TÉCNICO LISBOA

OptoPAD: dissecting the neuronal control of *Drosophila* eating habits by merging the flyPAD with optogenetics

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Thesis to obtain the Master of Science Degree in

Biomedical Engineering

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November 2015

Abstract

The goal of this project is to develop technology to manipulate genetically identified neurons and investigate the circuit basis of behaviour in *Drosophila melanogaster* using optogenetics. We use a combination of the FlyPAD (Fly Proboscis and Activity Detector – a behavioural system that detects the physical interaction of flies with food by capacitive-based measurements) and *Bonsai* (a visual programming framework for the acquisition and online processing of data streams), in order to build a new behavioural paradigm where flies choose between two sources of food with different "neuronal consequences" depending on their actions. We built a system (OptoPAD) capable of simultaneous recording of feeding behaviour and manipulation of neuronal activity in 32 individual flies expressing different opsins.

The new system makes use of a 32-arena FlyPAD system where a high-power RGBA LED illuminates each individual arena. Using *Bonsai*, each capacitance signal is processed in real time and when the fly behaviour matches predefined parameters, an activation message is sent to an Arduino board leading to the activation of the LED and hence triggering the desired optogenetic manipulation.

Using this system we demonstrate that activation of *Gr5a*-positive neurons triggers appetitive behavior and activation of *Gr66a*-positive neurons drives aversion from food. This system allows the dissection of the circuit basis of feeding homeostasis and the closed-loop manipulation of circuits to study aversive, appetitive or anticipatory behaviours in *Drosophila*.

Resumo

Este projeto tem como objetivo desenvolver um novo dispositivo que consiga manipular geneticamente neurónios e investigar o comportamento alimentar da *Drosophila melanogaster*, utilizando optogenética. Com o propósito de construir um novo paradigma comportamental onde moscas da fruta tenham a oportunidade de escolher entre duas fontes de comida com diferentes "consequências neuronais", usámos uma combinação do FlyPAD ("Fly Proboscis and Activity Detector" – um sistema de estudo comportamental, que deteta a interação das moscas com a comida por medidas capacitivas) com o *Bonsai* (uma interface visual para aquisição e processamento online de sinais). Construímos um sistema (OptoPAD) capaz de gravar o comportamento e manipular a atividade neuronal de 32 moscas em simultâneo.

Este novo sistema utiliza 32 arenas do FlyPAD, onde um "RGBA LED" de alta potência ilumina cada uma das arenas. O Bonsai processa em tempo real cada sinal capacitivo proveniente de cada arena e quando o comportamento da mosca é o esperado, uma mensagem de ativação é enviada para o Arduino, levando à ativação do LED, despoletando assim a manipulação optogenética.

Com este mecanismo conseguimos demonstrar que a ativação de neurónios que expressem recetores gustativos *Gr5a* induz comportamento apetitivo e a ativação de neurónios expressando *Gr66a* causa uma resposta aversiva em relação a uma fonte de comida. Este sistema permite a compreensão dos circuitos neuronais por trás da homeostasia alimentar e a manipulação em "closed-loop" de circuitos que permitam estudar comportamentos aversivos, apetitivos e antecipatórios em *Drosophila*.

Acknowledgments

I would like to thank Professor José Santos Victor, for overseeing this project and for the fruitful discussions and advices on the technical part of the work.

I would like to thank Doctor Carlos Ribeiro, for the irreplaceable inputs and comments throughout all this dissertation and for allowing me to join his group in the end of 2012, which made me grow not only as a scientist but as a person.

I would like to especially thank Doctor Pavel Itskov, who was truly my closest mentor since the beginning of my work at the Champalimaud Foundation. Doctor Itskov always believed in me and in my work and together we were able to overcome many difficulties and were able to show everyone the potential of our projects.

I would like to thank Gonçalo Lopes, João Frazão and Niccolò Bonacchi, for teaching me how to use *Bonsai* and for the productive discussions on how to build and optimize my *Bonsai* workflow.

I would like to thank Filipe Carvalho and Artur Silva, from the Hardware Platform, for helping me with my boards' designs and assemblage.

I would like to thank Matthieu Pasquet, who was the first engineer I met at the Foundation and who taught me what it really means to be an engineer.

I would like to thank all the members from the Ribeiro Lab for always being very supportive.

Finally, I would like to thank my family and close friends, for always being really interested and for listening to my enthusiastic explanations of my work.

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List of Acronyms

FlyPAD	Fly Proboscis and Activity Detector
PER	Proboscis Extension Reflex
CAFE	CApillary FEeding
FLIC	Fly Liquid-Food Interaction Counter
RMS	Root Mean Square
SEZ	SubEsophageal Zone
GRN	Gustatory Receptor Neuron
ReaChR	Red activatable Channelrhodopsin
DBS	Deep Brain Stimulation
FPGA	Field Programmable Gate Array
I^2C	Inter-Integrated Circuit
LED	Light Emitting Diode
RGBA	Red, Green, Blue, Amber
PSU	Power Supply Unit
FIR	Finite Impulse Response

1. Introduction

1.1. Motivation

Animals need to eat in order to survive, making feeding an essential component of animal behaviour. In fact, feeding is one of the few behaviours that can give us an insight into an animal's physiological status, since they tend to choose their diet according to their needs. Fruit flies (*Drosophila melanogaster*) have emerged as a powerful model to study the neuronal and molecular mechanisms underlying feeding behaviour as they can be easily tracked and genetically manipulated (Itskov & Ribeiro 2013); however, their small size makes it difficult to quantify their food intake.

In order to overcome this disadvantage, we have recently developed an automated, high resolution behavioural monitoring system called FIyPAD (fly Proboscis and Activity Detector), which uses capacitive-based measurements to detect the physical interaction of individual flies with food, generating metrics related to total food intake, hunger and satiation (Itskov et al. 2014).

In recent years, neuroscientists have developed different approaches in order to understand the relationship between neuronal activity and behaviour: one way is to look for correlations between neuronal activity and the behaviour of the animal: another is to monitor the effect of lesions on behaviour; and a third approach is to directly manipulate neuronal activity and correlate it with changes in behaviour. The latter methodology can be performed both in open- or closed-loop experiments. Open-loop means that neuronal activity in manipulated independently of the animal's behaviour; in contrast during closed-loop experiments, the behaviour of the animal determines the way in which neuronal activity is manipulated, allowing higher temporal precision of the manipulation (which animals can learn as it comes closer to the physiological situation where an action triggers a response) and fully autonomous neuronal manipulation.

1.2. Goal

The goal of this project is to build a behavioural setup capable of simultaneously recording feeding behaviour and manipulating the electrical activity of neurons in *Drosophila* in a closed-loop manner.

1.3. Structure

In this master thesis, I will start by reviewing the literature related to this work.

Secondly, I will describe the OptoPAD, a closed-loop system for simultaneous monitoring of feeding behaviour and optogenetic manipulation in freely moving adult flies that we designed and developed at the Champalimaud Foundation. I will describe the methods and materials used throughout this work, starting with the development of hardware, further software development using *Bonsai* (Lopes et al. 2014) and finally explaining the protocols of the experiments performed.

In the fourth chapter, validation of both hardware and software will be described before the actual experimental results. Together with technical descriptions of both hardware and software, I will present some experiments that serve as a proof-of-principle and elaborate on how this device can be used in future studies on neuronal control of feeding behaviour.

In the final chapter of this thesis, I will discuss the advantages and limitations of this system as well as the experiments I have performed using the OptoPAD.

2. State of Art

We can travel more than a century into the past and find authors interested in how the body maintains its stability in the face of a variable external environment. For example, Bernard in 1878 in his book “Leçons sur les phénomènes de la vie, communs aux animaux et aux végétaux” stated that “the conditions which must be maintained constant in the fluid matrix of the body in order to favour freedom from external limitations are water, oxygen, temperature and nutriment (including salt, fat and sugar)” (Bernard 1878; Cannon 1929). Indeed since the first definition of homeostasis, nutrition has always formed a core component of it. It is well known that animals, in order to survive and create healthy offspring (species maintenance), must eat. Moreover, they must eat according to their internal needs.

Understanding the way the brain is able to modulate eating behaviour according to its body needs is a way of getting closer to food related diseases, such as obesity, and possibly to finding some answers as to whether it has a neuronal explanation (Smith & Geary 2002). In order to discover solutions to these problems, scientists have used mainly rats and mice, as they are evolutionarily closer to humans, yet easier to study in a controlled setting than hominids.

Drosophila melanogaster, in turn, has emerged as a powerful model to study the neuronal and molecular mechanisms underlying feeding behaviour and many tools have been designed to help scientists study this behaviour that is shared by all living animals (Itskov & Ribeiro 2013).

2.1. What are the methods to measure feeding behaviour?

2.1.1. Humans

In order to study feeding behaviour it is of extreme importance to measure it quantitatively. The main problems when assessing the eating habits of humans is the lack of precision/accuracy and a great bias when doing “natural setting” (Free-living) studies on one hand and the less naturalistic approach when performing more controlled studies in laboratories as nowadays society and environment have a large influence on the eating behaviour of human beings (Blundell et al. 2010).

Inside the laboratory, appetite and food intake measurements rely on metabolic kitchens for accurate and hygienic food preparation, small rooms where participants test foods, standard operating procedures describing every step researchers will conduct (Gibbons et al. 2014) and visual analogue scales (Rogers & Blundell 1979; Stubbs et al. 2000) or electronic appetite rating systems (Delargy et al. 1996; Whybrow et al. 2006).

Alternatively, “natural setting” studies of eating behaviour in humans use data such as food acquisition in society or assessment of food consumption, calculated from a form of questionnaires (diet histories,

24-hour dietary recall, food frequency questionnaires), food records/diaries or food lists (Jeor 2005; Wansink 2009).

During the past few years, scientists have been more interested in a combination of the two methodologies, where under laboratory conditions eating while watching television or talking to other people is studied (Stroebele & de Castro 2004; Salvy et al. 2007).

In summary, these methodologies help researchers to draw phenomenological conclusions on human feeding habits and how the human organism responds to the ingestion of food. However, to better understand why feeding happens and how feeding preferences are coded in the brain, scientists turn their attention to animal models.

2.1.2. Rats and Mice

“Food consumption is episodic; it occurs in discrete bouts a certain number of times each day…” (Gibbons et al. 2014, p.G3), so, to be successfully studied, “food and water intake should be observed and recorded bout by bout” (Ulman et al. 2008).

In mammals, particularly in rats and mice, some studies on food consumption are based on periodic measurements of the difference between the food weight in the beginning and at the end of the experiment. Even though much progress has been made to improve these methods, the main disadvantages are the inability to distinguish bouts of feeding and errors in the weighing, as the animals may take food from the holders and not eat it or even urinate or defecate in their food. Nowadays there are more accurate methodologies that mitigate these problems. Researchers have changed from using periodic measurements to trip-switch devices.

Trip-switch devices are electronic circuits that when activated, disconnect power from a running machine so that the machine is stopped. These devices record timestamps of when the circuit was interrupted. Some examples of these are as follow:

- Beam break systems: where the time of the interruption of a light beam near the food is recorded. This system does not give any information about the quantity of food ingested as the animal may be standing near the food, therefore interrupting the beam, without eating it.
- Lick meters: when the animal licks from the water/fluidic food bottle, an electrical circuit is completed through its tongue, body and feet to the floor of the cage. This method is very reliable for measuring the timing of the feeding bouts. However, as the amount of fluid ingested per lick is not constant, a correlation between number of licks and quantity of fluid ingested during a bout is not possible.
- Tablet meters: often used for “learning” paradigms, where the animal has to press a bar in order for a defined weight of food to be dispensed. Bout timing is again disregarded, since, even though the food is available the animal may not eat it right away. This method only records the start time of bouts. The end time and duration cannot be measured (Ulman et al. 2008).

In order to obtain the most reliable characterisation of rodents’ feeding behaviour, it is best to use a combination of trip-switch devices, which give access to the detailed dynamics of feeding behaviour, complemented, where possible, with the measurement of food volume/mass.

2.1.3. Flies

Fruit flies (*Drosophila melanogaster*) have been a key model to study evolutionarily conserved biological processes such as ageing, nutrient sensing and feeding because of their short life cycle and their array of genetic tools to rapidly unravel the complexity of biological systems. Although this model has been widely used to study feeding behaviour, precise quantification of food intake has been very difficult to achieve, due to the small quantities flies eat and also due to their small size (hard to measure reliable weight differences) (Wong et al. 2008; Wong et al. 2009). During recent decades several methods have been developed to study different aspects of food intake and feeding behaviour in *Drosophila* (for review see Itskov & Ribeiro 2013).

Taste is an essential component of feeding behaviour. Gustatory perception in Drosophila is initiated when tastants contact with the Gustatory Receptor Neurons (GRNs), which transmit taste information to higher brain centres. Hodgson et al. (1955) developed a method to record how GRNs respond to taste stimulation: they recorded action potentials elicited by the application of a taste stimulus in a recording electrode (where the tastant solution is mixed with an electrolyte). They recorded the activity of these neurons on the tip of the labellum, where many neurons expressing gustatory receptors are localized (Hodgson et al. 1955).

Two decades after, Dethier published one of the landmark books of the field (*The Hungry Fly: A Physiological Study of the Behaviour Associated with Feeding*, 1976), where he described the most commonly used assay to study feeding behaviour in flies, or better said, to measure behaviour associated to feeding: the Proboscis Extension Reflex (PER) (Dethier 1976). This assay is based on the fact that by applying an appetitive or aversive stimulus to either the labellum or the tarsi of the fly, where the majority of the taste sensilla reside (Vosshall & Stocker 2007), the fly will extend or retract its proboscis, respectively. Although PER is a robust assay, is extremely reproducible and can be used to study learning and memory (DeJianne et al. 1985; Keene & Masek 2012), it does only poorly correlate with food ingestion (Itskov & Ribeiro 2013).

One of the mostly used methods to study feeding is the Two Color Choice Assay, described by Tanimura in 1982. By visually scoring the colour of the abdomen of the flies that were left to eat in an environment where one available food is dyed red and the other blue (non-absorbable dyes are used) one can get a quantitative readout of food choice (Tanimura et al. 1982; Ribeiro & Dickson 2010).

For absolute quantification of food intake, two other methodologies have been developed: measuring colorimetrically the homogenate of flies which had consumed solutions mixed with food-dyes (Tanimura et al. 1982) and similarly by monitoring ingested radioactively labelled food medium by scintillation counting (Thompson et al. 1991; Carvalho et al. 2005; Carvalho et al. 2006).

Unfortunately, these last three methods (Two Choice Assay, Colorimetric Quantification and Radioactive Quantification) do not allow dynamic monitoring of food intake across time as they rely on scoring groups of dead flies, and additionally do not take into account the food excreted by the flies (though isotope

labelling does account for permanent tissue incorporation) (Ja et al. 2007; Wong et al. 2008; Itskov & Ribeiro 2013).

One assay designed to measure ingestion of food in real-time by individuals or groups of fruit flies on the scale of minutes to days is the CAFE (CApillary FEeding) assay. In this method, flies are allowed to consume liquid food from graduated glass microcapillaries. The descent of the meniscus allows unambiguous measurements of consumption. It can be used to study food preference as multiple capillaries may be used (Ja et al. 2007). However, this methodology can only measure intake of liquid food, flies are forced to eat in an upside-down position, dynamic measurements have low temporal resolution (tens of minutes) and measuring behaviour of individual flies is very difficult (Itskov & Ribeiro 2013).

Information is getting more and more computerized. Thus, the automated and high-throughput gathering of data is becoming possible and scientists are developing methods that will allow them to get more digital data in less time.

Many such methods utilise video tracking to study flies' approach to food. Not designed to study feeding in particular, these methods give important information on the behaviour of flies during their lifetime (Zou et al. 2011). From the reviewed literature, the methodologies using video tracking, consider feeding as a constant (>5 seconds) position of the fly near the food source (5 and 6 mm) (Root et al. 2011; Zou et al. 2011; Zaninovich et al. 2013). As noted previously, the key caveat of this method is its inability to measure actual feeding behaviour, because flies might be resting, grooming or even sleeping near the food, which will be mistakenly considered as feeding.

Last year, two devices were published almost simultaneously that use a different approach to the fly's feeding behaviour. The first one, known by the acronym FLIC (Fly Liquid-Food Interaction Counter) is a complete hardware and software system for collecting and quantifying continuous measurements of feeding behaviours in the fruit fly. This system can record activity of multiple flies simultaneously. Flies are left to eat in one arena where the food source is surrounded by an electrode. When the fly touches the food, it completes a voltage divider circuit and the resulting voltage is detected on a 10MOhm resistor and recorded. Even though this methodology apparently shows high-throughput and reliable results there are some drawbacks.

On one hand the sampling rate used is not optimal, since the system can collect data as frequently as 500 samples per second, but for data analysis authors perform a running average over 100 samples in order to remove high-frequency noise. This protocol under-samples the signal to 5Hz, diminishing its resolution. On the other hand the way feeding behaviour is identified leaves a lot of margin for errors. "Feeding behaviours were identified by signal intensities that surpassed a defined threshold value above baseline." The long, high intensity signal was identified as feeding and ephemeral spikes were associated to tasting events. Again, the idea of the fly standing near and touching the food, resting or grooming will be misleadingly identified as feeding.

