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Gene Networks for Attention and Motor Control A Path from Drosophila to Humans

Ralph Greenspan
UNIVERSITY OF CALIFORNIA, SAN DIEGO

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14. ABSTRACT
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Summary

This grant effort was instrumental in promoting two main innovations. The first of these innovations is conceptual: we chose a simple instantiation of a fundamental problem, and describe an array of complementary approaches to enable its solution. We chose to study attention as an experimentally tractable and ethologically critical paradigm. We demonstrate by studying *Drosophila* tethered flight in a virtual reality arena, that flies possess the capability for top-down (endogenous or sustained) attention, assayed using distractible trace learning. Next, with the powerful genetic toolkit of *Drosophila* at our disposal, we performed a finer grained characterization of attentional neural circuitry involved, revealing concerted activity across brain regions to guide visual learning. Our success in a separate AFOSR DURIP award has further allowed us to combine the behavioral assay for attention with *in vivo* two-photon imaging to parameterize the response properties of specific neural structures during the distractible trace learning task.

The second of these innovations is technical: in this grant, we provide evidence supporting two novel technologies for long-term chronic brain imaging in naturally behaving flies. We strongly believe that development of such technologies is critical to fully understand the link between the perceptual state of the animal, its attentiveness and cognition. The first, Flyception, has begun to shed light on the neural dynamics of complex social behaviors that have otherwise remained inaccessible under conventional imaging paradigms. More importantly, our recent results offer the first glimpse of the impact of behavioral state modulation on neural circuit function. The second, FlyBAM, currently under development, will have an order of magnitude higher spatial resolution than Flyception, to enable activity monitoring at single-cell resolution on a brain-wide scale.

Trace conditioning in the attentive fly

Delay conditioning and trace conditioning (**Fig. 1**) represent different aspects of plasticity. Trace conditioning, the ability to hold a memory trace "in mind" during a time interval, is directly relevant to higher level cognitive functioning, especially when it is shown to be sensitive to distraction, a stimulus that intervenes in the time interval between the end of the conditioned stimulus (CS) and the beginning of the unconditioned stimulus (US). This gap distinguishes trace conditioning from classical delay conditioning, where the CS and US overlap. Distractible trace conditioning has been proposed as a behavioral paradigm for high-level attention (Carter et al., 2003). However, it is still unknown how the brain encodes CS traces and how they are associated with a US in trace conditioning. Additionally, in mice and humans, presentation of distractor stimuli selectively interferes with trace but not delay conditioning, suggesting a top-down attentional requirement for this type of learning (Han et al., 2003).

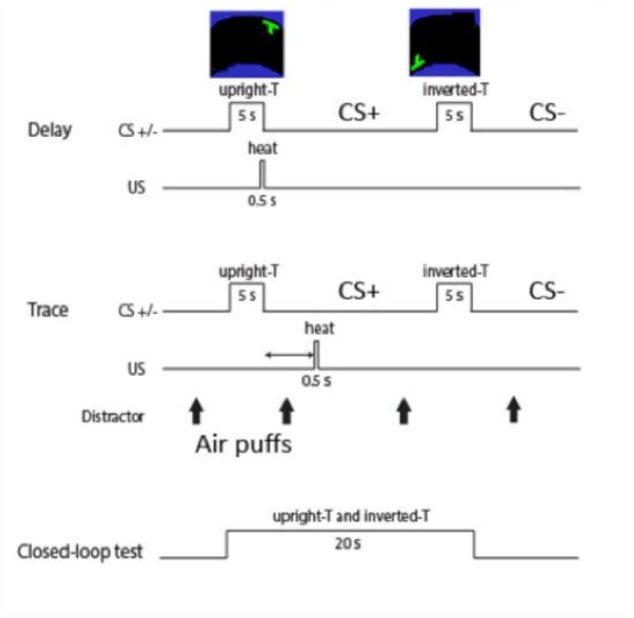

Figure 1: Delay and trace conditioning protocol for flies. Bottom row is the post-training test protocol.

As a necessary prelude to characterizing delay and trace conditioning in flies, we developed, during the current grant period, a spherical virtual-reality arena for tethered flies in flight. This setup employs a high-speed projector (912 x 1140 resolution, up to 4225 frames/sec refresh rate) to project computer-generated imagery on the walls of the sphere, immersing the enclosed tethered fly in a panoramic visual surround (**Fig. 2**). We coupled the flight simulator with an infra-red laser-based heat punishment and used gentle air puffs as a neutral distractor. Yaw-axis turning behavior was quantified by analyzing the amplitude difference between the left and right wing-beats using real-time machine vision techniques (Grover et al., manuscript under review) (**Fig. 3**).

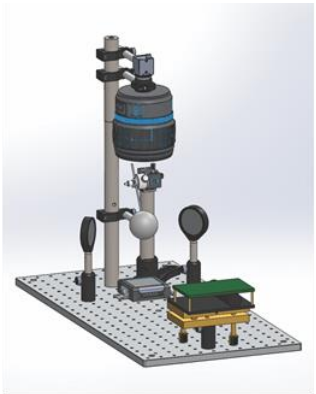

Figure 2: Tethered flight virtual-reality arena

In our experiments (**Fig. 1**), we present an image (an upright T shape) in the tethered fly's frontal field of view as the CS+ for 5 sec,

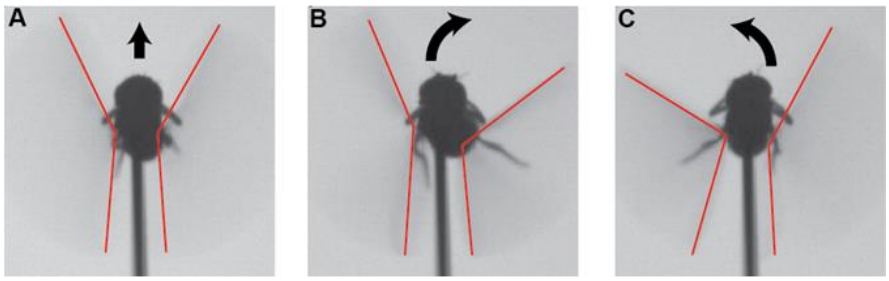

Figure 3: Yaw steering responses calculated by wing beat detection. (A) Forward flight with no torque, (B) Steering to right with clockwise torque, and (C) Steering to the left with counter-clockwise torque.

and an aversive heat stimulus (using an infra-red laser) as the US for 0.5 sec. As a control, a CS- stimulus (inverted T shape) is presented without any heat punishment. Variable trace intervals (2-40 sec) were tested with and without distractions. Post-training, both images are presented simultaneously 180 degrees apart to the fly in closed-loop mode, without the laser heat, and the fly's avoidance of the CS+ image as the tendency of orienting away from it, and towards the CS- image, is scored.

