Universal Tools for Analysis of Neural Function in Insects

Dianne Duncan, Quentin Coquerel, Ian Duncan, Barani Raman and Yehuda Ben-Shahar
Washington University Departments of Biology and Biomedical Engineering

The problem

Conservative estimates indicate that insects comprise about 40% of the total number of identified species on earth, and some sources place that value as high as 80% (1). Among these species are both those that are extremely beneficial to humans as pollinators and secondary food sources, and those that are profoundly detrimental as agricultural pests and disease vectors. Our goal here is to derive tools that can be used to interrogate neural activity in potentially ANY of the insects, in order to better understand, and perhaps modify, their behavior to the benefit of humans. We present here three ongoing molecular studies as well as a novel imaging system that we hope will be easy to use and applicable to most non-canonical insect species (and likely other arthropods as well). We are testing each of these techniques in both *Drosophila* and the orthopteran *Schistocerca americana* as proof of principle.

Solutions

The goal of our NSF NeuroNex Edge project is to develop physical and molecular tools for the analysis of neural function in diverse insect species. While much has been gleaned from studies in a few genetically tractable species, most notably *Drosophila*, the rich contributions of neural ethology in other arthropods (2,3) cannot be easily advanced without a similar robust molecular toolbox, particularly for species which have no whole genome information. To address this, we have begun to develop a suite of potentially universal molecular tools that will allow interrogation of neural function in a wide variety of insects, and likely be applicable to even more divergent arthropods. Preliminary data regarding three of these methodologies is presented here, along with a novel imaging technology which promises to greatly improve neural function analysis in live insects engaged in olfactory stimulation.

Conclusions

- Molecular and optical tools are being developed to allow for interrogation of neural function in insects
- The molecular tools should be applicable to species across multiple orders, whether or not a sequenced genome exists
- The optical system will allow fast, deep, highly resolved, two color imaging of brain activity in living insect transgenes with minimal perturbation of the subject during recording.

References

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Crispr/CAS9

We have chosen to take advantage of the wide conservation of the insect *white (w)* gene as a site for Crispr/CAS integration of the calcium marker GCaMP. *w* codes for an ABC-2 type transporter which is essential to transport pigment precursors to the pigment cells that promote eye pigmentation (4). When *w* is mutant, the eyes are still functional and the animals survive, but there is no pigmentation, and the eyes appear white. Genetic analysis suggests that *w* is present in many insect species across numerous taxa (5). To test this possibility, we have applied the same strategy that has proven to be successful for *Drosophila* to the orthopteran *Schistocerca americana* (The American Grasshopper). In this strategy, the *white* gene is replaced by a 'landing pad' for the phi C31 integrase that will result in animals with white eyes and concomitant expression of DSRed. Successful replacement of the cassette by phi C31 with a gene of choice will yield animals that can easily be identified by loss of DSRed expression.

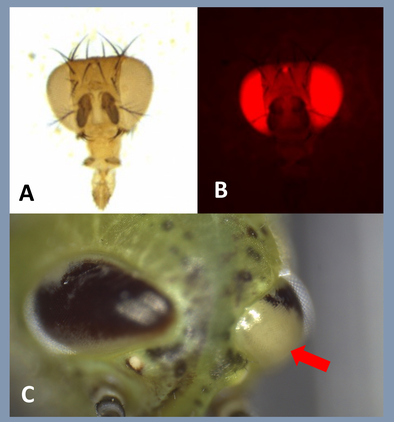


Figure 1.
A and B: *Drosophila* adult transgenic showing loss of *white* gene expression (A) and concomitant gain of DSRed (B) under the control of the eye promoter PXP3.
C: *Schistocerca* hatchling showing mosaic loss of *white* in patches in the eye (Arrow).

