AFRL-RW-EG-TR-2023-371
Neural Networking Transforming In House Visual Neurophysiology Research Capabilities

Lorian Schweikert
601 South College Rd
Wilmington, NC 19720

Nicholas I Rummelt

Air Force Research Laboratory
Munitions Directorate
101 West Eglin Blvd
Eglin AFB, FL 32542-5910

Sep 2021

Final Report

Controlled By: USAF AFRL/RW
CUI Category: CTI
Distribution Statement: A
POC: RWTC

DISTRIBUTION STATEMENT A – Approved for public release.

DESTRUCTION NOTICE – CUI documents may be destroyed by means approved for destroying classified information or by any other means making it unreadable, indecipherable, and unrecoverable.

WARNING – This document contains technical data whose export is restricted by the Arms Export Control Act (Title 22, U.S.C., Sec. 2751, et seq.) or the Export Administration Act of 1979 as amended (Title 50, U.S.C., App. 2401, et seq.). Violations of these export laws are subject to severe criminal penalties. Disseminate according to the provisions of DoD Directive 5230.25.

AIR FORCE RESEARCH LABORATORY, MUNITIONS DIRECTORATE
Air Force Materiel Command • United States Air Force • Eglin Air Force Base

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation; or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

This report was cleared for public release by XX Eglin Air Force Base Wing Public Affairs Office and is available to the general public, including foreign nationals. Copies may be obtained from the Defense Technical Information Canter (DTIC) (http://www.dtic.mil).

AFRL-RW-EG-TR-2023-371 HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

FOR THE DIRECTOR:

Signed

NICHOLAS I. RUMMELT, DR-03, DAF
Work Unit Manager, Computational
Engineering Sciences

Signed

KIRK J. VANDEN, DR-04, DAF
Technical Advisor (Acting),
Computational Engineering Sciences

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government's approval or disapproval of its ideas or findings.

NOTICE TO ACCOMPANY THE DISSEMINATION OF EXPORT CONTROLLED TECHNICAL DATA

1. Export of information contained herein, which includes, in some circumstances, release to foreign nationals within the United States, without first obtaining approval or license from the Department of State for items controlled by the International Traffic in Arms Regulations (ITAR), or the Department of Commerce for items controlled by the Export Administration Regulations (EAR), may constitute a violation of law.

2. Under 22 U.S.C. 2778 the penalty for unlawful export of items or information controlled under the ITAR is up to ten years imprisonment, or a fine of $1,000,000 or both. Under 50 U.S.C., Appendix 2410, the penalty for unlawful export of items or information controlled under the EAR is a fine of up to $1,000,000, or five times the value of the exports, whichever is greater; or for an individual, imprisonment of up to 10 years, or a fine of up to $250,000, or both.

3. In accordance with your certification that establishes you as a "qualified U.S. contractor," unauthorized dissemination of this information is prohibited and may result in disqualification as a qualified U.S. contractor and may be considered in determining your eligibility for future contracts with the Department of Defense.

4. The U.S. Government assumes no liability for direct patent infringement, or contributory patent infringement or misuse of technical data.

5. The U.S. Government does not warrant the adequacy, accuracy, currency, or completeness of the technical data.

6. The U.S. Government assumes no liability for loss, damage, or injury resulting from manufacture or use for any purpose of any product, article, system, or material involving reliance upon any or all technical data furnished in response to the request for technical data.

7. If the technical data furnished by the Government will be used for commercial manufacturing or other profit potential, a license for such use may be necessary. Any payments made in support of the request for data do not include or involve any license rights.

8. A copy of this notice shall be provided with any partial or complete reproduction of these data that are provided to qualified U.S. contractors.

DESTRUCTION NOTICE

FOR CLASSIFIED DOCUMENTS. Follow the procedure in DoD 5220.22-M. National Industrial Security Program. Operating Manual, Chapter **5**, Section 7, or DoD 5200.1-R. Information Security Program Regulation. Chapter 6, Section 7.

FOR UNCLASSIFIED, LIMITED DOCUMENTS. Destroy by any method that will prevent disclosure of contents or reconstruction of the document.

<table>
<tr><td colspan="4">REPORT DOCUMENTATION PAGE</td><td colspan="2">Form Approved OMB No. 0704-0188</td></tr>
<tr><td colspan="6">Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202- 4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</td></tr>
<tr><td colspan="2">1. REPORT DATE (DD-MM-YYYY)
30 Sep 2021</td><td colspan="2">2. REPORT TYPE
Final</td><td colspan="2">3. DATES COVERED (From - To)
17 May 2021 – 30 Sep 2021</td></tr>
<tr><td colspan="4" rowspan="3">4. TITLE AND SUBTITLE
Neural Networking Transforming In House Visual Neurophysiology Research Capabilities</td><td colspan="2">5a. CONTRACT NUMBER
FA8651-21-2-0002</td></tr>
<tr><td colspan="2">5b. GRANT NUMBER
N/A</td></tr>
<tr><td colspan="2">5c. PROGRAM ELEMENT NUMBER
N/A</td></tr>
<tr><td colspan="4" rowspan="3">6. AUTHOR(S)

