# Investigating genetic manipulations as a method to rewire moonwalker neurons in *Drosophila*

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# Contents



**Abstract**

Synthetic biology provides a framework to understand the connectivity of the nervous system and the mechanisms that drive behavior. Rewiring the nervous system using transgenics provides researchers with a tool to examine how developmental rewiring functions. This project aims to understand whether the moonwalker descending neurons (MDN) (activation sufficient for backward walking [1]) can be rewired using transgenic Drosophila. We considered seven genetic manipulations that can be activated optogenetically: TNT (positive control), GFP and IMP TNT (negative controls), Shal RNAi, Dorsal, Eag DN and Hunchback. Video recordings of flies in a thin rectangular arena were used to train a neural network to identify the forward or backward movement of the fly for stimulated and non-stimulated periods. The percentage of time spent walking backwards in stimulation was compared to the controls. No changes in behavior were detected, however this work can serve as a template to study the rewiring of neurons using other genetic manipulations in transgenic flies.

# 1 Introduction

Neural circuits have been studied extensively to better understand the underlying mechanisms that drive behavior. Rewiring the nervous system presents an opportunity to determine sufficiency and necessity of neural circuits in relation to specific types of behavior. Schafer et al. implemented this technique in C. elegans by introducing a new transgenic synapse and thereby synthetically modifying a neuron's connectivity [2]. This approach can be further extended to other organisms as a means to determine how changes to the connectivity of the brain can induce alterations in behavior. A study investigated the rewiring of neuronal pathways in ferrets and showed how their visual behavior could be mediated by retinal projections in the auditory thalamus and cortex [3]. The receptive fields of these rewired projections are initially identified, then a visual stimulus is presented to that part of the visual field and it is observed that the rewired ferrets perceived a visual stimulus rather

than auditory. The exact mechanism that drives the light induced sensations remained unclear to the authors, however they concluded that during development, sensory afferents can instruct their cortical target as to its eventual function [3]. The aim our project is to investigate whether it is possible to rewire neurons in the brain of Drosophila using genetic manipulation. Rewiring could include damaging neuronal pathways, promoting the creation of new pathways (compared to the wildtype flies), or changing the properties (for example, potassium conductance) of the neurons. The population of neurons studied are the moonwalker descending neurons (MDN). Bidaye, Salil S., et al observed that the firing of this population of these neurons are necessary and sufficient to induce backwards walking. Blocking MDNs were shown to have no effect on forward walking, however synaptic silencing of MDNs almost entirely blocked backward walking [1]. The same group also identified moonwalker ascending neurons (MAN) whereby these neurons play a role in inhibiting forward walking and consequently in inducing persistent backward walking. Using optogentics (expressing light sensitive protein channels in the neuron membrane), the flies can be very reliably forced to walk backwards upon stimulation by orange light. The channelrhodopsin used for the experiment is csChrimson, a protein that serves as an ion channel gate sensitive to red light.

### 1.0.1 Genetic modifications being studied

**TNT:** Expressing TNT (tetanus toxin) prevents firing of the neurons in which it is expressed by reducing neurotransmitter exocytosis (release into synapse). This acts as a positive control since moonwalker neurons will not be able to fire and thus there should be no change in behavior upon light stimulation [4].

**GFP:** Green Fluorescent Protein emits light when exposed to blue UV light, however it has no effect on neuronal firing. It thus acts as a negative control (moonwalker descending neuron activation should cause the normal effect of backward walking).

**IMP TNT:** The control used for TNT to ensure that the act of transgenic intervention does not have an effect, thus it too acts as a negative control (it has no effect on neuron firing) [4].

**Shal RNAi:** Shaker neurons have abnormal potassium channel conductance which result in delayed repolarization of the neuron [5].

**Dorsal:** A mutation to the transcription factor that allows for the dorso-ventral organization of Drosophila in embryos. Embryos form with both dorsal and ventral sides of the embryo exhibiting dorsal structure [6].

**Eag DN:** It encodes a protein channel which allows potassium and calcium ions to pass through. The ion conductance is controlled by cAMP and causes an alteration in the firing of neurons, producing spontaneous repetitive firing [7].