Nevertheless this method can be useful as it allows long term recordings of flies' interaction with food, allowing for example the study of circadian variation in "feeding" over a week (Ro et al. 2014).

The second system, the FlyPAD (fly Proboscis and Activity Detector) is an automated, high-resolution behavioural system, which uses capacitive-based measurements to detect the physical interaction of individual flies with food. When an animal, standing on one electrode that surrounds the food source, touches the food which is placed on a second electrode, with its proboscis or leg, changes in the dielectric constant between the two electrodes occur, thereby creating a deviation in the capacitive signal. This sensor has a resolution of 100Hz with a sensitivity of 1fF. One setup can record up to 64 channels simultaneously and independently. This means 32 flies in a two-choice task or 64 in a no-choice task. Furthermore this throughput can be augmented by connecting multiple setups to one computer, as many as USB ports the PC has.

This method makes use of an off-line (after the experiment) algorithm for detection of activity bouts (changes in the capacitive signal that represent the contact of the fly with the food, interacting either with its proboscis, leg or body).

Furthermore, due to the great sensitivity and time resolution this system allows, we were able to show that flies eat from solid sources of food in a very stereotypical way. In fact, flies may use a central pattern generator in order to control a feeding motor programme. Flies rhythmically extend and retract their proboscis on the food. This behaviour was reflected in a highly rhythmic, square wave-like pattern in the capacitance signal. The signal was processed using a “sip-detection” algorithm to study feeding behaviour of flies dynamically across time.

This method is presently one of the most sensitive methods to measure feeding behaviour of flies as it is sensitive to individual sips. Moreover it allows for high-throughput experiments as the data analysis takes much less time than with the “traditional” methods, which rely on manual scoring.

The main limitation of the FlyPAD is its inability to perform long-term experiments (more than 2 hours without disturbing the behaviour of flies); however, with the modification of the setup design this could be overcome (Itskov et al. 2014).

In summary, many methods have been designed and developed to better understand how animals eat, when they eat and what they eat (Itskov & Ribeiro 2013; Deshpande et al. 2014), however we will focus on the FlyPAD throughout this thesis, as it allows the analysis of motor output.

2.2. Genetic Manipulation in *Drosophila*

During the past century a vast arsenal of genetic tools have been developed by *Drosophila* geneticists, making the fruit fly “currently the model organism that allows the most sophisticated genetic manipulations of all higher eukaryotes” (Venken et al. 2011, p.202). Neuroscientists can use such genetic manipulations to gain control over neuronal populations either by silencing or activating them and study how that modulation influences behaviour. In fact, the broad use of *Drosophila melanogaster* in a variety of biological studies, in particular neuroscience, is mainly due to the straightforward ability to express and manipulate a gene of interest in a spatially controlled way in a transgenic animal (Pfeiffer et al. 2010).

The binary *UAS/GAL4* expression system is a methodology used to express a gene of interest at a desired time and place in *Drosophila*. GAL4 is a yeast *Saccharomyces cerevisiae* regulatory protein that binds to specific DNA sites called the upstream activation sequence (*UAS*) (Fischer et al. 1988). When a stock of flies expressing the driver (GAL4) in a particular neuronal population is crossed to one other stock that holds the activation sequence before the transgene of interest, that transgene (that might be a reporter gene, like the LacZ gene or the green fluorescent protein gene) will be expressed in the progeny in the targeted neurons (Brand & Perrimon 1993; Duffy 2002).

Over the last few decades many laboratories have been creating their own GAL4 lines, where this transactivator is expressed in diverse biological tissues and in particular neuronal populations. These lines relay on techniques that reliably integrate exogenous DNA into the germline of *Drosophila melanogaster* (Bachmann & Knust n.d.). In brief, vectors with P-elements that incorporate the GAL4 gene are injected to Drosophila embryos. Upon random transposition, genomic enhancers control the expression of GAL4 (Rubin & Spradling 1982; O'Kane & Gehring 1987; Brand & Perrimon 1993). Furthermore, one other technique has been recently used, in which Phage ΦC31 integrase is used to mediate unidirectional site-specific homologous recombination between its *attB* and *attP* recognition sites (Groth et al. 2004).

Ultimately, GAL4/UAS is the most widely used system for gene manipulation in *Drosophila*. However other similar binary systems are also used, like the LexA transactivator/LexA operator (Szüts & Bienz 2000; Lai & Lee 2006) or the QF transactivator/QUAS systems (Potter et al. 2010).

It is important to state that the transgene under control of the UAS (gene of interest) can encode proteins that supress the activity of neurons, optical sensitive proteins, fluorescent proteins, and even proteins that become fluorescent when the neuron is active (Venken et al. 2011).

2.3. Gustatory System in *Drosophila*

In order to survive, animals must distinguish between edible and contaminated food. Olfaction and taste play an important role on the differentiation of these products. On one hand, olfactory neurons are able to detect volatile cues in order to detect food, presence of predators or mates. On the other hand, the gustatory system recognizes soluble substances that elicit or supress feeding behaviour (Scott et al. 2001; Dunipace et al. 2001; Thorne et al. 2004; Marella et al. 2006).

Unlike vertebrates, which possess only one gustatory organ in the body, insects have their taste organs distributed over different parts of their body. Neurons expressing gustatory receptors (gustatory receptor neurons – GRNs) can be found in sensory bristles on the proboscis, internal mouthpart organs, legs and wings (Stocker 1994).

"The main taste organs are the labial palps, which are located at the distal end of the proboscis, and the labral and cibarial sense organs, which are located inside the pharynx." (Dunipace et al. 2001, p.822) In fact, each one of these organs has a variable number of taste *sensilla*, which contain the dendrites of GRNs. These *sensilla* are morphologically identified as short (S) and long (L), both with one

mechanosensory neuron and four chemosensory neurons (each one tuned either to sugar, water, low salt or high salt concentrations); or intermediate (I), with also one mechanosensory neuron but with two chemosensory neurons (that detect low salt and sugar in only neuron and high salt in the other) (Raphael Falk 1976; Shanbhag et al. 2001; Amrein & Thorne 2005; Hiroi et al. 2004) . Subsequently, these neurons project axons to the subesophageal zone (SEZ) – the first relay centre of gustatory information in the fly brain (Stocker & Schorderet 1981; Wang et al. 2004; Thorne et al. 2004).

By 2001, Clyne, Dunipace, Scott and co-workers analysed the *Drosophila*'s genome using a bioinformatics algorithm and identified all 68 GR genes (each one coding for a seven-transmembrane domain protein) (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001). These genes are expressed by GRNs and each cell can express multiple receptors (Thorne et al. 2004; Wang et al. 2004).

The fruit fly, like other insects and mammals, tends to search for sweet food and avoid bitter compounds as they are usually toxic (Vosshall & Stocker 2007). In *Drosophila*, this distinction has been reported to be mediated by many different GRNs, particularly by the activity of two different GRN populations that lack anatomical overlap (Dunipace et al. 2001; Wang et al. 2004; Marella et al. 2006). These cells are named *Gr5a*- and *Gr66a*-positive neurons. Studies have shown that flies lacking *Gr66a* expression fail to avoid bitter substances and that those lacking *Gr5a* are not attracted to sugar (Thorne et al. 2004). Furthermore, the segregated projection of these two types of neurons reveal that there is a spatial map of taste quality in the fly's brain (Wang et al. 2004). Ultimately, we can say that activation of *Gr5a* cells mediates attractive/appetitive behaviours while the activation of *Gr66a* cells mediates aversive responses (Marella et al. 2006).

2.4. What is Optogenetics?

2.4.1. History

Optogenetics refers "to the integration of optics and genetics to achieve gain- or loss-of-function of well-defined events within specific cells of living tissue" (Yizhar et al. 2011).

In 1971 and 1973 Oesterhelt and Stoeckenius (Oesterhelt & Stoeckenius 1971; Oesterhelt & Stoeckenius 1973), identified the first bacteriorhodopsin, a microbial light-activated ion pump. In the subsequent decades, the microbial opsin family that comprises this pump was later enlarged by hyperpolarizing light-driven chloride importers – the halorhodpsins (Matsuno-Yagi & Mukohata 1977) and by depolarizing light-gated cation channels – the channelrhodopsins (Nagel et al. 2002; Nagel et al. 2003). These findings by Nagel and his team were influenced by Hegemann's work, in which he discovered and characterized a *Chlamydomonas* photoreceptor that promotes positive or negative phototaxis (the movement of this green alga towards or away from light) (Hegemann 1991).

2.4.2. Different opsins

Opsin genes can be divided into two distinct superfamilies: microbial opsin genes (type I) and animal opsin genes (type II) (Fenno et al. 2011). Although these opsin families are very similar in structure, as

both encode a seven-transmembrane protein, type I is only found in prokaryotes, algae and fungi (Spudich 2006), whereas type II is present in higher eukaryotes, and is mainly responsible for vision (Sakmar 2002; Shichida & Yamashita 2003).

2.4.3. All-*trans* retinal

Each opsin requires a vitamin A-related cofactor (retinal) to form a functional molecule, the rhodopsin (Yizhar et al. 2011). Upon absorption of a photon, retinal isomerizes and triggers a sequence of conformational changes within the opsin partner, therefore allowing ions to cross through the cell's membrane (Fenno et al. 2011).

This process is slightly different across the various opsin families. On one hand, type II opsin genes encode G protein-coupled receptors and bind retinal in the 11-*cis* configuration while in the dark. Retinal isomerises to the all-*trans* configuration upon illumination, which induces the conformational changes of the protein. After photoisomerisation, the retinal-protein linkage is hydrolysed, free all-*trans* retinal is released from the protein and is replaced by a new 11-*cis* retinal molecule for later signalling (Hofmann et al. 2009). On the other hand, type I opsins encode proteins that utilise retinal in the all-*trans* configuration, which photoisomerises upon photon absorption to the 13-*cis* configuration. The activated retinal molecule in type I rhodopsins does not dissociate from its opsin protein, but thermally reverts to the all-*trans* state (Haupts et al. 1997).

2.4.4. Optogenetics in Neuroscience

Due to a variety of obstacles to the use of these microbial opsins, for a long time researchers postponed the experimental implementation of optical control of neurons. These presumptions include arguments such as: photocurrents wouldn't be strong and fast enough to efficiently control neurons; microbial membrane proteins would be toxic and have low expression levels in mammalian neurons; and an additional cofactor (all-*trans* retinal) would have to be added to any intact-tissue experimental system (Yizhar et al. 2011). Yet, in 2005, Boyden and Deisseroth demonstrated that the introduction of microbial opsin genes (in particular channelrhodopsin-2) (Nagel et al. 2003) to mammalian neurons could reliably generate defined sequences of spikes or synaptic events in response to light with millisecond-timescale temporal resolution (Boyden et al. 2005).

Additionally, Deisseroth and Zhang showed a year later that mammalian brains, and indeed all vertebrates, have sufficient all-*trans* retinal for microbial opsins to be successfully used as a single-component tool (Deisseroth et al. 2006; Zhang et al. 2006).

From 2005 onwards, many improvements have been made so that experiments can be tuned up and opsins used to achieve the desired physiological effect, the desired kinetic properties and the required wavelength, power and spatial extent of the stimulation. Nowadays there are over twenty single-component optogenetic tools (Yizhar et al. 2011; Fenno et al. 2011).

Most experiments require a specific *in vivo* delivery procedure of the optogenetic tool. In mammals (rats, mice and primates) the most commonly used techniques to deliver the opsins to targeted cells are: viral

promoter targeting, projection targeting, transgenic animal targeting and spatiotemporal targeting (Yizhar et al. 2011). In brief, viral expression systems depend on the injection of a viral vector (for example, lentiviral vectors or adeno-associated viral vectors) that carries the genetic material of the microbial opsins into the targeted mammalian cells (Hendrie & Russell 2005). For projection targeting, microbial opsin gene products are used, which traffic down dendrites or axons and create light-sensitive projections (Gradinaru et al. 2010). For transgenic animal targeting, it is possible to use mouse transgenic lines directly expressing opsin genes under local promoter-enhancer regions (Wang et al. 2007). Finally, spatiotemporal targeting relies on electroporating optogenetic constructs into mouse embryos in utero (Petreanu et al. 2007). The two last techniques present some advantages compared to the viral infections, as opsins are expressed from the time of birth (Fenno et al. 2011).

Unlike mammals, flies and worms do not possess sufficient levels of endogenous retinal for opsins to function in all cell types. Therefore, supplementary retinal in the food is needed to enable the use of optogenetic tools in these animals (Zhang et al. 2010). In order to use opsin genes in Drosophila, one needs to generate transgenic animals, by injecting a construct into the fly which, when incorporated, will allow expression of opsin genes under the control of UAS, either using a P-element insertion (Rubin & Spradling 1982) or site specific integration using Phage ΦC31 integrase system (Groth et al. 2004).

There are various single-component optogenetic tools available (Yizhar et al. 2011; Fenno et al. 2011). Nonetheless, I will give a more detailed insight on two particular tools: ReaChR and Chrimson channelrhodopsins.

Although flies are an incredible model to dissect neuronal and molecular mechanisms underlying behaviour or circuit function, the use of optogenetics in adult flies has been difficult to implement due to two main reasons. The majority of channelrhodopsins available are blue light-sensitive channels. Flies can see blue light (Stavenga 2002), so stimulation might induce visually driven behavioural artefacts, as optical stimulation in such small animals will inevitably reach the eye. On the other hand, blue light is strongly absorbed by the cuticle of the fly, which may prevent activation of the channels if the targeted neurons are not close to the surface (Inagaki et al. 2014).

In the absence of proper optogenetic tools, researchers started using a thermosensitive cation channel (dTRPA1) (Rosenzweig et al. 2005) for neuronal activation in freely moving animals (Venken et al. 2011). However this method lacks both temporal and intensity precision and might lead to confounding results in behavioural studies due to temperature changes (Inagaki et al. 2014).

In 2014, two new red-shifted channelrhodopsins were described and from then on, have been quickly adopted by *Drosophila* researchers.

One of them, engineered by Roger Tsien and his team in the University of California, is called ReaChR (Red activatable Channelrhodopsin), which has improved membrane trafficking, greater photocurrents and fast kinetics when compared to a previously described red spectral shifted channelrhodopsin (C1V1(E122T)). Its maximum response is given at ~590nm. This opsin was proposed to be a robust means to stimulate neurons with red-orange to red light (Lin et al. 2013). In fact, this tool was used

successfully to dissect the neuronal control of Drosophila's male courtship, using 1.1 mW/mm^2 (Inagaki et al. 2014).

The other red-shifted channelrhodopsin was described by Klapoetke and colleagues at MIT, who discovered multiple channelrhodopsin homologs from different species, via *de novo* sequencing. The new red-light drivable channelrhodopsin, discovered from the species *Chlamydomonas noctigama*, was nicknamed Chrimson and, with a spectral peak at 590nm, was described as "45nm more red-shifted than any other previously known channelrhodopsin". This tool was effectively tested in Drosophila by measuring the percentage of flies expressing Chrimson in *Gr64f* neurons (sweet taste receptors) that elicited PER, when stimulated (with 0.015 mW/mm^2) with amber light (617nm). Chrimson proved to be a reliable and fast means to optogenetically stimulate flies (Klapoetke et al. 2014).

2.5. What is "Closed-loop"?

"Closed-loop" refers to the control systems that uses the output of a system to control or modify the input. These systems measure the error between the desired and the actual state of the plant and uses it to alter the signal of the input (Oppenheim et al. 1997, chap.11). An example of a closed-loop system is the human ability to control hand motion when grasping an object. The input signals to the moving hand are modified by the visual feedback, as the directional movement of the hand is clearly different if we have our eyes opened or closed.

Closed-loop systems are widely used in electronics, for example the control system of "cruise control" that is used in cars, in order to maintain a specific speed. In science, and in particular in Neuroscience, "closed-loop" systems have been taking a role in studies made with non-human animals as well as in clinical applications in humans.

From the clinical point of view, closed-loop systems have been used to optimize rehabilitation in patients with movement disorders like dystonia and Parkinson's disease. Deep Brain Stimulation (DBS) has been an important method that shows improvement on the physical symptons of these diseases. As these diseases are progressive and dynamic, the feedback control of DBS has been very helpful in the protocol optimisation. Also, this adaptive control allows "a transformative way toward individually tailored rehabilitative therapeutics" (Afshar et al. 2012; Broccard et al. 2014), not only for movement disorders, but also for psychiatric diseases, pain or epilepsy (Carron et al. 2013).

Closed-loop experiments in Drosophila have manly been used to study optomotor responses, where various visual stimuli lead to different motor behaviours, for example, following the movement of a vertical bar. These motor responses are usually studied in the context of walking or flying. Researchers can study locomotion either in freely moving or tethered flies.

Studying freely moving animals (walking or flying) requires video tracking of the animal, where its position serves as input for modifications either in the visual stimulus (projection or display panels) (Schuster et al. 2002; Rohrseitz & Fry 2011), in the direction or strength of wind in a wind tunnel system (Fry et al. 2008) or even in the target of one laser in order to heat parts of fly's body (Bath et al. 2014).

In setups where the fly is tethered, closed-loop systems have received more specialisation and development.

