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Miniature Insect Model for Active Learning

DELIVERABLE 3.3 Activation patterns in operant learning

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Abstract: We have developed an assay that reliably records neural activity in genetically targeted neurons of freely moving Drosophila larvae. We have recorded the spontaneous activity of neurons involved in the learning process, such as Kenyon cells (KCs) and Dopaminergic (DA). Also, we have recorded the neural activity of KCs after stimulation, correlated to their change in movement. A draft paper discussing this work in detail is attached (see D3.3_Appendix), which has been recently been re-submitted after revision with Scientific Reports.

In order to develop a reliable assay to monitor neural activity from intact and freely behaving larvae, we carefully calibrated the following parameters:

Larval pre-treatment

The larval treatment before the analysis considering in particular:

- Amount of CTZ to administer to the animal
- The age of the animal at the time of analysis
- The time of CTZ incubation
- The copies of the calcium reporter protein GFP-Aequorin (AEQ)
- The number of animals to analyse at the same time
- The type of stimulus to present to the animal

Nevertheless, all these parameters need to be carefully regulated according to the neurons we are examining. For example, to monitor neural activity from KCs (about 300 neurons) of one larva we incubated *201y/+;AEQ/+* embryos in 250uM CTZ food for 3 days; whilst to record DAs activity (about 100 neurons) from a single larva we fed *TH/+;AEQ/+* larvae embryos with 625uM CTZ food for 3 days (Fig. 1).

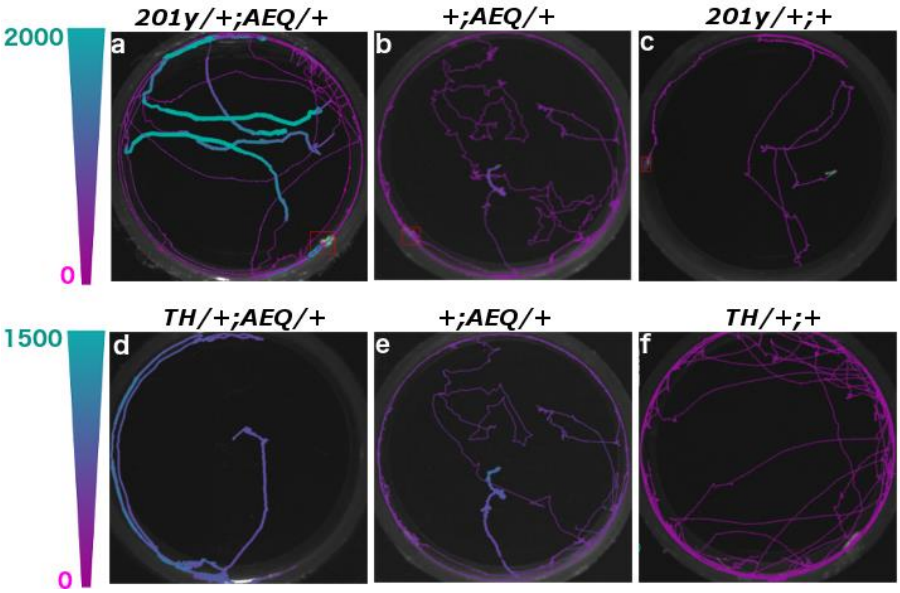

Figure 1: Kenyon cells and Dopaminergic neuron show spontaneous activity. Representative images of larval trajectories with biololuminescence value colour-coded according to heatmap (max. set to 2,000 au in (a-c), and to 1,500 in (d-f)). The line width is increased to enhance contrast between high and low values are informative of the bioluminescence value. (a-c) 201y-targeted KCs show higher activity compared to the +;AEQ/+ (n = 6) control, the same is observed in TH-targeted DAs' (controls: +;AEQ/+ and TH/+;+) (d-f).Bioluminescence frame length = 2,000 ms. Video frame = 50 ms.

The Apparatus

The building up of a specific apparatus *ad-hoc* to analyse larval neural activity and behavior, was crucial for the development of the assay. It requires both a photomultiplier tube (PMT) to record the bioluminescence and a camera to capture the larval movements. The PMT passively captures any photon, regardless the wavelength, thus, it should work in complete darkness to make sure it records, specifically, the bioluminescence signal. On the other hand, we need to illuminate properly the larvae to make videos. We solved this issue in collaboration with Drs. K. Lagogiannis and B. Risse (Webb Lab, UEDIN) using a system of filters for different light wavelengths combined to a "FIM" larval illumination (Fig. 2). All the

main elements of the apparatus were custom-designed and 3d printed making the entire system very cost-effective.