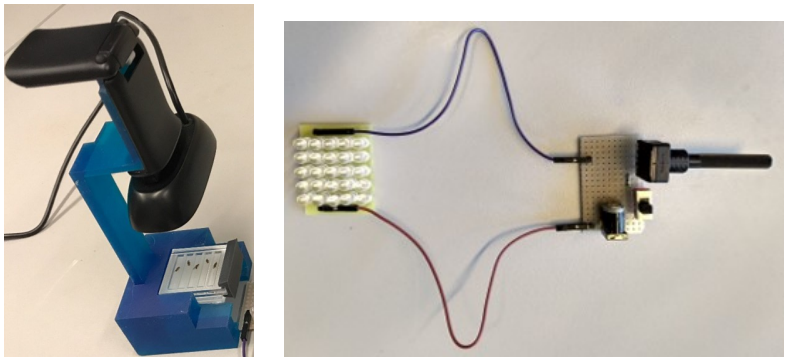
**Hunchback:** The hunchback transcription factor controls abdominal structure in embryos. The mutated flies thus exhibit altered anatomy, for example with shortened abdominal sections [8].

Note the effects described above are observed when the genes are expressed in embryonic Drosophila, the effects of expressing these genes in the moonwalker neurons is unknown

# 2 Methods

## 2.1 Experimental setup

The experiment was conducted using the setup shown in Figure 1. The camera, a Logitech $c270^{TM}$, was used to record 4-5 flies in a 3cm x 3cm arena at a rate of 10 fps. The arena is sub-sectioned into 5 chambers separating the flies. The rig was positioned to have minimal light variation on the arena. Each arena was shook slightly before trial recordings to move the flies to more optimal positions (away from the walls). The recordings were done for 5 seconds before, 5 seconds during, and 5 seconds after light stimulation. The 5 second duration was chosen as it was observed that the flies began to move forward instead of backward after around 8 seconds of stimulation in the negative control trials and we wanted to record only data while the stimulation was eliciting the expected behavior. The light intensity was set to maximal amplitude for all experiments. Each type of fly was recorded for 10 trials which were performed with approximately a 30 second interval between trials. The transgenic flies studied include: IMP TNT (negative control), GFP (negative control), Shal RNAi, dorsal, TNT (positive control), Eag DN, and hunchback.



(a) Camera and arena. (b) Circuit for light stimulation.

Figure 1: Experiment setup [9]

## 2.2 Data processing

Two different methods were explored for identifying the fly positions and their direction of travel. The first was to use image processing and the second was to train a neural network.

### 2.2.1 Image Processing

The first method used OpenCV to identify the centroid and heading. Since the lighting conditions across the arena were quite variable, adaptive thresholding was used to distinguish the fly from the background. Then to remove additional objects (from edges and dark patches in the arena), morphological filtering was used to create a mask, along with an algorithm to remove edges (long lines). Once the flies were isolated, they needed to be edited such that only the main shape of the body was left (removed legs and accentuated distinction of the head shape from the body, see Appendix: Figure 7b). To account for wings being detected, a threshold was placed on object areas so they were excluded and the heading direction was separately evaluated if the wing was part of the object (see Appendix: Figure 7c). The resulting image allowed for fairly accurate representation (92.5% of the flies correctly identified, although not reflective of shape quality) with visual inspection needed to confirm shape quality.

In order to evaluate the fly's head direction, we developed two independent methods. Both methods leverage the fly's body asymmetry (across the shortest axis) and assume that the center of mass is closer to the lower extremity rather than near the head. The first method consisted of determining a contour that envelops the fly and then measuring the maximum distance (representing heading direction) between the center of mass and the calculated contour points. The second method encases the fly's body in a bounding box, splits this box in two equal halves and counts the pixel density in each half. The head should be contained in the half with the smaller pixel density. Although these two methods provide reliable detection of the fly's heading in principle, the results obtained were unreliable due to the inconsistency of the fly's shape after image preprocessing.