PiggyBac with defined promoters

In species where *w* cannot be utilized either because there is insufficient gene conservation or eye pigmentation is generated in a different manner, the PiggyBac system of transposition can be utilized. PiggyBac has proven to be remarkably promiscuous, working in a wide variety of insect cells. The OpIE2 Baculovirus promoter has been shown to express within hours after transfection in insect cells (6), and recently has been used to generate stable transgenes from Aedes aegypti (7). In *Schistocerca* embryos, OpIE2 driving DSRed can be detected in extraembryonic cells as early as 4 days post injection (Figure 2A-B), making it a reliable and robust marker for transient transfection which is then integrated stably into the host genome at high frequency. We have generated a plasmid, pMCSG6sOD, that has a large multiple cloning site upstream of the coding sequence for GCaMP6s in an OpIE2-DSRed background (Figure 2C). With *Schistocerca*, we are testing the ubiquitous promoters from *PolyUbiquitin*, muscle *actin* and *alpha tubulin*, and the pan-neuronal marker *nSynaptobrevin*.

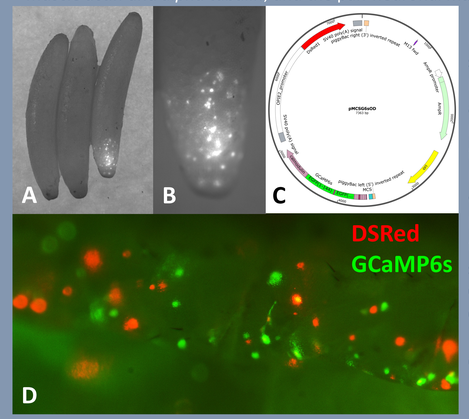


Figure 2. A-B: Embryo showing robust DSRed signal 4 days after injection. C: Map of the promoter tester plasmid. Functional promoters will drive GCaMP6s. D: *Schistocerca* transgene hatchling showing DSRed and GCaMP6s expression. GCaMP here is driven by a smaller version of the OpIE2 promoter.

PiggyBac as an Enhancer Trap

For species which have no genome information, a final molecular tool is being developed to utilize the PiggyBac integrating plasmid to be used in a screen for enhancers with useful expression patterns. This method has been used to great success in other species such as *Drosophila* (8), and will be facilitated by the early expression of DSRed to choose only those embryos that have potential for integration. Figure 3 shows the strategy for generating this plasmid; we are taking advantage of studies of the OpIE2 promoter to choose deletions that have been shown to eliminate OpIE2 enhancer function (6). By whittling down the size of the OpIE2 promoter just until DSRed expression is eliminated, we hope to define a minimal promoter that can then be cloned into the MCS of pOsG6sOD to generate an enhancer-tester.

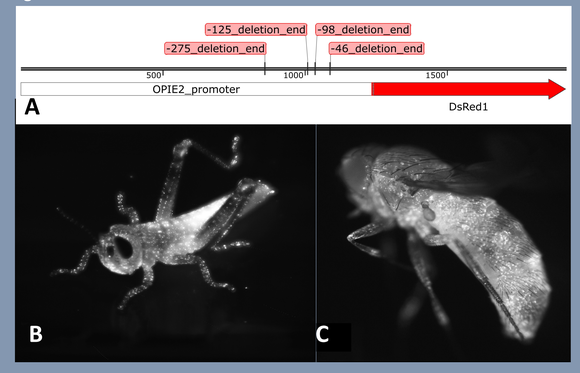


Figure 3. A: Deletion sites that have been shown to modulate IE2 expression . B-C: Expression of OpIE2-DSRed in *Schistocerca* and *Drosophila* transgenes injected with the same plasmid. The signal is confined to the same cell type (plasmatocytes) in each species.