No Authors.</td><td colspan="2">5d. PROJECT NUMBER
N/A</td></tr>
<tr><td colspan="2">5e. TASK NUMBER
N/A</td></tr>
<tr><td colspan="2">5f. WORK UNIT NUMBER
W1R8</td></tr>
<tr><td colspan="4">7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Wilmington
601 South College Rd
Wilmington, NC 19720</td><td colspan="2">8. PERFORMING ORGANIZATION REPORT NUMBER

N/A</td></tr>
<tr><td colspan="4" rowspan="2">9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Research Laboratory
Munitions Directorate
101 West Eglin Blvd.
Eglin AFB, FL 32542</td><td colspan="2">10. SPONSOR/MONITOR'S ACRONYM(S)
AFRL/RWTC</td></tr>
<tr><td colspan="2">11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-RW-EG-TR-2023-371</td></tr>
<tr><td colspan="6">12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release.</td></tr>
<tr><td colspan="6">13. SUPPLEMENTARY NOTES SUBJECT TO EXPORT CONTROL LAWS
DISTRIBUTION STATEMENT INDICATING AUTHORIZED ACCESS IS ON THE COVER PAGE AND BLOCK 12 OF THIS FORM. DATA RIGHTS RESTRICTIONS AND AVAILABILITY OF THIS REPORT ARE SHOWN ON THE NOTICE AND SIGNATURE PAGE.</td></tr>
<tr><td colspan="6">14. ABSTRACT
The major goal of the project was to develop a new center of expertise called the Neurophysiology Core at the AFRL RW/RWWI Natural Systems Sensing Laboratory (NSSL) at Eglin AFB. My aim was to establish four cutting-edge research methodologies at the NSSL, both building upon existing approaches (e.g., electrophysiology) and introducing new approaches to the lab. Establishing the Neurophysiology Core was aimed at serving an immediate goal of studying polarization vision in flying insects (e.g., tabanid flies and others) and a future goal of understanding sensory processing in these systems, generally. This work had four objectives for establishing the four following methods at the NSSL: The products of this effort were 1) establishing the proposed Neurophysiology Core methods at the NSSL, 2) developing four protocols associated with those methods, 3) recording and archiving of four master lectures on the Neurophysiology Core methods, 4) cross-training of AFRL RW/RWWI personnel and AFRL Scholars on the Core methods, 5) mentorship of AFRL Scholar research involving the Neurophysiology Core, and 6) preliminary data collection using each of the four methods as it relates to tabanid polarization vision.</td></tr>
<tr><td colspan="6">15. SUBJECT TERMS
Connectome, neural models of polarization vision, neural models of insect vision, neurophysiology of polarization vision</td></tr>
<tr><td colspan="3">16. SECURITY CLASSIFICATION OF:</td><td rowspan="2">17. LIMITATION OF ABSTRACT

SAR</td><td rowspan="2">18. NUMBER OF PAGES

21</td><td>19a. NAME OF RESPONSIBLE PERSON
Nicholas I. Rummelt</td></tr>
<tr><td>a. REPORT
Unclassified</td><td>b. ABSTRACT
Unclassified</td><td>c. THIS PAGE
Unclassified</td><td>19b. TELEPHONE NUMBER
(850) 883-0886</td></tr>
</table>

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

Section **Page**

List of Figures vi

INTRODUCTION 1

Protocol I: Electroretinography 2

Protocol II. Neural Tract Tracing within Insect Brains for Imaging by Fluorescence Microscopy 3

Protocol III: Immunohistochechemistry of Target Proteins in Insect Brains for Imaging by Fluorescence Microscopy 9

Protocol IV: PTA-staining for Enhanced-Contrast Imaging of Eyes/Neural Tissue by Micro-CT 13

LIST OF FIGURES

Page

Figure 1 The four research methodologies established at the NSSL by this effort…………………..1
Figure 2 Optical train of the updated electroretinography..3
Figure 3 Recording chamber of the updated electroretinography 3
Figure 4 Insertion of lipophilic neural tract-tracing papers into the eyes of a tabanid fly.......8
Figure 5 Successful fluorescent tracing of photoreceptor pathways and ventral regions of tabanid eyes 8
Figure 6 Successful immunohistochemical staining of target revealing neuronal architecture of the tabanid brain..12
Figure 7 Coronal section of the right eye of a tabanid..12

Introduction

1. Major Goals and Objectives: The major goal of the project was to develop a new center of expertise called the Neurophysiology Core at the AFRL RW/RWWI Natural Systems Sensing Laboratory (NSSL) at Eglin AFB. My aim was to establish four cutting-edge research methodologies at the NSSL, both building upon existing approaches (e.g., electrophysiology) and introducing new approaches to the lab. Establishing the Neurophysiology Core was aimed at serving an immediate goal of studying polarization vision in flying insects (e.g., tabanid flies and others) and a future goal of understanding sensory processing in these systems, generally. This work had four objectives for establishing the four following methods at the NSSL:

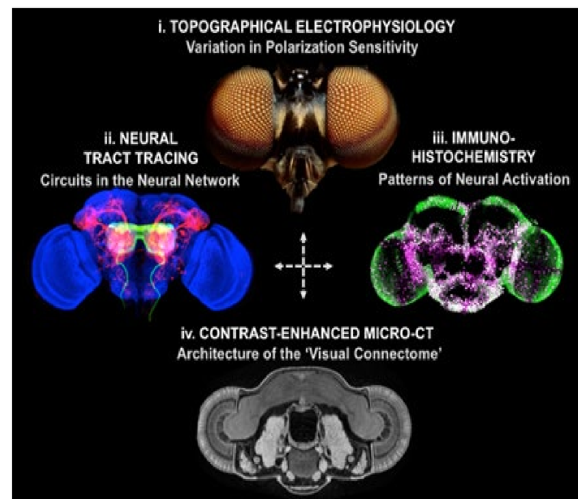

Fig. 1 The four research methodologies established at the NSSL by this effort.