### 2.2.2 Neural Network

An alternative method to detect the fly's heading direction was to implement a neural network and to train it on labelled data, namely, head and body end points. With a total of approximately 10500 images to be processed, we randomly selected 105 images from all of our data and manually labelled them using annotation tools as seen in Figure 2. The selected images accounted for variations in position, orientation and lighting conditions. For each image, a bilateral filter was applied to smooth the image whilst preserving the edges. Then we split each frame into stripes each corresponding to a single fly. Given the small sample size, the data was augmented by applying vertical and horizontal flips to original images. A CNN with auxiliary losses was trained on the augmented data (approx. 2000 samples) for 40 epochs. Predictions could then be made on the rest of the dataset as seen in Figure 3. The centroid position was determined by taking the difference in $x$ position between the predicted head and bottom positions. Only the $x$ position was used for simplification given that the fly cannot move forwards or backwards along the $y$ axis. Once all the data had been annotated, movement was measured as the difference in centroid position between the current frame and 10 frames before. Given that there are small fluctuations in the centroid position irregardless of movement due to image pre-processing, a minimum change of $10^{-3}$ of the picture width was used to indicate that there was no motion. Movement in the same direction as

the heading direction was labelled as +1, in the opposite direction as -1 and no movement as 0. The magnitude of change in either direction was not measured. Using this information, the walking behavior could be quantified by counting the sequence of movements during the trial. Furthermore, the number of backward or forward movements could be extracted for specific period (i.e. during stimulation time only).

Given the reliability of the neural network model and inconsistency of the traditional image processing method, we proceeded with the former for the remainder of the data analysis.

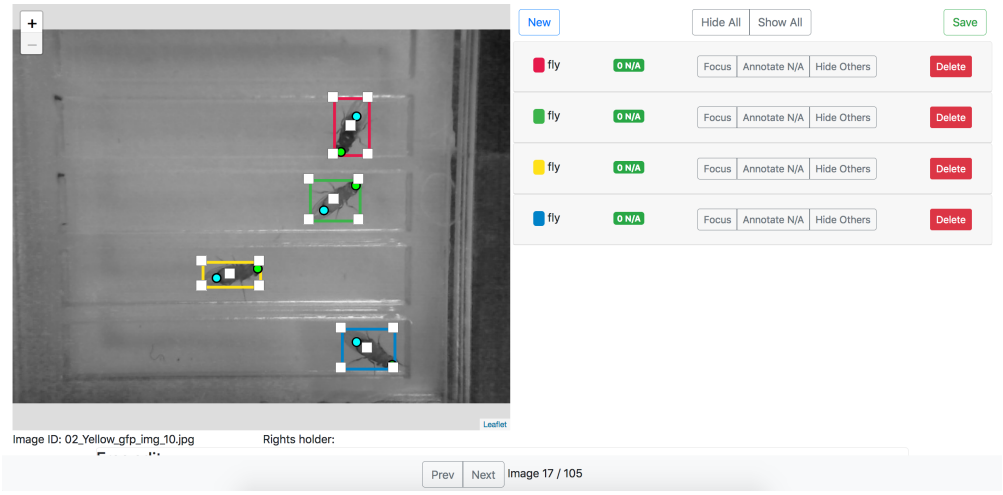



Figure 2: Labelling software used for training. Data included in training are head and wing positions of each fly (bounding box not used)

