the trachea aside instead of removing them. However, removing some of it is unavoidable in order to expose the AOTu.

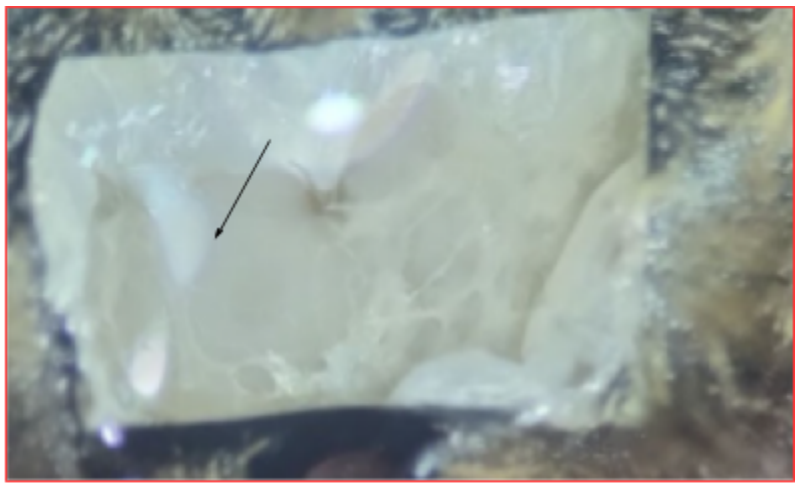


Figure 3.6. An image under the microscope of the honey bee brain depicting the approximate injection site (indicated by the arrow). This dye then travels through the vITT to the AOTu on the other side.

4. Protocol for applying Kwik-Sil and coverslip
    - A small piece of tissue paper is then used to absorb most of the hemolymph.
    - Kwik-Sil (a translucent, medium-viscosity silicone adhesive) is applied above the exposed brain and covered with a coverslip.
    - During the initial learning period of this thesis, transparent coverslips were used. This was later switched to coverslips painted over with black acrylic paint. The reason for doing so was to further block out all light that might interfere and enter the sensitive two-photon microscope setup.
    - Now the coverslip is tightly pressed on the applied Kwik-Sil and the wax on the bee holder. This is one of the most challenging steps of this process, and one that I had to do countless times to get the hang of. The biggest problem in this process is the formation of air bubbles. The process is described in more detail below.
    - Gloves are worn for this procedure to avoid the coverslip sticking to my fingers.
    - A small amount of Kwik-Sil is poured onto a surface and mixed gently to prevent air bubbles from forming. This is done to ensure high-quality scanning under the microscope.
    - Now, a drop of Kwik-Sil is taken on a forceps and applied to the exposed brain region.
    - A clean coverslip is placed above the Kwik-Sil that has been applied.

- The back of the forceps is then used to press down on the coverslip on the wax so that it sticks in place. For this, the amount of wax on the bee mount needs to be sufficient enough. An iron solder (heated up to 50° Celcius) can be used to melt the wax a little if the coverslip doesn't stick to the wax.
- If bubbles creep in and nothing can be done to remove them, a last try can be made to remove the coverslip, wait a couple of minutes, and then remove the dried Kwik-Sil using forceps. The kwik seal usually comes out easily if the haemolymph was not completely dried out. The whole process is then tried again.
- At last, all the prepared bees are kept in a covered box with a small opening. This is intended to mimic a bee's natural hive and keep the bee relaxed.

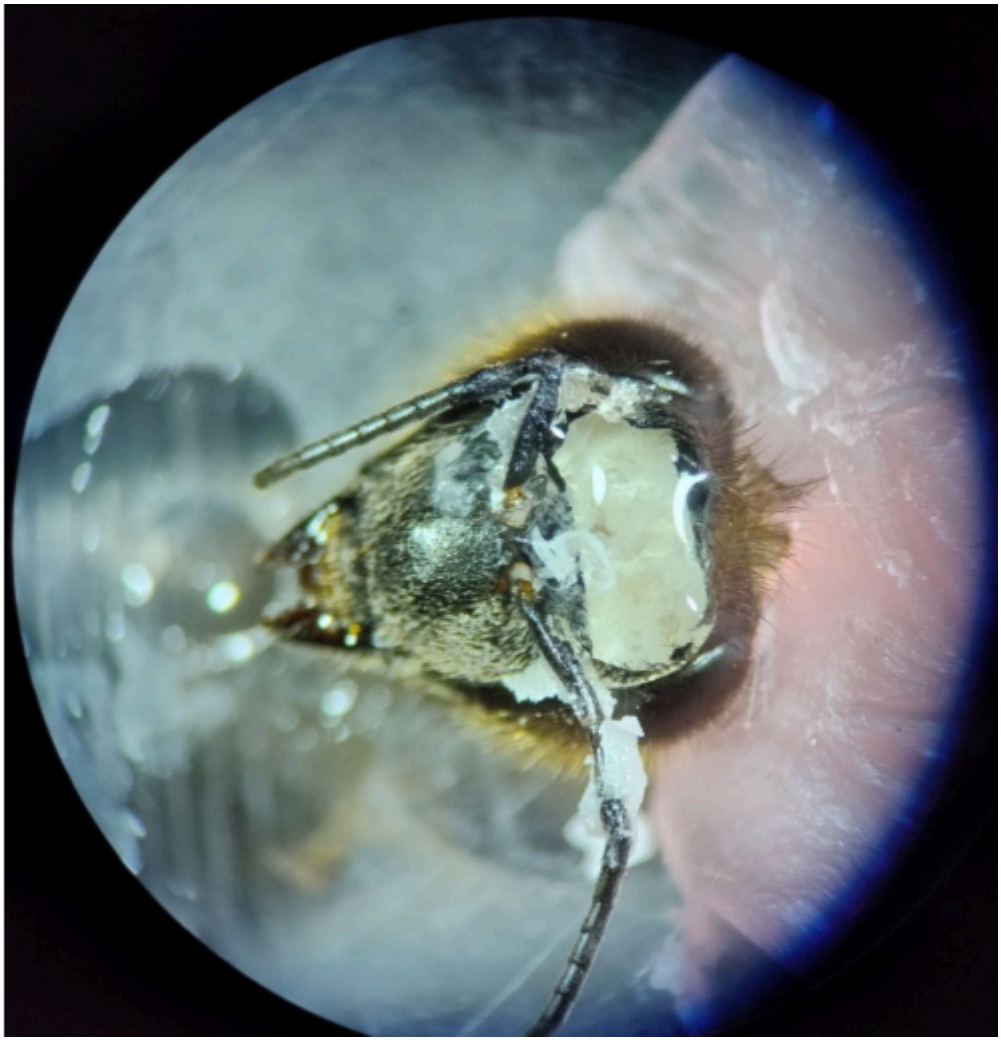


Figure 3.7 A look under microscope at a bee with a window in the head capsule.