i. Topographical electrophysiology to measure variation in polarization sensitivity over the eye surface. *The goal was to expand NSSL's current electroretinography (ERG) and StarGate rigs, which measure the electrical responses of eyes to light, by adding circularly polarizing filters and performing a combination of intracellular and extracellular recordings for assessing polarization sensitivity.*

ii. Neural tract tracing to reveal circuit pathways connecting polarization detection to processing centers in the brain. *A series of neuronal tracers, capable of labeling neural circuits occurring within and between neural cells, were injected into the photoreceptive units (i.e., ommatidia) of insect eyes to help visualize the connectivity of the polarization vision network.*

iii. Immunohistochemistry to reveal patterns of neural activation and thus, the functional organization of the polarization vision network. *A conserved immediate early gene called Hr38 serves as a biological marker of activated neurons. We worked to reveal patterns of information processing in polarization vision by working to develop an effective antibody for HR38 visualization and by applying other antibodies known to reveal neuronal architecture.* iv. Contrast-enhanced micro-CT via phosphotungstic acid (PTA) staining and image segmentation to visualize the structural architecture of the visual system 'connectome'. *We worked to produce a three-dimensional visual blueprint of the polarization vision neural network.*

2. Accomplishments: The products of this effort were 1) establishing the proposed Neurophysiology Core methods at the NSSL, 2) developing four protocols associated with those methods, 3) recording and archiving of four master lectures on the Neurophysiology Core methods, 4) cross-training of AFRL RW/RWWI personnel and AFRL Scholars on the Core methods, 5) mentorship of AFRL Scholar research involving the Neurophysiology Core, and 6) preliminary data collection using each of the four methods as it relates to tabanid polarization vision. The methodological protocols and associated preliminary data are as follows:

Protocol I: Electroretinography – Vi3 Automated Scan Static Polarizer File Setup

Step 1: Set Scan Parameters: start and end wavelengths and interval wavelength ○ make sure shutter position zeros = true zero on the motor/filter combos

Step 2: Set Shutter times: accommodation and monochromator shutter open time

Step 3: Set Accommodation start delay and accommodation end delay ○ 5 second delay between accommodation and monochromator flash is a nice start point.

Step 4: Set 'time between measurements' – both allows motor to move and allows eyes to recover - see note - must be longer than time it takes for motor to move.

Step 5: Set number of times open (is for mono shutter) = how many flashes at a given wavelength that you want

Step 6: Set mono start delay and end delay – amount of time record on the oscilloscope before and after flashes from both shutter

- If using the polarizer – set polarizer angle to true angle. When scanned finishes, change the folder name to include the polarizer angle information.

Step 7: Hit the white arrow to run the file.

Step 8: Hit Go scan

- At the end of the day, return to the vi1 (manual monochromator and motor control) and zero out all motors.

<u>Setting up a LUT file</u>

Step1: Open vi1 file; open to set monochromator wavelength

Step 2: Open Spectrasuite software, load calibration files and set parameters the same for both files.

Step 3: Set rig to max intensity of 350nm (weakest increment) – must set NDF to clear zone - 30deg (0 to 90 is clear).

Step 4: Measure transmission at that max intensity of 350nm = the goal photon flux at each increment.

Step 5: Move to the next increment and take measurement. Then look at reference table to estimate NDF degrees to balance transmission. Repeat for every increment.

Step 6: Press stop, exit vi file – run an automated scan to make sure photon flux is equal across wavelengths.

END PROTOCOL

Fig. 2 Optical train of the updated electroretinography (ERG) rig. This effort led to 1) rebuilding of the ERG rig after it was first transported to the NSSL, 2) creation of new look-up tables (LUTs), 3) insertion of a rotating lineaer polarizer in the optical train, and 4) the design and creation of an electrode set for differential electrophysiological recording.

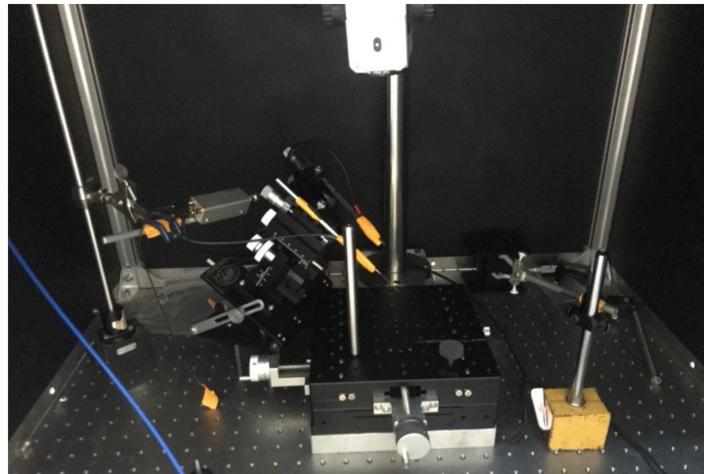

Fig. 3 Recording chamber of the updated electroretinography (ERG) rig. A custom electrode set allowing differential recording (i.e., recording vs reference and ground) was installed and optimized for recording from insect eyes. The rig is now outfitted for ERG recordings of polarization sensitivity. Topographical measures, such as from the dorsal vs. ventral eye aspects, are now possible.