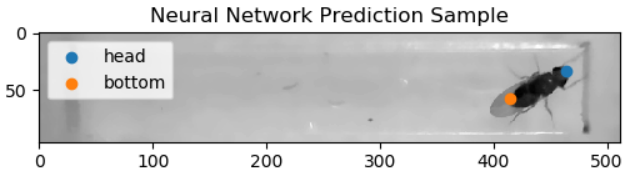
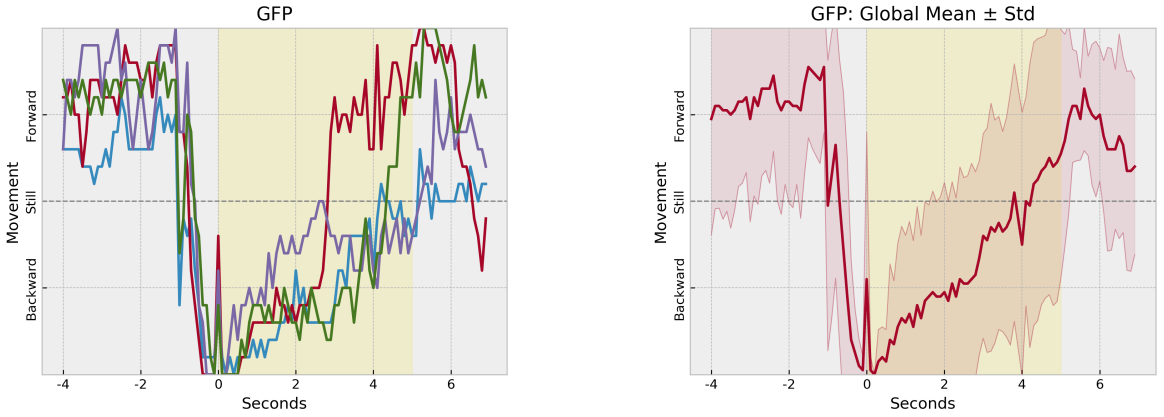



Figure 3: Visualization of neural network prediction for head and body end positions

## 3 Results

The data collected allowed us to quantify the walking behavior of the fly with and without optogenetic stimulation. All flies were considered for data analysis irrespective of their level of activity for a given trial. For each fly, the sequence of motion was recorded for the entire trial. For each genetic manipulation, ten trials were ran in order to obtain a sufficiently large sample size to fully capture the behavior. Each trial was centered together based on the start of the stimulation time and from this the average walking sequence for each fly was taken across trials (Figure 4a). The walking behavior for the negative control (GFP) is presented in Figure 5 and illustrates three general be-

haviors: forward walking before stimulation followed by backward walking during stimulation and returning to forward walking during stimulation. Although the exact magnitude is not recorded, it can be observed that backward movement is most prominent at the start of the stimulation and decreases as stimulation time continues. This is primarily explained by the fact that the fly has reached the wall and has no space to continue moving backwards. An alternative reason may be due to the dynamics of the moonwalking ascending neurons as discussed previously. These set of neurons were shown to play a role in the persistence of backward walking. It can be observed that near the end of stimulation, some flies begin to walk forward, which may indicate that those set of neurons are not being activated. In contrast, the TNT positive control exhibits a different set of behavior whereby forward movement is maintained throughout the trial even during the stimulation period. This behavior occurred as expected given that expressing TNT silences MDN firing, which were determined to be necessary for backward walking. Flies recorded from the other trials (Shal RNAi, Dorsal, Eag DN and Hunchback) exhibited similar walking behavior to the negative control as seen in Figures 6 and 4b. One potential significant difference however is a lower variability in the ShalRNAi and Dorsal trials as seen in Figure 6. This can be intrepreted as flies with ShalRNAi and Dorsal transgenes performed more backward walking during the stimulation period than any other type. It is difficult to claim that these differences are significant given that this discrepancy may have arisen due to the particular set samples that we used (only completed 10 trials). Based on the results obtained, it is difficult to conclude that the genetic manipulations selected contributed to the rewiring of MDN neurons such as to cause a modification in the behavior seen in the negative control.




(a) Each line represents the average walking sequence of one fly per chamber across ten trials.

(b) Average and standard deviation for all trials and all flies.

Figure 4: The average walking behavior per fly during one cycle (before, during and after stimulation) for the GFP negative control.