3.4 Imaging and Visual Stimulus Protocol

1. Mounting bees under the microscope

   A two-photon microscope is used to image the prepared bees. Care is taken not to use too much light as the PMTs (Photo Multiplier Tubes) are quite sensitive and can be damaged by excess light. The software used is Prairie View 5.8. Once all the equipment is switched on, we put the bee under the microscope. We then put a drop of deionised water on the coverslip and slowly adjust the water-immersion microscope so that the brain region is in focus.

2. Procedure for Two-photon scans

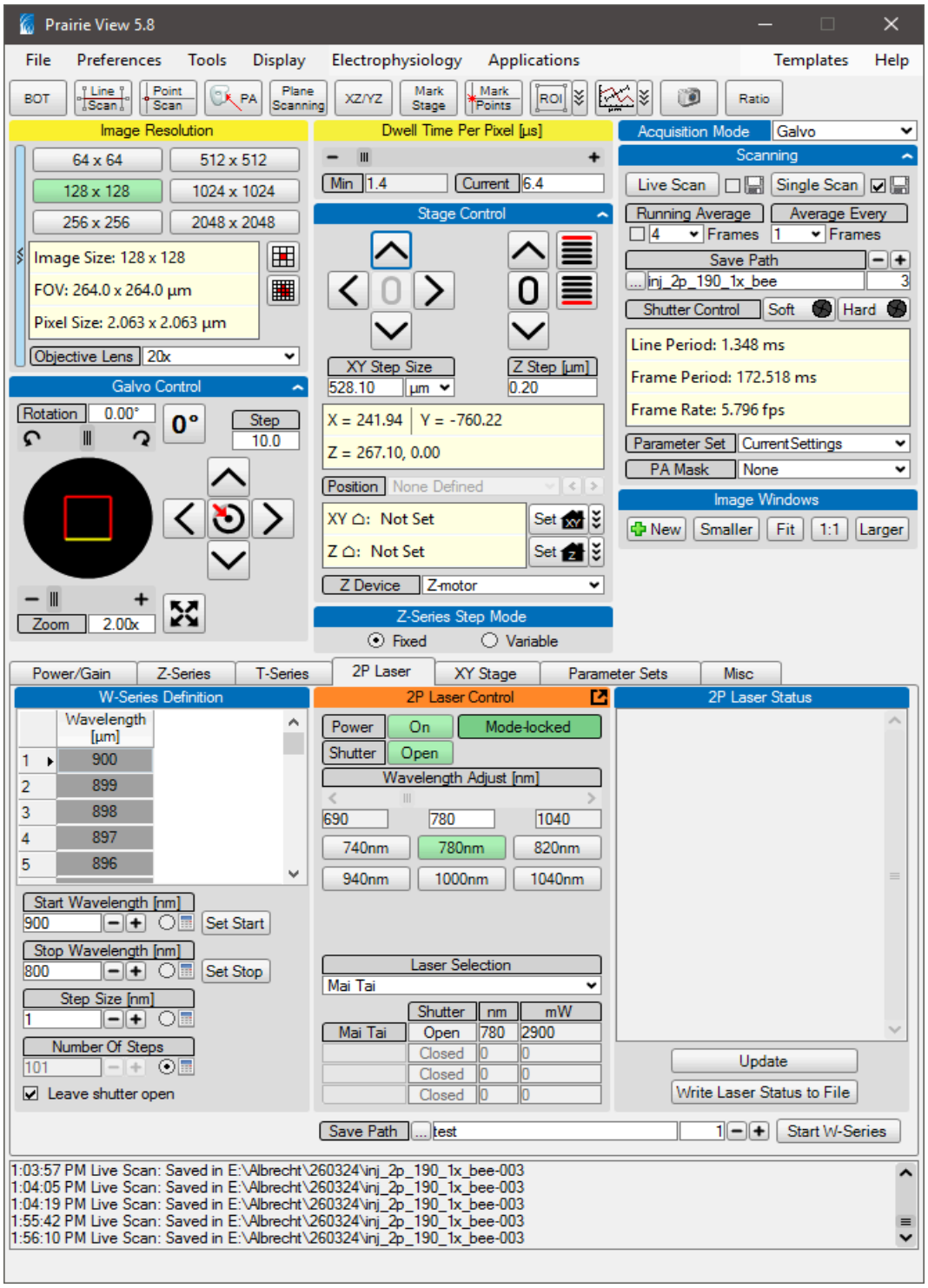


Figure 3.8 The Prairie View Software. This software is used to directly control all the functions of the two-photon microscope.