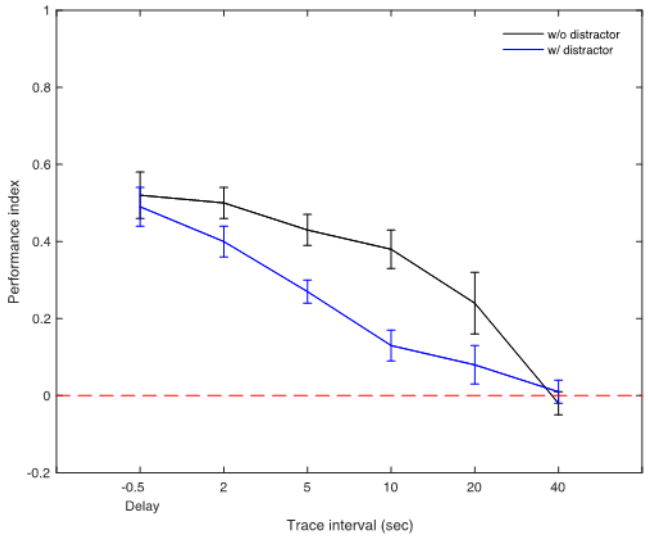

Figure 4: Delay and trace conditioning results. Performance index corresponds to anti-fixation of the CS+ stimulus. Mean and s.e.m, N=20.

We have now found that *Drosophila* is capable of distractible trace conditioning. As can be observed from our results, wildtype flies learn to associate the CS+ and US stimuli during both delay and trace conditions, up to a trace interval of ~20 sec (**Fig. 4, black curve**). Our data also suggests that much like mice and humans, distractors during the training period interfere with trace but not delay conditioning in flies (**Fig. 4, blue curve**).

Moreover, we have now also shown which specific brain regions in *Drosophila* are required differentially for delay conditioning, trace conditioning and for the distractibility of trace conditioning (**Figs. 5 and 6**). We used the Gal4-UAS system to genetically express an inwardly rectifying K+ channel, Kir2.1, to suppress neural activity in disparate neural structures in the fly brain and screen for effects on attentional behavior.

Under delay conditioning, both with and without distractions, flies with neural activity silenced in the Fan-shaped Body (FB) and to a lesser extent, Ellipsoid Body (EB) substructures

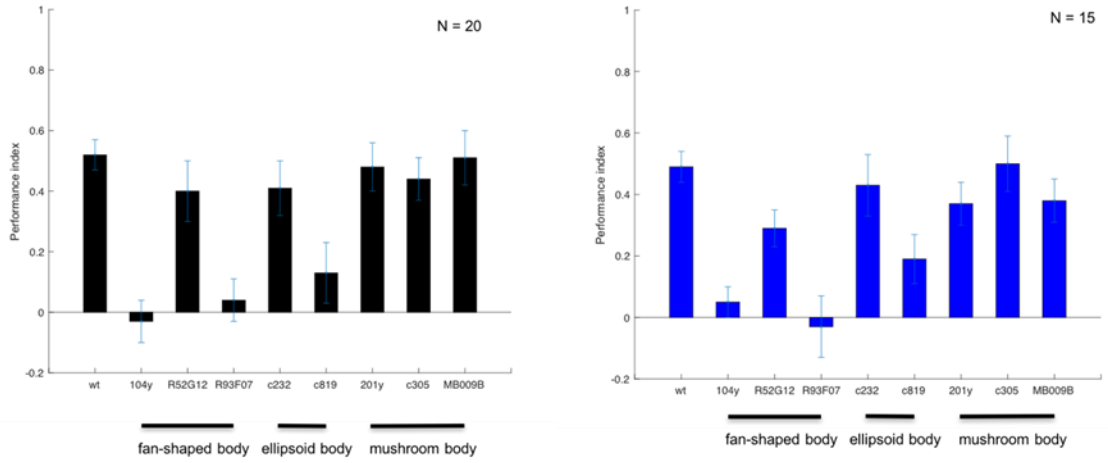

Figure 5: Delay conditioning results of targeted silencing using Kir2.1 of CX and MB neuron types without (left panel) and with (right panel) distractions.

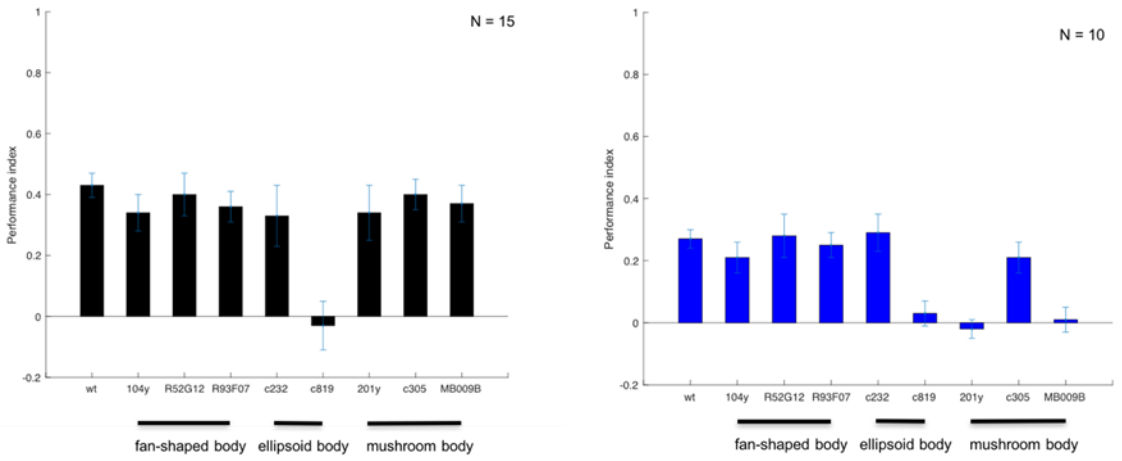

Figure 6: Trace conditioning (with 5 sec trace interval) results of targeted silencing using Kir2.1 of CX and MB neuron types without (left panel) and with (right panel) distractions.

of the CX displayed lower performance indices (**Fig. 5**). MB silenced flies displayed no deficits in delay conditioning, both with and without distractions.