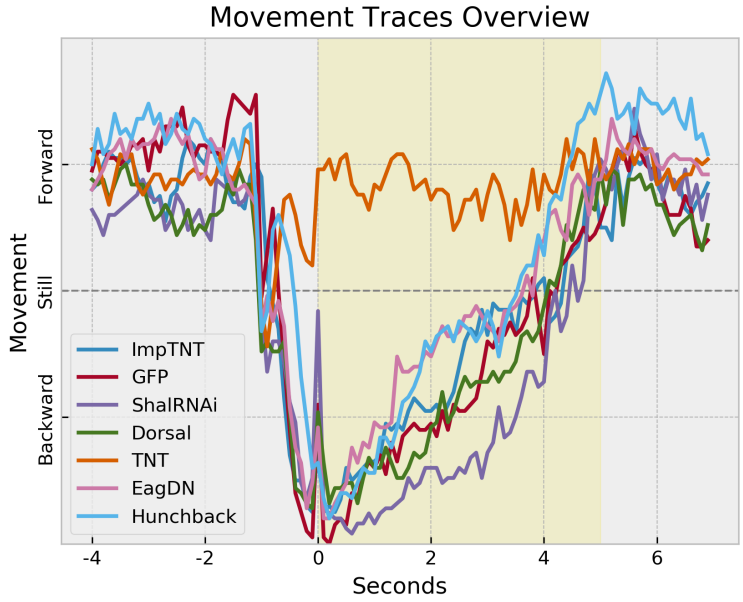



Figure 5: The average walking behavior during one cycle for different transgenic fly type

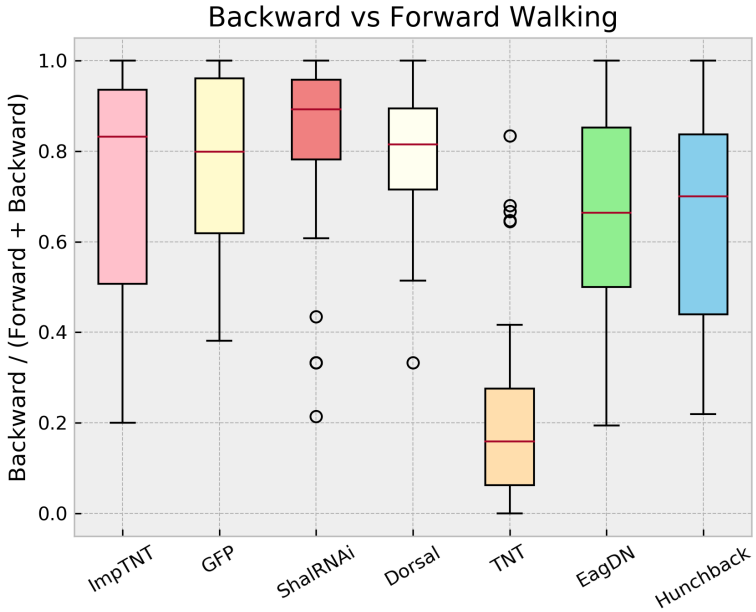



Figure 6: Boxplot comparing the duration of backward walking for the different transgenic flies during the stimulation period.

## 4 Discussion

There are several areas of the experiment setup and processing that could potentially be improved. The arena placement was not completely consistent, thus the borders were not always in the same location. Improving on this could allow us to hard-code a method to split up the individual

compartments, reducing background complications. A different solution could be to draw coloured contours over the individual arena edges for easy detection in the image processing stage. Another issue was the large variability in lighting conditions between the stimulated and non-stimulated flies. The stimulated fly images were higher quality since the light was directed through the arena towards the camera, providing great contrast and low noise. Using a light source which is also under the arena for the non-stimulated recordings (not including red wavelengths) would provide more consistent and higher quality images. With the improved contrast, a thresholding algorithm could identify the wings (very clear in the stimulated period) which could then be used to identify the head direction of the fly. Alternatively, we could only concentrate our analysis on the stimulation period.

The image processing technique could still be a viable method with a few modifications. The accuracy of the two direction identification methods by themselves (longest length from center to contour and ratio of black to white pixels within halves of a bounding box) were insufficient. However, you could define two halves of the fly (across shortest axis) and categorize the resulting direction (as left or right) from the different methods. Then 3 different methods for direction identification could be compared and the final decision taken as the best 2/3 to create a more robust algorithm. Furthermore, we observed that the identification of the centroid position was more accurate using the image processing techniques compared to the neural network. Therefore, one consideration would be to combine both techniques: centroid position by image processing and heading direction using neural networks.