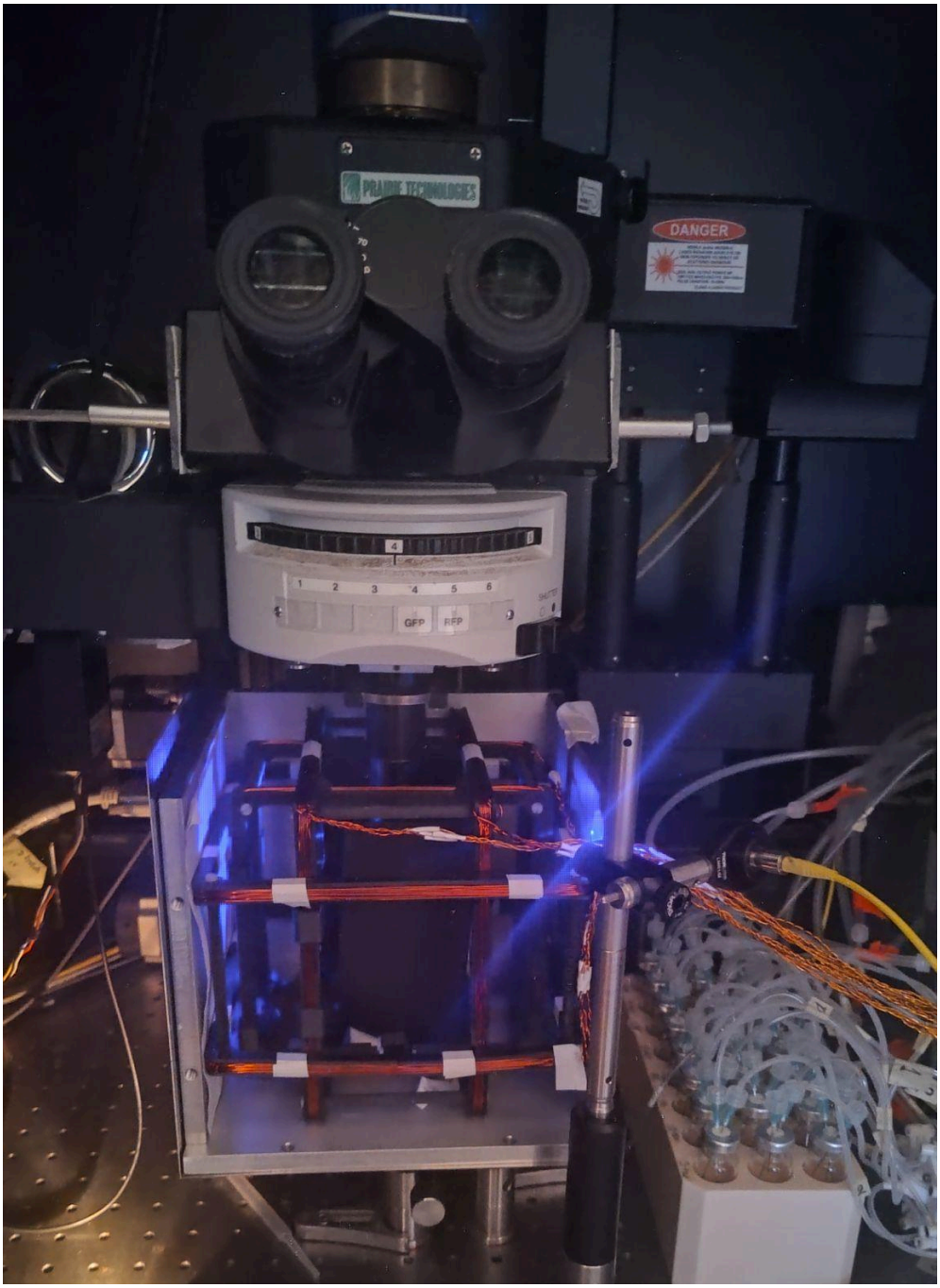
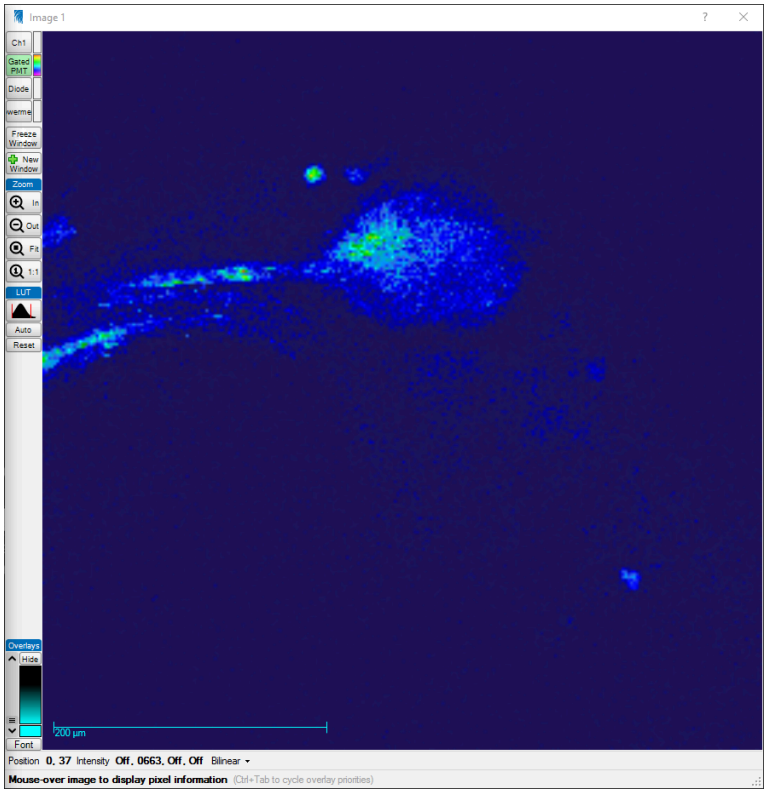


Figure 3.9 The two-photon microscope (Manufacturer: Bruker; Model: Ultima 4) with the visual laser stimulus on. The laser (illumination on the right) passes through a hole in the aluminium shield, reflects on the backside, and falls on the opposite side (illumination on the left) of this shield, creating a homogenous scattering

It is essential that the intensity of the two-photon microscope is not too high, as it might cause photobleaching and also damage the tubercle.

The microscope door is then closed, and the Livescan button is used to scan and locate the desired AOTu region. Some live staining images of the AOTu are shown below (Figure 3.10 and Figure 3.11)

(a)



(b)

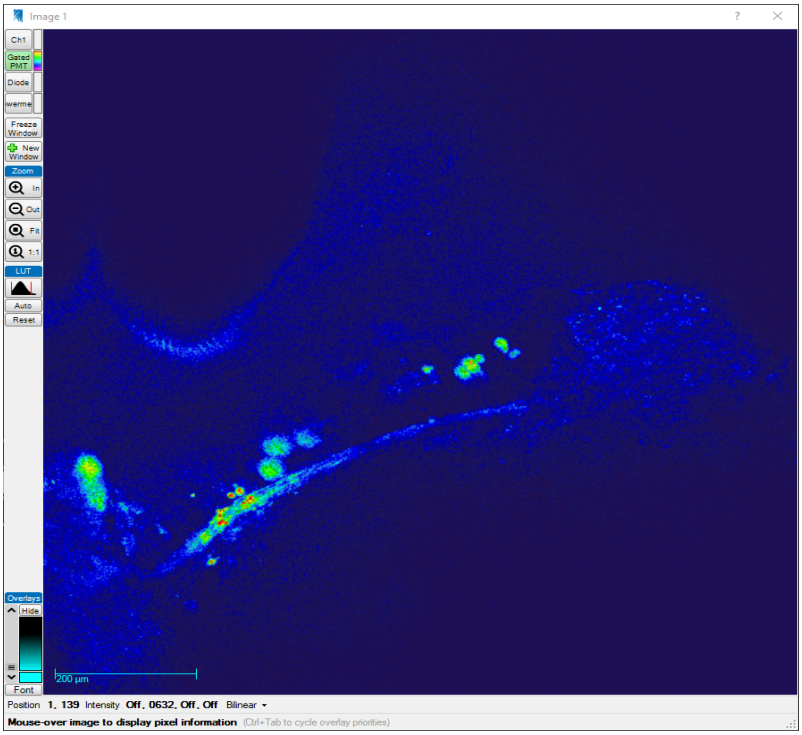


Figure 3.10 (a)Live two-photon calcium fluorescence images of the AOTu (b) Live two-photon calcium fluorescence images of the vITT connecting the bilateral AOTu region

3. Visual stimulus protocol:

A blue stimulus laser (473 nm wavelength) is turned on. The laser power can be set to 50mW. In most experiments, about 5-20 mW of laser power was used. The laser light is reflected by an aluminium wall and falls on a white sheet of paper. This is done to create uniform light scattering to mimic the blue light in the sky.