Protocol II. Neural Tract Tracing within Insect Brains for Imaging by Fluorescence Microscopy

Gelatin Coating Slides

1. Place glass microscope slides in dipping racks
   - o purchased slides should be glass microscope slides (uncoated) with correct thickness for the microscope (i.e., #1 thickness slides; 25 x75 mm slides & #1.5 coverslips; 24 x 60 mm)

2. Clean slides in sonicating bath: fill sonicator with tap water and .5 scoopula of Alconex and set agitation for 30 minutes.

3. Remove racks from sonicator and rinse with DI water.

4. Dip slides in 70% ethanol
   - o 30ml DI + 70ml pure ethanol ratio at a final volume required to cover the slide racks in the dipping container

5. Place slides in a warm incubating oven to dry thoroughly (e.g., overnight)

6. Make a 0.5% gelatin solution (final volume 1000ml)
   6a. heat 500 ml of deionized (DI) water to 60°C on a heat plate (with stir bar)
   6b. slowly add 5g of porcine gelatin in the warmed water and let dissolve 6c.
   let it dissolve and add DI water to reach a final volume of 1000ml.

7. Pour warmed gelatin solution into a dish.

8. Place slide rack(s) into the solution and tap on the bottom of the dish to remove air bubbles.

9. Let racks sit for 5 minutes.
   - o use a transfer pipette to pop bubbles at surface of solution so when rack is removed, they are bubble free.

10. Dry overnight in a 55°C oven.

11. Repeat 2x for a total of 3 separate gelatin-solution dips.

12. Store coated slides in a slide box at room temperature.

Preparing Solutions

1. Make 0.1M phosphate-buffered saline (10x PBS) as a concentrated stock solution.
   1a. see manufacturer's instructions for recipe.
   1b. current supply requires pouring 1 pack of PBS solute into 1000ml of DI water.

2. Make 0.01M phosphate-buffered saline (1x PBS) as a working stock solution 2a. mix 100ml 10x PBS into 900ml DI water (1000ml final volume).

3. Make 4% paraformaldehyde in 0.01M phosphate-buffered saline (4% PFA in 1x PBS) 3a. dilute concentrated PFA (e.g., 16% or 20% PFA) from ampoules into 1x PBS 3b. use "concentration1volume1 = concentration2volume2" (c1v1=c2v2): 20% PFA concentration (50 ml of PFA) = 4% PFA concentration (X volume of 1xPBS); 1000 = 4x is 250 ml final volume; thus 50 ml of 20% PFA + 200ml of 1xPBS = 250ml volume of 4% PFA

Sample Preservation

1. Remove head from insect.
2. Under a dissection scope, dissect the brain from the head for fixation.
   - using microdissection scissors and fine forceps remove the back of the head then tease the brain tissue away from the eyecups, then cutaway the eyecups isolating the retinas, optic lobes and rest of the brain in one intact piece.

3. Place brain in fixative (4% PFA in 1x PBS) in a vial for 24 to 48 hrs on a shake plate at room temperature; the volume of fixative should be ~6x times the volume of the head allowing for good perfusion.

4. After a maximum of 48hrs in fixative, either place the sample in 1x PBS for long term storage (in 4°C) or begin neural tract tracing protocol below.
   - note, leave a few drops of fixative in the vial with the 1x PBS to inhibit bacterial growth.

Insert NeuroVue Lipophilic Tracer

1. Under a dissection scope, cut small triangular pieces of the NeuroVue papers for insertion into the insect brain.
   - use microdissection scissors and fine forceps to cut the NeuroVue papers and clean your tools with 70% ETOH between handling different NeuroVue paper dyes.

2. Insert the cut NeuroVue paper into the region of interest.
   - under a dissection scope, use fine forceps to insert the sharpest corner of the cut triangle into your sample where desired.

3. Return your sample to 4% PFA in 1x PBS and place in the incubating oven set to 37°C.

4. Let sample incubate for 1 to 3 weeks.
   - attempt to check the distance of dye tracing by imaging the intact specimen under the. fluorescence microscope at low magnification.

Note, an alternative method to using NeuroVue is the microinjection of DiI dye into the tissue using a fine gauge needle. DiI dye may coagulate within the needle, so consider intermittent dipping of needle in a hot bath.

Sample Preparation and Sectioning

1. After the incubation period, prepare the sample for cryosectioning by removing the NeuroVue papers and placing the sample into a 30% sucrose solution in 1xPBS 1a. mix 30g of sucrose into a final volume of 100ml of 1x PBS.

2. Remove excess fixative from the head using blue roll, then place into sucrose solution for overnight incubation at room temperature.
   - To avoid osmotic shock in sucrose, consider serial incubations of 20% then 30% sucrose.
   - Tissue should sink when adequately infiltrated with sucrose.