Data analysis was performed on all flies, however the differences observed from the different genetic modifications may be more representative if we pre-selected flies that exhibited movement during stimulation. From initial observation, it appeared that a significant portion of flies remained stationary during stimulation primarily because they were already stuck at the wall. You could quantify the number of flies to which this applies to and determine if it is significant enough to skew the results, then apply a filter to discard such flies from final data analysis.

Data analysis could also be improved by extracting more detailed parameters from the recorded data. In our project, we focused solely on whether the fly moved forward, backward or remained still from one frame to another. We could provide a more detailed analysis of the walking behavior by recording velocity, rotation following a similar model as Sen et al [10]. They provided a more comprehensive analysis of the backward walking behavior by providing features such as total backward locomotion, backward turn and rotation angle.

There were no significant changes observed from the genetic modifications with the interventions studied, however other transgenic flies may rewire the Drosophila brain. Further studies could observe many different transgenic flies: with a more automated set up it would be possible to study a much higher number of flies. The genetic modifications may also have an effect in other neuron populations even though they had no effect in the moonwalker neurons. Thus it could be interesting to study the same mutations in other neuron populations. Related to this idea, the experiment only studied the forward or backward walking behavior of the flies. The mutations could have effected

other behaviors or the walking behavior on a more subtle scale. Studying other factors such as the leg movement may then provide additional insights.

Although we did not detect changes in behavior with the particular mutations studied, it does not eliminate the possibility of genetic rewiring. Rewiring can aid in behavioral studies and provide more precise information on the role of different circuits in the brain.

## References


[1] Bidaye, Salil S., Christian Machacek, Yang Wu, and Barry J. Dickson, "Neuronal control of drosophila walking direction," *Science*, 2014, dOI: 10.1126/science.1249964.

[2] Ithai Rabinowitch William R Schafer, "Engineering new synaptic connections in the c. elegans connectome," *Worm*, 2015, dOI: 10.4161/21624054.2014.992668.

[3] Von Melchner, Laurie, Sarah L. Pallas, and Mriganka Sur, "Visual behaviour mediated by retinal projections directed to the auditory pathway," *Nature*, 2000, dOI: 10.1038/35009102.

[4] Sean T. Sweeney, Kendal Broadie, John Keane, Heiner Niemann, Cahir J. O'Kane, "Targeted expression of tetanus toxin light chain in drosophila specifically eliminates synaptic transmission and causes behavioral defects," *Neuron*, vol. 14, p. 341, 1995.

[5] Mark A. Tanouye, Alberto Ferrus, Shinobu C. Fujita, "Abnormal action potentials associated with the shaker complex locus of drosophila," *Neurobiology*, vol. 78, no. 10, p. 6548, 1981.

[6] Christiane Nusslein-Volhard, "Maternal effect mutations that alter the spatial coordinates of the embryo," 1979, academic Press Inc.