A MATLAB script is used to connect the laser, the two-photon apparatus, and the computer. (This script has been made by previous PhD and Master's students and Dr Haase). The same script is then used to run 15 trials consisting of a visual laser stimulus (Figure 3.12), collecting a time-series recording of neural activity throughout the trial interval (in .tif image format).

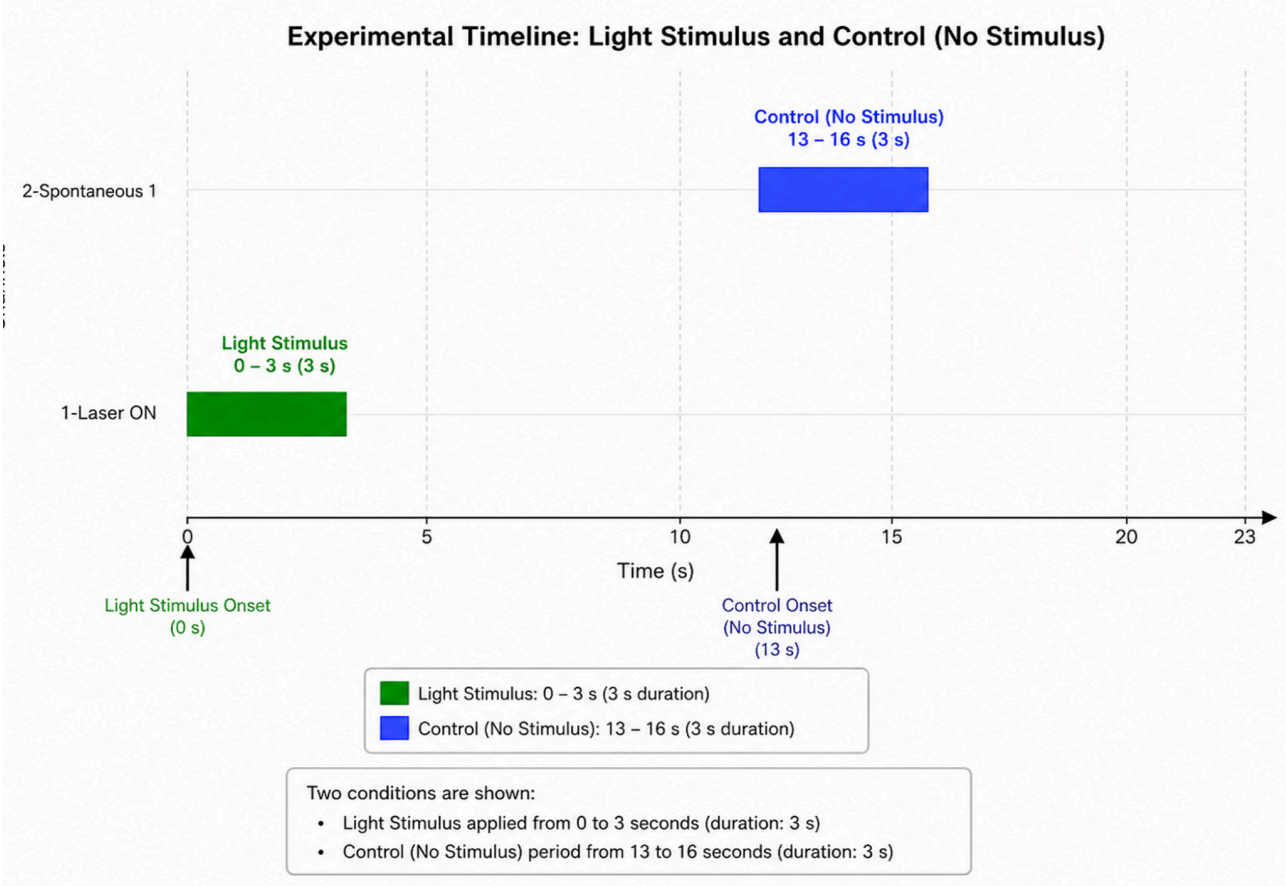


Figure 3.12 A trial consists of a laser ON stimulus and a spontaneous (control) signal (both 3 seconds) with periods of 10 seconds of rest in between them. In addition, the first trial was preceded by a 10-second delay for habituation.

The trial consists of a brief laser stimulus, on for 3 seconds, followed by 10 seconds of rest. The next 3 seconds (No stimulus) are now treated as a control to compare the neural response to the visual stimulus. The next 10 seconds are again rest. Thus, we get a stimulus (laser on) and a control response (spontaneous 1) for each trial. Before the 15 trials begin, a 10-second delay is given for the bee to become habituated to its environment. (Figure 3.12)

3.5 Data Analysis

A multi-region analysis of the time series recordings is performed using another MATLAB script, in which the temporal fluorescence response of two regions of interest (ROIs) is captured in TIF File format. All fluorescence images were averaged to obtain a mean projection image, which was then used to select different regions of interest (ROI): the AOTu (ROI 1) and a control background region (ROI 2) (Figure 3.13).

The fluorescence responses of each trial are normalised to the pre- stimulus fluorescence, and their normalised fluorescence change ($\Delta F/F$) is then plotted for each region and each time period (laser on and spontaneous 1). The calcium fluorescence response ($\Delta F/F$) of the different regions is then plotted on four graphs: a)AOTu (region 1) and laser on; b) background(region 2) and laser on; c)AOTu (region 1) and spontaneous 1; and d) background(region 2) and spontaneous (Figure 4.1).
The averages of normalised fluorescence activity is then compared across all trials at the start, during, and after the visual stimulus to establish or detect a proper stimulus response.