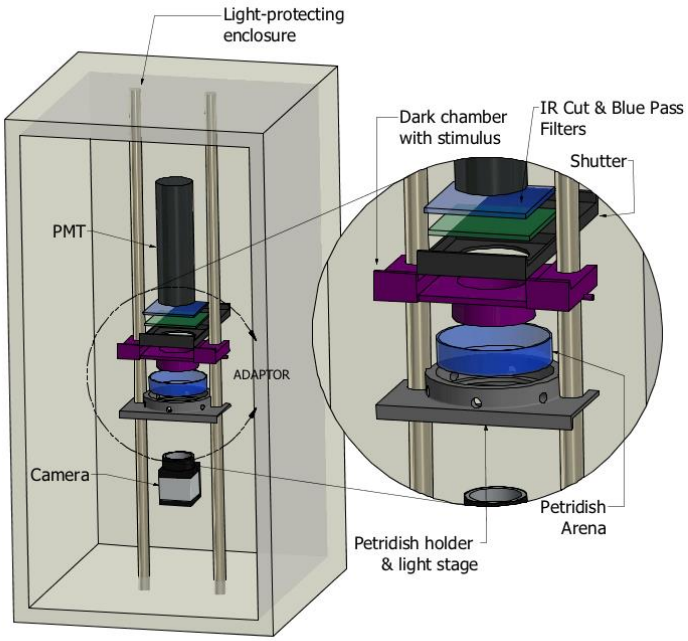

Figure 2: The developed apparatus. A light secure box contains the PMT and the camera, with the experimental stage between them. Light filters, a small chamber and shutter system protect the PMT from unwanted light signals. The petridish is illuminated by side mounted IR, which is reflected from the larva down to the camera.

Stimulus

Since this assay aims to monitor the effect of inputs coming from the environments on the neurons and behaviour at the same time, we examined the possibility to monitor changes in neural activity after stimulation. Thus, as a proof-of-concept we chose CO_2, that it is known to activate KCs in adults, can be easily checked over-time using a CO_2 sensor, and, also, it causes an evident effect on behaviour effect: the larva falls asleep (Fig. 3). A gas stimulus delivery system was designed and incorporated into the system.