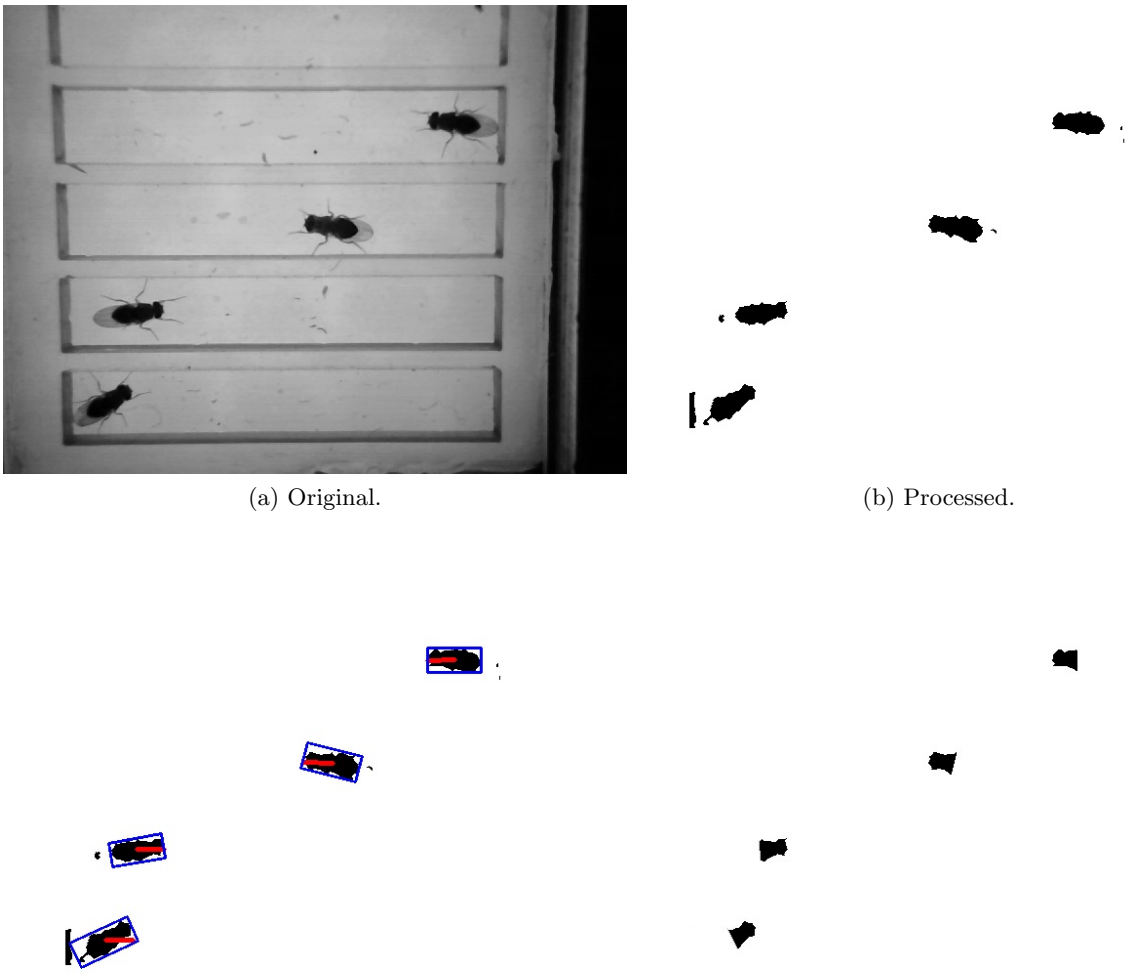
[7] Andrea Bruggemann, Luis A. Pardo, Walter Stuhmer, Olaf Pongs, "Ether-a-go-go encodes a voltage-gated channel permeable to $k^+$ and $ca^{2+}$ and modulated by camp," *Nature*, vol. 365, p. 445, 1993.

[8] Ruth Lehmann, Christiane Nusslein-Volhard, "hunchback, a gene required for segmentation of an anterior and posterior region of the drosophila embryo," *Development Biology*, vol. 119, p. 402, 1987.

[9] Pavan Ramdya, "Week 10: Mini-project experimental rig construction," 2019, controlling behavior in animals and robots course notes.

[10] Sen, Rajyashree, Ming Wu, Kristin Branson, Alice Robie, Gerald M. Rubin, and Barry J. Dickson, "Moonwalker descending neurons mediate visually evoked retreat in drosophila," *Current Biology*, 2017, dOI: 10.1016/j.cub.2017.02.008.

# 5 Appendix



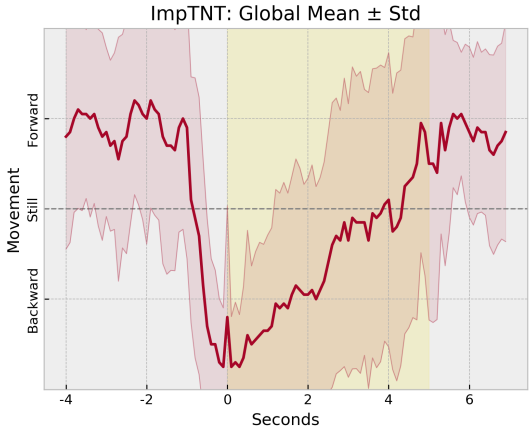
(a) Original.

(b) Processed.