3. Make an albumin-gelatin solution for tissue embedding; this step gives the sample integrity for sectioning.
   3a. mix 4.2g porcine gelatin + 60ml 1x PBS + 1 egg yolk and heat to 40°C – keep warm on stir plate or in water bath; 1 week shelf life in fridge.
   3b. use a transfer pipette to put some mixture in a petri dish, place the sample on top, then cover with more mixture and place the petri dish in the fridge.
   3c. once coagulated, add more mixture using the transfer pipette while the sample stays in the fridge, repeat until thoroughly coated.
   3d. use a razor blade to cut a square around the sample and flip over onto more mixture.
   3e. coat the back of the sample thoroughly by adding coats of the mixture in the fridge.
   3f. again cut a square around the sample and place back into 30% sucrose and store in the fridge (short-term) until ready for cryosectioning.

4. Section your sample using OCT mounting medium (Tissue Tek) on the Leica Cryostat; load 6-8 µm thickness sections onto the gelatin-coated microscope slides.

5. Dry slides overnight at room temperature in a slide folder.

Counterstaining or Preparation for Coverslipping

1. Consider counterstaining the sections to provide background contrast and morphological context for neural tract tracing. The counterstain must also be fluorescent.
   - consider completing anti-alpha-tubulin immunohistochemistry as a counterstain. Currently, our NeuroVue dyes are ‘red’ and’ maroon’, thus the secondary antibody for visualizing our alpha tubulin antibody should be green, such as an Alexafluor 488 goat anti-mouse antibody.
   - see immunohistochemistry protocol for details.

2. To prepare for coverslipping, flood slides with fixative (4%PFA in 1x PBS) for 1 hr.
   - place slides on a paper towel under the hood.
   - use a transfer pipette to flood the slides; the liquid will adhere by capillary action.
   - this step ensures that sections adhere to the slide.

3. Complete 4x washes of slides for 15 min each.
   - place slides in tall plastic wash containers filled with 1x PBS for 15 minutes.

Coverslip Slides for Imaging

1. Remove excess 1x PBS from the slides by tapping slide edge on a paper towel.

2. Pipette 75 µl of Slowfade Gold antifade reagent with DAPI in a string of dots along the edge of the slide, adjacent to the sections.
   - this is a special mounting medium that extends the life of the fluorophores and makes nuclei fluoresce blue.

3. Lower a coverglass onto the slide; do so at an angle to push the DAPI along the sections without creating air bubbles.

4. Store slides in a slide book (in the fridge and in darkness) until ready to image.

END PROTOCOL

Fig. 4 Insertion of lipophilic neural tract-tracing papers into the eyes of a tabanid fly. The reagent NeuroVue Red has been inserted into the dorsal aspect of the eyes and NeuroVue Maroon has been inserted into the ventral aspect of the eyes.

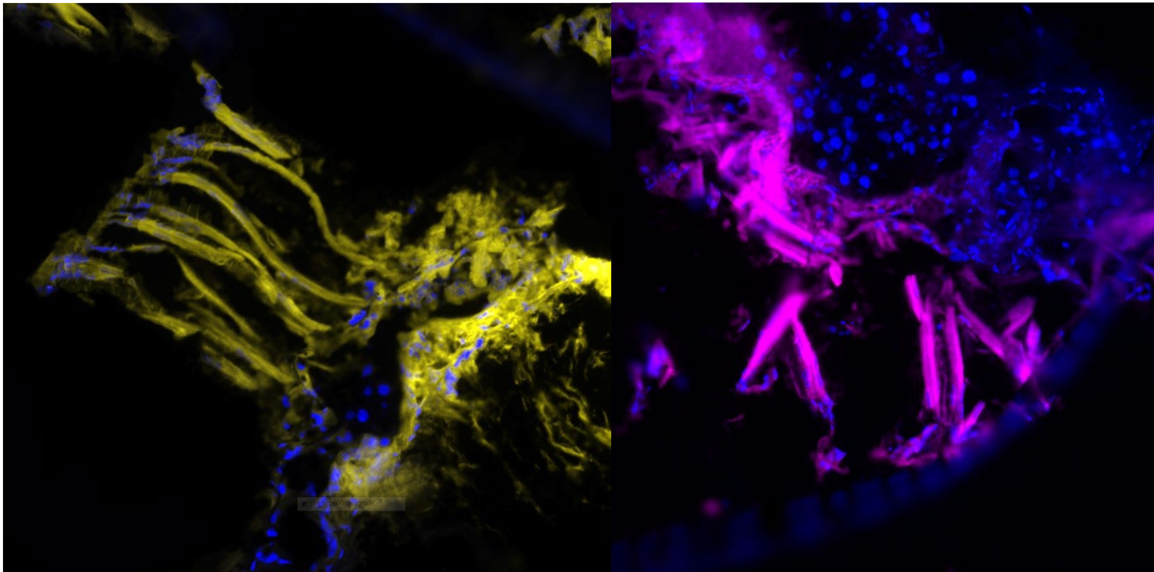

Fig. 5 Successful fluorescent tracing of photoreceptor pathways from dorsal and ventral regions of tabanid eyes. Left: NeuroVue Red and Right NeuroVue Maroon.

Protocol III: Immunohistochemistry of Target Proteins in Insect Brains for Imaging by Fluorescence Microscopy

Prepare slides, required solutions, and tissue sample as outlined in the neural tract tracing protocol above.