When studying walking in Drosophila, flies are left to walk on an air-suspended ball. The rotation of the ball is recorded and the path the fly takes is integrated in a virtual map of the movement of the fly. The fly is able to navigate in a virtual world as its movement is being mapped onto the projected visual stimulus, thus closing the loop (Seelig et al. 2010; Bahl et al. 2013; Moore et al. 2014; Paulk et al. 2015).

The same virtual world navigation occurs in studies using flight simulators, where the fly is suspended and either measurements of the yaw torque (Götz 1964; Heisenberg & Wolf 1979) or of wing beat amplitude and frequency (Gotz 1987; Lehmann & Dickinson 1997; Lehmann & Dickinson 1998; Dickinson 1999; Fenk et al. 2014; Stowers et al. 2014), serve as input for the navigation in the virtual reality that, is being presented to the fly.

Furthermore, closed-loop systems in Drosophila play a key role in studying memory and learning in flies. Examples of conditioning in flies, are the flight simultator developed by Wolf and Heisenberg in 1990 (Wolf & Heisenberg 1990), where the fly's yaw torque is input for modifications in the visual stimulus, and the heat box by Wustmann in 1996 (Wustmann et al. 1996), where the position of the fly serves as input to control a heater that functions as an aversive stimulus.

3. Materials and Methods

We built a new setup based on the previous behavioural measurement system, the FlyPAD (Fly Proboscis and Activity Detector). The FlyPAD is composed of 32 individual arenas (8x4), each one hosting one capacitance sensor for two independent channels, one board hosting the Field Programmable Gate Array (FPGA), that with the implementation of an I^2C (Inter-Integrated Circuit) protocol allows the simultaneous recording of the 64 channels, streaming them to the PC via USB port (Fig. 3.1). The computer is able to receive the data from the USB port using *Bonsai*, a "modular, high-performance, open-source visual programming framework for the acquisition and online processing of data streams" conceived and developed at the Champalimaud Foundation (Lopes et al. 2014). To save the data from the FlyPAD, *Bonsai* allows an interface to record the capacitance signal at 100Hz.

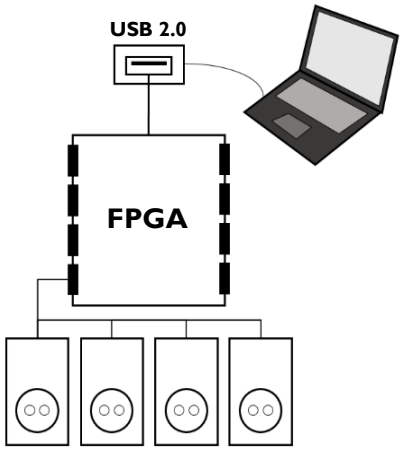

Fig. 3.1 Schematic of the FlyPAD
(adapted from Itskov et al. 2014)

Figure 3.2 represents the concept of the new behaviour recording and manipulation setup, from now on called OptoPAD.

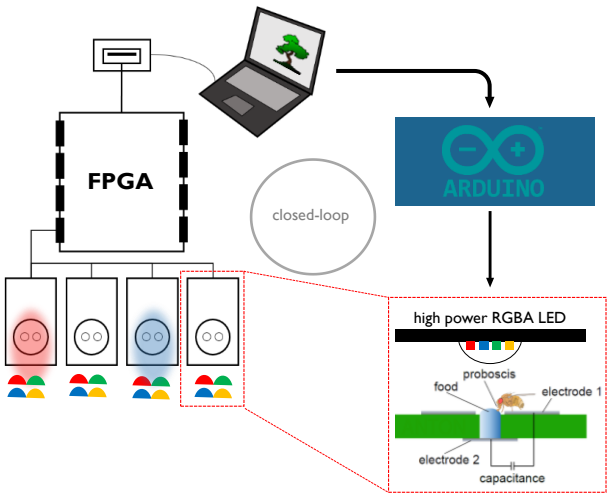

Fig. 3.2 Schematic of the OptoPAD
From the FlyPAD schematic previously shown, to the clockwise direction we find the PC running Bonsai and receiving the capacitive data stream; Bonsai is able to send activation messages to different pins of the Arduino (the actuator), which will activate the different colours of the LEDs on top of the FlyPAD behavioural arenas where the animals are interacting with food.

Throughout this chapter I will give a detailed explanation of each part of Fig. 3.2, starting with the hardware.

3.1. Hardware

Hardware development can be divided into two parts: development of the board hosting the LED for light stimulation and development of the board hosting the controller (Arduino) that distributes the power for light activation.

3.1.1. OptoPAD LED Board

As stated in the goal of this project, we wanted to make sure that this setup could be used with any opsin, independently of its activation wavelength. Because of this we made sure to use a Light Emitting Diode (LED) that would emit in any visible wavelength. We chose the High Luminous Efficacy 10W RGBA LED from *LedEngin, Inc.* (ref. no. LZ4-00MA10). The optical and electrical characteristics at 25ºC are stated in the following table.

Table 3.1 Optical and Electrical characteristics of LZ4-00MA10 *(adapted from LED documentation)*

	Parameter	Symbol	Typical				Unit
			Red	Green	Blue	Amber	
Optical	Luminous Flux (@ I_F=700mA)	Φ_V	87	120	32	736	lm
	Dominant Wavelength	λ_D	625	523	460	590	nm
	Viewing Angle[1]	$2\Theta_{1/2}$	95				Degrees
	Total Included Angle[2]	$\Theta_{0.9}$	115				Degrees
Electrical	Forward Voltage (@ I_F=700mA)	V_F	2.4	3.9	3.5	2.5	V
	Temperature Coefficient of Forward Voltage	$\Delta V_F/\Delta T_J$	-1.9	-2.9	-3.0	-2.8	mV/ºC
	Thermal Resistance (Junction to Case)	$R\Theta_{J-C}$	1.8				ºC/W

In order to use 32 of these LEDs, we need 22.4A (700mA x 32) and a power of 320W (10W x 32) if all the LEDs would be activated at the same time.

[1] Viewing Angle is off axis angle from emitter centreline where the luminous intensity is ½ of the peak value.
[2] Total Included Angle is the total angle that includes 90% of the total luminous flux.

We decided to use the Corsair CS750M ATX Power Supply, an external power supply unit (PSU) that could give us a Maximum Load of 25A and a Maximum Combined Wattage of 130W in each DC Ouput of 3.3V and 5V.

Subsequently, we designed a control circuit for the multiple LEDs using transistors as switches. The control of the active state of the transistors would be set by a constant potential input to their gate pins. The transistors were chosen based on the voltage needed to activate them and on the drain current they sustain. We decided to use a MOSFET N-Ch that needed 1.8V limit tension and has 1.1A drain current which is enough to activate our LEDs (0.7A). In order to control the gate pin voltage of each transistor, we used the digital control of the Arduino which changing from low state to high state gives a voltage difference from 0 to 5V, respectively. Arduinos are microcontroller boards that can be programed or serve as a circuit controller that receives information online from one computer (https://www.arduino.cc).

Calculation of Resistor value:

$$R_{LED\ colour} = (V_{dd} - Forward\ Voltage_{LED\ colour}) \times I_F \qquad (1)$$

These calculations were performed taking into account the information in the documentation of the LEDs, listed above. The final version of the circuit was designed using Eagle 6.2.0 software. V_{dd} for LED_{Red}, LED_{Green} is 5V and for LED_{Blue}, LED_{Amber} is 3.3V. (Fig. 3.3)

Table 3.2 Resistors Values

	Value [Ω]
R1	3.3
R2	0.5
R3	1
R4	0.5
R5	2

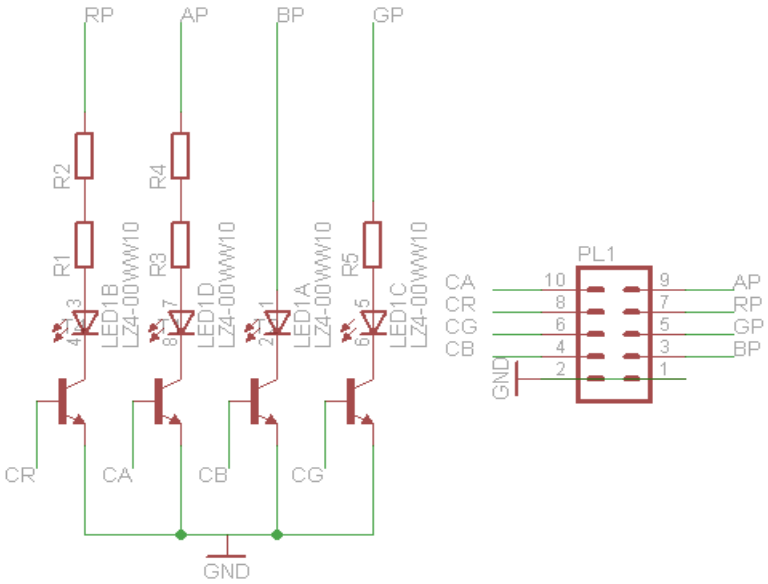

Fig. 3.3 Schematic of the OptoPAD LED Board

RP – Red Power; AP – Amber Power; BP – Blue Power; GP – Green Power; CR – Control Red; CA – Control Amber; CB – Control Blue; CG – Control Green

Finally, using the same Eagle software we designed a board hosting the LED and the four controlling transistors. (Fig. 3.4)

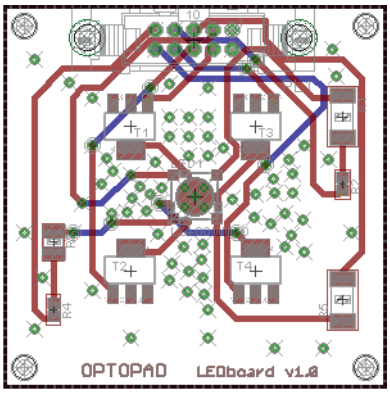

Fig. 3.4 OptoPAD LED Board Design

The multiple vias that connect the top extended ground to the bottom one serve to dissipate as much heat as possible from the activated LED. This way we avoided the installation of a heat-sink.

The dimensions of this board were set so that it can be placed exactly on top of the FlyPAD arena with the help of 4 screws and nuts, not needing any additional structure. Furthermore, the LED is positioned in order to precisely face the center of the arena. (Fig. 3.5)

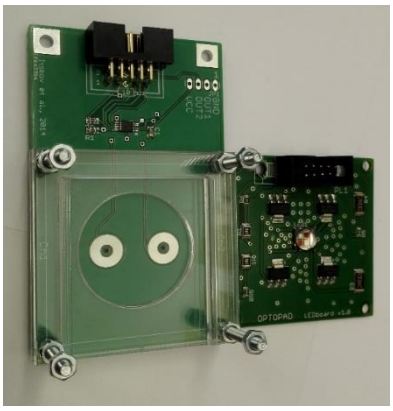

Fig. 3.5 FlyPAD arena and OptoPAD LED Board side by side to depict the similar dimensions

3.1.2. OptoPAD Main Board

We needed a board that would host the Arduino and would distribute all the power to the different OptoPAD LED Boards.

In order to allow the manipulation of 32 flies in individual arenas, we needed 128 digital pins (32 x 4) to control the 4 transistors on each of the 32 OptoPAD LED Boards. We decided to use an Arduino Mega 2560 which has 54 digital input/output pins. By using three of these boards we could control all the LEDs (3 x 54 = 162 > 128).

To power the LEDs we connected the OptoPAD Main Board to the Corsair CS750M ATX Power Supply, which distributes the electricity to the OptoPAD LED Boards. As this power supply is modular, it gave us the necessary flexibility to use any power cables. As we needed input voltage of 3.3 V and 5 V to power our LEDs we decided to use SATA Power Cables. (Fig. 3.6)

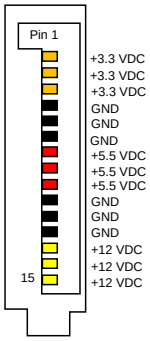

Fig. 3.6 Serial ATA (SATA) Power Connector pinout.

Ultimately, we can say that the OptoPAD Main Board is an interface board that distributes power and control inputs to the OptoPAD LED Boards.

Figure 3.7 shows the schematics of the OptoPAD Main Board and Figure 3.8 depicts the actual board design. Both were produced using Eagle 6.2.0.