When tested under a trace conditioning protocol (with a 5 sec trace interval), strikingly, FB silenced flies exhibited wildtype-like behavior, and flies with silenced neural activity in the EB, in particular R2 neurons, displayed a complete loss of trace learning (**Fig. 6, left panel**). When distractions were added to the trace conditioning protocol, again, silencing EB neurons caused a deficit in trace learning behavior. However, unlike delay learning with distractions, silencing of the MB neurons, in particular the gamma lobes of the MB, caused a loss of trace learning, suggesting perhaps the role of the MB as a gating mechanism to memory formation (**Fig. 6, right panel**).

Our most convincing evidence to date of the necessary architecture for attention-like processes points to the central complex (CX) and the mushroom body (MB) structures (de Bivort & van Swinderen, 2016). We have begun characterizing the role of these specific sub-structures in the brain using an *in vivo* calcium imaging preparation. We have successfully setup a two-photon microscope that can be rotated in space to gain optical access without significant perturbation to the fly's body position. A custom stage promotes natural flight behavior in the tethered fly, which receives a projector-based visual stimulation much like the tethered flight arena mentioned above. This setup has enabled us to measure neuronal activity under more realistic conditions for the fly and characterize visual response properties under both open and closed loop visual stimulation paradigms.

Our results suggest that EB activity is correlated with presentation of the CS stimulus. We have therefore begun to visualize the formation of the stimulus trace in EB neurons, as well as the effect distractions have on EB activity (**Fig. 7a**). We find that the CS stimulus trace persists as a slowly decaying activity trace in the EB and can last up to several seconds, and that distractions shorten the decay of EB neural activity while also lowering their overall intensity (Grover et al., manuscript in preparation) (**Fig. 7b, c**).

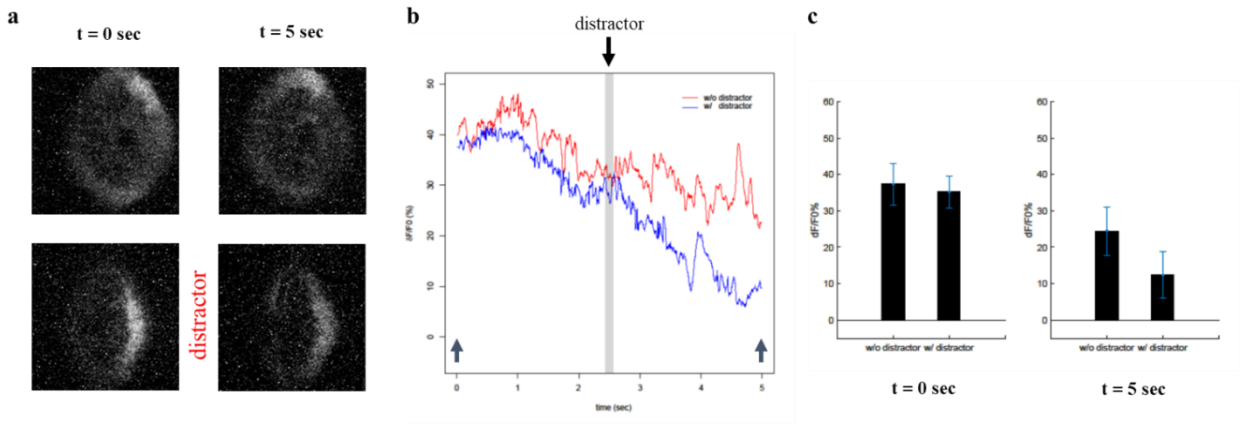

Figure 7: EB stimulus trace: (a) raw activity recording to CS+ stimulus in EB at 0s (left) and 5s (right). Distractor is introduced in bottom panel. (b) dF/F activity values of peak activity in EB with distractor (blue) and without distractor (red). Distractor is presented at gray bar timepoint. (c) Preliminary results of effect of distraction on EB activity, mean with s.e.m. of dF/F activity at 0s (left) and 5s (right), N = 3.

Dopaminergic neurons (DANs) are required for long term potentiation of the synapses that specify the association between the conditioned stimuli and the valence of approach (appetitive) or avoidance (punishment) (Aso et al., 2014ab). In the fly olfactory circuit, DANs act as positive or negative reinforcers. For instance, repeated punishment in the presence of an odor progressively weakens the synapses between odor coding and output neurons in the MB, reinforcing the negative association ultimately leading to a learnt avoidance response. This general mechanism appears to hold true even for the case of visual learning. Our results indicate that a complete silencing of DANs in the brain show a total loss of delay/trace learning (Xi et al., 2008; Zhang et al., 2007) (**Fig. 8**). In addition to the MB, regions in the CX are also heavily innervated by DA terminals, however their role in visual object learning has yet to be identified (Mao & Davis, 2009).

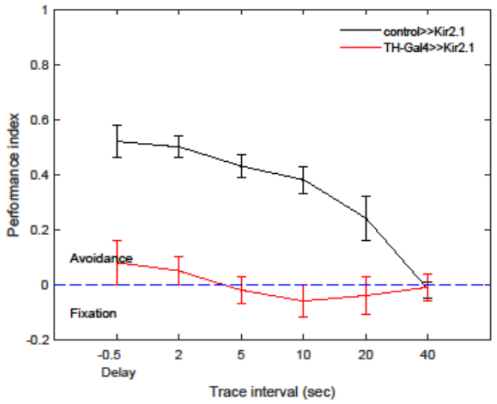

Figure 8: Inactivation of dopaminergic neurons (red) leads to loss of delay and trace learning compared to control (black). Mean and s.e.m., n = 20

By examining tyrosine hydroxylase (*TH-Gal4*) expression and anti-TH immunoreactivity in the *Drosophila* brain, one subtype of the PPM3 cluster of dopaminergic neurons has been shown to project to the FB (**Fig. 9a**) and another subtype of that same cluster was found to project to the EB (**Fig. 9b**) (Mao & Davis, 2009). Thus, FB and EB could be viable candidate structures where CS and US signals merge, and we are currently confirming this possibility.

