

***GCaMP Fluorescence is Not Increased in the Antennal Lobe of Drosophila  
in Response to Odor and Electrical Stimulation***

***Michael Claffey and Tristan Shuman***

***University of California, San Diego***

***Department of Physics, Department of Psychology, and Graduate Program  
in Neurosciences***

***The ability to locate food, avoid predators, and even to mate often requires olfactory signals to be correctly interpreted. Thus, olfactory perception is integral to survival in many species including drosophila. The fly brain is an attractive system to study because it can produce complex behaviors with a simple circuitry. Mapping this circuitry and investigating how a fly can produce such complex behaviors with limited synaptic inputs has become a topic of great interest. In the fly, olfactory signals are first recognized by sensory hairs on the antenna and these hairs contain projection neurons that link to one of 43 glomeruli in the antennal lobe in the brain. Recently, Wang et al. (2003) developed an imaging technique utilizing a transgenic calcium sensor (GCamp) located in projection neurons that could be imaged using 2-photon microscopy. They found that at ethologically relevant odor intensities, the pattern of activation in glomeruli was very sparse. Each odor activated only one or two specific glomeruli. This result indicated that these animals were able to code for specific odors simply by activating particular neurons, rather than activating a diverse pattern of neurons.***

***The G-CaMP system is uniquely qualified to address how olfactory information is encoded. The G-CaMP transgene contains a spliced copy of eGFP fused to the calcium binding site of calmodulin and to a M13 fragment of myosine light chain kinase. Under baseline conditions, very little Ca<sup>+</sup> is bound to the GCaMP structure and thus, very little fluorescence is emitted. When Ca<sup>+</sup> enters the cell, however, the conformational change caused by the binding of Ca<sup>+</sup> to the calcium binding site significantly increases the fluorescence. Thus, this system emits fluorescence only in the presence of calcium and can be used as a measure of neural activity.***

***The current study sought to answer 3 questions. First, could we replicate the detection of GCaMP expressing olfactory interneurons (Wang et al. 2003) using an epifluorescent microscope setup? Second, could we detect different patterns of activation in the glomeruli in response to odor delivery? Third, could detect activation in response to direct stimulation of the antenna nerve?***

## **Methods and Materials**

### ***Apparatus***

A microscope with an electron-multiply charged couple device (EMCCD) was used to obtain images of the preparation. Brightfield images used an incandescent lightbulb powered by a variable power supply which illuminated the preparation from the side opposite of the objective. Epifluorescent images used a broadband fluorescent lamp that illuminate the preparation through the objective. A series of filters ensured that light incident on the preparation was primarily limited to the excitation frequency of GCaMP (peak at 485 nm) while light entering the EMCCD was limited to the emission frequency of GCaMP (peak at 530 nm). The excitation light first passed through a 490DF20 filter before reflecting off a dichroic mirror towards the objective. Light returning from the objective was transmitted through the same dichroic mirror and then through a 536DF32 emission filter. A 4x air objective was used to acquire images in which the entire brain preparation was in the field of view while a 40x water-immersion objective was used to magnify the glomeruli alone. The microscope also contained a 1.25x inline magnification lens.

The EMCCD was an Andor iXon (Belfast, Northern Ireland) with 1004 x 1002 pixels comprising a 8 mm by 8 mm imaging area. Effective resolution with the 4x objective, as measured by imaging a metric ruler, was approximately 1000 pixels per millimeter. The CCD temperature was maintained at -20 degrees Celsius. Images were acquired using Andor's Solis imaging software. All images were acquired using a background correcting feature which removed the DC-offset of each pixel. Brightfield images were typically acquired using a 100 ms exposure time. Epifluorescent images with the 4x objective, used to localize the glomeruli, were taken with 5 second exposure and 8x8-pixel binning. Epifluorescent images with the 40x objective, used to analyze glomeruli fluorescent intensity, were taken in 1-second exposure sequences with 4x4-pixel binning.

The operation and coordinated timing of the equipment was controlled using custom code written in Matlab (The Mathworks, Natwick, MA). The computer running Matlab used a digital acquisition card with 8 BNC ports to send TTL signals to the EMCCD, a shutter blocking the florescent light source, a picospritzer (described below) and an electrical stimulator (described below).

Odor stimulation was achieved through two lines of compressed air. The first line was used as a dilution and flowed straight onto the prep, providing 90% of the air flow. This allowed for a minimal change in air pressure when switching between odor and non-odor

stimuli. The second line passed through a computer controlled valved that allowed switching between an odor and non-odor air flow. The odor air flow was directed through a vial containing the odor, and then combined with the dilution line. The non-odor air flow was directed straight to the dilution line. Two odors were utilized during these experiments. The first odor was Palmer's cocoa butter formula (E.T. Browne Drug Co., Inc., Englewood Cliffs, NJ) and the second odor was a rose scent (1,2-Propanediol, Sigma-Aldrich, St. Louis, MO).

