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Robust Assay to Study Associative Learning in *C. elegans*

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Abstract

Aging is a natural process that leads to a decline in physical and often mental abilities. It also leads to risk of degenerative diseases such as dementia. This project designed a system to allow for study of the effects of aging through use of associative learning in *C. elegans*. This system includes a microfluidic device that allows for study of adult *C. elegans* where previously devices only allowed for larval and smaller adult *C. elegans*. A light device was created to provide conditioned stimulus whose behavioral response could be mediated by pairing with attractive chemical stimuli to test associative learning. The designed system is capable of versatile experiments in which degree and direction of associative learning can be assessed and compared across ages, gender, and genetic mutants including Alzheimer's.

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Chapter 1: Introduction

1.1 Motivation of the Project

People are living longer lives than their ancestors, and are discovering the problems that come with a long life. One of these problems is the neural decline that occurs as people age, whether it be from natural aging processes or from degenerative diseases such as dementia, Parkinson's, or Huntington's. Currently, over 12.5% of Americans are over the age of 65 and are considered elderly [1]. Long life comes with many side effects including increased risk for Alzheimer's, other forms of dementia, Parkinson's disease, and Motor neuron disorders. Currently, there are 1 in 3 elderly Americans suffering from dementia, 1 in 10 elderly Americans suffering from Parkinson's, and 1 in 100,000 Americans suffering from Motor neuron disorders [2-4]. These diseases slowly strip away a patient's ability to live a normal and productive life. Eventually, the patient is even robbed of their own memories and personality. They are ravaged, both physically and mentally, which has a detrimental effect not only on the patient, but on their family and friends.

In an attempt to solve this devastating problem, a large amount of research has gone into investigating how the human brain changes in an effort to minimize mental decline. Some research has discovered issues as severe as impairment in episodic memory, most likely linked to an issue in encoding memories, caused by advanced age [5]. This is caused when the brain cannot distinguish the difference from one event to that of a similar, previous one. Additionally, it was discovered that when an older adult is presented with similar stimuli their brains processes both of the stimuli in the same manner [6]. In other words, the brain generalizes between similar stimuli and is less sensitive to differences. In contrast, in young adults oftentimes even small

differences in stimuli cause large changes in brain region reactivity [6]. For older adults, this lack of distinction leads to confusion, the inability to distinguish people and places, and a general decline in ability to perform everyday tasks. This also means that after a certain point humans lose self-sufficiency. This causes their ability to live a full, productive life to decline and lowers their standard of life. Ultimately, this decline in the standard of living caused by old age is what this project is trying to address. Through exploring the aging process and how the brain degrades, the team ultimately hopes to gain more knowledge on how this degradation can be prevented.

Animal models can recreate specific human disorders and diseases. These models are a very informative area of study that have already granted researchers insight into the process of aging [7]. Alzheimer's and other disorders can be induced in animal models, then they can be tested against healthy specimens. This disease inducement allows control of the exact stage and progression of the disease, which is an impossibility with human patients. Additionally there are a multitude of different factors, such as pathological, environmental, and social, that can affect the human aging process. In model animals, all these aspects can be controlled, ensuring that the observed effects are solely those of biological aging [7].

The animal model, *C. elegans*, are especially easy to control and model the human neural system especially well. In addition, the actual neuron's structure in *C. elegans* is quite similar to humans, the aging stages are alike, though much shorter, their genome and neural net has been entirely mapped, and many neurological diseases can be induced through mutant strains. As a result, many researchers use *C. elegans* to study the aging process of humans, in addition to neural diseases. All of these factors make *C. elegans* an ideal model animal for this project, which is why the team chose to use this animal as the subject of their research.