Over here, the fura-2 dextran dye shows an inverted calcium response to increased neuronal activity. This increase is because, as calcium rushes into neurons during increased neuronal activity, the extracellular calcium concentration and, subsequently, the fura-2 bound to calcium ions decrease.

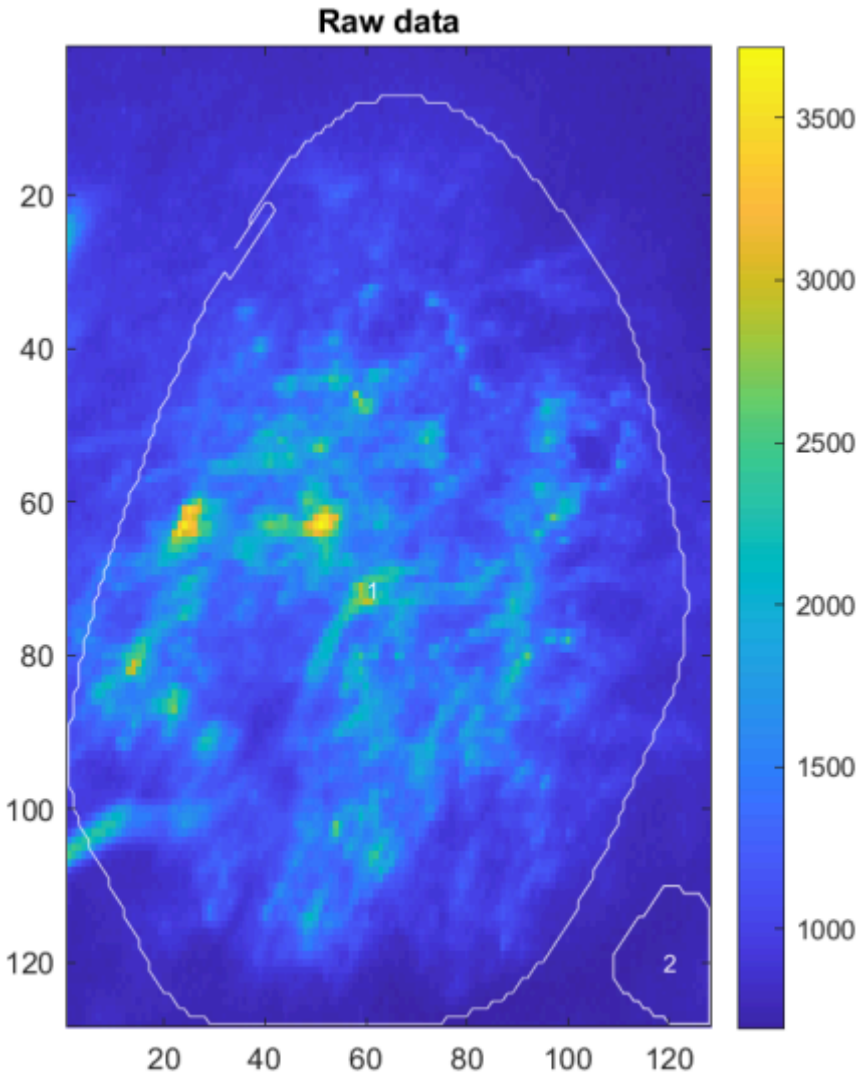


Figure 3.13 Region 1 covering the AOTu and Region 2 covering a background area in order to detect visual (blue) laser light penetrating the two-photon apparatus

A high-pass filter is used in addition to remove slow baseline drifts, like fluorescence decay due to photobleaching. Additionally, individual select trials can be removed to improve results.

4. Results and Discussion

Two-photon microscopy also enables structural imaging of the neural tissue. Calcium fluorescence images at different depths of one such AOTu are shown in Figure 4.1. At approximately 40 microns depth, the Major Unit Dorsal Lobe and the Major Unit Ventral

Lobe can be visibly distinguished. The visibility of these distinct lobes at this depth confirms successful staining of the AOTu, ensuring we targeted the correct anatomical region rather than adjacent brain tissue.

