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# Unlocking pan-neuronal expression in mosquitoes

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**The ability to express a gene in all neurons is a crucial tool for studying the nervous system. Zhao et al., 2021 unlock genetic access to all neurons in mosquitoes by generating the first pan-neuronal transgenes in this non-model insect.**

As vectors for a multitude of diseases, mosquitoes are a worldwide threat to human health. To find a human and bite, a mosquito's nervous system receives sensory information, interprets that information, and ultimately decides on a course of action (Konopka et al., 2021). Understanding how the mosquito's ~220,000 neurons accomplish such complex tasks can lead to strategies to combat mosquito attraction and biting (Raji and Potter, 2021). However, the brain of a mosquito has been a black box, with few genetic tools available to unlock the mysteries hiding within. Zhao et al. (2021) now report the generation of the first pan-neuronal transgenic lines in *Aedes aegypti* mosquitoes, enabling genetic access to all neurons in this mosquito species.

When introducing a new genetic method into insects, previous work from *Drosophila melanogaster* is often used as a guide. This model organism has been an efficient and facile system for developing genetic methods since the early 1980s (Rubin and Spradling, 1982). In *Drosophila*, the first widely used pan-neuronal line was derived from the *embryonic lethal abnormal vision* (*elav*) promoter, an RNA-binding protein specifically expressed in neurons (Robinow and White, 1991). Similarly, pan-neuronal transgenes in *Drosophila* have been generated by using enhancers + promoters from genes involved in neuronal function, such as the synaptic proteins neuronal synaptobrevin (nSyb), synaptotagmin1 (Syt1), and bruchpilot (brp) (Riabinina et al., 2015). The generation of pan-neuronal transgenes in *Drosophila* is straightforward: the upstream region of a neuronal gene (typically sequences immediately upstream of the ATG start site to the preceding gene) is cloned 5′ to a gene of interest. This strategy works well with a compact genome like that of *Drosophila* (~221 Mb), but has proven less successful in the expanded genomes of mosquitoes (Riabinina et al., 2016); the *Aedes aegypti* genome, for example, is ~1,300 Mb. This necessitates the use of the CRISPR/Cas9 system to generate a knockin at a gene of interest to capture the necessary genomic regulatory regions that drive tissue-specific expression (Kistler et al., 2015).

In this work (Zhao et al., 2021), the authors confirmed neuronal enrichment of *nSyb*, *Syt1*, *elav*, and *brp* by using RNA sequencing (RNA-seq) of *Aedes aegypti* brains compared with that of non-neuronal tissues. Interestingly, transcription expression amounts varied, and *nSyb* and *nSyt1* were the most highly expressed and *brp* was the least highly expressed. These data implicated *nSyb* or *Syt1* as potential targets for generating robust pan-neuronal effectors. The authors chose to focus on *Syt1* because efficient guide RNAs could not be found to target *nSyb*.

Choosing *Aedes aegypti Syt1* as the pan-neuronal target gene was the initial step in generating a pan-neuronal transgene; the second step was deciding which effector gene to express. To accomplish their goal of generating a transgenic pan-neuronal activity monitor, the group decided to use the effector GCaMP6s, a modified version of green fluorescent protein (GFP) that fluoresces more brightly in the presence of calcium, and thus acts as a fluorescent proxy for neuronal activity (Chen et al., 2013). The authors were faced with another challenge: how to express GCaMP6s without affecting the function of the *Syt1* gene. After all, disrupting the function of a pan-neuronal gene would likely lead to a decrease in fitness. To accomplish this, the authors targeted the end of the coding region of *Syt1* and replaced the stop codon with *T2A-GCaMP6s*. The T2A peptide causes the ribosome to skip peptide bond formation and allows multiple proteins to be expressed from the same transcript (Diao and White, 2012). Although the knockin was successful and the mosquitoes were healthy, expression of GCaMP6s was weak, despite using three copies of T2A-GCaMP6s. The authors reasoned this was because GCaMP6s was distributed throughout the neuron. So they developed a new strategy: instead of expressing GCaMP6s as a separate protein, they would fuse it to the synaptotagmin protein itself to concentrate GCaMP6s to pre-synaptic sites (Figure 1). This strategy was successful, and the authors generated healthy *Aedes aegypti* mosquitoes that expressed GCaMP6s in all neurons at amounts sufficient to perform calcium imaging. Indeed, the authors could identify regions of the antennal lobes, the primary center of olfactory signals in the brain, which were activated by different odors. The *Syt1:GCaMP6s* transgenic *Aedes aegypti* strains can be used to examine neuronal activity throughout the mosquito brain.

The authors next turned their attention to the more challenging goal of enabling genetic access to all *Aedes aegypti* neurons. To allow the greatest amount of flexibility and versatility, the authors decided to introduce a binary expression system. Binary expression systems utilize two components: the first component is an exogenous transcription factor, and the second component is an effector gene whose expression is controlled by the