The current gold standard for studying *C. elegans* involves microfluidic devices [8-12]. These devices take all the factors that influence *C. elegans* into account. They are designed so that there is a constant laminar flow of fluid through the device, allowing for the worms, which are highly sensitive to pressure changes, to inhabit an environment with no changes in pressure. With this laminar flow, stimulants can be introduced in a controllable manner and can be turned into pulse or gradient formation in order to elicit stimuli responses from the *C. elegans*. Device design can also control the movements of the *C. elegans* using the peg based design, discussed further later in this paper, which allows for easier automated tracking and data gathering [13]. The data gathering is completed by analyzing video data for the *C. elegans*' motion.

However, there is currently one issue preventing this project from using *C. elegans* as a model for human aging. There is no system currently capable of studying *C. elegans* in the manner necessary to determine the effects of aging on the neural network. In order to study the effects the team will need to measure both the learning capabilities, i.e. cognitive function, of the *C. elegans* and the physical changes in the neural net that occur. The first problem is that older *C. elegans* cannot fit into the current microfluidic environment owned by the Quantitative Neurotechnology Lab (QNTL), the location of the team's research, without damaging it. This is due to the fact that the device was designed for young adults. As a result, before the examination of the animals began the team had to redesign the device in order to compensate for the unique size and strength of the older adult *C. elegans*. The second issue is that there is no current way to experiment with associative learning, arguably the simplest way to measure cognitive ability, in *C. elegans*. This is due to the fact the system can currently only present liquid chemical stimuli and as associative learning involves simultaneous presentation of stimuli these chemicals would mix and run the risk of simply forming a new stimuli. Thus the team must devise a system that

allows for simultaneous presentation of two distinct stimuli. These changes will be detailed later in this report.

1.2 Scope

C. elegans testing often consists of experiments that are conducted in agar plates. This environment is not a suitable environment as stronger concentrations of stimuli must be used to offset evaporation. Using an overly strong concentration, even of an attractive stimulus, can easily repel the *C. elegans*. Additionally, the stimuli is stationary and cannot be used to manipulate the animals. Thus this inhospitable environment created the need for another environment to be developed. Microfluidic environments were developed to contain and test *C. elegans*. They are the current gold standard, and do not have the problems that agar plates face. However, the majority of these environments are designed for animals that are in their young adult stage or younger. Since the project revolved around studying aging, and therefore needed to test fully grown, adult *C. elegans*, the team chose to design a microfluidic testing device suitable for fully grown adults. To accomplish this, the team had to consider what dimensions the device needed to have in order to allow the adult animals to move naturally. The team also had to redesign any areas that the adults could push through with their superior strength.

The qualitative method most researchers use to judge the reaction of the *C. elegans* is their movement in relation to a gradient stimulus. A strong backwards motion indicates repulsion and a strong forwards movement indicates attraction. Adult *C. elegans*, unlike their younger counterparts, do not move much. As a result, it can be very difficult to judge the response of adult *C. elegans* to a stimulus. Due to this issue, adult *C. elegans* have not been extensively studied. To advance this area of knowledge, the team designed a microfluidic device with a pulse

delivery system to accommodate the adults' lack of movement. This system floods the device with a stimulus for a predetermined amount of time. As a result, the adult *C. elegans* do not have to travel to or from the stimulus to show their preference as they must with a gradient device. Instead, with the pulse, they will be surrounded with the stimulus and can indicate their preference with small forward or backwards movements.

To ensure the experiments had an innovative aspect, the team decided to use light as a stimulus for prompting associative learning. Very few researchers have used light as a stimulus with *C. elegans*, and none have used light to explore associative learning thus far. Light is generally perceived as a negative stimulus by the soil-dwelling nematodes, as it is a signal that they are on the surface and are not buried in the dirt as they desire to be [14]. In learning experiments, the team used an intensity and wavelength that would elicit low response levels, and paired the weakly negative light stimulus with a strong positive stimulus. The goal was to have the *C. elegans* learn to associate the light with a positive stimulus and overcome their natural instincts to reverse and avoid the light.