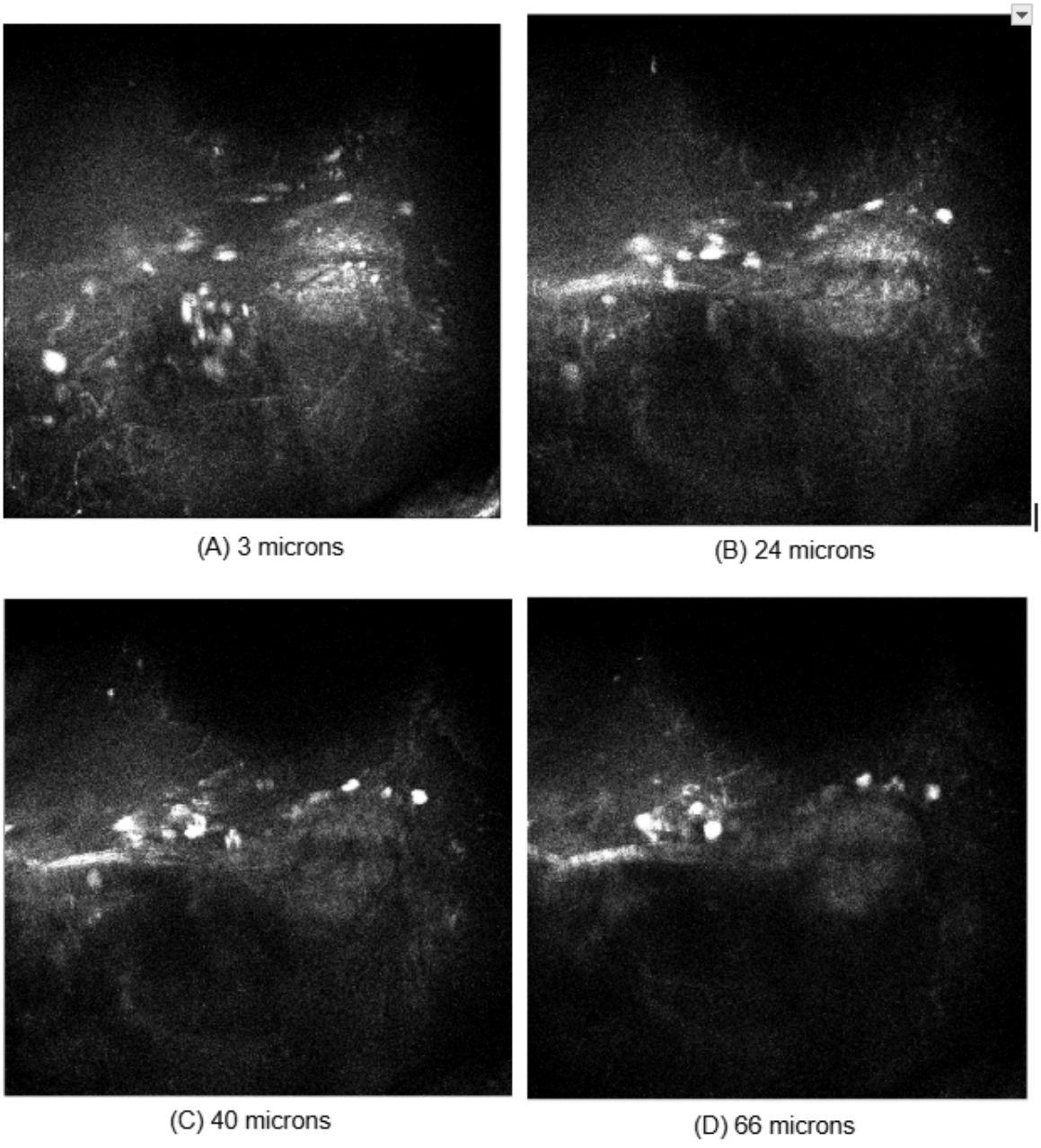


Figure 4.1 Calcium fluorescence scans of AOTu at various depths. These show the AOTu structure in a high three-dimensional resolution. In (C) and (D), the two hemispheres, the Major Unit Dorsal Lobe and the Major Unit Ventral Lobe, are visibly distinguishible

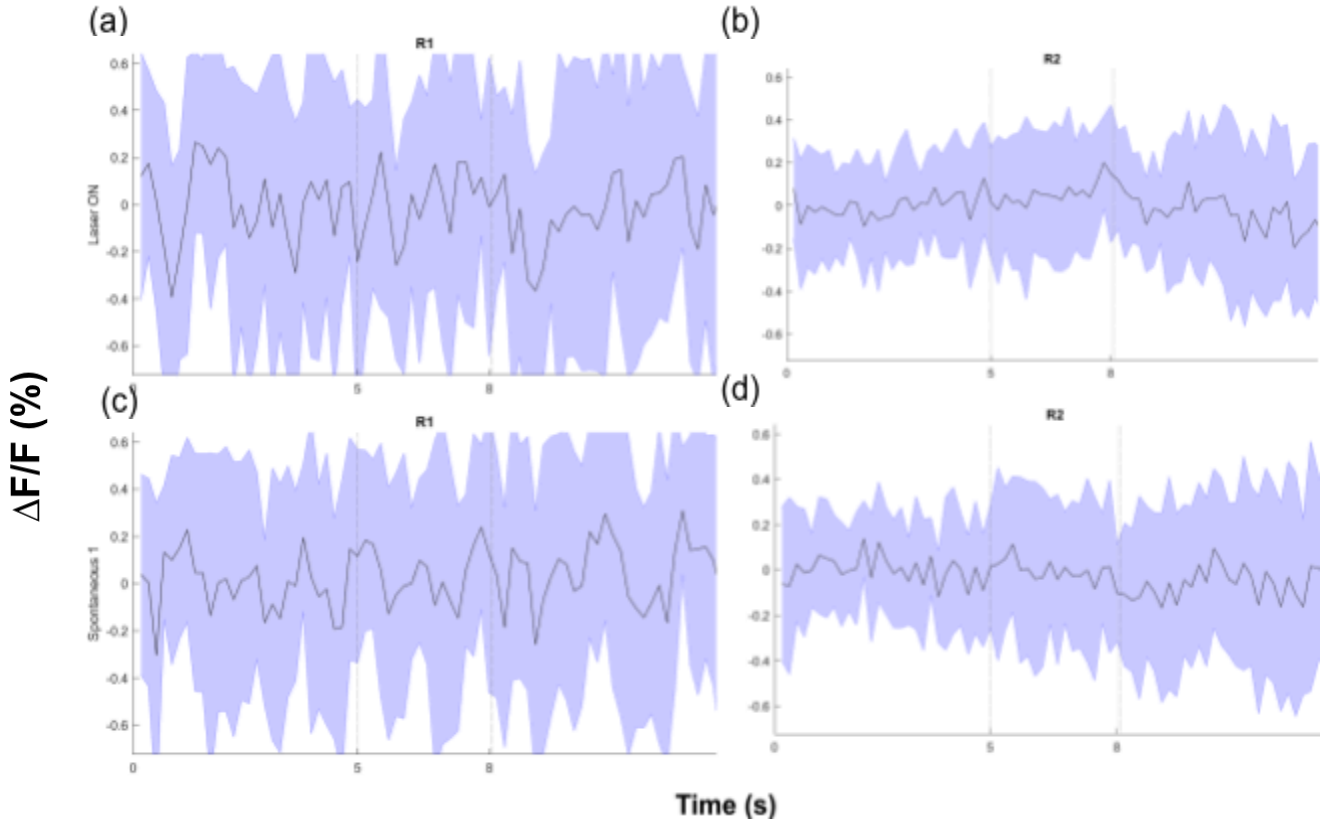


Figure 4.2 Neuronal fluorescence activity responses in each region and time period. The visual stimulus and the control stimulus are activated at t = 5 s and end at t = 8 s. a)AOTu (ROI 1) and laser on, b) background(ROI2) and laser on, c)AOTu (ROI 1) and spontaneous 1 and d) background(ROI2) and spontaneous.