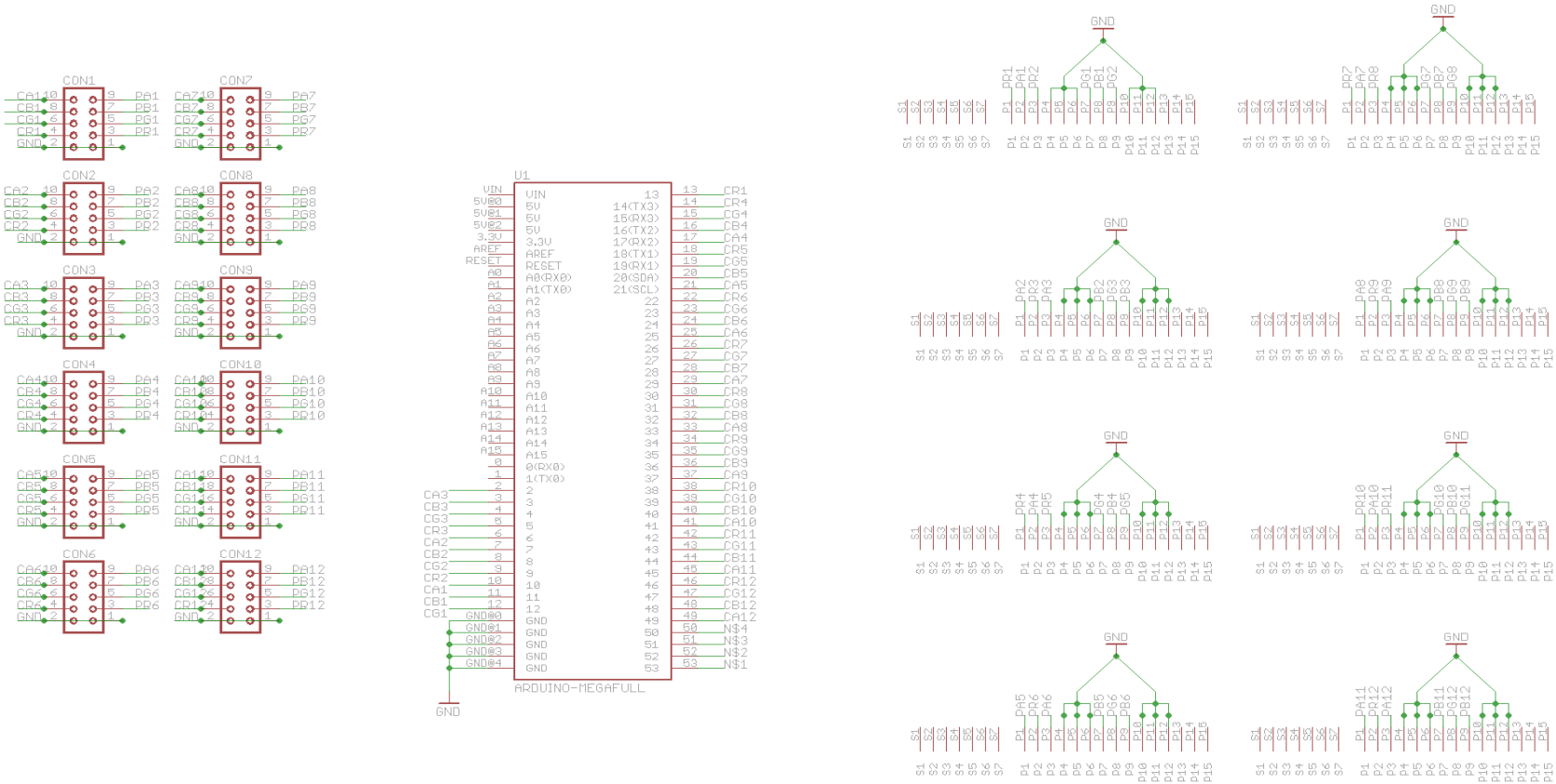

Fig. 3.7 OptoPAD MainBoard schematics

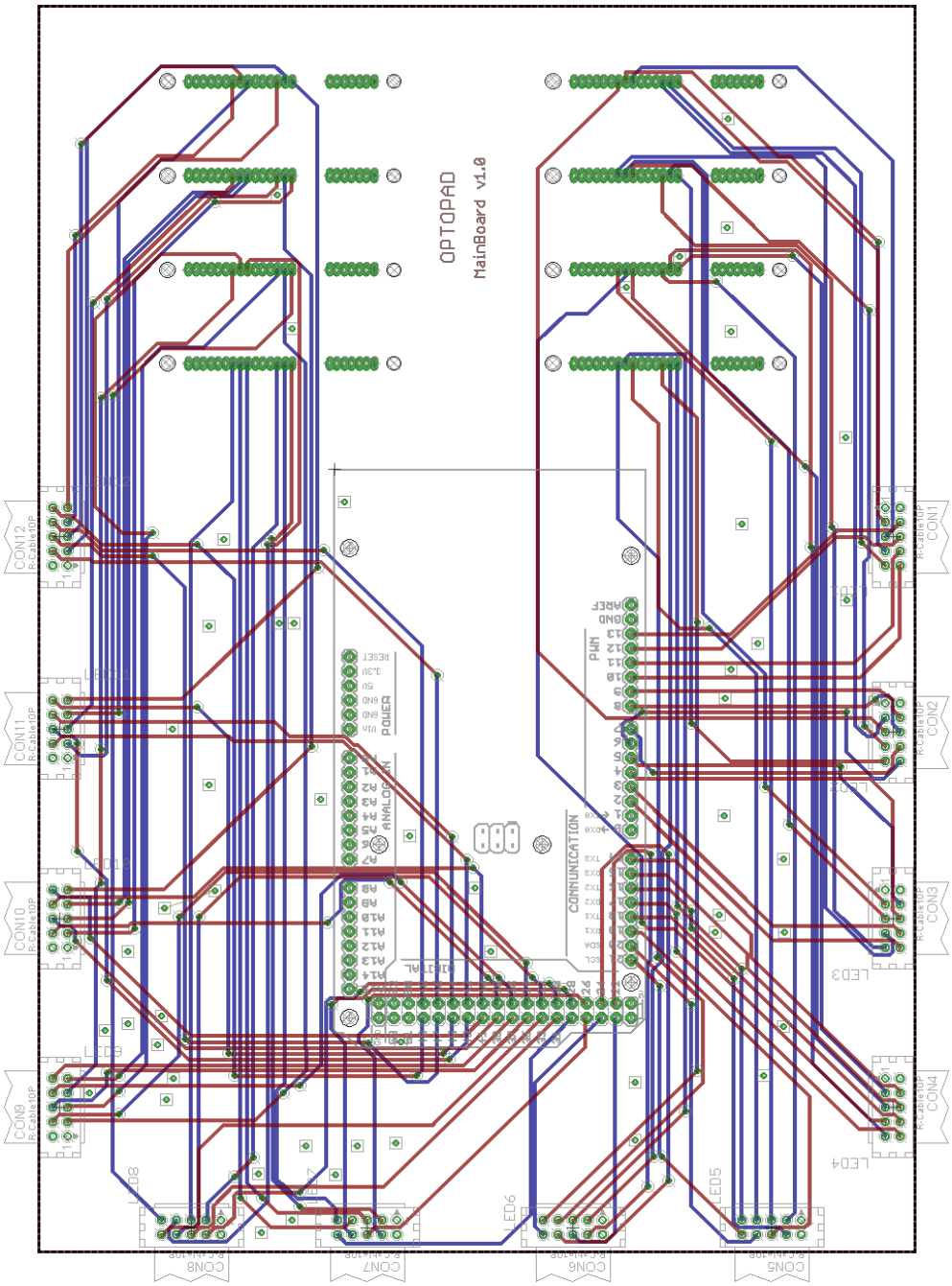

Fig. 3.8 OptoPAD MainBoard Design

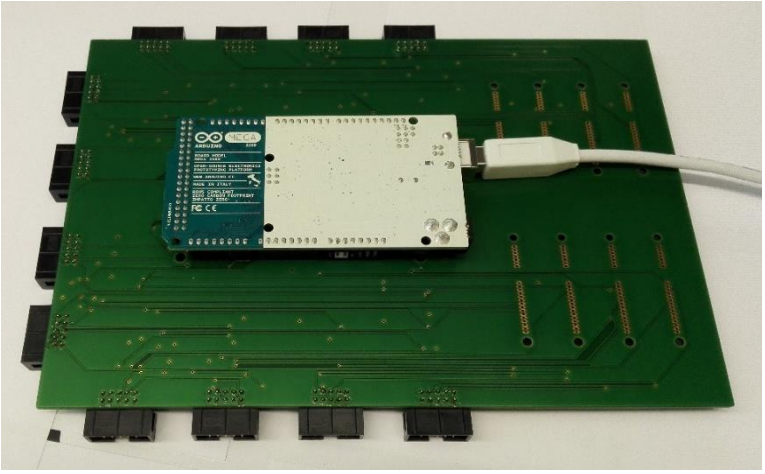

Fig. 3.9 OptoPAD MainBoard

Each one of the three OptoPAD Main Boards (Fig. 3.9) is able to control and power 12 OptoPAD LEDBoards. A schematic of the whole system is depicted on Figure 3.10.

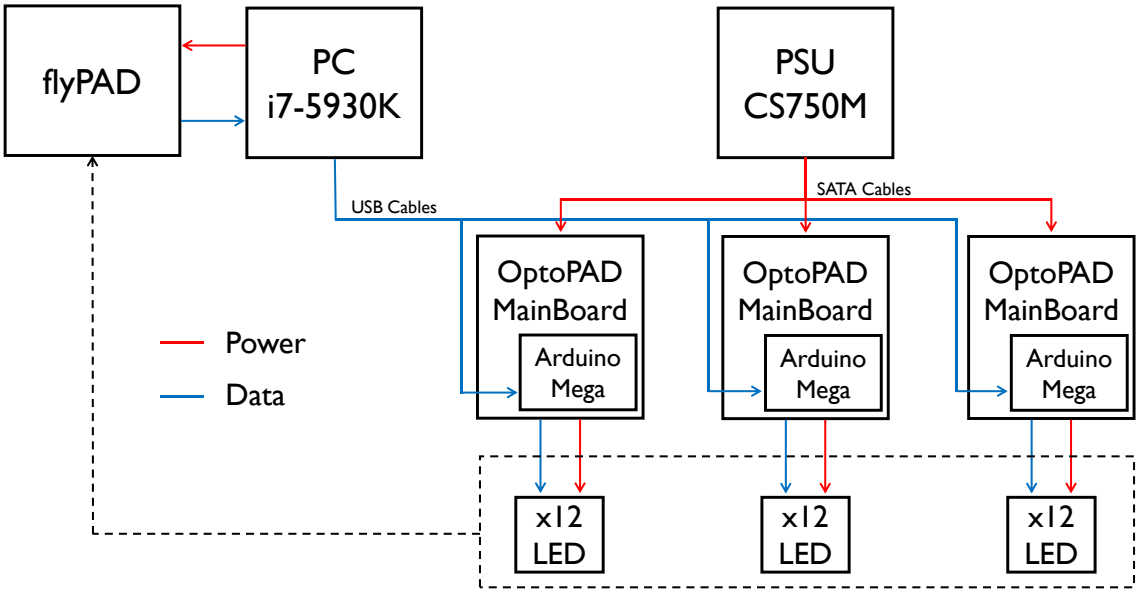

Fig. 3.10 Complete system schematics

3.2. Software

After the hardware was designed and assembled we developed the software to run the closed-loop experiments. The software can be divided into three parts: activity bout detection algorithm, experiment structure builder and the complete *Bonsai* workflow.

3.2.1. Activity bout detection algorithm

Unlike the offline activity bout detector for the FlyPAD written in MATLAB, which takes into account the capacitance signal of all the experiment in order to detect activity bouts (whenever the fly interacts with food), we needed an online real-time detector for the OptoPAD. Taking into account that the data stream of the FlyPAD is designed to enter the computer via a *Bonsai* workflow, and knowing that this visual framework is able to process data in real time, we used this software to design our activity bout detection algorithm and further protocols.

We wanted to associate the optogenetic stimulation to behaviour of flies on food, be that either tasting or feeding. An activity bout is defined as any active interaction between the fly and the food. As soon as the fly touches the food, a change in the capacitance signal of the FlyPAD is detected.

We created a workflow in *Bonsai* capable of processing each channel of the FlyPAD independently. Figure 3.11 illustrates the Activity Bout Detection Algorithm built in *Bonsai*.

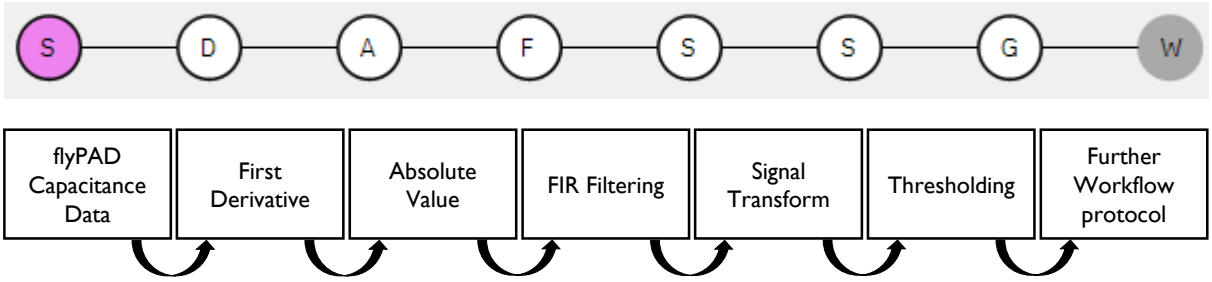

Fig. 3.11 Bonsai: Activity Bout Detection Algorithm

This figure represents the activity bout detection algorithm that is performed for every channel of the FlyPAD (64 signal parallel processing). Firstly, the pink “S” represents the Source, which in this case is one of the capacitance signals coming from the “FlyPadSource”. To this signal we primarily apply the derivative of two consecutive samples (“D” node). With this, the sharp positive and negative peaks of this transformation are related mainly to the onset and offset of sips. Next, in order to amplify the signal the “absolute value” transformation is applied to it, making every negative value become positive (“A” node). Consequently, the data is filtered (“F” node). This filter is a “FIR filter” (Finite Impulse Response) that, with a kernel of only ones, performs an addition on the values inside that kernel window. From the “F” node we get an “OpenCV.Net.Mat” output. After the next “S” node which is a “Sum”, performing a sum of one sample, the result being that sample value, we get an “OpenCV.Net.Scalar” output. The following “S” node is just an output selection of the previous one: “Source.Val0” being a double. Lastly, performing a “greater than” comparison (“G” node) we can identify activity bouts.

The last node “W” is a node that transmits the signal to further processing in the workflow.

In this algorithm, there are two parameters (FIR filter kernel window size on the “F” node and threshold value on the “G” node) that can be tuned to optimize this protocol of detection. However this matter will be further discussed in the Results chapter.

3.2.2. Experiment Structure Builder

From the Activity bout detection protocol the signal is transformed into a digital data stream where the high and low states mean the fly is interacting or not with food, respectively. Each experiment can be run in each independent arena, being defined by 8 parameters:

[colourCh1, colourCh2, delayCh1, delayCh2, sustainCh1, sustainCh2, probabilityCh1, probabilityCh2]

Figure 3.12 represents how an experiment in one arena runs.

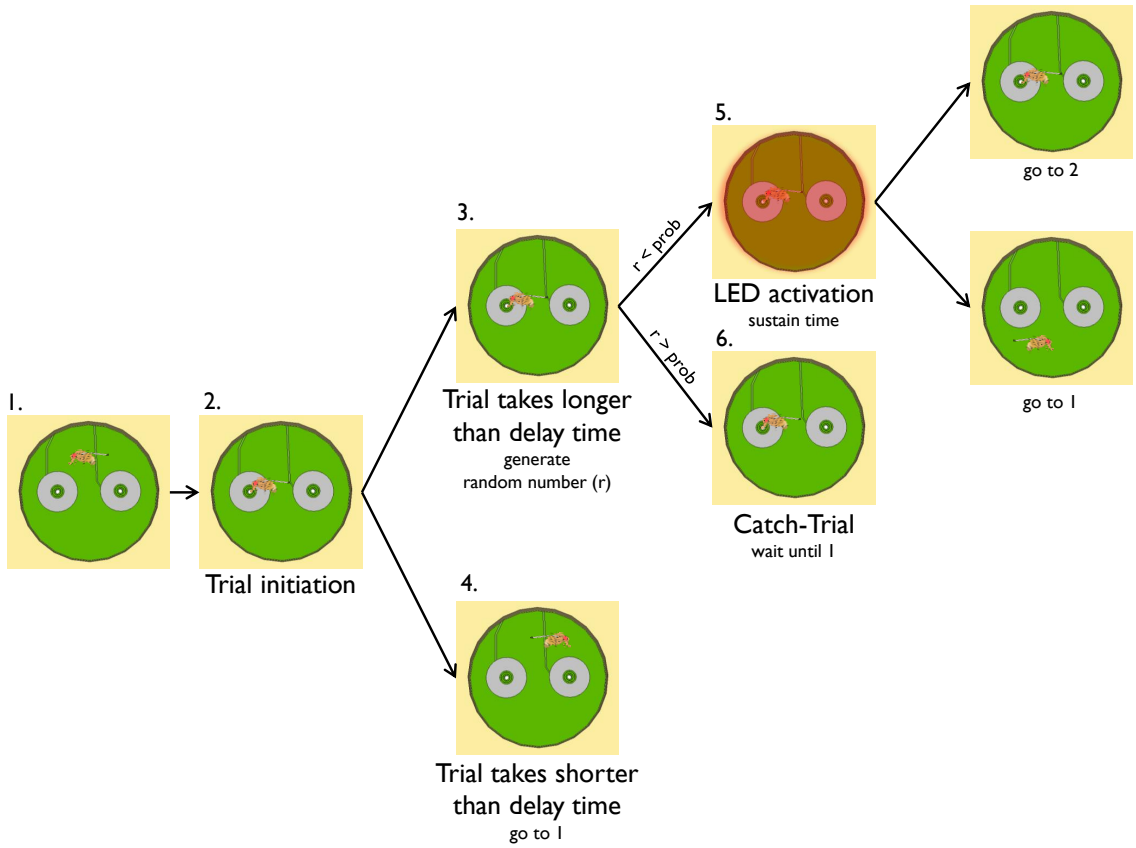

Fig. 3.12 Experiment structure

1. Fly walks in the arena; 2. Fly finds a food source; 3. Fly keeps interacting with food for longer than the "delay" time; 4. Fly does not interact with food for longer than the "delay" time; 5. LED is active for "sustain" time; 6. Catch-Trial: system waits until activity bout is over

We wrote a function in MATLAB (Appendix 1) to build a 32x8 matrix that represents the 32 arenas of one setup and the eight parameters of the experiment structure. This function saves the personalized matrix into a ".dat" file to be read by *Bonsai* in the LED activation protocol.

This system is able to activate opsins sensible to diverse wavelengths (capable of stimulating at four different wavelengths: 460 nm – blue, 523 nm – green, 590 nm – amber and 625 – red). The experiment structure takes into account the multiple LED colours installed in the OptoPAD LED Board, being set with different values on the colourCh1 and colourCh2 parameters. These two parameters can be set to five modes: '0'-no light; '1'-red; '2'-blue; '3'-green; '4'-amber.

In order to build a flexible setup, where we could study learning and memory, the feature of "catch-trials" was essential. A catch-trial is a part of the experiment which, in this case, after a complete trial where

the fly interacts with food for longer than the set delay time, no optogenetic stimulation will occur but the behaviour of the animal is still recorded. The last two parameters of the experiment structure (probabilityCh1 and probabilityCh2) define the percentage of trials that should give rise to stimulation. This parameter can be understood as “probability of stimulation” and is set to a number between 0 and 1 (percentage/100). The remaining trials will give rise to “catch-trials”.

3.2.3. Bonsai Workflow

We can detect in real time if the fly is interacting with food and we have defined how an experiment should look in terms of its structure. The next step is to integrate all this information and build a workflow in *Bonsai* that would run these experiments.

The complete *Bonsai* workflow (detailed explanation in Appendix 1) is depicted on figure 3.13.

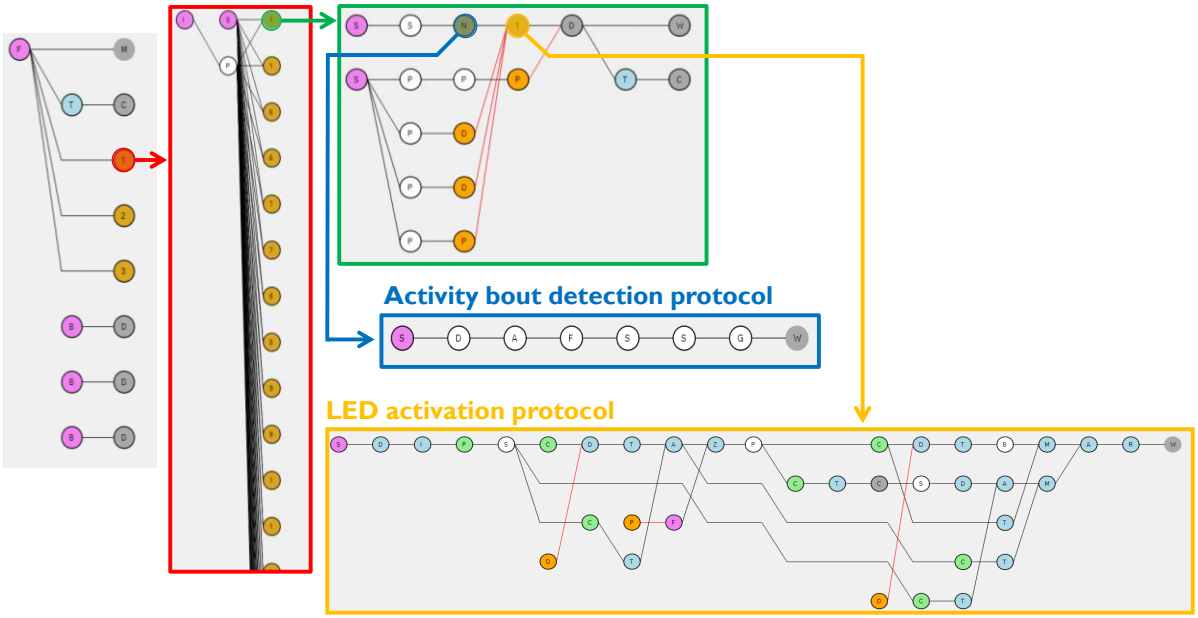

Fig. 3.13 Bonsai: Complete Software Workflow

OptoPAD's software runs as follows: the real time capacitance data from the FlyPAD enters the computer via Serial communication; the capacitance signal from each of the 64 channels is processed in parallel in *Bonsai*, where the activity bout detection algorithm detects if the fly is interacting with the food; *Bonsai* imports the "experiment structure" file, created in MATLAB; *Bonsai* runs the LED activation algorithm according to the experiment structure explained in figure 3.12 (Fig. 3.14).