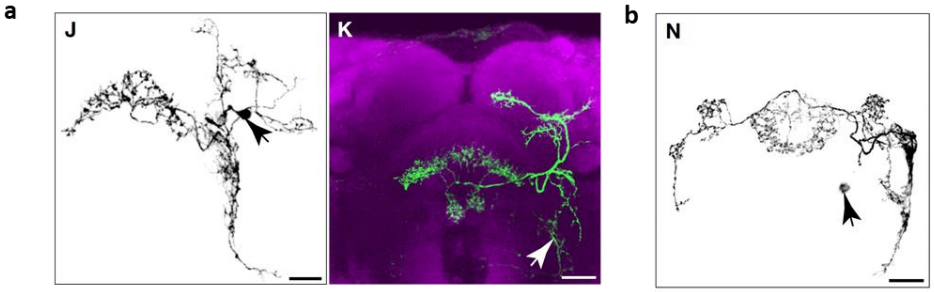

Figure 9: PPM3 cluster of dopaminergic neurons projecting to FB (a) and EB (b)

Impact of perceptual state on attentiveness and cognition

Both in vertebrates and invertebrates, various neuromodulators have been implicated in the regulation of higher cognitive function (Bargmann & Marder, 2013). For instance, Dopamine has been shown to modulate a wide array of behaviors and cognitive processes such as selective attention (van Swinderen & Brembs, 2010), arousal (Ueno et al., 2012), aggression (Alekseyenko et al., 2013) and mating drive (Zhang et al., 2016) to name a few (Riemensperger et al., 2011). Octopamine has been implicated in modulating gain control of sensory stimuli (Suver et al., 2012), while distinct serotonergic neurons can act as inhibitory modulators of specific behaviors and induce quiescence (Pooryasin & Fiala, 2015). Neuropeptides such as neuropeptide F have been shown to play a role in reward-based learning (Shohat-Ophir et al., 2012). *Therefore, a complete understanding of how attention regulates information flow in the brain would require addressing the link between the perceptual state of the animal and its attentiveness.*

In the tethered fly visual experiments described in the previous section, the object of the fly's attention was inferred by a flight bias towards or away from the presented stimulus. Furthermore, these behavioral techniques simultaneously coupled with neurophysiological readouts provide critical supporting evidence for attention-like mechanisms in the brain. However, to study how underlying perceptual state might affect attention, one must first possess the capability to systematically alter the animal's perceptual state, and then assay for the attentional consequences.

To accomplish this, we need to resolve two major challenges. *First, a fly needs to be kept alive for days after surgically removing the cuticle and creating an optically clear window to the brain.* This is because the invasive nature of *in vivo* imaging preparations could affect the brain state of the flies. *Second, development of an imaging system that allows for chronic imaging with minimal undue effects to their internal brain state.*

We recently developed a method to create an optically clear window on the fly's head and perform chronic brain imaging, similar to cranial window implantation in rodents (Trachtenberg et al., 2002) (**Fig. 10**). The advantage of this technique is that it seals the brain (after removing the cuticle) with a transparent silicon elastomer allowing operated animals to be untethered after surgery and subjected to behavioral tasks while allowing for direct optical access to the brain (Grover et al., 2016). As a result, fruit flies can be made amenable to functional brain imaging in real-time or in time-lapse over the course of seconds to days.

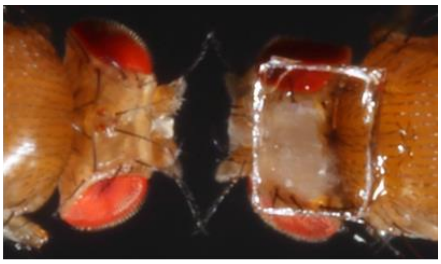

Figure 10: Comparison of a normal fly with one where the optical window is surgically created

The second challenge involves imaging brain activity in an animal repeatedly over a period of days. Conventional methods of *in vivo* calcium imaging, including those described in the previous section, have necessitated immobilizing the animal's head to a fixture, followed by removing of the cuticle (to expose the brain) and bathing of the head capsule in saline to prevent desiccation (Seelig et al., 2010). A caveat of this approach, however, is that the fly will only survive for a few hours at best, and is therefore not suitable for chronic and repeated imaging. Some previous studies have attempted to couple creating a chronic imaging window in the fly head with a technique to reversibly attach the animal to a fixture for imaging purposes (Huang et al., 2018). While this technique allows for repeated imaging of the same fly over a long period of time, the animal would still need to be anesthetized before every imaging session to secure it for

positioning under the imaging device. The undue effects of anesthesia might be limited if the imaging sessions are few and far between, however, studying changes to the fly's brain state might require finer time-lapse imaging to capture subtle changes in patterns of brain activity. *Therefore, an approach to image brains from naturally behaving flies would be required to accelerate our understanding of the neural basis of complex behavior.*

We recently developed such a method, 'Flyception', which for the first time enabled studying neural dynamics in freely behaving flies (Grover et al., 2016). It does so with a galvanometer-mirror based real-time tracking system that accurately locates the brain of a freely walking fly on multiple imaging sensors at 1,000 frames/sec (**Fig. 11**).