Immunohistochemistry Protocol

1. Flood slides with fixative (4%PFA in 1x PBS) for 1 hr.
   - place slides on a paper towel under the hood and use a transfer pipette to flood the slides; the liquid will adhere by capillary action.
   - this step ensures that sections adhere to the slide.

2. Complete 4x washes of slides for 15 min each.
   - place slides in tall plastic wash containers filled with 1x PBS for 15 minutes.
   - during these washes start the next two steps!

3. Make the 'CBT' (solution for diluting the antibodies; making a final volume of CBT needed to flood each of your slides (1 slide requires 0.5 ml of CBT); each slide will be flooded with antibodies/CBT twice. To make a ~10ml volume (enough for the full protocol for 10 slides):

   8a. mix 5 ml 0.5% λ- carrageenan + 5ml 1x PBS + 0.1g of protease free bovine serum albumin (BSA) + 30 µl of Triton-X; slowly mix BSA and stir until in solution.

   8b. place in fridge until needed.

4. Prepare the primary antibody dilution. To do this, dilute the primary antibody in CBT to the required concentration (often reported in the literature). The ratios below are shown in microliters (µl).
   - mouse, anti-alpha-tubulin 1:100 (Lin & Strausfeld 2013)
     Tubulin (-alpha) Antibody (12G10 alpha-tubulin) - DSHB (uiowa.edu)
   - mouse, anti-allatostatin 3:1000 (Lin et al 2021)
     https://dshb.biology.uiowa.edu/5F10
   - rabbit, anti-FMRF-amide 3:1000 (Lin et al 2021)
     https://www.immunostar.com/shop/antibody-catalog/fmrf-amide-cardio-excitatorypeptide-antibody/

EXAMPLE: To calculate the dilution, first I note the required final volume of CBT to cover my slides. Remember, 0.5ml if required to flood each slide. Thus if I have 10 slides, I will need a total of 5ml of CBT for the primary antibody step. (Note, the secondary antibody step will require an additional 5ml of CBT the next day). Of my 10 slides, I set two aside for control where I will omit primary antibody and flood them with CBT only. Thus, I have 8 slides requiring primary antibody and a total of 4 ml or (4000 µl) of CBT. To apply the mouse, alpha tubulin antibody at 1:100 concentration; first, pipette 4ml of fresh CBT into a vial, then pipette the required volume of the alpha tubulin antibody for a 1:100 concentration: 4000 µl of CBT/100 = 40; therefore, 40 µl of the antibody is required for 1:100. If I was double labeling with another

primary antibody. I would disregard the insertion of the alpha tubulin antibody and calculate the necessary dilution for the second antibody according to the final volume of CBT. Thus, both antibodies mix together and are simultaneously applied to each slide.

5. Flood slides with primary antibody for an 8hr overnight incubation.
   - o remove the slides from the wash container and place them on the antibody loading board, with the sample sections facing downward. Then, apply the antibody/CBT mixture under each slide using a P1000 pipette set to 500 µl (or 0.5ml).
   - o place in a ‘hydration chamber,’ which is a Tupperware containing wet paper towels. Store this container in the dark overnight.

6.Complete 4x washes of slides for 15 min each for 15 min each.
   - o place slides in tall plastic wash containers filled with 1x PBS for 15 minutes.
   - o during these washes start the next step!

7.Prepare the secondary antibody dilution o Alexafluor 555 goat anti-mouse (1:500)
   - o Alexafluor 488 goat anti-rabbit (1:500)

EXAMPLE continued: All slides (test and no-primary control) should be covered with a secondary antibody dilution in CBT. Thus, for 10 slides, I require 5ml (or 5000 µl) total volume of CBT. For a 1:500 dilution, mix 10µl of Alexafluor 555 goat anti-mouse (which will bind the mouse, alpha tubulin antibody) into the 5000 µl of the CBT.

8. Flood slides with secondary antibody for an 1hr incubation
   - o remove the slides from the wash container and place them on the antibody loading board, with the sample sections facing downward. Then, apply the antibody/CBT mixture under each slide using a P1000 pipette set to 500 µl (or 0.5ml).
   - o place in a ‘hydration chamber,’ which is a Tupperware containing wet paper towels. Store this container in the dark during the incubation.

9. Complete 4x washes of slides for 15 min each.
   - o place slides in tall plastic wash containers filled with 1x PBS for 15 minutes.

<u>Coverslip Slides for Imaging</u>

1. Remove excess 1x PBS from the slides by tapping slide edge on a paper towel.

2. Pipette 75 µl of Slowfade Gold antifade reagent with DAPI in a string of dots along the edge of the slide, adjacent to the sections.
   - o this is a special mounting medium that extends the life of the fluorophores and makes nuclei to fluoresce blue.

3. Lower a coverglass onto the slide; do so at an angle to push the DAPI along the sections without creating air bubbles.