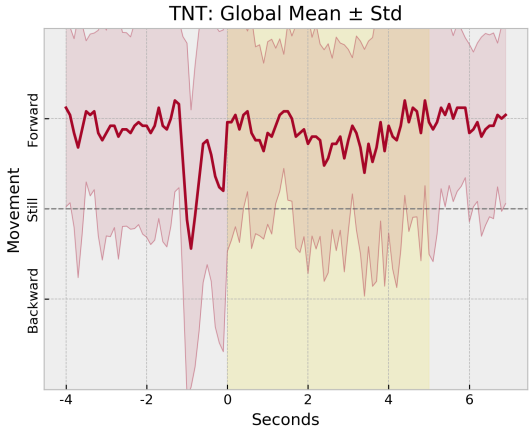
(c) Length detection algorithm.

(d) Black to white ratio algorithm.

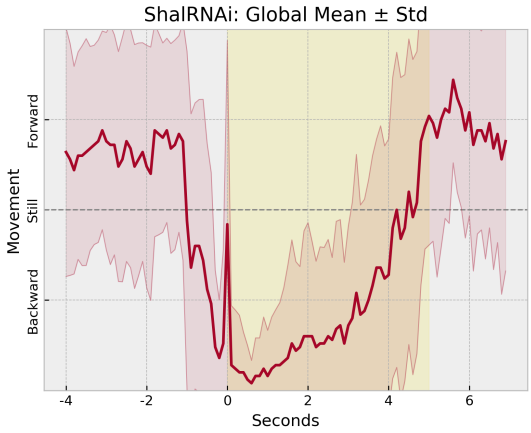
Figure 7: Example image in IMP TNT recording for image processing method.



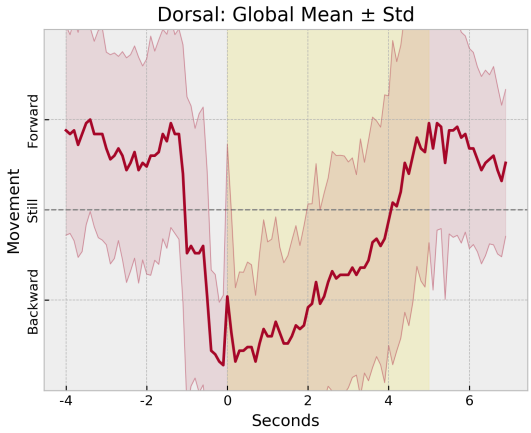

(a) IMP TNT.



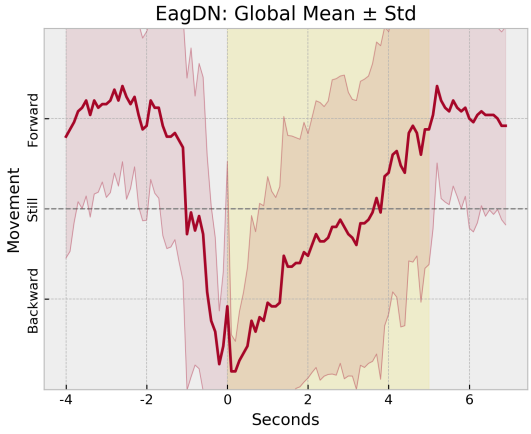

(b) TNT.



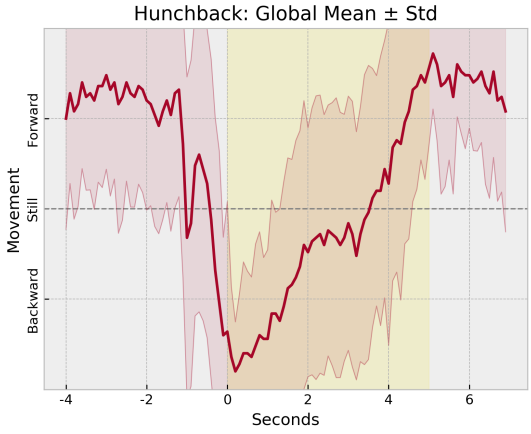

(c) Shal RNAi.




(d) Dorsal.




(e) Eag DN.




(f) hunchback.

Figure 8: Average movement over all flies and trials for each genetic modification.