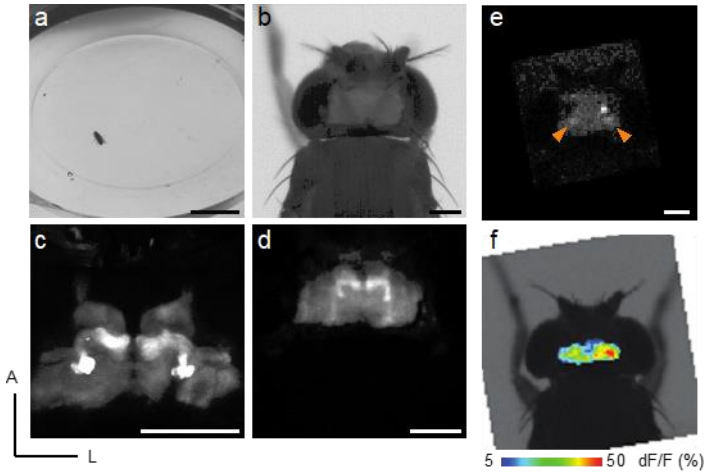

Figure 11: Flyception free walking brain imaging system for fruit flies. a) behavior arena, b) imaging window on the head, c) confocal image of the brain, d) fluorescence brain image obtained with flyception, e-f) GCaMP activity in male fly's lateral protocerebrum during courtship. Scale bar, 200um

Although this technique shed light on behaviors that had otherwise remained inaccessible under conventional imaging preparations, it was not without its limitations. For instance, the tracking algorithm relied on detecting the contour of the fly head, and contacts between flies would therefore create a challenge. Also, the cameras were focused to a fixed plane, and any behavior that caused the fly's head to deviate from that focal range was not captured. As such, it was not possible to study brain activity in flies at all stages of the mating sequence, especially during copulation where flies are not only in physical contact with each other, but also the male mounted on the female moves out of the cameras' focal range. During the current grant period, we have successfully developed 'Flyception2', a new system that features three major enhancements to expand the range of behaviors accessible under the untethered imaging paradigm (Grover et al., manuscript in preparation).

First, we sought to improve the robustness of the tracking algorithm, especially when flies made physical contact with each other. To do so, we developed a method that tracks a retro-reflective marker placed on the coverslip over the fly's head. The marker consisted of three 30 um diameter glass beads hemi-spherically coated with aluminum attached along the edges of the imaging window in a triangular pattern. The retroreflective marker reflected the overhead collimated infra-red light directed at the fly through the galvanometer mirror system back to the tracking camera. The tracking algorithm detected the illuminated markers as three bright spots on an otherwise dark background, the centroid of which was used as an estimate of the center of the fly head to update the galvanometer mirrors (**Fig. 12**).

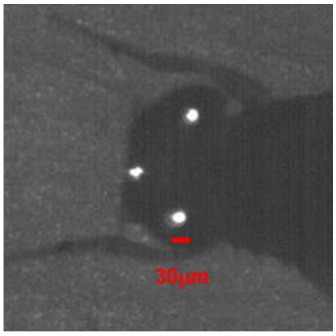

Figure 12: Tracking the fly in Flyception2 with microspheric markers

There are five principal advantages of this marker-based tracking approach over the previously employed contour-based tracking. First, it is computationally less expensive; tracking continues to function at a 1 KHz update rate and potentially even beyond. Second, it requires a smaller field of view to initiate tracking, allowing for higher magnification or faster frame rate tracking. Third, since the algorithm does not rely on the fly's outline, it robustly tracks the head even in situations where the fly makes physical contact with other flies (e.g., during courtship and mating). Fourth, the retroreflector sends the incident light back to the source regardless of the angle of incidence (for up to about 30 degrees), making it possible to image the brain even when the fly tilts its head. Fifth, retro-reflection is highly energy efficient compared to other light emitting methods (e.g., fluorescence), and is more suitable for high-frame rate, low-exposure applications.

The second feature is a fast-synchronous electronic focus control of the tracking and fluorescence paths to keep the fly head in focus at different z-planes over a range of a few millimeters at 50 um steps. By tapping into the autofocusing capabilities of the macro lenses used in our setup, we were able to develop a command set that controlled the ultrasonic motors in the lens' assembly, much like the way commercial single-lens reflex cameras interface with attached lenses. This form of focus control offers far better precision, repeatability, synchrony, speed and cost effectiveness compared to moving the tracking and fluorescence cameras and attached fixed focal lenses with linear stages. Coupled with the marker-based tracking, it has enabled us to monitor brain activity of the male fly during copulation, when he mounts the female, a behavior that has remained inaccessible under any imaging paradigm.

The third enhancement is the addition of a second excitation laser light path (561 nm) and emission light splitting optics to enable ratio-metric imaging in the fly. In this study, we image calcium-dependent GCaMP (green) and calcium-independent tdTomato (red) fluorescence simultaneously at 50 Hz with a single sCMOS camera; however, the system is amenable to modification for fluorescence markers of other wavelengths. This has made activity measurements more robust against motion artifacts and allowed for comparison of neural activity between different behavioral states and postures.

With the new methods in place, we examined brain activity in male flies engaged in various stages of the courtship and mating process. We examined the activity patterns of a cluster of ~20 male-specific dorsal posterior *fruitless* gene-expressing neurons, namely the P1 interneurons, that are known to respond to female contact (Hoopfer et al., 2015). We used P1a split-Gal4 to express GCaMP6s and tdTomato specifically in the P1 interneurons, and performed ratio-metric imaging of these neurons in male flies. Consistent with previous results, we observed increased activity in P1 neurons as the male exhibited courtship behavior, approached and contacted the female (**Fig. 13, middle row**). Due in large part to the marker-based tracking, we were able to reliably observe complete interaction sequences of the two flies. For instance, we observed that if the female was indifferent towards the male and walked away, P1 activity tended to subside to baseline levels. However, if the female remained interested, P1 activity tended to get brighter as the male continued courting the female.