4. Store slides in a slide book (darkness) until ready to image.

END PROTOCOL

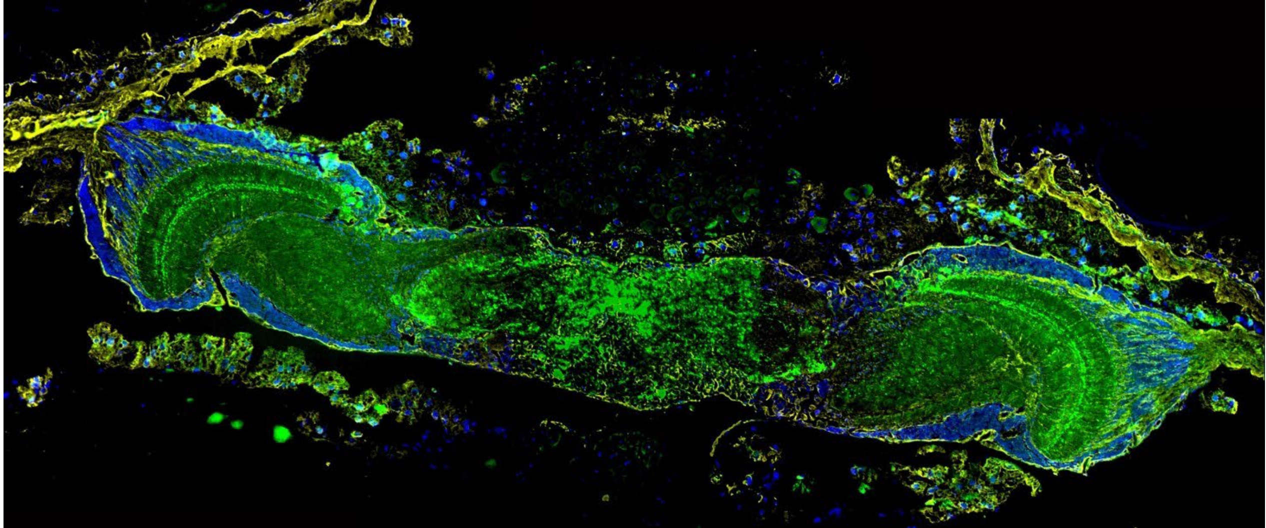

Fig. 6 Successful immunohistochemical staining of target revealing neuronal architecture of the tabanbid brain. Coronal section of the eyes and brain are shown. The structural protein alphatubulin is fluorescing yellow, the neuropeptide FMRF-amide is fluorescing green, and DAPI- nuclei stain has stained cell nuclei blue.

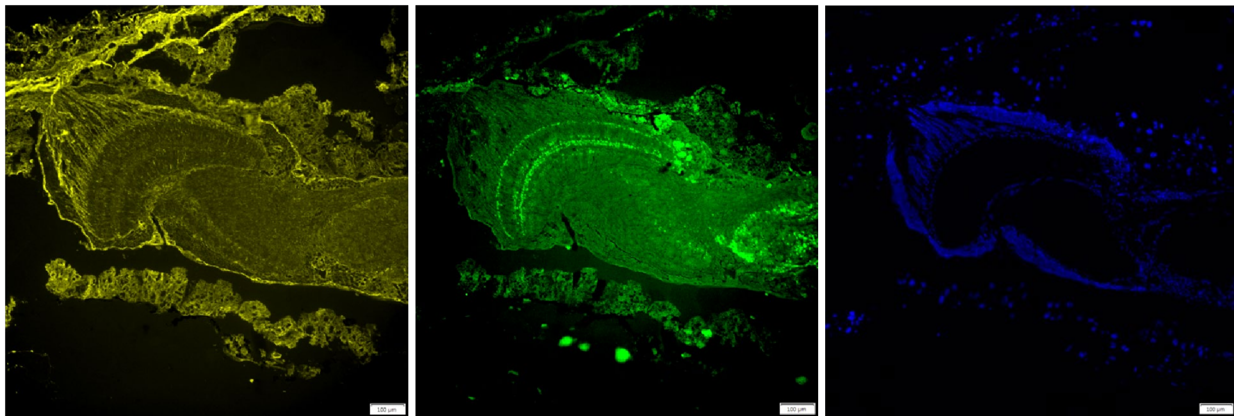

Fig. 7 Coronal section of the right eye of a tabanid is shown. Fluorescent channels are separated. The structural protein alpha-tubulin is fluorescing yellow, the neuropeptide FMRF-amide is fluorescing green, and DAPI- nuclei stain has stained cell nuclei blue.

Protocol IV: PTA-staining for Enhanced-Contrast Imaging of Eyes/Neural Tissue by Micro-CT

Removing the head and long-term storage

1. Remove head from insect.

2. Under a dissection scope, remove antennae, mouthparts, and any extra tissue using microdissection scissors; this allows fixative and PTA stain to adequately enter the brain.

3. Place head in boiling deionized (DI) water for 30 seconds.
   - this helps to prevent brain shrinkage when the head is stored in ethanol for a prolonged period of time; not necessary when examining visual structures/eyes.

4. Place head in 70% ethanol until the time you wish to stain the head.
   - 70% ethanol = 30ml pure ethanol + 70 ml deionized water.

Staining

1. Remove head from ethanol and remove excess ethanol using blue roll.

2. Place head in a vial with 1% PTA solution in 70% ethanol.
   - 1% PTA in 70% ethanol = 1g of phosphotungstic acid (PTA) in 100 ml of 70% ethanol.

3. Store at room temperature (ideally on a shake plate) for 7-14 days.

4. Prepare head for scanning by removing from the PTA solution, removing the excess solution with blue roll and placing the head in the smallest possible vial in 70% ethanol.

5. If head appears insufficiently stained after scanning, place the head back into the PTA solution to ensure effective staining.