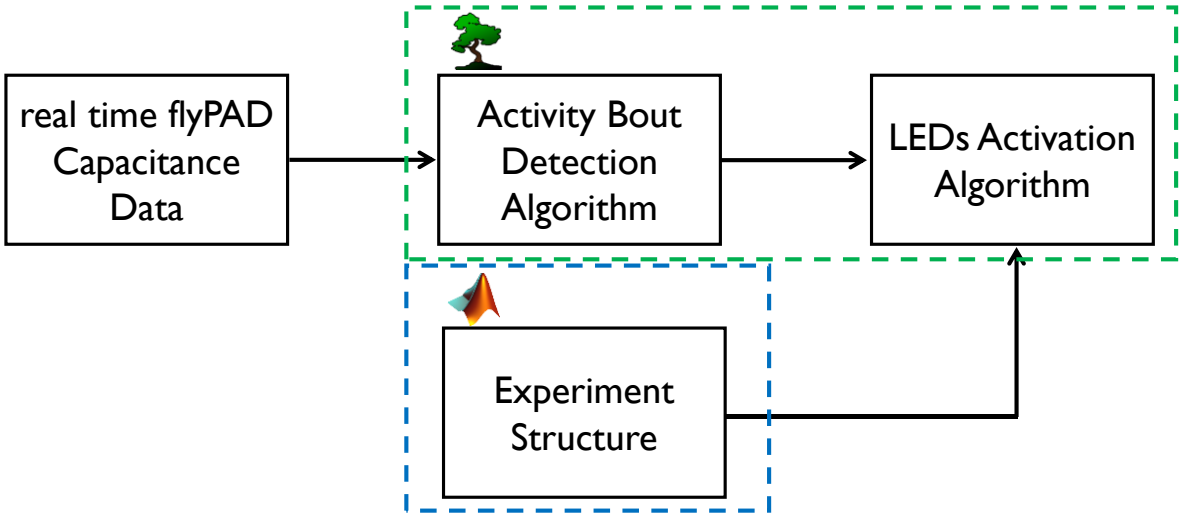

Fig. 3.14 Data flow in the complete OptoPAD system

3.3. Experiments

3.3.1. Stocks

Drosophila stocks were maintained on standard medium (containing, per litre, 80g cane molasses, 22g sugar beet syrup, 8g agar, 80g corn flour, 10g soya flour, 18g yeast extract, 8ml propionic acid and 12ml nipagin (15% in ethanol)) at 18ºC with 12:12 light/dark cycle. The following lines were used:

1. *Gr66a-Gal4/Cyo*
2. *Gr5a-Gal4 (II); Gr5a-Gal4 (III)*
3. *w-;;UAS-ReaChR/TM6 Sb Tb*
4. "virginized" stock *+/yhs-hid;;pUAS-Chrimson-mVenus/TM3, Sb*

3.3.2. Appetitive Behaviour Experiment Design

Our first experiment was designed to test if we could induce appetitive behaviour by activating *Gr5a* (sugar) positive neurons.

Gr5a-Gal4 (II);Gr5a-Gal4 (III) males were crossed to *+/+;+/+;pUAS-Chrimson-mVenus/TM3, Sb* virgins and always kept in the dark. Flies were left to mate at 25ºC for three days. Parents were discarded and on the 14th day after crossing progeny were sorted against balancers and males were selected.

Gr5a-Gal4 (II)/+;Gr5a-Gal4 (III)/pUAS-Chrimson-mVenus (*Gr5a*>Chrimson) males were starved for 24h in a vial with paper towel soaked with a 5mL aqueous solution of 400µM all-*trans* retinal.

After these 24h, flies were tested in the OptoPAD with both sources of food containing a solution of low sucrose concentration (5mM sucrose solution). We used a low sucrose solution so that the food would not be very appetitive *per se* but would be appetitive enough to elicit rare feeding events. Our hypothesis was that we could manipulate the fly's feeding behaviour to eat more from a source of food that would trigger sweet sensors via optogenetic stimulation than from a similar source without the stimulation.

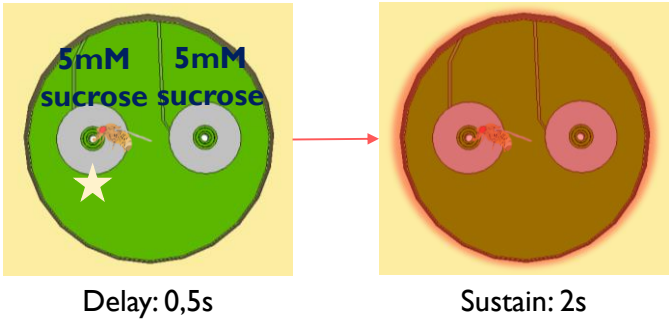

Fig. 3.15 Appetitive Behaviour Experiment Schematics
Flies interacting with the channel marked with the star for longer than 0.5s would get red light stimulation for 2s. Both sources of food contained 5mM sucrose solution

3.3.3. Aversive Behaviour Experiment Design

In the second set of experiments, we wanted to test if by activating *Gr66a* (bitter) positive neurons, flies would show aversive behaviour towards the source of food associated with the activation.

Gr66a-Gal4/Cyo;+/+ males were crossed to *w-;+/+;UAS-ReaChR/TM6 Sb Tb* virgins and always kept in the dark. Flies were left to mate at 25ºC for three days. Parents were discarded and on the 14th day after crossing progeny were sorted against balancers and females were selected.

Gr66a-Gal4/+;UAS-ReaChR/+ (*Gr66a*>ReaChR) females were deprived from protein for 3 days in a vial with paper towel soaked with a 5mL aqueous solution of 100mM sucrose and 400µM all-*trans* retinal. To these female flies, 3 males were added to the vials to make sure the females were mated.

After 3 days, flies were tested in the OptoPAD setup with both sources of food containing a 10% yeast solution (a highly appetitive food for female flies).

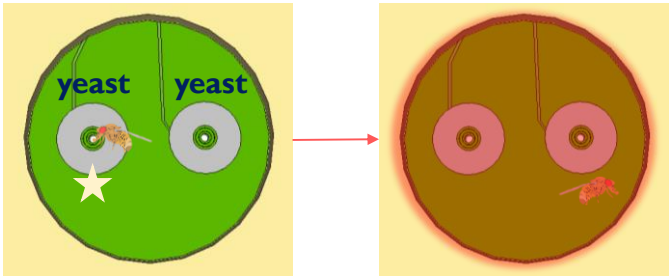

Fig. 3.16 Aversive Behaviour Experiment Schematics

Flies interacting with the channel marked with the star for longer than 1s would get red light stimulation for 2s. Both sources of food contained 10% yeast solution

3.3.4. Preference Shift Experiment Design

The following experiment is a variation of the previous one. If flies show aversive behaviour when *Gr66a*-positive neurons are activated, could we shift preference of food choice while the assay is running?

To enable this experiment we had to add a feature to the *Bonsai* workflow, so it could select, according to the run time of the experiment, from which "experiment structure file" it would read.

Gr66a-Gal4/Cyo;+/+ males were crossed to *+/+;;pUAS-Chrimson-mVenus/TM3, Sb* virgins and always kept in the dark. Flies were left to mate at 25ºC for three days. Parents were discarded and on the 14th day after crossing progeny were sorted against balancers and females were selected.

Gr66a-Gal4/+;pUAS-Chrimson-mVenus/+ (*Gr66a*>Chrimson) females were deprived from protein for 3 days in a vial with a paper towel soaked with a 5mL aqueous solution of 100mM sucrose and 400µM all-*trans* retinal. To these female flies, 3 males were added to the vials to make sure the females were mated.

After 3 days, flies were tested in the OptoPAD setup with both sources of food containing a 10% yeast solution. In this experiment we changed the structure of the experiment across channels with a periodicity of 5min blocks.

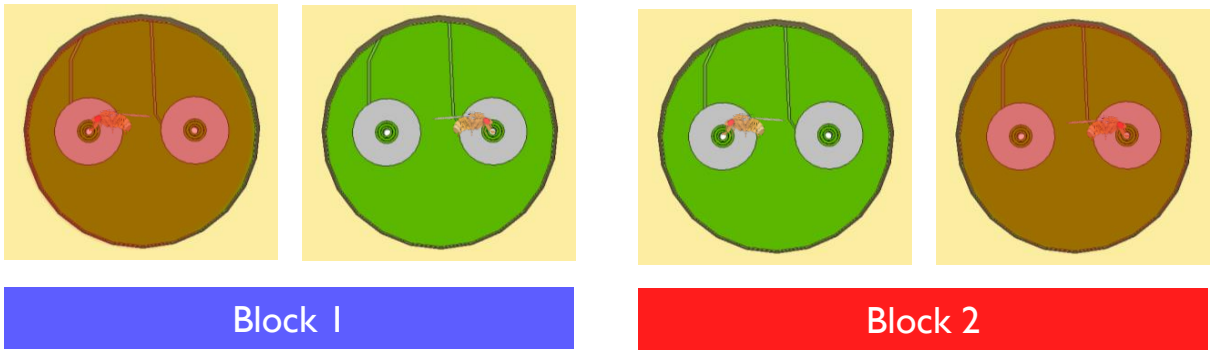

Fig. 3.17 Shift Preference Experiment Schematics

Block 1 – in the first 5min flies interacting with the channel 1 for longer than 0.5s would get red light stimulation for 2s. Block 2 – in the second 5min flies interacting with the channel 2 for longer than 0.5s would get red light stimulation for 2s. After 10min the experiment continues running Block 1 again, followed by Block 2, repeatedly until the end of the 60 minutes. Both sources of food contained 10% yeast solution

4. Results

In this chapter I will firstly discuss the results we collected to validate the hardware and software design. In a second part, I will describe the experiments we performed to test the behaviour of flies upon gustatory manipulation.

4.1. Hardware and Software Validation

Firstly, we had to optimise our activity bout detection algorithm. We optimized our protocol by minimising the difference between the number of identified activity bouts by the offline data processing algorithm and our new *Bonsai* workflow, by coding it into MATLAB. This way we could manipulate the parameters (in particular, the FIR filter window length and the value of the threshold) with the same data set in order to discover which pair of values would be optimal.

Figure 4.1 depicts a surface plot with the difference in the number of activity bouts detected by the offline and online algorithms.

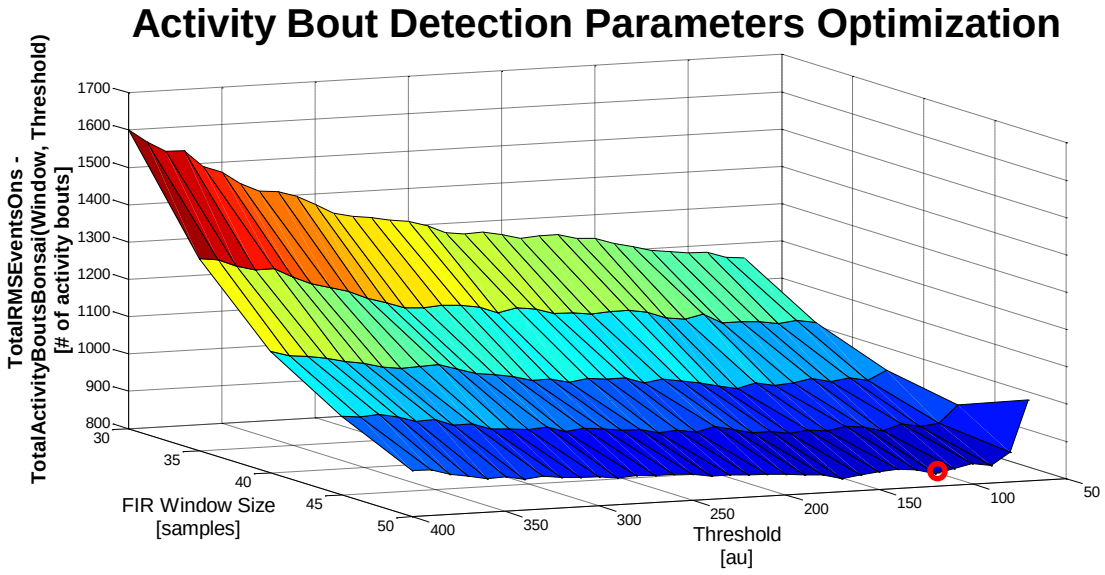

Fig. 4.1 Activity Bout Detection Parameters Optimisation

The red circle highlights the coordinates where the difference between the two protocols is minimum: FIR Window Size 50 samples; Threshold 120 units.

When analysing figure 4.1 one could argue that the tendency of the difference values suggests that if we used bigger FIR windows we would find a better minimum. However, we decided that we shouldn't consider FIR window sizes bigger than 500ms as the bigger the FIR window is, the more you postpone the offset of an identified activity bout.

As depicted in the figure a minimum difference was found with FIR Window Size equal to 500ms and Threshold value equal to 120 units (red circle).

Using these values we could reliably identify activity bouts as shown in figure 4.2.

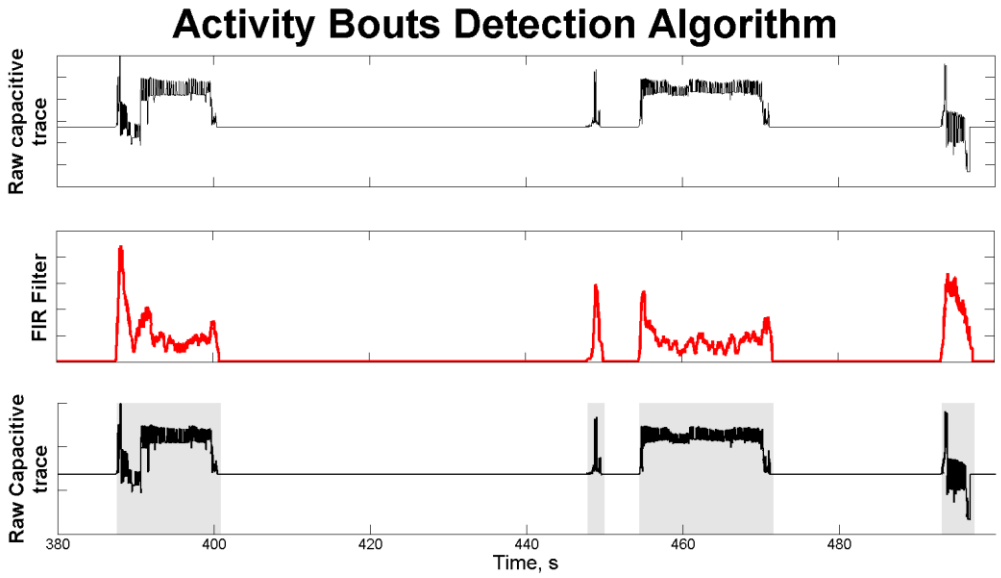

Fig. 4.2 Activity Bouts Detection Algorithm

The first plot shows a detail of the raw capacitance signal of a fly interacting with food; the second is the same signal depicted previously already processed by the algorithm until the FIR filtering (derivative, absolute value and FIR filter); the final plot represents the same capacitance signal with the grey shadows identifying activity bouts.

We next set onto study the latency of our system. The software has computing time that delays the delivery of the message. Furthermore, electrical circuits are not 100% efficient when transmitting signals due to physical properties of electrical components and wires.

Taking into account these two considerations we were able to calculate our system's Latency.

In MATLAB we wrote the same processing algorithm that *Bonsai* performs for detecting activity bouts (Appendix 3). This code would process data offline (data that was previously collected using the OptoPAD). This way we knew when the LED would turn on in optimal situations, in other words, when the LED was expected to turn on.

As shown in the Appendix 1, *Bonsai* saves the timestamps of the most relevant actions: Capacitance Signal timestamp – whenever a sample is collected from the FlyPAD; Digital Output timestamp – whenever a message is sent to the Arduino boards to set the states of the pins to high or low; and Catch Trial timestamp – whenever the computation of the catch trial protocol is True.

To calculate the Latency, we installed a phototransistor connecting the two electrodes of one channel of our sensor. *Bonsai* recorded the data from both channels but only the activity in the channel without the phototransistor would trigger LED activation. This phototransistor was chosen to be sensitive to the wavelength of the Red colour of the LED (sensitive to 570nm). When the light would be triggered by the signal of the recording channel we would get a saturation of capacity in the other channel as its electrodes were now connected by the phototransistor.

Using MATLAB we were able to identify the samples when the phototransistor was on (*i.e.* the LED was also on). Calculating the difference between these samples and the expected samples when the LED

was supposed to turn on resulted on a mean latency of 86.5ms (minimum – 5 samples; maximum – 12 samples).