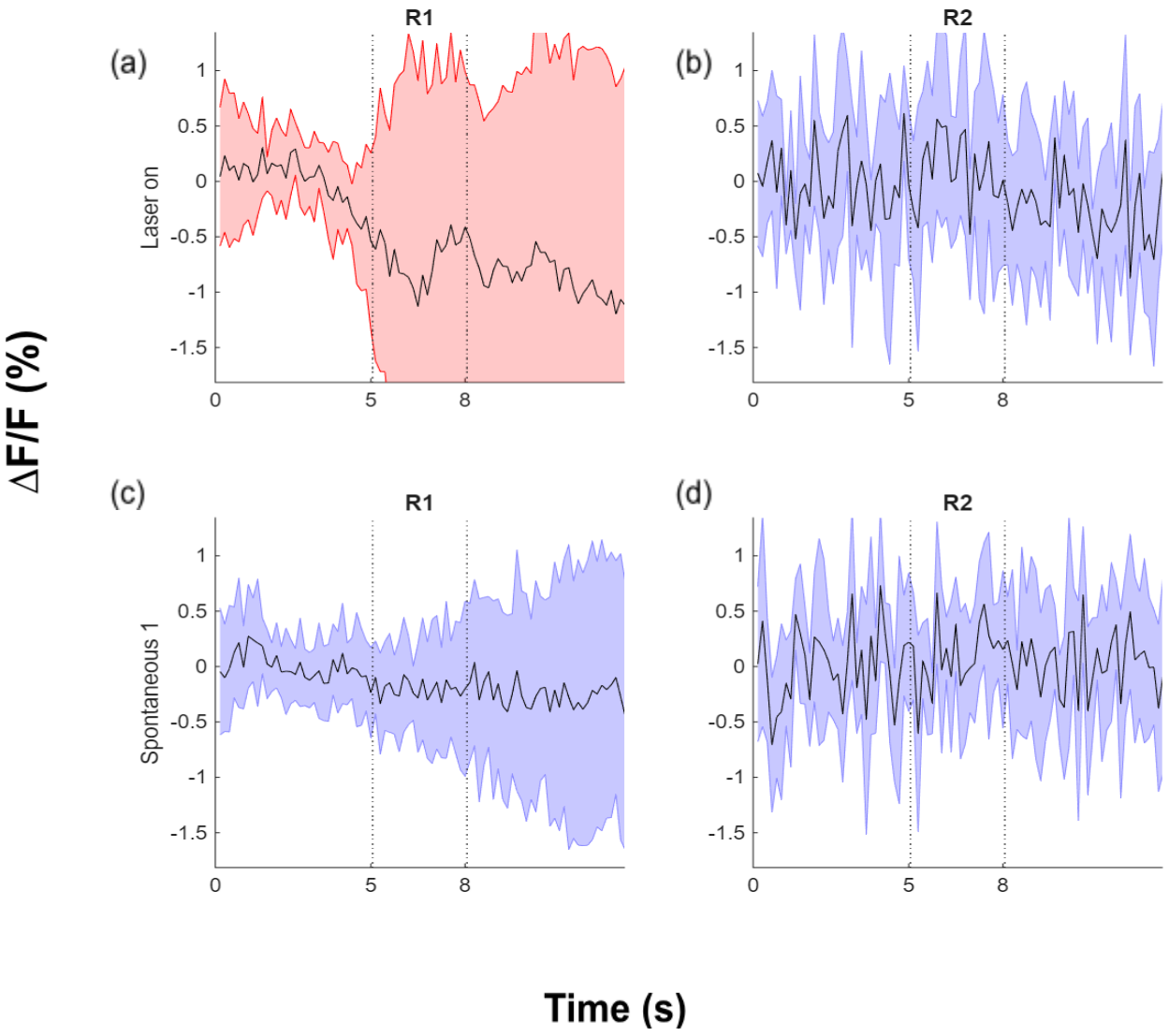


Figure 4.3 Neuronal fluorescence activity responses in each region and time period. The visual stimulus and the control stimulus are activated at t = 5 s and end at t = 8 s. a)AOTu (ROI 1) and laser on, b) background(ROI2) and laser on, c)AOTu (ROI 1) and spontaneous 1 and d) background(ROI2) and spontaneous.
a)AOTu (ROI 1) and laser on, b) background(ROI 2) and laser on, c)AOTu (ROI 1) and spontaneous 1 and d) background (ROI 2) and spontaneous.
This is one of the instances that suggests a potential visual response, as we see a notable change in ΔF/F (%) after stimulus onset and offset (~0.5%) in the AOTu (R1 or ROI 1, upper left). However, due to extreme standard deviation, this could not be used further.