For electrical stimulation, a glass pipette with an opening that was approximately 80 micrometers in diameter was used as a section electrode to secure the antenna nerve. A variable voltage stimulation unit was used to deliver current to the preparation. The prototypical stimulation was a train of 10 pulses at 100 Hz with a 1 ms pulse width. Stimulation parameters were varied throughout the experiment to deliver different voltages (2 - 99 volts), reverse polarity, increase pulse width to 2 ms or increase the duration of the pulse train.

### ***Preparation***

Transgenic flies expressing GCaMP in the olfactory project neurons, as described in Wang et al. (2003), were obtained from Jing Wang. All flies each contained four copies of the G-CaMP 1.3 transgene. These flies ***have G-CaMP expression limited to the projection neurons emanating from the antenna and projecting onto the glomeruli.***

Flies were decapitated and all head cuticle and connective tissue was removed. For olfactory experiments, an antennae-brain preparation was utilized. For electrical stimulation experiments, the antennae were separated from the brain to expose the antenna nerve. This dissection was performed in calcium-free adult hemolymph like (AHL) solution in order to reduce neural activity. AHL contained 108 mM NaCl, 5 mM KCl, 2 mM CaCl, 8.2 mM MgCl<sub>2</sub>, 4 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM trehalose, 10 mM sucrose, and 5 mM HEPES. The solution was titrated with NaOH to a pH of 7.4. Prior to imaging, calcium-free AHL was replaced by calcium-containing AHL.

### ***Procedure***

After dissection, the preparation was placed under the brightfield microscope using the low magnification objective (4x) to localize the fly brain. A fluorescent image was captured in order to locate the glomeruli of interest (Figure 1, see below). Fluorescent images at this magnification were captured using 5-sec exposure time and high levels of binning (8x8) in order to localize the strongest signal. Once centered, the high magnification objective (40x) was inserted and fluorescent images were captured using 3-sec exposure time and medium levels of binning (4x4; Figure 2, see below). The focus was then increased or decreased in ten unit increments until an optimal arrangement of glomeruli were visible and in focus.

To test ***whether we could detect different patterns of activation in the glomeruli in response to different odors***, we began by taking three 1-sec images of the baseline fluorescence. After two minutes, a 200ms spritz of odor was puffed onto the sample. We waited 800ms to allow diffusion of the odor to reach the sample, and then collected three 1-sec images. This cycle was then repeated five times, with two minutes between each cycle.

To test ***whether we could detect any activation in response to direct stimulation of the antenna nerve***, several different protocols were attempted with varying voltages, polarity, pulse width and duration of the pulse train. For each protocol, we took a series of seven 1-sec images with stimulation occurring after three seconds.

## ***Analysis***

To analyze the effect of dispersing odor over the preparation, we compared intensity within a selected region of interest (ROI) for odor versus no odor. A rectangular region of interests (ROI) was manually drawn around the glomerulae. The mean intensity of this ROI was calculated for each image. The mean ROI intensities for images taken after odor delivery were compared to ROI intensities taken without odor delivery using a 1-tailed independent t-test.

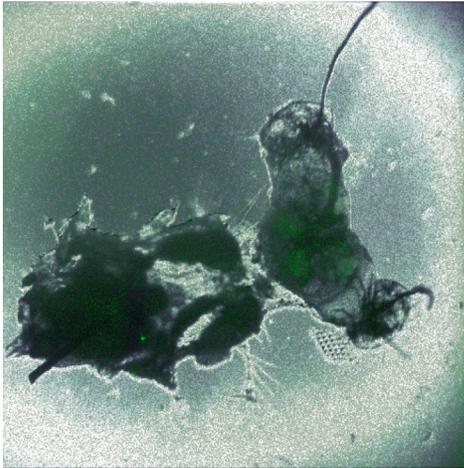
To analyze the effect of electrical stimulation, we again used mean intensities of ROIs. In addition to selecting an ROI around the glomerulae, we also selected an ROI of roughly equal size in the background image where no fluorescent was evident. This ROI would be used to control for DC drift in the camera. The mean intensity of both the glomerulae and background ROI were computed for each 1-sec image and these values were plotted across time for each set of stimulation parameters to visually identify a possible effect of stimulation.