Although this latency seems long for a behavioural setup, assuming that the fly's stereotypical eating pattern is performed at 5Hz, with this latency we are activating a neuronal consequence with a precision of ±1 sip. Furthermore, the FlyPAD system streams data to the Serial Port in batches of six samples, giving rise to the variability observed in the calculated latency. Improving the communication protocol between the FlyPAD and the computer would lead to a decrease of the latency of at least 60ms.

4.2. Results of the Experiments

4.2.1. Appetitive Behaviour

In this experiment our hypothesis was that pairing food ingestion with artificial activation of *Gr5a*-positive neurons would elicit appetitive behaviour. In order to test whether this was the case, we allowed flies to choose from two different sources of food. Even though both sources had the same composition (5mM sucrose solution), one of them was paired with red light stimulation for 2s, half a second after the fly would start interacting with it.

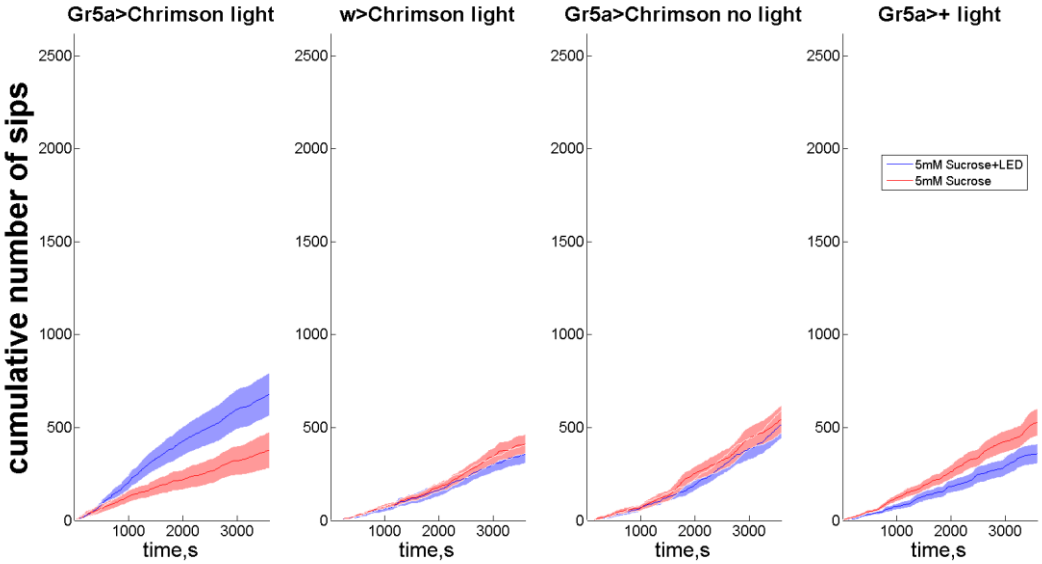

Fig. 4.3 Cumulative number of sips across the experiment condition and the different controls

The blue trace represents the cumulative number of sips in the source of food coupled with the red light and red is the same parameters in the source of food with no light stimulation. Line represents the mean and the shading the standard error of the mean (Gr5a>Chrimson light, n=31; w>Chrimson light, n=32; Gr5a>Chrimson no light, n=31; Gr5a>+ light, n=31).

We found that the experimental flies prefer to eat from the source of food that was paired with the optogenetic stimulation (Fig. 4.3): they had more sips in this source compared to the source that was not paired with light. This effect was only present in flies expressing Chrimson in the *Gr5a*-positive neurons. In all controls no difference was observed between the two food sources in terms of number of sips.

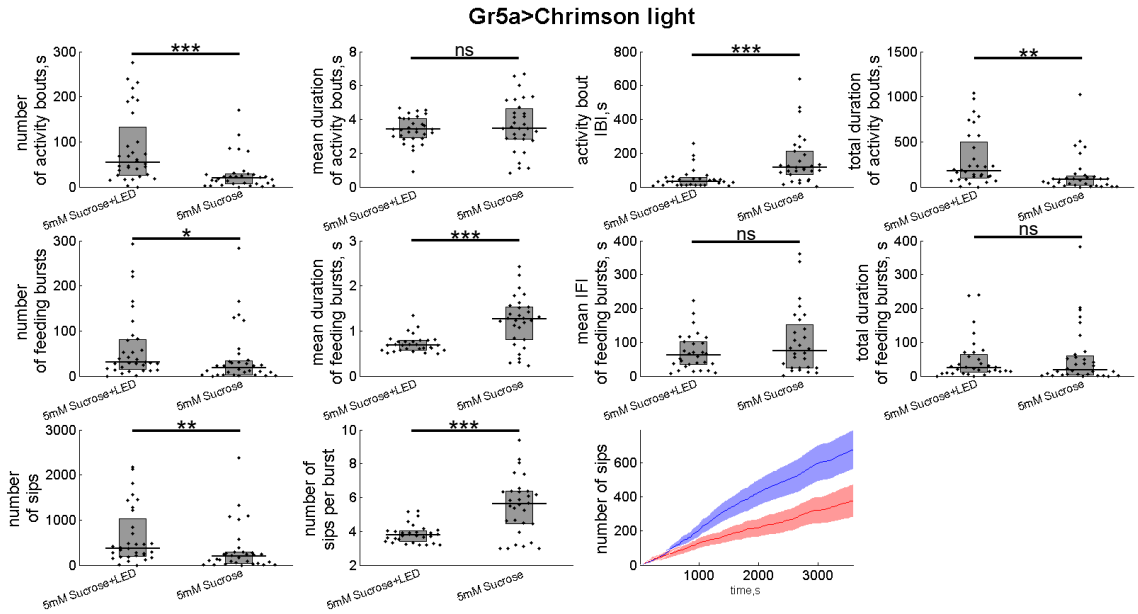

Fig. 4.4 Feeding microstructure of flies expressing Chrimson on Gr5a-positive ***neurons***

*Comparison between the behaviour of flies when stimulated for 2s with red light after 0.5s interacting with food in one channel, against the feeding behaviour in the other channel with the same food but with no optogenetic stimulation. Box plots display the median and interquartile range. ns – not significant (P>0.05), * – P≤0.05, ** – P≤0.01, *** – P≤0.001, significance was tested by Wilcoxon Rank-Sum test with Bonferroni correction, n=31.*

Several parameters in the microstructure of feeding strengthen the conclusion that pairing food exploration with activation of *Gr5a* neurons is appetitive (Fig. 4.4). The number of activity bouts, number of feeding bursts and number of sips in the light-paired channel are greater than in the channel with no light activation. Furthermore, the activity bout Inter-Bout Interval is smaller in the channel that triggers the light stimulation when compared to the other source of food, meaning that the probability of returning to the food paired with LED activation is higher. However, some metrics support the opposite conclusion as the average time flies spend eating (mean duration of feeding burst) and also the number of times flies sip within the feeding burst is smaller in the optogenetic stimulation channel. In addition, flies harbouring only *UAS-Chrimson* without the Gal4 also decrease the average time of feeding burst and the number of sips per burst (Fig. 4.5), which can be explained by an unspecific expression of Chrimson in other neurons that might trigger a startle response. Furthermore, the shortening of the feeding burst may also be explained by the PER triggered by the activation of the *Gr5a* neurons, which could interrupt the ongoing feeding burst (Inagaki et al. 2014).

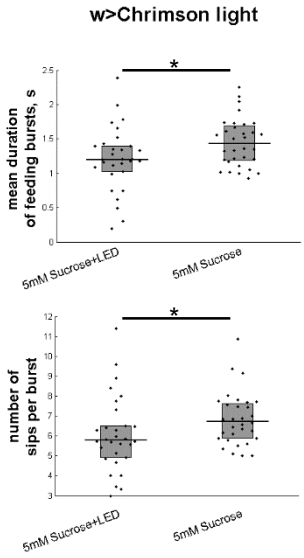

Fig. 4.5 Mean duration of feeding bursts and number of sips per burst of Chrimson control flies

*Tested for the choice between 5mM sucrose paired with red light and just 5mM sucrose. Box plots display the median and interquartile range. * – P≤0.05, significance was tested by Wilcoxon Rank-Sum test with Bonferroni correction, n=32.*

Another way to represent this elicited appetitive behaviour is the cumulative preference index through the whole experiment (Fig. 4.6), clearly showing a robust and early change in preference by *Gr5a* neuron activation.

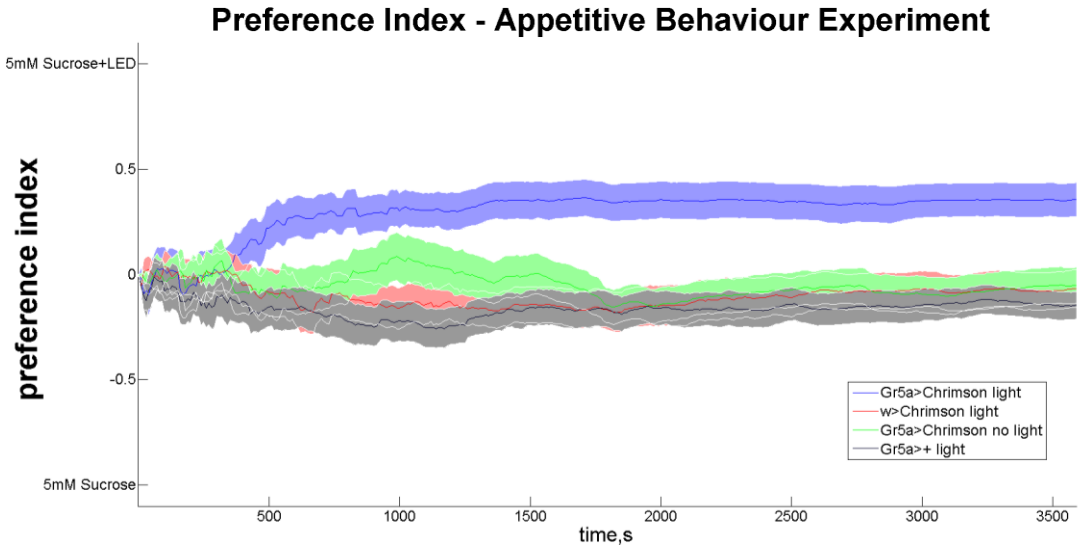

Fig. 4.6 Cumulative preference index – Appetitive Behaviour

Line represents the mean and the shading the standard error of the mean (Gr5a>Chrimson light, n=31; w>Chrimson light, n=32; Gr5a>Chrimson no light, n=31; Gr5a>+ light, n=31).

We can therefore conclude that it is possible to induce appetitive behaviour in flies using this setup and pairing the exploration of a food source with *Gr5a* neuron activation, increases the attractiveness of that food source, as shown in the increased number of sips and a higher frequency of visits.

4.2.2. Aversive Behaviour

In the “Aversive Behaviour” experiment our hypothesis was that pairing food ingestion with artificial activation of *Gr66a*-positive neurons, would elicit aversion towards a source of food. We tested this hypothesis by allowing flies to choose from two different sources of food. Even though both sources had the same composition (10% yeast solution), one of them would lead to activation of the red LED for 2s after one second of interaction with that food.

One second after the initiation of an activity bout (when the light is turned on), experimental flies (expressing ReaChR in *Gr66a*-positive neurons) interrupt the stereotypical pattern of eating in the channel coupled with light (Fig. 4.7). Control flies, however, show no signs of disturbance (i.e. they continue eating) when the red light is shining (Fig. 4.8).

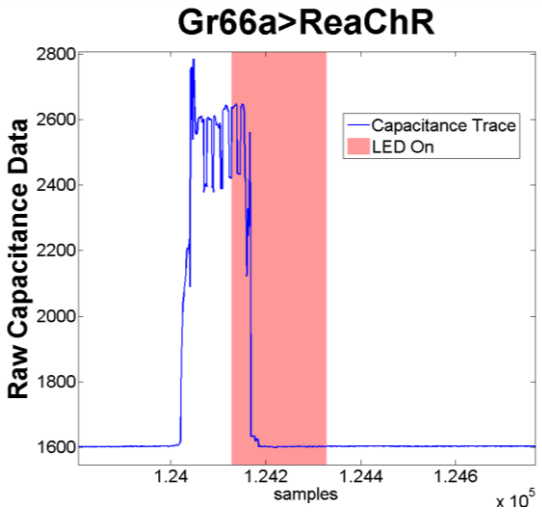

Fig. 4.8 Activity bout trace of a fly expressing ReaChR in Gr66a-positive neurons

Red shade depicts when the LED was switched on.

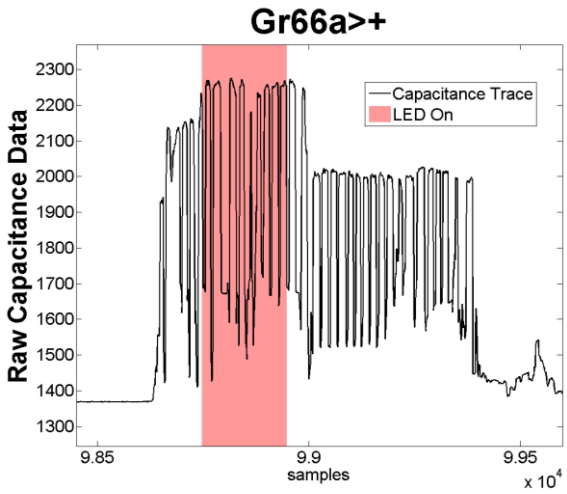

Fig. 4.7 Activity bout trace of a Gal4 control fly

Red shade depicts when the LED was switched on.

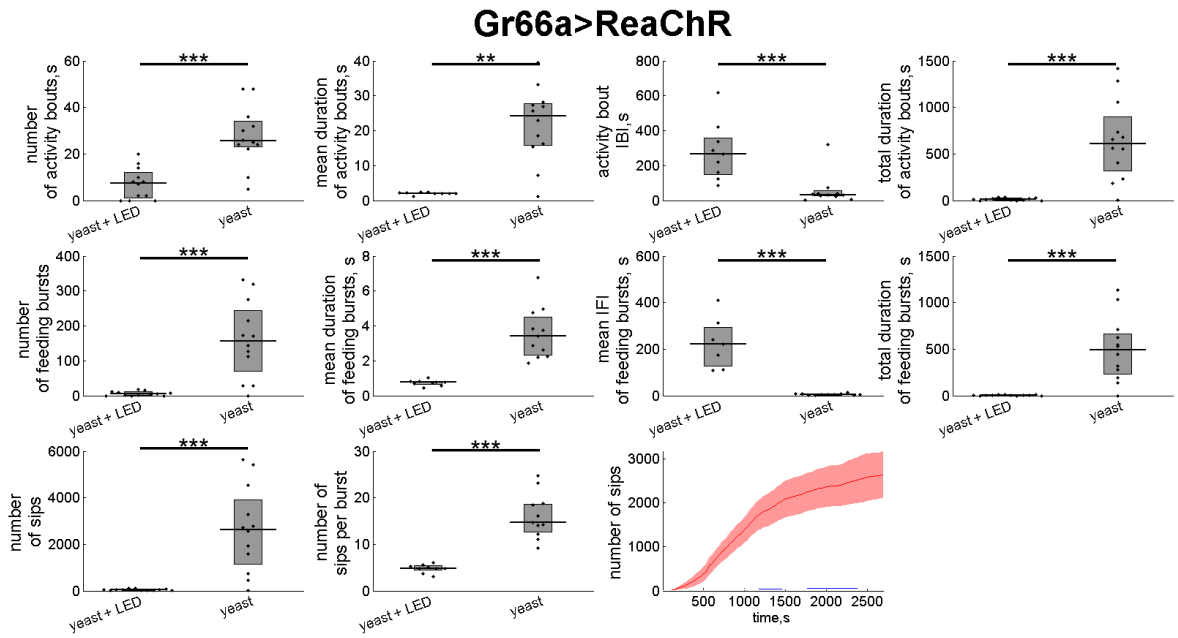

Fig. 4.9 Feeding microstructure of flies expressing ReaChR in Gr66a-positive neurons

*Comparison between the behaviour of flies when stimulated for 2s with red light after 1s interacting with food in one channel, against the feeding behaviour in the other channel with the same food but with no optogenetic stimulation. Box plots display the median and interquartile range. ** – P≤0.01, *** – P≤0.001, significance was tested by Wilcoxon Rank-Sum test with Bonferroni correction, n=12.*

Consistent with triggering aversive behaviour, all feeding microstructure parameters are altered when comparing both sources of food (Fig. 4.9). These results provide evidence that we are able to elicit aversive behaviour with this setup: flies decrease the number of activity bouts, feeding bursts and sips upon light stimulation. Also, flies decrease the average time they spend interacting with food (mean duration of activity bout) or eating (mean duration of feeding burst), increase the Inter-Bout intervals and Inter-Feeding intervals (i.e. decreased probability of returning to the food) when optogenetic stimulation occurs.