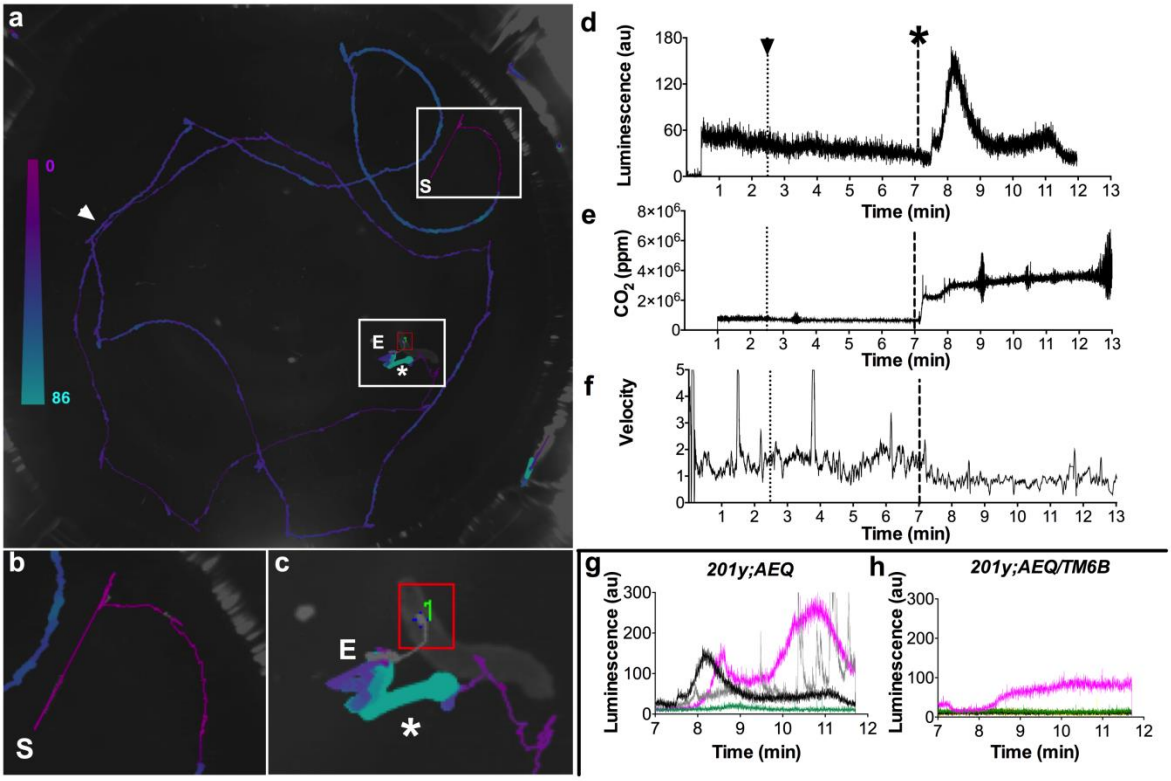

Figure 3 Simultaneous recording of behaviour and bioluminescence responses to stimulation. The 201y;AEQ larva was left to freely move on the arena for 13 min., during which we recorded bioluminescence, CO2 levels and a video. At 30 sec. the shutter was opened, at 1 min. the CO2 sensor was switched on, at 2 min. 30 sec. (arrowhead) 200 ml air was pumped slowly into the chamber, and at 7 min. (*) 200 ml CO2 was pumped slowly into the chamber. (a) Example image of larval trajectory. The line is coloured according to the displayed heatmap. (b) Enlarged image of the initial part of the experiment during which the shutter is closed and, therefore, the luminescence signal is low. (c) Enlarged image of the larva and trajectory during CO2 stimulation. Here the carbon dioxide leads to high bioluminescence and the animal stops. S = start and E = end. (d-f) Aligned plots showing neural activity (d), stimulus (e) and behaviour (f). (g-h) KC neural activity emitted after CO2 stimulation of 201y;AEQ (g) and 201y;AEQ/TM6B larvae (h). Bioluminescence and CO2 frame length = 100 ms. Video frame = 50 ms.

Next steps

In the original proposal, the objective for this deliverable was to measure “activation patterns in operant learning”. Developing the bioluminescence assay to work on freely moving larvae required the careful analysis of a number of factors that impacted this plan. On the one hand, all the technical aspects of the apparatus and, on the other hand, the adaptation of behaviour experiments to the apparatus. All this work took more time than expected to develop a reliable and useful assay, thus, delaying the analysis of specific neuron activation during the associative learning. Moreover, the ability to observe operant learning in larva at all remained doubtful.

Instead, we plan to evaluate the learning performance of the *Drosophila* strain expressing the calcium reporter (GFP-Aequorin) following an established associative learning paradigm from LIN (Fig. 4). We reviewed the available options regarding the odour delivery, but we opted for the odour containers, as they are visible in the recorded videos and they represent in a clean way to deliver the odour within the apparatus during the analysis.

While performing the learning experiment outside the apparatus is meant to establish the learning degree of the used larvae during the final “test-phase”, the associative learning experiment within the bioluminescence apparatus aims to monitor the activity of specific neurons involved in learning during both training- and test-phases.

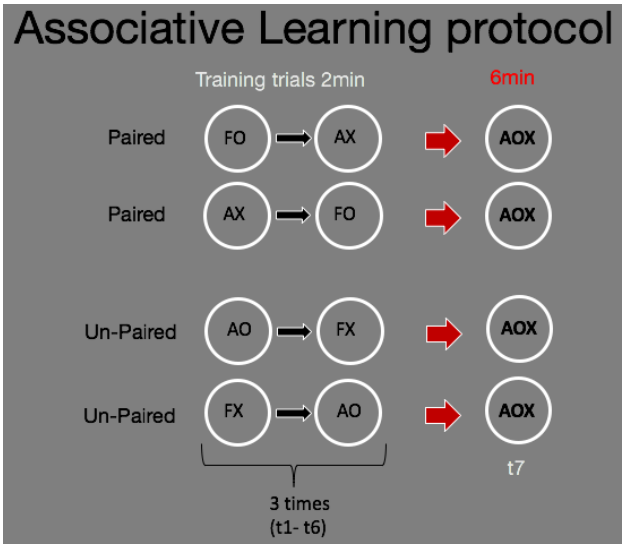

Figure 4 Learning protocol to be used in planned experiments with the apparatus