Little is known about neural dynamics in copulating flies and roles of neurons implicated in courtship during this process, primarily because it has been impossible to visualize brain activity in flies engaged in mating with *in vivo* imaging techniques. The addition of electronic focusing adjustability of lenses in our setup allowed us to maintain focus on the male fly's brain as he mounted the female and observed activity of P1 neurons during copulation from the same flies we used for recording courtship behavior. Strikingly, these neurons were inactive during

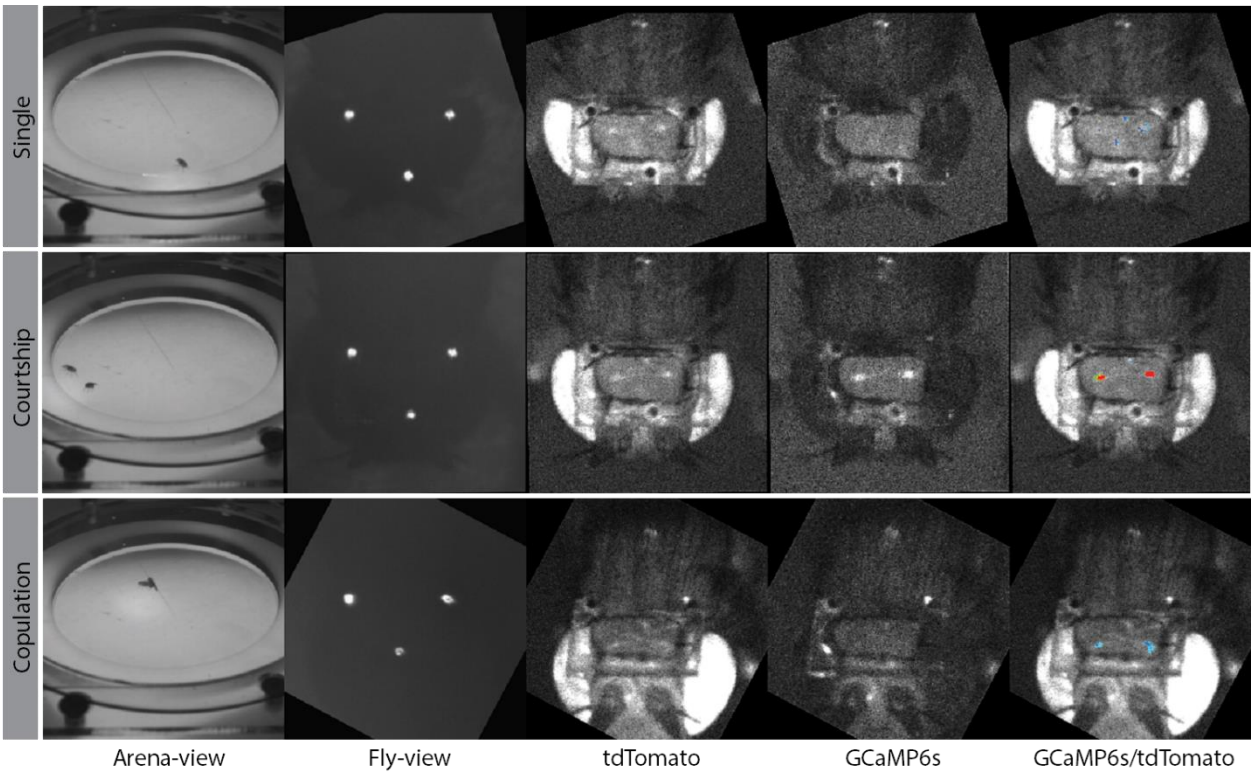

Figure 13: Flyception2 imaging of P1 interneurons in a single fly (top row), during courtship of a female (middle row), and during copulation (bottom row).

copulation (**Fig. 13, bottom row**), despite physical contact with the female that drives activity in these neurons during courtship. Furthermore, we find that P1 activity returns post-copulation when the male approaches and contacts the same female, however, not with the same vigor as prior to mating. These results suggest that P1 neurons are perhaps modulated by the behavioral state of the animal.

Next, we imaged a population of ~8 mAL GABAergic neurons that are known to play an inhibitory role during courtship and are also known to contact P1 interneurons (Zhang et al., 2018). The role of mAL, however, during copulation has so far remained unknown. We confirmed sustained activity in these neurons during copulation, suggesting a role in inhibition of P1 during copulation (**Fig. 14, right**). These results offer a glimpse into the neuromodulatory alteration of circuit function based on behavioral state, and critical to our study on attention.

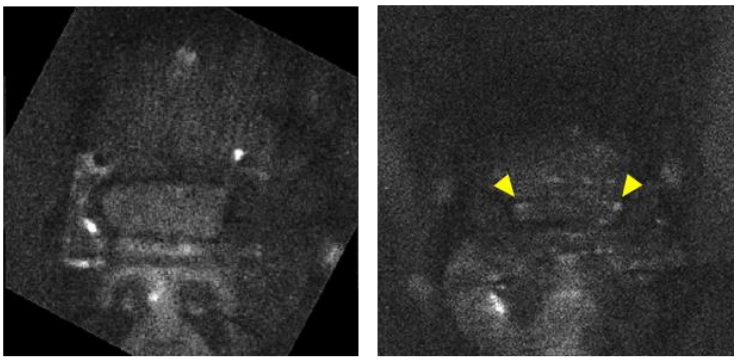

Figure 14: Flyception2 imaging showing lack of P1 activity (left) and mAL activity (right, location indicated by yellow arrows) during copulation.

We could in principle use this imaging technique to perform chronic time-lapse imaging of basal brain state, however, the imaging resolution of Flyception is limited (~30 µm) and individual neurons in proximity to each other cannot be resolved. Therefore, in addition to improving the Flyception system for low-magnification applications, we have also begun building a novel imaging

platform, FlyBAM, that has an order of magnitude higher spatial resolution than our original Flyception system to enable activity monitoring at single-cell resolution on a brain-wide scale. The necessary (and costly) components for creating an initial prototype of this novel microscope were made possible by an AFOSR DURIP grant awarded to us during the 2016-17 fiscal year, and as we illustrate below, we have been successful in demonstrating its feasibility in meeting our initial goals set for this grant period. This system, once ready, will also play a major role in another effort that we are a part of -- an AFOSR MURI project to study the role of sleep/wake homeostasis on energy consumption/regeneration in the fly brain. As detailed below, this cannot be achieved by simply modifying the existing Flyception system but rather requires an entirely new optical and mechanical design as well as novel tracking and image analysis algorithms.