6. Once stained, place back in to 70% ethanol until you are ready to scan.
   - unlike iodine stain, PTA stain does not easily overstain tissue or leech from the sample when removed from stain.

END PROTOCOL

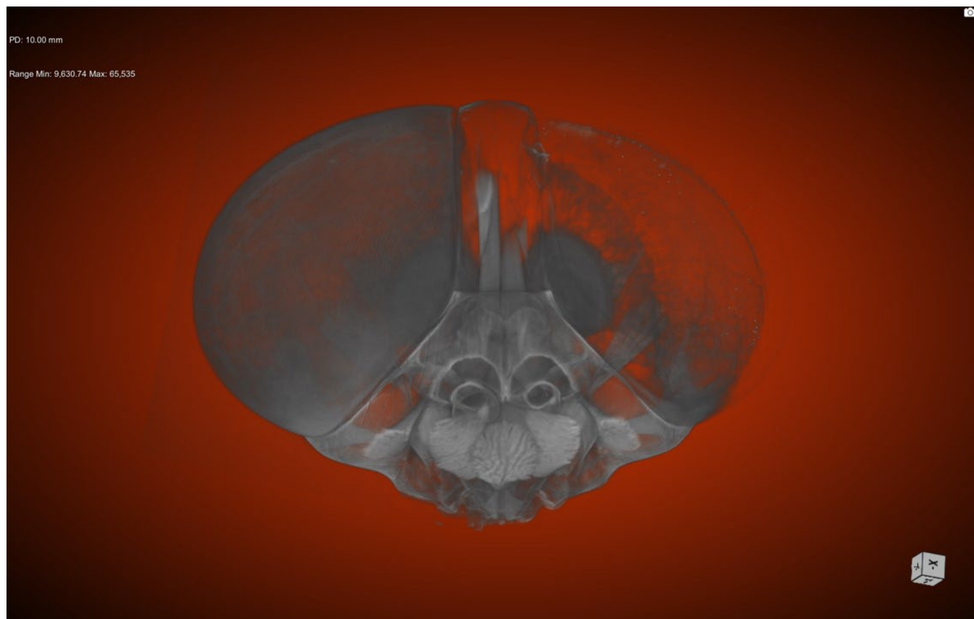

Fig. 8 Micro-CT imaging of tabanid eyes and brain following contrast enhancement by 1% PTA stain. The internal and external morphology of the eyes, as well as subdivisions of the brain are distinguishable. Partial segmentation of these structures shown on left eye.

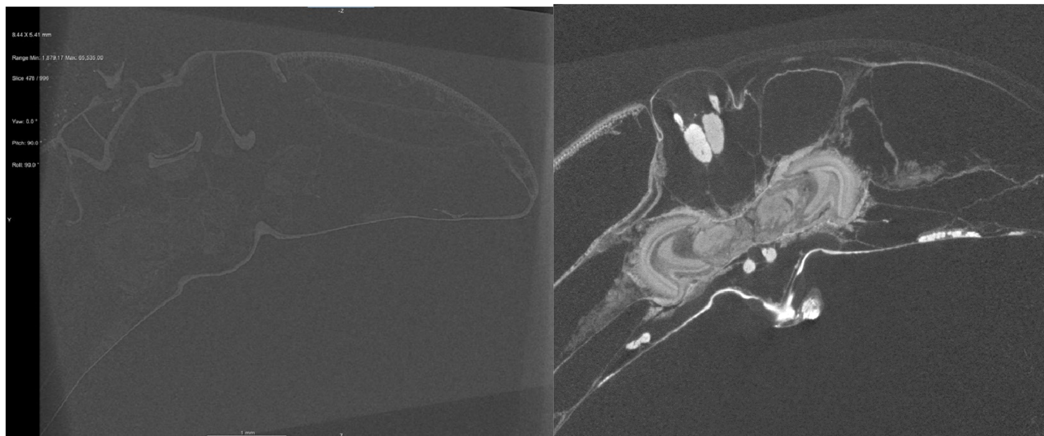

Fig. 8 Comparison of micro-CT visualization of tabanid brain with or without contrast enhancement. All scan parameters were held constant. Left: Micro-CT scan of tissue without contrast enhancement. Right: Micro-CT scan of tissue after a 1-week incubation in 1% PTA stain.

3. **Training Opportunities Under the Award:** Training opportunities under the award consisted of cross-training of AFRL RW/RWWI personnel and AFRL Scholars on Neurophysiology Core methods and mentorship of AFRL intern research involving the Neurophysiology Core.

4. **Dissemination of Results:** The four master lectures and four developed protocols on the Neurophysiology Core methods were archived to be openly accessible by NSSL personnel and AFRL scholars, present and future. Preliminary data on tabanid polarization vision has been archived for future publication with continuing work.

5. **Plans:** There are no research plans for the next reporting period as this is a terminal report.

6. **Honors:** During the 10-week reporting period, no honors or awards were received by the PI.

7. **Technology Transfer:** There were no technology transfer activities associated with this award.

8. **Participants:** Dr. Lorian E. Schweikert, Principal Investigator, 1.5 person-months of effort.

9. **Students:** There were no students or other personnel supported by this award.

10. **Products:** The products of this effort are the four master lectures and four developed protocols on the Neurophysiology Core methods.