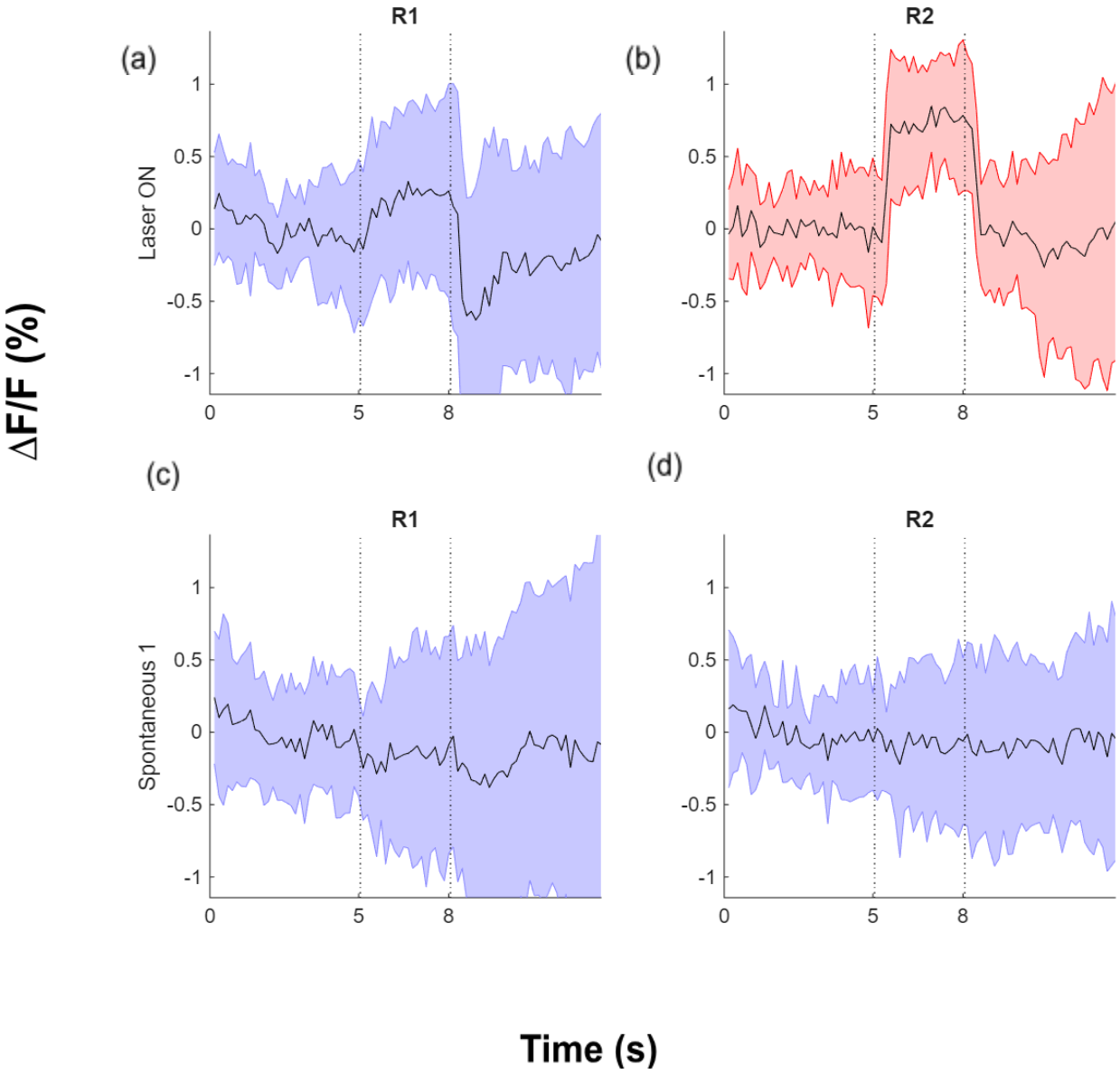


Figure 4.4 Neuronal fluorescence activity responses in each region and time period. The visual stimulus and the control stimulus are activated at t = 5 s and end at t = 8 s. a)AOTu (ROI 1) and laser on, b) background(ROI2) and laser on, c)AOTu (ROI 1) and spontaneous 1 and d) background(ROI2) and spontaneous.
This is a prime example of how visual stimulus 'leakage' might mask the response. During laser on, we observe an uneven increase in fluorescence at visual onset (t = 5 s) in the AOTu (R1) and the background (R2). This leads us to speculate that there's likely a high leakage of visual stimulus light in the preparation (as seen by control R2), and the low increase in the R1 region may be attributed to the decrease in the fluorescence due to increased neuronal activity. This is further supported by a strong decrease in the visual offset response (t = 8 s) in R1.

Here, we examine neuronal activity (marked by a sharp decrease in fluorescence) in the AOTu (ROI 1) when the visual stimulus (a blue laser of max power 50 mW) is turned on and then off during trials (Figure 3.13). We are looking for the phasic ON-OFF responses typically observed in visual processing units.

A sharp decrease in the fluorescence response is expected here, as shown by Oesterle (2026), when the visual stimulus is introduced and removed (Figure 4.5 shows a sharp increase because Oesterle has inverted the recordings), i.e., we expect a significant drop in fluorescence in the AOTu during the onset of visual stimulus response (Figure 4.3 (a), Figure 4.4 (a), and Figure 4.5 (a)) **.** However, despite multiple trials, this response could not be reliably observed and distinguished.

One highly plausible explanation is that the fluorescence signal might be masked by the visual stimulus laser light “leaking into” the recorded fluorescence response. These can be seen in many of the result plots (Figure 4.4)**.** Hence, a significant effort in the lab went to improving the shielding to prevent this leakage. For example, a black sheet of paper was added to absorb as much light as possible and to block reflected light from entering the microscope aperture.

One result showed a highly probable response, which may have been masked due to the ‘leakage’ of stimulus light, as shown in Figure 4.4. This result suggests that a visual stimulus response is quite likely achievable with further development of the technique. However, even after multiple trials, another response wasn’t clearly observable or distinguishable, so progress towards magnetic stimulus tests couldn't be made.

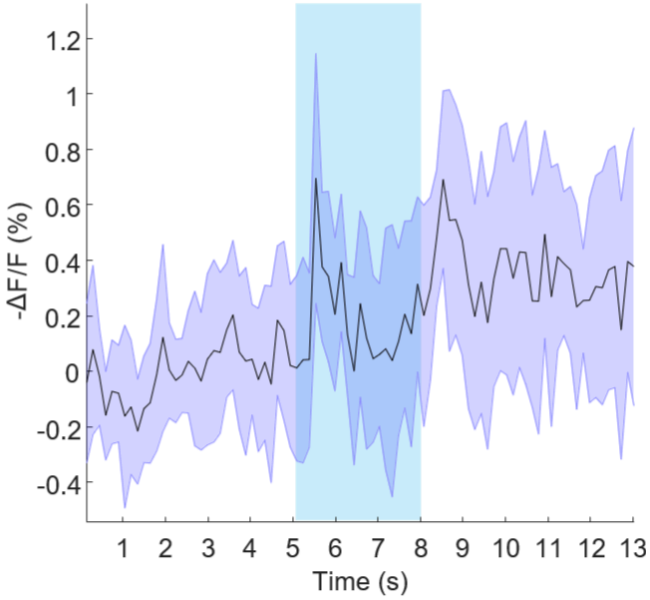


Figure 4.5 Expected fluorescence change in activity in the AOTu during the onset and immediately after the visual stimulus. The fluorescence response over here is inverted. (Oesterle, 2026)

Another likely reason could be winter. By the time I became proficient, it was winter, and bees are significantly less active in these months. In the months of December and January, bees do not leave their hives, and need to be removed by opening the hives. These cold temperatures could severely damage these bees when opening the hive or transporting them. And possibly, I might be leaving the bees in the ice for too long to anaesthetise them. This problem was noted before, and drastically reducing exposure to ice and insulating them during transport considerably increased their survivability the next day. However, this still did not result in manifesting a visual response.

The most plausible reason, however, is the inadequate quality of preparation. Movement might not be properly constrained, giving rise to significant variations in the measured activity. Brain damage is also likely. The AOTu is situated right below the brain surface. This means there is a high likelihood of damaging the AOTu during removal of trachea and salivary glands or during injection. It is also possible that the visual stimulus (laser) might not be powerful enough to induce a response from the bee. This might be even more

likely, given that the laser power was often reduced to avoid leakage of laser light into the neural activity response.

Too much two-photon excitation power, and lower dye concentration can also lead to a higher noise-to-signal ratio (Oesterle, 2026) and obscure the signal. Habituation is also an issue: after multiple trials, bees become accustomed to the visual stimulus and stop responding. Oesterle (2026) themself used only four bees for their magnetic stimulus responses after receiving sufficient and satisfactory visual responses; this shows how difficult it has been to obtain a confirmatory visual response.

All things considered, it is likely that a combination of several factors above is responsible for the absence of a confirmatory visual response. By honing and further developing techniques for higher-quality preparation, better shielding, and summer preparation, the next step is to obtain a consistent visual response and move further towards investigating magnetoreception.

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