Developing the new microfluidic environment device, light device, and approach for understanding how adult *C. elegans* react to stimuli was driven by the opportunity for improving upon technology. No suitable devices for testing the phototaxis of adult *C. elegans* in response to light stimuli existed, and there was a need for that type of device in order to experiment with *C. elegans* of all ages and for the ability to study associative learning. This need existed due to the knowledge that could be gained concerning human aging and how the neurons' properties change as they age, to accomplish this, the team met goals that were established early in the design process. These goals are as follows:

One goal was to design a microfluidic device suitable for adult *C. elegans*. To make the device suitable for adults, the team used a pulse method to deliver stimulants. Additionally, the team chose device dimensions that allow adult *C. elegans* the ability to move naturally. The design of the worm barrier does not allow adult *C. elegans* to use force to escape the device. These choices created an environment that could accommodate the testing of adult *C. elegans* with chemical stimuli.

Another goal the team met was testing the device with liquids prior to being used for experiments involving *C. elegans* to ensure that it possessed a few key characteristics. The device could not leak, allow fluid evaporation or the formation of permanent air pockets. The team also had to check that the design had a smooth, laminar flow of fluid through the microfluidic channels. If these needs were not met, the adult *C. elegans* would act in an unnatural way, causing the data to be inaccurate.

Testing of the microfluidic device with *C. elegans* was an extremely important goal that had to be accomplished early on. This was done to ensure that the device met the design goals, including not allowing *C. elegans* to escape and creating an environment where *C. elegans* had the ability to move naturally. It was essential that the team determine if the adult *C. elegans* were compatible with the device before running the actual experiments. If the design and dimensions were not suitable for the *C. elegans*, they would not be able to provide useable data. By running tests without stimuli, specifically to observe how the *C. elegans* interacted with the designed environment, the team was able to determine whether any further device changes needed to occur.

A highly prioritized goal was the construction of the light device which created a stimulus that could be applied independently from the chemical stimuli and thus used in

conditioning experiments. Both construction and testing of the light device to ensure that the wavelength and intensity were appropriate and could be adjusted were an important to achieving this goal. If the wavelength and intensity could not be adjusted, it was a possibility that the pre-set values would not cause a reaction or would cause too strong of a reaction in the *C. elegans*. Before beginning the behavior experiments, the team performed several preliminary experiments to find the best wavelength and intensity to elicit a response from the *C. elegans*.

The team also had a goal of conducting learning experiments with wild type N2 adult *C. elegans* that would demonstrate how aging affected learning. To accomplish this, the team needed to design associative learning experiments that could be compared to prior experiments done with young adult *C. elegans* in the QNTL and by other researchers using light as a stimulus with young adults. Comparing the associative learning ability of younger and older animals would allow the team to determine how age affected the learning process and addresses the overall goal of the project.

1.3 Methodology Overview

The first step in the project was to design a successful microfluidic device for testing adult *C. elegans*, in order to compare the learning patterns between them and their young adult counterparts. The success of the device was quantified by several parameters listed below. The device had to be able to accommodate adult *C. elegans* and controlled enough to draw comparisons between it and the young adult device. The device also had to meet several standards set by the previous devices. It had to allow for the natural movement and observation of adult *C. elegans*. This parameter was evaluated by a comparison to an agar plate control, a flat petri dish with agarose media in it which allows for unimpeded movement. Furthermore it was

compared with how locomotion in young adults change from agar plates to microfluidic devices, and locomotion of adults on the current microfluidic devices. Some variation was to be expected due to the different morphology of adult *C. elegans* when compared to young adults. This design consideration was secondary to the definition of arena boundaries. In previous devices, the larger and stronger adults were capable of escaping the experimental arenas, invalidating experiments. The new device had to be designed so that the adults were contained within their arenas. Additionally, it had to be transparent to allow for observation and data collection of the *C. elegans*. Finally, the device had to keep *C. elegans* viable, i.e. the material must be neutral, and had to be experimentally similar to previous devices. If these parameters were met, the device would be deemed successful.