We plan to achieve this through three technical innovations. First, by fabricating a fiducial marker, similar to Flyception2, that enables accurate detection of the fly head at high-speed (> 1kHz) (**Fig. 12**). The marker will be used to determine the x-y coordinate, the rotation angles (pitch, yaw, and roll), and the z-axis position of the fly head. This information will be used not only for tracking the fly in real-time but also for post-acquisition image processing in which we perform image deblurring, image registration, super-resolution, and fluorescence intensity quantification. The advantage of this approach is that a fiducial marker can help a tracking algorithm for any model organism to which the marker can be attached. For example, this should enable tracking of zebrafish larvae as well as fruit flies, thereby widening the scope of the technology. We have conducted pilot experiments to image micro-spherical fiducial markers placed in a triangular pattern along the edges of the coverslip with a 10x magnification objective lens.

Second, development of a novel microscope mechanism that allows for fast translational motion of the objective lens over a moving fly. For XY movement, we have developed a periscope mechanism that is comprised of two pairs of mirrors coupled with rotary bearing joints (much like a human arm) between the tube lens and the objective. This way one can move the objective lens without altering the image axis. This configuration is critically important to minimize load of the moving parts and achieve accurate tracking, because it decouples the objective optics from the rest of the imaging system, while maintaining a fixed distance between

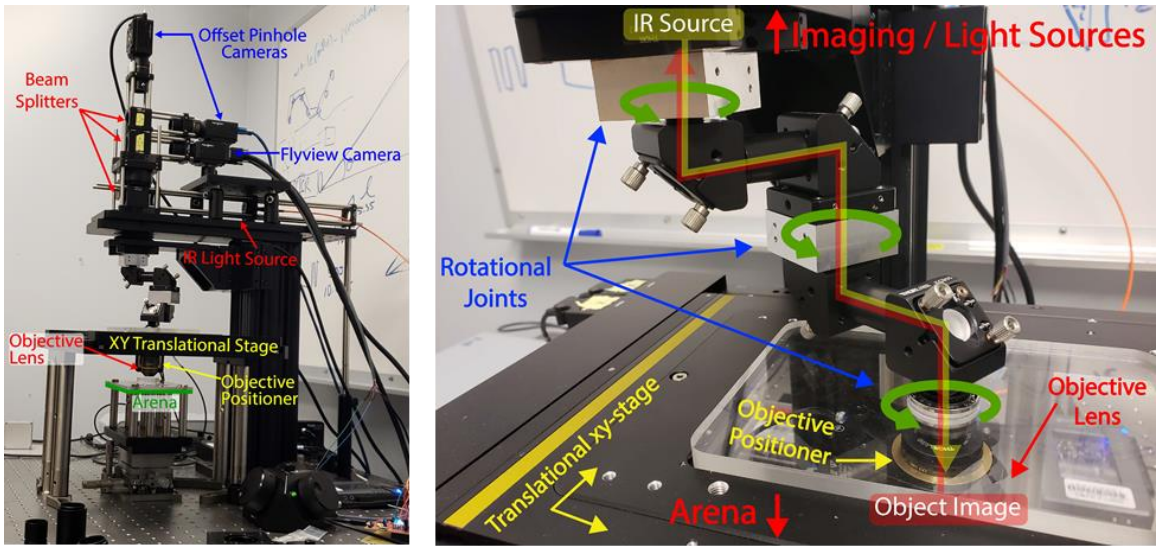

Figure 15: Left, translational microscope prototype under construction in our lab. Right, periscope mechanism attached to xy-motorized stage, with z-piezo driven custom autofocus

them, and allows for fast and accurate motion of the objective lens via a motorized stage. As a result, the system will be highly flexible (e.g., it can be combined with a variety of advanced imaging techniques such as confocal microscopy, two-photon microscopy, and Light Field Microscopy). For accurate estimation of movement in the z-axis, we have developed a method that uses two off-center pinhole-modulated cameras to provide accurate and fast focus information (Guo et al., 2015). In this method, by placing a pinhole at the Fourier plane, focal position can be recovered by calculating the phase correlation of the two-corresponding pinhole-modulated images. One advantage of this system is that by deploying a small-sized pinhole in both cameras, autofocusing can reach the millimeter range, orders of magnitude longer than the objective's depth of field. Another benefit of this method is that it gives directionality about the z-axis. In other words, we can tell whether the object is farther or closer from the focal plane. We have successfully developed and incorporated both the periscope mechanism and z-autofocus module into our microscope prototype with preliminary success at tracking a fly and are at present optimizing the tracking for various motion profiles relevant to natural fly movement and behavior (**Fig. 15, and Fig. 16**).

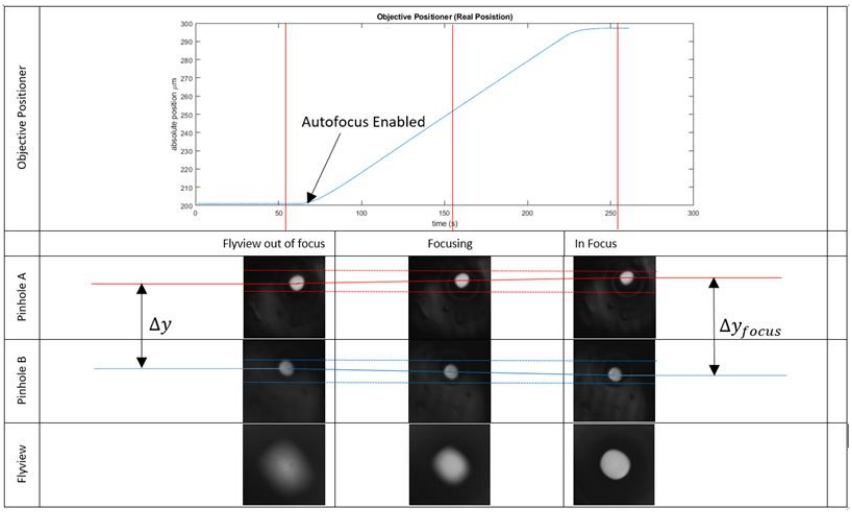

Figure 16: Pinhole modulated camera views (A & B) illustrating focused (increased depth of field) but translationally shifted bead due to it being out-of-focus in xy-tracking camera that lacks the pinhole.