A new Two-photon Single-Objective Dual-excitation Lightsheet Microscope (2PSODLM) is being developed to perform functional volumetric imaging of genetically labelled neural populations in locust brains:

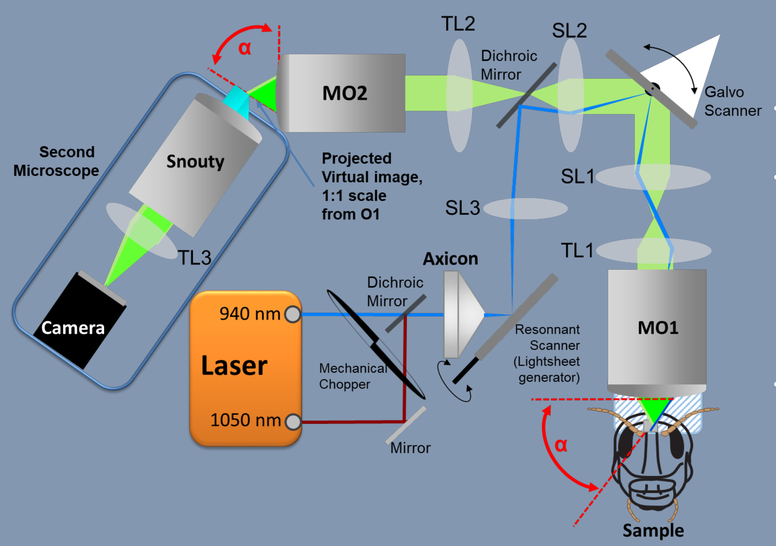
- Fast volumetric imaging of small in vivo tissue with limited access
- Simultaneously monitoring 2 distinct neuronal population,
- High temporal and spatial resolution (20 volumes per second at 60x60x100µm, and sub micrometer resolution in 3 dimensions)
- 2P ultrashort pulsed lasers improve tissue penetration and contrasts.
- Using a bessel beam to scan and create the lightsheet gives a more homogenous lightsheet along the projection axis, with the property of self-regeneration, limiting shadow projection commonly found in lightsheet microscopes

The 2PSODLM is composed of two microscopes in series:

•**Microscope #1** (from main objective 1 (**MO1**) to main objective 2 (**MO2**)) generates and scans the lightsheet at the sample with a 38° angle (α) via the single objective **MO1** into the brain. The light emitted from the sample is then collected at **MO1** and projected onto **MO2** at a 1:1 scale, free of optical aberrations, with the same angle α.

•**Microscope #2** (in blue box, on the left) captures and magnifies the virtual image formed after **MO2** with the virtual image in the same plan as the focus plan, avoiding any optical aberrations.

2PSODLM Design and Principles of Operation



- **Lightsheet generation:** Two ultrashort pulsed laser beams of 940 (GCaMP) and 1050nm (RCaMP) are merged and sent through an **Axicon** to generate the Bessel beam. A 12KHz **resonant scanner** generates the lightsheet. The scan lens (**SL3**) determines both depth of penetration and thickness of the lightsheet, and thus the Z axis resolution of the system.
- Note that the laser beam is shifted from the center of **SL3**, projecting the lightsheet with an angle α at **MO1** into the locust brain.
- **Correcting optical aberration:** The plane of excitation is projected with an angle α from the plane of focus of the objective. The signal fluorescence will therefore be emitted from a plane extending both above and under the plane of focus. This creates optical aberration degenerating the signal along the collecting optic path. The solution is to build a symmetric optic pathway by the scanning mirror, reversing the optical operation (MO1-TL1-SL1 ≡ SL2-TL2-MO2). The virtual image projected at **MO2** is therefore equivalent to the image at **MO1**, ready to be magnified.
- **Signal Magnification:** The second microscope forms an α angle with **MO2** axis, so the virtual image coincide with the "**Snouty**" lens focus plane. The **Snouty** lens allows for very close reimaging from **MO2** projection thanks to the specific glass piece added, allowing the capture of more emission signal compared to regular objectives. Tube lens 3 (**TL3**) and Snouty magnifications determine the total magnification of the microscope. The final image is projected on the camera sensor.