## **Results**

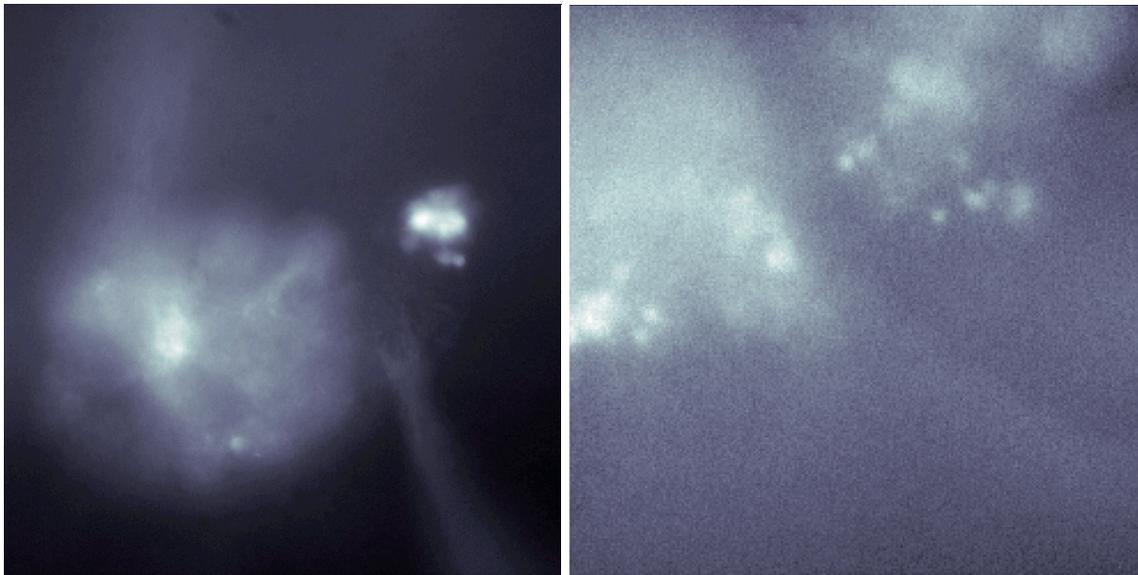
### **Question 1 – Can baseline fluorescence be detected?**

The first question we sought to answer was if we could detect G-CaMP expressing olfactory projection neurons using an epifluorescent microscope. Figure 1 shows a fluorescent image, seen in green, overlaid onto a brightfield image using low magnification. Fluorescence is clearly visible in the olfactory bulb and projection neurons. At high magnification, numerous glomeruli are visible (Figure 2). When the focus is adjusted to bring an optimal number of glomeruli into the focal plane, a distinctive pattern of glomeruli is evident. This background expression of G-CaMP in olfactory glomeruli clearly demonstrates that we were able to localize these inputs with an epifluorescent microscope. The main drawback of this setup is the background noise

produced by out of focus glomeruli. This problem could be resolved with a confocal or 2-photon microscope.



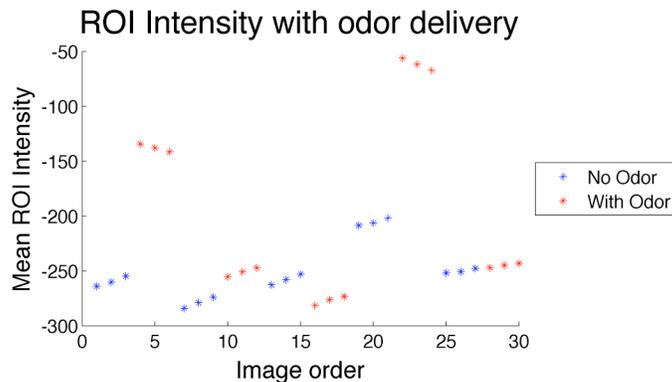
**Figure 1.** The C-shaped olfactory projection neurons clearly display baseline fluorescent activity. This composite image was created by overlaying a low magnification brightfield image with a fluorescent image. The fluorescent image was colorized as green for visual contrast. The right portion of the fly preparation is the brain; the left portion is the antenna and proboscis assembly. Width of field of view is approximately 1 mm; each CCD pixel imaged approximately 1 micrometer of preparation.



**Figure 2.** Distinct glomerulae are visible at higher magnifications. Width of field of view is approximately 100 micrometers; each CCD pixel imaged approximately 2.5 micrometer of preparation.

## Question 2 – Does odor delivery increase fluorescence?

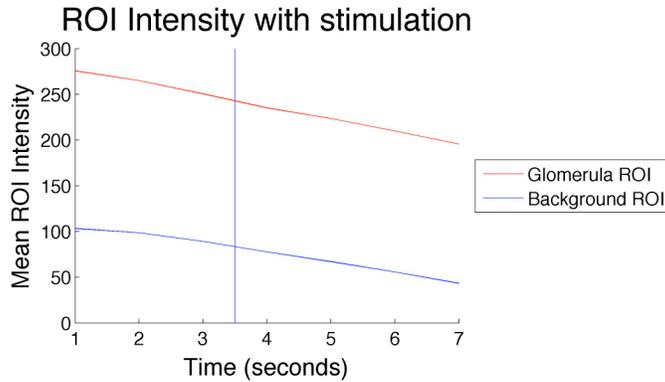
For the images collected during the odor delivery experiment, an ROI was manually drawn around the glomerulae and the mean intensity of the ROI was calculated for each image. These intensity means are shown in Figure 3. After either odor delivery or a recovery period without odor, three consecutive images were taken. From this figure it is apparent that intensity was dramatically greater for odor versus without odor for the 1st and 4th round of odor delivery. An dependent t-test of ROI intensity revealed that odor was significantly greater than without odor,  $t(14)=3.2$ ,  $p < .01$ . Intensity values are arbitrary units due to background correction.