Third, development of a computational framework to achieve high-resolution volumetric activity imaging from the data inherently subjected to motion issues. This will include 3D deconvolution, 3D image de-blurring, 3D image registration, 3D super-resolution, and fluorescence intensity quantification. The innovative aspect of our approach is that it takes advantage of the information obtained from the 2D fiducial marker for processing volumetric fluorescence images. Because the fiducial marker is sampled at a much higher frequency than the fluorescence images (1kHz vs 100Hz), it can provide an accurate 4D estimation of the fly brain that can be used for reconstructing dynamic fluorescence intensity data (Bergamasco et al., 2016). Our pilot experiments with off-the-shelf maximum likelihood (ML)-based blind de-blurring method (Light Microscopic Images Reconstructed by Maximum Likelihood Deconvolution) (Ma et al., 2015) gave promising results with our flyception system and we plan to build on those methods for the high-magnification imaging system.

References

Alekseyenko, O. V., Chan, Y. B., Li, R., & Kravitz, E. A. (2013). Single dopaminergic neurons that modulate aggression in Drosophila. *Proceedings of the National Academy of Sciences*, 201303446.

Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guérin, G., ... & Rowell, W. J. (2014). Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. *Elife*, *3*, e04580.

Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T. T., ... & Rubin, G. M. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife*, *3*, e04577.

Bargmann, C. I., & Marder, E. (2013). From the connectome to brain function. Nature methods, 10(6), 483.

Bergamasco, F., Albarelli, A., Cosmo, L., Rodola, E., & Torsello, A. (2016). An Accurate and Robust Artificial Marker Based on Cyclic Codes. *IEEE Trans. Pattern Anal. Mach. Intell.*, *38*(12), 2359-2373.

de Bivort, B. L., & van Swinderen, B. (2016). Evidence for selective attention in the insect brain. *Current opinion in insect science*, *15*, 9-15.

Carter, R. M., Hofstötter, C., Tsuchiya, N., & Koch, C. (2003). Working memory and fear conditioning. *Proceedings of the National Academy of Sciences*, *100*(3), 1399-1404.

Grover, D., Katsuki, T., & Greenspan, R. J. (2016). Flyception: imaging brain activity in freely walking fruit flies. *Nature methods*, *13*(7), 569.

Guo, K., Liao, J., Bian, Z., Heng, X., & Zheng, G. (2015). InstantScope: a low-cost whole slide imaging system with instant focal plane detection. *Biomedical optics express*, *6*(9), 3210-3216.

Han, C. J., O'Tuathaigh, C. M., van Trigt, L., Quinn, J. J., Fanselow, M. S., Mongeau, R., ... & Anderson, D. J. (2003). Trace but not delay fear conditioning requires attention and the anterior cingulate cortex. *Proceedings of the National Academy of Sciences*, *100*(22), 13087-13092.

Hoopfer, E. D., Jung, Y., Inagaki, H. K., Rubin, G. M., & Anderson, D. J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in Drosophila. Elife, 4, e11346.

Huang, C., Maxey, J. R., Sinha, S., Savall, J., Gong, Y., & Schnitzer, M. J. (2018). Long-term optical brain imaging in live adult fruit flies. *Nature communications*, *9*(1), 872.

Ma, Z., Liao, R., Tao, X., Xu, L., Jia, J., & Wu, E. (2015). Handling motion blur in multi-frame super-resolution. In *Proceedings of the IEEE Conference on Computer Vision and Pattern Recognition* (pp. 5224-5232).

Mao, Z., & Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. *Frontiers in neural circuits*, *3*, 5.

Pooryasin, A., & Fiala, A. (2015). Identified serotonin-releasing neurons induce behavioral quiescence and suppress mating in Drosophila. *Journal of Neuroscience*, *35*(37), 12792-12812.

Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., ... & Hirsh, J. (2011). Behavioral consequences of dopamine deficiency in the Drosophila central nervous system. *Proceedings of the National Academy of Sciences*, *108*(2), 834-839.

Seelig, J. D., Chiappe, M. E., Lott, G. K., Dutta, A., Osborne, J. E., Reiser, M. B., & Jayaraman, V. (2010). Two-photon calcium imaging from head-fixed Drosophila during optomotor walking behavior. *Nature methods*, *7*(7), 535.

Shohat-Ophir, G., Kaun, K. R., Azanchi, R., Mohammed, H., & Heberlein, U. (2012). Sexual deprivation increases ethanol intake in Drosophila. *Science*, *335*(6074), 1351-1355.

Suver, M. P., Mamiya, A., & Dickinson, M. H. (2012). Octopamine neurons mediate flight-induced modulation of visual processing in Drosophila. *Current Biology*, *22*(24), 2294-2302.

Trachtenberg, J. T., Chen, B. E., Knott, G. W., Feng, G., Sanes, J. R., Welker, E., & Svoboda, K. (2002). Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature*, *420*(6917), 788.

Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., & Kume, K. (2012). Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. *Nature neuroscience*, *15*(11), 1516.

van Swinderen, B., & Brembs, B. (2010). Attention-like deficit and hyperactivity in a Drosophila memory mutant. *Journal of Neuroscience*, *30*(3), 1003-1014.

Xi, W., Peng, Y., Guo, J., Ye, Y., Zhang, K., Yu, F., & Guo, A. (2008). Mushroom bodies modulate salience-based selective fixation behavior in Drosophila. *European Journal of Neuroscience*, *27*(6), 1441-1451.

Zhang, K., Guo, J. Z., Peng, Y., Xi, W., & Guo, A. (2007). Dopamine-mushroom body circuit regulates saliency-based decision-making in Drosophila. *science*, *316*(5833), 1901-1904.

Zhang, S. X., Rogulja, D., & Crickmore, M. A. (2016). Dopaminergic circuitry underlying mating drive. *Neuron*, *91*(1), 168-181.

Zhang, S. X., Miner, L. E., Boutros, C. L., Rogulja, D., & Crickmore, M. A. (2018). Motivation, Perception, and Chance Converge to Make a Binary Decision. Neuron, 99(2), 376-388.