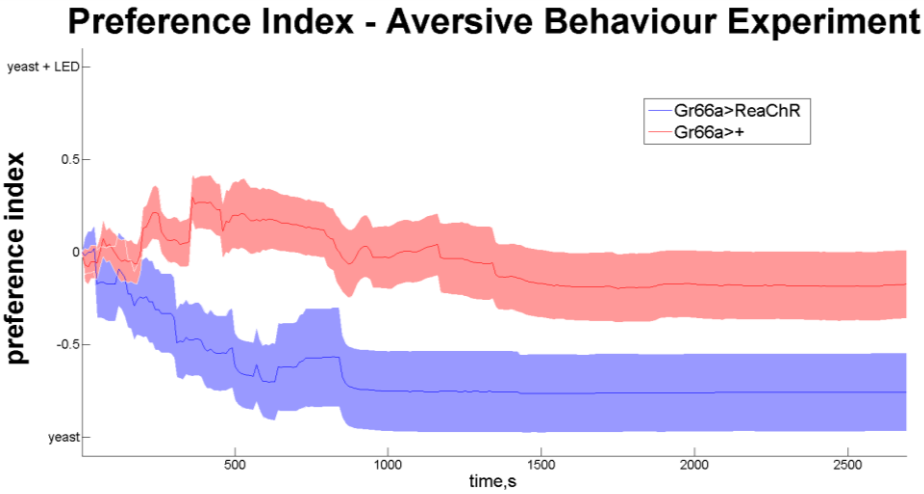

Fig. 4.10 Cumulative preference index – Aversive Behaviour

Line represents the mean and the shading the standard error of the mean (Gr66a>Chrimson, n=12; Gr66a>+, n=12).

In the control experiment we performed, no statistically significant changes were found, which again strongly suggests that flies in our setup do not have any bias towards or against red light.

Therefore, with this setup we are able to manipulate behaviour and impose aversiveness to a source of food (Fig. 4.10).

4.2.3. Preference Switch

Optogenetic activation of *Gr66a*-positive neurons elicits an aversive response towards the source of food paired with the stimulation. Our hypothesis for this experiment was that with periodic change of the contingency (which food source triggers light stimulation), flies would be able to change their preferred food source, showing evidence that we could dynamically manipulate preference between food sources.

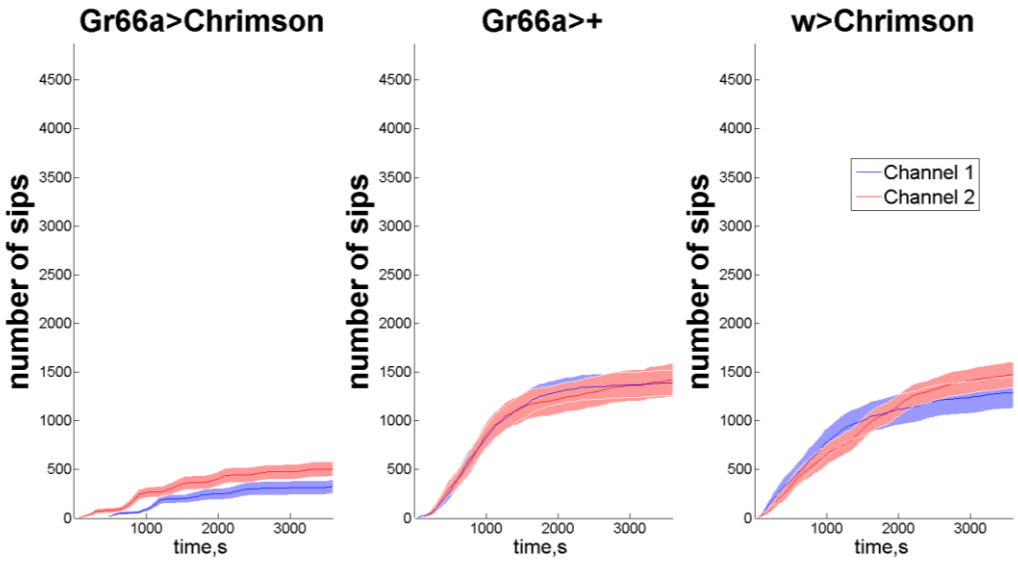

Fig. 4.11 Cumulative number of sips across the experiment condition (Gr66a>Chrimson) and the different controls

The blue trace represents the cumulative number of sips in channel 1 and red is the same parameter in channel 2. Line represents the mean and the shading the standard error of the mean (Gr66a>Chrimson, n=42; Gr66a>+, n=43; w>Chrimson, n=38).

In this experiment light was paired only with a source of food (channel 1) for five minutes, where in the subsequent five minutes only channel 2 would be associated with optogenetic stimulation, returning to the channel 1-activation protocol afterwards. Whereas the controls never change the way they eat, experimental flies stop eating from the source of food that triggers the light (Fig. 4.11).

Flies expressing Chrimson in *Gr66a*-positive neurons tend to eat less (close to 1000 sips in total), compared to the control flies of this experiment (close to 3000 sips in total). In fact, the plateau of the cumulative number of sips, which is usually correlated to satiation, in the control flies, occurs at a lower number of sips counting in the experimental flies. This result shows evidence that, even though we thought the plateau was associated with satiation, these flies are not able to find the best feeding strategy in order to reach satiety, due to the unpredictable contingency of the experiment.

We counted the number of sips performed by the experimental flies in batches of 10s across the experiment (Fig. 4.12). When light is paired with activity on channel 1, flies have more sips per 10s on the other channel (channel 2), and vice-versa. In the transitions of “blocks”, the sip count per 10s from the channel paired with light rapidly decreases, whereas in the other channel increases.

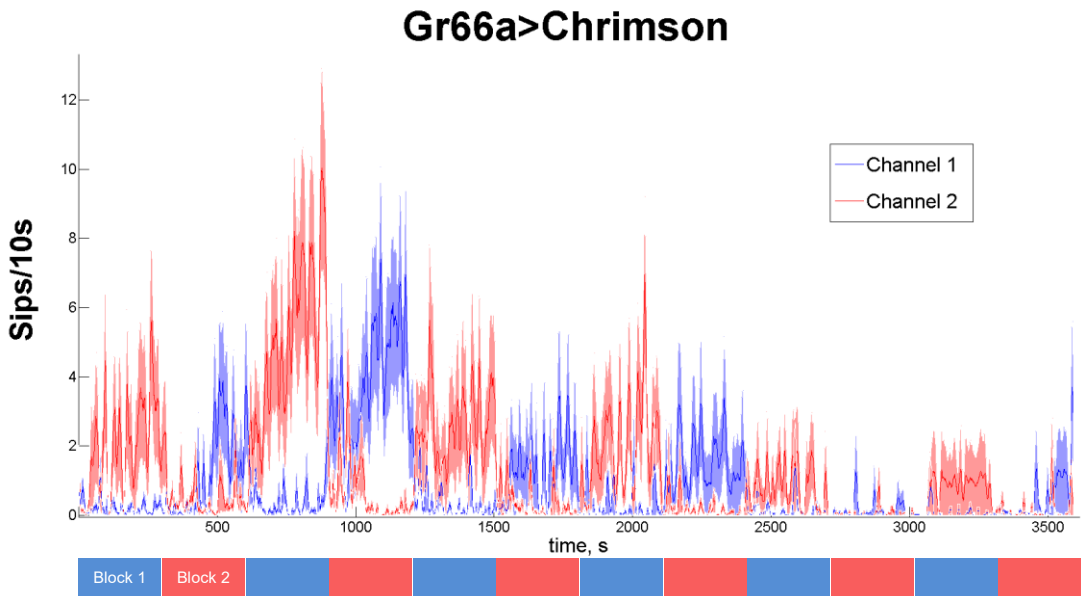

Fig. 4.12 Number of sips per 10s

The blue trace represents the number of sips per 10s in channel 1 and red is the same parameters in channel 2. Line represents the mean and the shading the standard error of the mean (Gr66a>Chrimson, n=42) The blue rectangles in the bottom represent when the experiment was in “block 1” and the red rectangles depict the time spent in “block 2”.

With this result we were able to demonstrate that we could dynamically manipulate *Drosophila* preference between food sources, through optogenetic activation of *Gr66a*-positive neurons.

5. Discussion

We developed a new behavioural setup that allows recording of feeding and closed-loop optogenetic manipulation of neurons in Drosophila. With this system, we were able to induce appetitive behaviour towards a source of food by stimulating *Gr5a*-positive neurons and to induce aversive behaviour through activation of *Gr66a*-positive neurons.

Despite most studies that use flies and manipulation of GRNs being also directed to the study of learning and memory (Keene & Masek 2012; Masek et al. 2015), all the setups used so far work in an open-loop manner, where flies cannot associate their own actions to a reward or punishment. The developed setup is a behavioural system that can be used to study memory and learning in a more naturalistic environment in comparison to previous setups, in which flies are not tethered and are freer to interact with food.

In this context, the OptoPAD may allow researchers to explore many different questions regarding whether the activation of a particular neuronal population will trigger different feeding behaviours.

5.1. Hardware and Software

The developed setup is capable of recording and manipulating the activity of 32 flies simultaneously. However, and knowing that this system makes use of 4 USB Ports (1 for the FlyPAD and 3 for the Arduinos), one can scale-up the amount of flies studied according to the number of available USB Ports in the computer. The throughput is enough to have a significant number of experiments performed in a working day.

With the OptoPAD, one can use nearly any available channelrhodopsin, as the system is equipped with 4 colour LEDs in all the behaviour arenas. One limitation is that with the hardware and software developed so far, the LED can only be off or at maximum intensity: it is therefore not possible to vary light intensity. This caveat could be overcome by adding potentiometers that control the current which passes through the LEDs (hardware solution) or by implementing a power width modulation that would control the LEDs' duty cycles (software solution). Once being able to activate more than one colour at the same time and change the intensity of each of the colours, it would be possible to emit at any visible wavelength.

Regarding the results described in this work, the resolution of stimulation is around 86.5ms (average latency of 8.65 samples). Although this value might seem too long for behaviour experiments, we know that fly's stereotypical movement of sips happen at about 5Hz, meaning that flies extend and retract their proboscis in periods of 200ms. Therefore, we are stimulating flies with the precision of ±1 sip. Furthermore, the FlyPAD system streams data in batches of six samples, thus giving rise to the variability of latency (minimum – 5 samples; maximum – 12 samples). The system's latency can be reduced by improvement of the protocol that streams data from the FlyPAD to the Serial Port. By re-writing this algorithm, OptoPAD's latency could be dramatically reduced, by at least 60ms (6 samples).

We performed an optimisation of Bonsai parameter to better detect the activity bouts. This optimisation resulted in a FIR filter window size of 50 samples and a threshold of 120 units. When executing the optimisation, we chose to test only FIR windows sizes up to 50 samples, since by using bigger windows we would postpone the offset of the identified activity bout. There is one example that demonstrates the problem of bigger window sizes. For example, if we set the delay (activity bout minimum duration in order to trigger stimulation) to 1s and the fly only interacts with food for 500ms, as the FIR filter is set to a window of 50 samples (500ms), the fly will get light stimulation as the filter is summing across 500ms. At the limit, the last sample of the interaction will be enough for the sum of the following samples to be above the threshold (0.5s interaction + 0.5s filtering = 1s activity bout). Nonetheless, this problem does not exist for activity bouts that last longer than the delay time.

Further optimisations could be implemented. The output of the FIR filter could be divided by the number of samples. This way we would have a normalized output value and the threshold would not need to be so high in order to detect activity bouts. Furthermore, instead of having a lot of computations such as the derivative, absolute value and the FIR filter, we could use a band pass filter or a pattern recognition filter that would detect groups of square wave-like signals at approximately 5 Hz (sips).

5.2. Experiments

Our results are in line with the current knowledge, as activation of *Gr5a*-positive neurons cause flies to stay in the location that was paired with activation, whereas the activation of *Gr66a* neurons led to the opposite effect (Marella et al. 2006). However in this study there are a lot of possible reasons for the modulation of preference. These flies could have stayed in the preferred substrate eating, grooming, sleeping or even laying eggs. In order to disentangle these possible explanations, we decided to run experiments that would allow us to understand what are the consequences of the activation of those neurons on feeding behaviour.

5.2.1. Appetitive Behaviour

We were able to create a “virtual reality” for the fly where one source of food was more appetitive than other, even though they were the same solution (5mM sucrose). Almost all the results support this conclusion. In fact, the number of activity bouts, feeding bursts and sips was augmented in the source of food that would trigger the optogenetic stimulation (activation of *Gr5a*-positive neurons) and the Inter-Bout interval was diminished in the same source of food, meaning that flies had higher probability of returning to the “sweeter” food than going back to the food that did not trigger light. Nevertheless, the duration of feeding bursts and number of sips per burst were reduced in the “light/sweet” source of food. This result stands in apparent disagreement with the conclusion of inducing appetitive behaviour. However, flies that only harbour *UAS-Chrimson* show the same phenotype reducing their feeding burst durations on the source of food with light stimulation. This result seems to show that these flies have unspecific expression of the channelrhodopsin, which by triggering other neurons can explain the interruption of feeding. In previous studies, continuous light stimulation of *Gr5a*-positive neurons expressing ReaChR induces PER in the beginning of the stimulation (Inagaki et al. 2014), which may

lead to the interruption of the ongoing feeding burst. In addition to that, one might speculate that while the light is active, flies do not feel the need to perform the motor program of feeding as their “sweet” gustatory neurons are already active, even with no more food being ingested.

5.2.2. Aversive Behaviour and Preference Switch

In relation to the last two experiments they can be discussed together, since, even though the targeted neurons express different channelrhodopsins (ReaChR and Chrimson), we can draw the same conclusion from their results: by activating *Gr66a*-positive neurons, flies show aversive behaviour towards the food paired with the stimulation.

We demonstrated that the feeding behaviour was interrupted as soon as these neurons were activated. All the features of the microstructure of feeding were significantly different from the behaviour on the source of food that would not trigger the stimulation. The number of activity bouts, feeding bursts and sips were drastically diminished in the “light/bitter” source of food. Flies have a lower probability of returning to the spot of food that will trigger light and also have a lower probability of repeating a feeding burst there (longer Inter-Bout interval and Inter-Feeding interval). Also, the amount of time they spend eating from that food source (that triggers light) is minimal when compared to the other source.

Furthermore, we show that by reversing the contingency of the experiment, flies rapidly change their preferred food source. When analysing the “Preference Switch” experiment after the “Aversive Behaviour”, we were expecting to observe the same overall results over both parts. However, the stagnation of the number of sips displayed by the experimental flies was an unexpected result. In fact, through all experiments we performed, flies show a plateau on the cumulative number of sips that can be explained by satiation (Itskov et al. 2014). In this case, we observe that the control flies perform close to 3000 sips before showing satiation (figure 4.11). Nonetheless, the observed “satiation plateau” of the experimental flies occurred close to 1000 sips. This also stands in contrast to the experiment in which the aversive channel was not switched (figure 4.9). Therefore the “Preference Shift” design leads to a premature cessation of feeding, suggesting that this plateau is not related to satiation. A possible explanation could be a learned helplessness effect (Yang et al. 2013), as flies might not being able to find the strategy to deal with the unpredictable contingency of the experiment.

6. Conclusion and Future Work

One of the main goals of behavioural neuroscience is to understand how neuronal circuits in the brain drive behaviour. What is needed in the environment of the animal so that one particular neuronal population gets activated and a specific response is triggered?

Feeding is one of the main behaviours in animal's repertoire as it is essential to maintain homeostasis and, ultimately, survival. This is why basic research on this topic is not only essential for Biology but also for Public Health. Only by studying feeding behaviour can scientists gain deeper understanding of feeding related diseases, such as obesity and anorexia nervosa.

The OptoPAD was developed not only to reach a deeper understanding on the mechanisms of feeding behaviour, but to also be used in the study of memory and learning in *Drosophila*. In summary, we have created a new behavioural setup that allows recording of feeding behaviour of flies and the manipulation of their behaviour in closed-loop. One fully functional system is composed of 32 high power LEDs, 128 transistors, three Arduino Mega boards, one Power Supply Unit and one FlyPAD system. With this device, we were able to manipulate circuits to study aversive or appetitive behaviours in *Drosophila*. We were capable of reproducing previous published results on the activation of neurons expressing the two gustatory receptors we used. Activation of *Gr5a*-positive neurons triggers appetitive behaviour and activation of *Gr66a*-positive neurons causes an aversive response towards food.

OptoPAD requires some improvements, particularly regarding its software. Modifying the activity bout detection protocol by designing a filter that would be specific to the stereotypical feeding behaviour of flies would be the first step of improvement. This would not only enable the OptoPAD to be a feeding recording and manipulation setup, but would help us to understand if flies are able to change strategies of feeding in a "sip" scale. However, this does not mean that the existing software is not efficient *per se*.

The next step towards studying learning and memory in *Drosophila* with this system is to find a new behavioural paradigm in which flies can learn the contingency of the experiment. From preliminary data collected using the OptoPAD, we could find features of behaviour that suggest that flies can learn to change their feeding motor pattern, conditioned by an aversive optogenetic stimulation. Furthermore, the OptoPAD allows the creation of a "virtual reality" environment where one source of food is more available, if it has a low probability of triggering an optogenetic punishment; or less available, if it has a high probability of activating a punishment. We can create predictable or unpredictable environments for flies by changing the contingency of availability with time or probability of stimulation.