Prototyping of potential designs was conducted through SolidWorks which was chosen due to SolidWorks being the most commonly known CAD software in use on WPI campus. To accommodate for the lower mobility of adult *C. elegans*, a pulse device was designed. The experimental design of this device was broken down into three steps. First the *C. elegans* were introduced to a neutral substance like S. Basal and given time to adjust to the new environment. Secondly the arenas were flooded with a stimulant and the *C. elegans* response was recorded through a camera. Finally arenas were then flooded with the same neutral substance and worm reactions recorded. Afterwards these videos were analyzed with the MATLAB script – ArenaWormTracker, whose script is available in Appendix H. This program tracks the movements, concentrations, ethograms and location of the *C. elegans* over the course of the experiment. Forward movement in response to stimulus introduction signifies an attraction and reverse signifies a dislike. Experiments to study learning, such as this one, can introduce a

neutral substance with an attractant or repellant and see the difference of association development between young adults and adults.

Another important step the team had to complete was designing and constructing the light device. The most important parameter that had to be met for the device to be successful was that the device was controllable. The team had to be able to precisely control the light's intensity, wavelength, and the timing of it turning on and off. To control the intensity, the team inserted a user replaceable resistor into the circuit. The team then used a light intensity meter to measure the intensity output. To ensure the intensity was consistent across the device, the light rays had to be parallel. To create this effect, the team used a system consisting of a LED, a diffuser lens, and condenser lenses, as can be seen in *Figures 4.5* and *4.6*. The wavelength was controlled by ordering a LED that would provide an acceptable wavelength. This could be adjusted by simply switching out the LED in the device, as the wavelength does not need to change during experimentation, just between experiments. To control the timing, the team utilized a MATLAB script that had been setup to control whether the stimulus valve was on or off, script available in Appendix B. This ensured that the delivery of the light stimulus was exact and controlled and could be pored with the chemical stimuli precisely. Another important characteristic of the light device was that the device be compatible with the existing setup. Specifically, the team did not want the light device to impede the data collection performed by the camera lens directly above the microfluidic device. To combat this issue, the team designed a cage structure to hold the light device at an angle on the side of the experimental setup.

In order to validate this system a series of experiments were conducted to ensure that associative learning was possible with the designed setup. These experiments were deemed a success if there was a change in the behavior of *C. elegans* in response to the light once it had

been associated with the positive chemical stimulus. Preliminary reaction testing to gauge the proper intensity and wavelength was performed in agar plates and worm reaction was gauged through qualitative review of video.

1.4 A Look Ahead

Much research has been done on the nervous system of *C. elegans* since Brenner's initial research of the genetics of nematodes in the early 1970s [16]. Most recent studies suggest that *C. elegans*, with their simple nervous system, are very suitable to model human system [17]. There have been many studies conducted that focused on the learning paradigms and aging of *C. elegans* and their behavioral changes under different stimuli [18]. In Chapter 2, the team will further review the past and ongoing research about *C. elegans* and the psychological and physiological changes when the animals are exposed to a new environment, or when the current living system is exposed to different conditions. Chapter 2 also introduces the need of microfluidic devices and their functions.

Based on the background research and client meetings, the team was able to clearly state the client statement and the need of the project. The team created an objectives tree as seen in *Figure 3.1* and prioritized the objectives based on the need and group discussions. Additionally, the constraints were analyzed for the engineering design part of the project and the devices and their performances were thoroughly researched. This allowed the team to create a better and more useful prototype.

The project consisted of two main parts, designing both a microfluidic and light device and designing experiments for validation of the system and for use of the system for study of aging. For the engineering design aspect, the team designed a polydimethylsiloxane (PDMS)

microfluidic device for adult *C. elegans*. The new microfluidic device was based on a microfluidic device that is currently used in the laboratory for a younger adult group of *C. elegans*. It should be noted that the microfluidic device for younger adult *C. elegans* was not suitable for studying adult *C. elegans* because *C. elegans* get much bigger in size as they age. The light device consisted of a controllable LED and four lenses (two condensers, a diffuser, and a plano-convex lens) as can be seen in *Figures 4.5* and *4.6*. This device was built so that the light could be controlled by MATLAB, intensity was controllable via a resistor, and the resulting circle of light would be large enough to cover the entire microfluidic device. The main objectives of the project were to make the device safe for the user and the *C. elegans* and to have a reliable device functions to allow accurate experiments.