**Figure 3.** Two instances of odor delivery resulted in substantially increased intensity in glomerulae ROI. The experiment consisted of sets of 3 1-second images following either odor delivery (red dots) or a baseline period without odor delivery (blue dots).

## Question 3 – Does electrical stimulation increase fluorescence?

For each combination of electrical stimulation parameters, a graph of glomerulae and background ROI mean intensity was generated in real time. Figure 4 shows a typical example in which a downward trend in both glomerulae and background ROI intensity is evident across time. This downward trend was attributed to a constant DC shift in the camera. Visual inspection did not detect any upward shift following electrical stimulation in either the glomerulae or background ROI. There were no detectable changes across the following adjustments to stimulation parameters: voltage at 2, 10, 20 and 99 volts; normal and reversed polarity; pulse width of 1 and 2 ms; pulse count of 10 (train duration of 100 ms) and 100 (train duration of 1000 ms).



**Figure 4.** No detectable increase in mean intensity of the glomerular ROI was observed following stimulation across a wide variety of parameters. This graph is an example of a single stimulation experiment. Time of electrical stimulation is indicated by the vertical line. The downward trend in both ROIs was attributed to a DC shift in the CCD.

## Discussion

We set out to examine how flies encode olfactory signals using a G-CaMP calcium indicator system localized to olfactory projection neurons. First, we asked if we could replicate the detection of olfactory glomeruli using an epifluorescent microscope setup. We successfully found G-CaMP fluorescence in the glomeruli of olfactory projection neurons in our setup. We were able to localize these glomeruli and measure baseline fluorescence. Next, we asked if we could detect distinct patterns of glomeruli activation in response to different odors. In our initial analysis during the time of the experiment, we did not detect any differences from baseline after an odor was applied. With the belief that the preparation was not responsive to odors, we opted to use electrical stimulation as a more reliable means to elicit a response. We were unable to observe a response to the electrical stimulation.

It was not until after completion of data collection that a new analysis of the odor experiment revealed a significant difference. The data are sparse and the effect appeared sporadically, so it is not convincing. Had we realized this potential effect at the time, we would have repeated the experiment to gather more substantial data.

There are a number of possible reasons why we did not see an increase in fluorescence after electrical stimulation. First, most of our dissections were completed by people without extensive experience in this exact preparation. The tools used were oversized and may have made it difficult to produce a viable prep. In addition, the AHL was not verified as being viable, as no evidence of cellular processes were evident. Thus, it could be that either the prep or the way we kept the prep was not adequate to keep that tissue alive long enough to see a significant signal. We could only focus on a limited number of glomeruli at any given time, and thus we may not have been observing the right focal plane to see a strong response. This is a disadvantage of our setup over confocal or 2-photon microscopes that can scan in an added dimension and easily look across multiple

planes. For electrical stimulations, there is no way to verify that sufficient current was ever passed through the stimulating electrode to elicit a response. One possibility is that the electrode was too large. The olfactory nerve was much smaller than the stimulating electrode and may not have made the necessary connection in order to create a large enough current for a calcium signal to be observed.

A number of technical issues limit the scope of our results. First, using an epifluorescent microscope inherently comes with a tradeoff in the focus of the z-plane. Thus, increased noise was present due to out of focus signal. Second, we had numerous issues with our odor and electrical stimulation setup. For the odor experiment, these included using an air puffing system with a submerged prep, possibly using insufficient concentrations of odor, and not being able to sufficiently flush the odor from the dish for repeated trials. For the electrical stimulation, these included using too large of a stimulating electrode. In addition, our CCD camera was inconsistent in its photon count. This was caused by a technical issue within the camera that was not corrected for. This is unlikely to be the cause of our lack of signal, however this did make it difficult to interpret between experiments as the signal was not consistent.

It is unfortunately that the odor effect was not recognized at the time of data collection. This would have altered the focus of the project to explore the issues of response magnitude, time course and selectivity as done in past research (Wang, et al, 2003; Root et al., 2007; Shang et al., 2007). However, the perceived need to turn to electrical stimulation provided us with hands-on experience in an additional technique. Regardless of the data and final conclusions, the project provided applicable experience across microscopy, specimen preparation and electrophysiology.

## References

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