The OptoPAD could be used to study operant conditioning in flies. Genetic modifications of the memory centres of Drosophila (*e.g.* the Mushroom Body) will allow studies on how and where flies store particular memories, how they can remember and, hopefully, serve as a model for the study of neurodegenerative diseases, such as Alzheimer's Disease.

As a future Biomedical Engineer I feel that this project helped me discover what a true engineer should be like: he finds and builds solutions to problems, always bearing in mind that technology is extremely

important for innovation. Technology makes us work faster, easier and achieve results that few years ago where unconceivable of achieving. With the most recent medical devices, it is possible to allow an amputee to get back on their feet and walk, replace malfunctioning cardiac valves or even use brain waves to control mechanical arms to help tetraplegics to perform daily tasks. It is our job to use the available technology to build a better world with more quality of life, better health and, most of all, use globalisation in favour of respect and equality.

7. References

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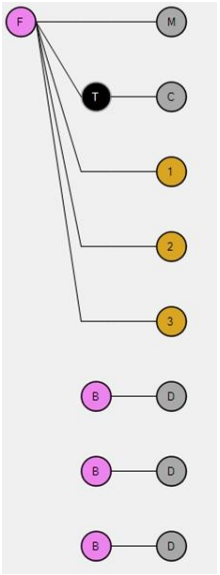
Appendix 1

In this Appendix I explain how all the *Bonsai* workflow works. I will focus on one channel as all the other 63 follow the same computation.

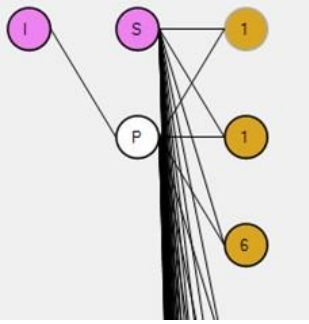
Appendix Fig. 1 represents the first screen of the *Bonsai* workflow.



Appendix Fig. 1 Bonsai: first workflow screen

The first “F” pink node represents the “FlyPadSource”, where all the capacitance data from the 64 channels is streamed. The next “M” node in the first line is a “MatrixWriter” where the data is saved on a file called “CapacitanceData”. The second line represents a “T” and a “C” node. The “T” refers to “Timestamp” which records the timestamp for each element produced by the sequence, meaning that a timestamp is produced at a frequency of 100Hz. Finally the “C” is a “CsvWriter” that stores this info into a “.csv” file called “CapacitanceTimeStamp”. The three brown nodes (“1”, “2” and “3”) are NestedWorkflows that encapsulate a more complex workflow inside them representing the three Arduino Mega boards of the setup. In order to initiate the three Arduino boards before the experiment starts, we send a “Boolean” “B” to each of the “DigitalOutput”s, depicted as the three pairs of nodes presented in the bottom of Appendix Fig. 1. This procedure must be done before further processing as we want to avoid the initialisation of the Arduino boards to be perform while the FlyPAD is already collecting data. It is important to say that this initialisation protocol takes more or less 5s per board.

We will now enter in the board “1” “NestedWorkflow”. (Appendix Fig. 2



Appendix Fig. 2 Bonsai: part of board “1” workflow

The pink “S” is the Source from the previous workflow (capacitance signal). The pink “I” is an integer source just to be able to call the following “P” “PythonTransform”:

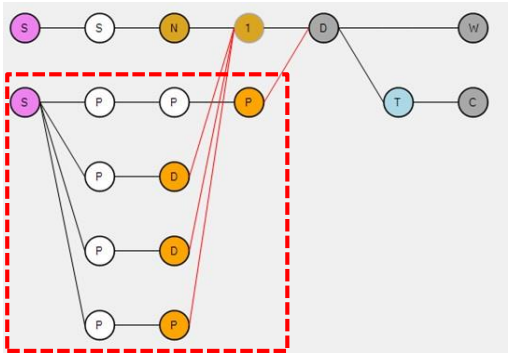
```
import clr

def getOutputType():
  return clr.GetClrType(list)

def process(input):
  with open(r'config/experiment.dat') as f:
    f=[x.strip() for x in f if x.strip()]
    data=[tuple(map(float,x.split())) for x in f[0:]]
    length = len(data)
  return data
```

As the code suggests, the PythonTransform imports the experiment structure that is saved in the `r'config/experiment.dat'` pathway (created in MATLAB - Appendix 2). Both the capacitance signal from the “S” source and the experiment structure from “P” node are transmitted to 24 subsequent NestedWorkflows that represent each one of the 24 channels of the 12 arenas that are connected to the first Arduino board.

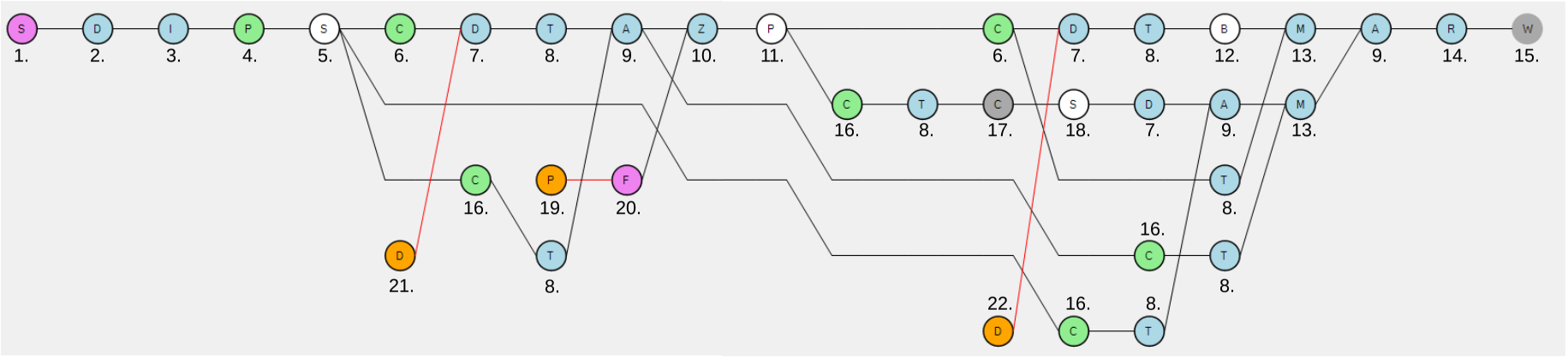
Appendix Fig. 3 represents the first NestedWorkflow of Appendix Fig. 2 (first brown 1 node). This NestedWorkflow is called “**1_1**” representing Arena **1** Channel **1**.



Appendix Fig. 3 Bonsai: “1_1” workflow

Nodes highlighted in the dashed red square integrate the data from the “experiment structure” file into the Bonsai workflow

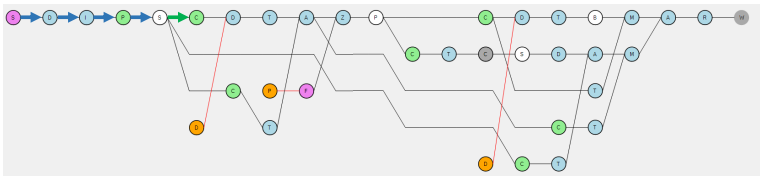
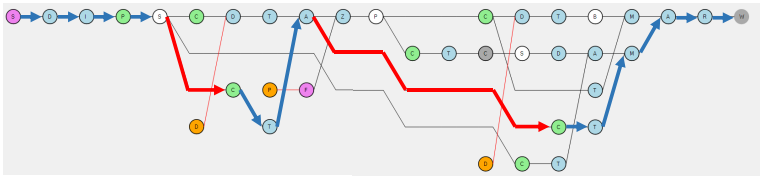
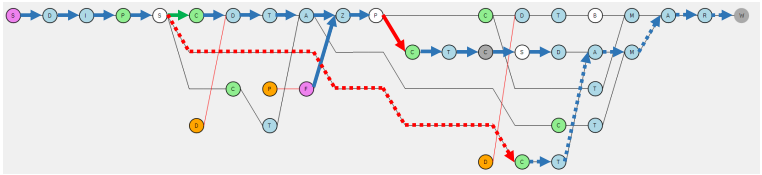
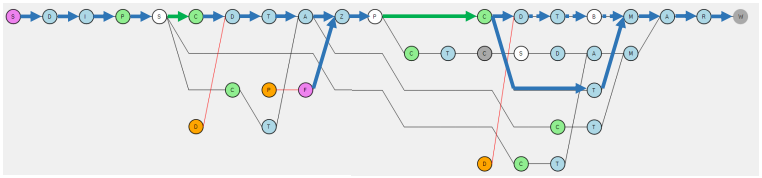
The first “S” pink node is called “Source1” and represents the capacitance data from the previous workflow, which is transmitted to the next “S” being a “SelectChannels” node, which in this case selects the channel 1 from the first arena. The brown “N” node is representing the activity bout detection processing node that was described in section 3.2.1, in which the capacitance signal is processed and in the end, the output determines if the fly is interacting with the food in this channel or not. Subsequently, the next brown “1_1” node receives this information that ultimately leads to LED activation. The grey “D” represents the DigitalOutput, which sends the message to activate or deactivate the transistor controlling the correct colour of the LED on Arena 1 to the correct Arduino pin. This information is saved in addition to the timestamp “T” by the “C” node, the CsvWriter, calling this file “DigitalOutputTimeStamp_01_01_.csv”.



Appendix Fig. 4 Bonsai: "1_1" workflow that depicts the LED activation protocol

1. Source1 represents an input sequence inside a nested workflow; 2. DistinctUntilChanged ensures that only distinct contiguous elements are propagated; 3. Index records the zero-based index of elements produced by an observable sequence; 4. PythonCondition is a Python script used to determine which elements of the input sequence are accepted; 5. Source.Value selects inner properties of elements of the sequence; 6. Condition (true) filters the elements of an observable sequence that are True; 7. Delay delays the notification of values by the specified time interval; 8. Take returns one element from the start of the sequence; 9. Amb propagates the observable sequence that reacts first; 10. Zip combines values from the source sequences whenever all of the sequences have produced an element; 11. PythonTransform is a Python script used to process and convert individual elements of the input; 12. BitwiseNot applies a bitwise complement on elements of an observable sequence; 13. Merge merges any number of observable sequences into a single observable sequence; 14.Repeat repeats the observable sequence indefinitely; 15. WorkflowOutput represents the output sequence inside a nested workflow; 16. Condition (false) filters the elements of an observable sequence that are False; 17. CsvWriter sinks individual elements of the input sequence to a text file; 18. Source.Value selects inner properties of elements of the sequence; 19. Probability 1_1 (Value) represents a property that has been externalized from a workflow element; 20. Float represents a workflow property containing a single-precision floating-point number; 21. DueTime Delay 1_1 (DueTime) represents a property that has been externalized from a workflow element; 22 DueTime Sustain (DueTime) represents a property that has been externalized from a workflow element

Appendix Table 1 Possibilities of Trials

Fly interacts with food		-
1. Fly interacts with food but stops before the “delay” time elapses		DigitalOutput **False**
2. Catch-Trial		DigitalOutput **False**
3. Complete Trial		DigitalOutput **True**

Note: Green Arrows – “True” Statements, Red Arrows – “False” Statements, Full Arrows – normal data flow, Dotted Arrows –data flow that occurs delayed in time

If a fly interacts with food the workflow presented on Appendix Fig. 4 is initiated. In other words, all trials are initiated, as showed in the first image of Appendix Table 1, with the fly touching the food satisfying the first Condition. From then on, there are three different possibilities on the behaviour of the fly.

The fly may stop interacting with the food before the “delay” time elapses. This way, while the true signal is still in the delay node, the false condition is triggered and travels across the workflow faster sending a false statement to the DigitalOutput, which, being already false, doesn’t change its state and the LED remains off. Subsequently, the system waits for a new trial.

If the fly keeps interacting with the food for longer than the “delay” time, then two other possibilities remain.

In the externalized property “Probability 1_1” we have defined the amount of trials that should lead to LED activation. The following code is run in the PythonTransform (Appendix Fig 4 – 11.):

```
import clr
from System import Random

def getOutputType():
    return clr.GetClrType(bool)

generator = Random()

def process(input):
  a=generator.NextDouble()
```

```
if (a<=input.Item2):
     return True
else:
     return False
```

When generating a random number between 0 and 1, we have defined that if that number is smaller than our specified probability, then that trial should lead to a “complete trial” with LED activation, otherwise a “catch-trial” should be initiated.

If the trial is a “catch-trial” (2. Catch-Trial – Appendix Table 1), the Timestamp of the trial is saved in a file called “CatchTrial_01_1_.csv” and a long delay (10min) is initiated. A continuous read-out of the capacitance signal guarantees that when the running activity bout terminates the 10min delay is aborted (dotted arrows) and a false statement is written in the DigitalOutput, therefore, waiting for a new trial.

On the other hand, if the random generated number is smaller than the Probability value (3. Complete Trial – Appendix Table 1), the True statement is propagated and takes two ways of the processing workflow. One of these statements is sent to the DigitalOutput, activating the LED, while the other remains in a delay mode as long as the defined “sustain” time. When this delay elapses, the True statement gets inverted to a False statement that leads again to the DigitalOutput (dotted arrows) and the LED is turned off.

Appendix 2

```
function [] = create_experiment(varargin)
%create_experiment Creates the structure of an experiment with 32 arenas
%   Creates experiment structure up to three different conditions.
%
create_experiment(beginArenaCondition1,endArenaCondition1,[colourCH1,colour
CH2,delayCH1,delayCH2,sustainCH1,sustainCH2,probabilityCH1,probabilityCH2],
%
beginArenaCondition2,endArenaCondition2,[colourCH1,colourCH2,delayCH1,delay
CH2,sustainCH1,sustainCH2,probabilityCH1,probabilityCH2],
%
beginArenaCondition3,endArenaCondition3,[colourCH1,colourCH2,delayCH1,delay
CH2,sustainCH1,sustainCH2,probabilityCH1,probabilityCH2])
%
%       Example of an experiment with only one condition
%           create_experiment(1,32,[1,0,2,3000,2,0,0.5,1])

data = cell(32,8);

if size(varargin,2)==3

    for i=varargin{1}:varargin{2}
        data{i,1} = varargin{3}(1);
        data{i,2} = varargin{3}(2);
        data{i,3} = varargin{3}(3);
        data{i,4} = varargin{3}(4);
        data{i,5} = varargin{3}(5);
        data{i,6} = varargin{3}(6);
        data{i,7} = varargin{3}(7);
        data{i,8} = varargin{3}(8);
    end

elseif size(varargin,2)==6

        for i=varargin{1}:varargin{2}
            data{i,1} = varargin{3}(1);
            data{i,2} = varargin{3}(2);
            data{i,3} = varargin{3}(3);
            data{i,4} = varargin{3}(4);
            data{i,5} = varargin{3}(5);
            data{i,6} = varargin{3}(6);
            data{i,7} = varargin{3}(7);
            data{i,8} = varargin{3}(8);
        end

        for i=varargin{4}:varargin{5}
            data{i,1} = varargin{6}(1);
            data{i,2} = varargin{6}(2);
            data{i,3} = varargin{6}(3);
            data{i,4} = varargin{6}(4);
            data{i,5} = varargin{6}(5);
            data{i,6} = varargin{6}(6);
            data{i,7} = varargin{6}(7);
            data{i,8} = varargin{6}(8);
        end

elseif size(varargin,2)==9
```

```
        for i=varargin{1}:varargin{2}
            data{i,1} = varargin{3}(1);
            data{i,2} = varargin{3}(2);
            data{i,3} = varargin{3}(3);
            data{i,4} = varargin{3}(4);
            data{i,5} = varargin{3}(5);
            data{i,6} = varargin{3}(6);
            data{i,7} = varargin{3}(7);
            data{i,8} = varargin{3}(8);
        end

        for i=varargin{4}:varargin{5}
            data{i,1} = varargin{6}(1);
            data{i,2} = varargin{6}(2);
            data{i,3} = varargin{6}(3);
            data{i,4} = varargin{6}(4);
            data{i,5} = varargin{6}(5);
            data{i,6} = varargin{6}(6);
            data{i,7} = varargin{6}(7);
            data{i,8} = varargin{6}(8);
        end

        for i=varargin{7}:varargin{8}
            data{i,1} = varargin{9}(1);
            data{i,2} = varargin{9}(2);
            data{i,3} = varargin{9}(3);
            data{i,4} = varargin{9}(4);
            data{i,5} = varargin{9}(5);
            data{i,6} = varargin{9}(6);
            data{i,7} = varargin{9}(7);
            data{i,8} = varargin{9}(8);
        end

else
    error('Verify your input')
end

data

dlmwrite('C: (…) \config\experiment.dat',data,'\t');
end
```

Appendix 3

```
fir_coeff=ones(1,50);


diffCapData=[];
diffCapData=diff(CapData(2,:)); % CapData(2,:) is the raw signal coming
from the first arena, second channel (where the capacitance signal was
processed using Bonsai)
absCapData=[];
absCapData=abs(diffCapData);
firCapData=[];
firCapData=conv(fir_coeff,absCapData);
activate=[];
activate=gt(firCapData,120);
diffactivate=[];
diffactivate=diff(activate);
activitybouts=[];
activitybouts=find(diffactivate>0);
expectedLedON=[];
expectedLedON=activitybouts+(0.5*100); % 0.5s was the delay set to perform
the experiment with the phototransistor (multiplied by 100 to compute
samples and not seconds)
```