The final design of the microfluidic device considered the physiology of adult *C. elegans*, their body mass and dimensions, and how much force they potentially produced while moving around. This allowed the team to make the new microfluidic device more resistant to internal force and to minimize the strength and mobility issues within the device. Another main focus of the design was to allow natural movements for *C. elegans* in the environment so that there was no influence from the external environment on the locomotion of the worms. The microfluidic device had laminar liquid flow through the microfluidic channels; this allowed the behavior and movement of *C. elegans* to not be interfered with by the controlled media. The quality and effectiveness of the prototype were evaluated and validated by completing preliminary testing and validation experiments, as described in Chapter 5.

The final design for the light device was heavily influenced by making it compatible with the existing experimental setup. The team wanted the same MATLAB script that controlled the liquid release valves to control whether the light was on or off, which would ensure the stimuli

were paired accurately. Additionally, the team did not want the light device to get in the way of the camera that was recording the experiments. As a result, the team designed a structure to hold the light device at an angle on the side of the experimental setup. The light still covered the entire microfluidic device but did not impede data collection. The reasoning behind using the three lenses and a diffuser was to ensure that the light intensity was evenly distributed across the device.

The second main focus of the project was to design experiments to validate the final device for associative learning capabilities and to compare the associative learning capabilities of older and younger adult *C. elegans*. These experiments involved both normal and mutant *C. elegans*. The findings from the validation studies allowed for further study to move ahead in future projects and for many possibilities in testing *C. elegans* become possible. The experiments to compare young adult and older adult *C. elegans* gave a guideline for the future experimentation using the system designed and for results to be accurately analyzed and compared.

Chapter 2: Literature Review

2.1 Significance

This project is concerned with the neurological and cognitive changes that occur from the natural process of aging and the effect degenerative diseases can have on this process. As we age, behavior and decision making changes. This is caused, or reflected, by changes in the brain. Aging is generally linked with a mental, and definitely physical, decline. This includes reduced control of motor functions, increased difficulty processing diverse stimuli, and general decline of mental speed [20]. As the average human lifespan increases, more of a human's life is spent in this advanced age state. The potential for severe mental and physical decline harshly limits what these older humans are capable of, making them dependent on others. This becomes even worse when affected by the debilitating diseases such as dementia and can have severe emotional effects on not only the patient but the family and friends as well.

When studying aging, a variety of approaches have been used such as psychological studies, post-mortem autopsies, neuroimaging, and use of human models to name a few. Through these various approaches a range of discoveries about the human brain's aging process have been discovered.

In psychological studies it has been found that there is severe impairment in episodic memory, linked to an issue in encoding memories [5]. This is in part due to failure to perceive distinctness. In other words, with increased age the brain ceases to distinguish the difference in one event from that of a similar, previous one. Additionally older adults have a tendency to not only code material into previous memories, they also tend to recognize novel stimuli as similar stimuli they have seen before [5]. This degradation of accurate processing was shown

particularly in a study that used pairings of novel-novel stimuli and novel-similar stimuli and recollection thereafter of these stimuli. Those in the novel-similar condition were less able to distinguish what occurred in the first and second section [5]. This deficit in processing clearly shows a breakdown in a specific type of processing center in the brain, though what this section is exactly is unknown.

Autopsies can often be used to find the physical changes in the brain, such as region specific loss of brain matter, plaque buildup on neurons, or tumors and unnatural growths. However, there are some faults with this method. The medical examiners are looking at one point in this person's life, the development of these changes cannot be shown from a single point in a lifetime. Additionally the information on the behavioral changes that went along with these physical changes is all but a mystery.