presence of the introduced transcription factor (Riabinina et al., 2015). An additional advantage of a binary expression system is that gene expression of the effector is amplified due to transcriptional enrichment. In *Drosophila*, three binary expression systems have been widely adopted: the GAL4/*UAS* system, the QF2/*QUAS* system, and the LexA/*LexAOp* system (Riabinina et al., 2015). Encouraged by their results with *Syt1*, and prior success of the QF2/*QUAS* system in *Aedes aegypti* and *Anopheles coluzzii* mosquitoes (Matthews et al., 2019, Riabinina et al., 2016), the authors decided to target *T2A-QF2* to the *Syt1* gene. The authors further decided to include *QUAS-GCaMP6s* as part of the target; this was a time-saving strategy to generate a fully functional QF2 + *QUAS* effector. The authors found robust expression of GCaMP6s in larval neurons; however, mosquitoes did not survive past the larval stage. Multiple factors might have caused this: (1) pan-neuronal expression of QF2 might be lethal to the *Aedes aegypti* mosquito, (2) amplified pan-neuronal expression of GCaMP6s might be lethal, or (3) the knockin was dominantly disrupting the function of the *Syt1* gene. To address these possibilities, the authors decided to swap out QF2/*QUAS* with GAL4/*UAS* components. This indeed resulted in healthy mosquitoes, but also led to weak and inconsistent GCaMP6s labeling. These results suggested that (1) QF2 and/or high amounts of GCaMP6s might be a major cause of lethality, (2) the GAL4/*UAS* system does not work effectively in *Aedes aegypti* neurons, and (3) the knockin strategy was unlikely to be the cause of the lethality. To address these additional challenges, the authors needed to revise their strategy.

A key insight made by Zhao and colleagues was to choose a different pan-neuronal gene to be targeted. Instead of aiming for the highest expression possible, the authors reasoned they could target *bruchpilot*, the lowest expressed pan-neuronal gene they examined, and then rely on effector expression amplification made possible by the use of a binary expression system. Next, they utilized a weaker version of the QF2 transcription factor, QF2$^{w}$, which had been shown in *Drosophila* to function effectively while also increasing the health associated with QF2 pan-neuronal expression (Riabinina et al., 2015). Third, they uncoupled the *QUAS-GCaMP6s* from the targeting construct and instead used an independent transgenic insertion to be crossed to their pan-neuronal *QF2*$^{w}$ strain (Figure 1). These efforts proved successful. The *brp-T2A-QF2*$^{w}$ strain was healthy, and robustly induced expression from *QUAS-CD8:GFP*, *QUAS-Syt1:tdTomato*, and *QUAS-GCaMP6s* transgenes. From these experiments, the authors demonstrated versatile genetic access to all neurons in *Aedes aegypti* mosquitoes.

In summary, the new pan-neuronal reagents developed by the McBride group should accelerate discovery into the function and/or anatomy of neurons in the *Aedes aegypti* brain or sensory tissues.

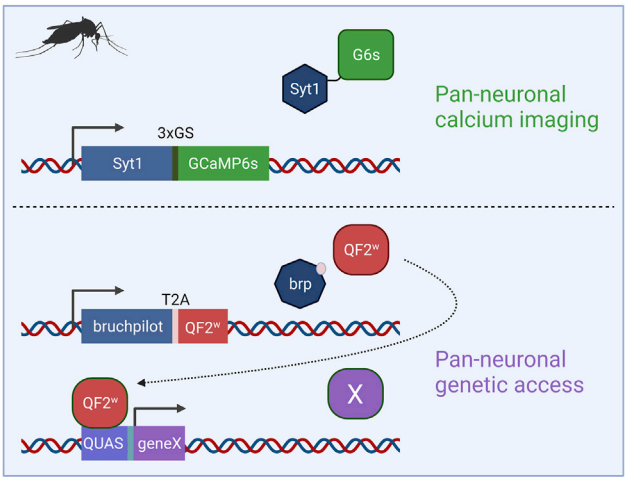


**Figure 1. Pan-neuronal expression systems in *Aedes aegypti* mosquitoes**

Shown on top, GCaMP6s (G6s) is targeted to the endogenous *Synaptotagmin1* (*Syt1*) genomic locus, creating a Syt1:3xGS:GCaMP6s protein. 3xGS encodes a flexible linker connecting the two proteins. This construct allows all neuronal activity to be monitored by calcium imaging. Shown on the bottom, a multipurpose pan-neuronal expression system is generated by targeting *T2A* and a *QF2*$^{w}$ transcription factor to replace the stop codon of the *bruchpilot* (*brp*) genomic locus. This leads to expression of pre-synaptic brp protein and a functional QF2$^{w}$ transcription factor, which binds *QUAS* enhancer sequences to drive expression of *QUAS-geneX* effectors. This allows versatile genetic access to all *Aedes aegypti* neurons. Figure created with Biorender (https://biorender.com/).

These reagents have recently been used to identify the sensory responses of stylet neurons toward blood and sugar during blood-feeding in *Aedes* mosquitoes, yielding insights into how this tissue responds during biting of a human host (Jove et al., 2020). They can also be utilized to examine how different brain regions respond to stimuli. For example, these new reagents could be used to identify how various odors are represented by the neuronal activity of the olfactory system, and lead to insights into how mosquitoes discriminate humans from other animals. The *brp-T2A-QF2*$^{w}$ transgene enables flexibility to express a variety of *QUAS-effector* genes for a multitude of purposes. This could include expression of effectors like CaMPARI to fluorescently label neurons on the basis of activity, photo-activatable GFP to label neurons on the basis of their location, RNAi constructs to knockdown gene expression, or multi-color reporters to label the anatomy of brain neurons. These new pan-neuronal strains are likely to become commonly used reagents in the growing *Aedes aegypti* genetic toolbox and the strategies employed to generate pan-neuronal lines could also guide the development of similar reagents in other non-model insects, such as *Anopheles gambiae* mosquitoes.

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