It has been found that when an older adult is presented with similar stimuli their brains processes them the same [6]. In other words the brain generalizes between similar stimuli and is less sensitive to differences. For younger adults even small differences in stimuli presented with specific changes in brain region reactivity depending on the stimuli [6]. This shows that this is an effect that occurs with age rather than a person to person difference. Another neuroimaging study which concerned the effect of multi-tasking on different ages showed that there was a greater strain on older adult's brains when trying to walk while doing a second, more mentally consuming, task. The young adults brain activated only in those areas that were necessary for the walking and the mentally consuming task. The older adults, in addition to having a much stronger response in those areas, also have reactions in additional areas [19]. This supports the findings above as well as showing that tasks in general become more difficult for the elderly to manage and that focusing on even something as simple as walking, a basic instinct, interferes

with our ability to think and perform tasks. However one interesting finding in this study was discovered when comparing the older and younger adult's performance. In both older and younger adults their performance on both tasks fell when they were multitasking in comparison to the performance of those tasks individually. The interesting part is what activity suffered more, the walking or the mentally consuming task. It was found that in younger adults, their performance in the mentally consuming task was very good, however their walking speed and consistency suffered. The opposite was true of the adults. Their walking was still quite good but their performance on the mentally consuming task fell [19]. This suggests that it is not an overall degradation in brain function we have to worry about but a degradation in certain regions or in certain types of neurons. What types of neurons and regions still needs further investigation.

This project will be attempting to perform this investigation. All psychological changes have a corresponding physical alteration. The team wishes to provide a model for studying the neurodegeneration that occurs in aging. However, humans are highly complex organisms with a multitude of systems and biological components acting together. There are hundreds of types of neurons with an assortment of functions and a variety of specific centers in the human brain [20]. Many things can affect the behavior of a human being even day to day, never mind over the course of a lifetime. Hormone levels, fatigue, immune reactions, environment, and age can all affect how a person develops over time. So in order to see how age itself is a factor on the physical changes, and thus behavioral changes, in the brain, this project must look at the factor of aging isolated from the other factors. In order to do this the team will need a simpler system to study than that of a human. Thus this project will focus on model animals, specifically *C. elegans*.

Use of human models is a very informative area of study. Through its use, it has been discovered that there is a loss in neural density in the medial temporal lobe, resulting in recognition memory, as we age. This was an unknown fact previously with many theories on which brain regions this sort of memory concerned [7]. One area that is particularly useful is in comparison of normal aging brains to those of sick aging brains [7]. In early Alzheimer's, for example, it is often hard to distinguish the progression of the disease from the normal mental decline of an aging. In fact often there are almost no signs until the later stages of the disease. This makes studies of the human brain unreliable as some "normal" brains may in fact be diseased [7]. This is where animal models can come in. Alzheimer's and other disorders can be induced into these models and then tested against healthy brains guaranteeing that the comparison of samples really are healthy or diseased.

Additional benefits to animal models are that there are a multitude of different factors, such as pathological, environmental, and social, that can affect the human aging process, but in model animals all these things can be controlled for [7]. It is important to separate the aging process out from the rest in order to examine what can be done to improve our cognitive decline. In humans it is often hard to know where to begin treating them as there are so many factors that affect their behavior. *C. elegans* are particularly beneficial because their environment is constant, they have instinct based rest cycles, and their immune response and hormone production is either negligible or easily controlled for [21 ,22]. Thus when testing the *C. elegans* it is truly possible to attribute their change in behavior and neural make up to the fact that they are aging.

C. elegans are a good model for humans for several reasons. First, they have 302 neurons and remain capable of relatively complex behavior [17]. That is to say, they are able to make rudimentary decisions, allowing observation of changes in this behavior to predict how various

stimuli affect behavior. Second, there are many biological processes that are analogous between *C. elegans* and humans. For example drug tests performed on *C. elegans* are predictive of reactions in humans [17]. Third is the fact that the entire genome and neural net has been mapped, making it a highly accessible model [23]. Fourth, is the similar growth style, with the “childhood” taking up a similar percentage to that of a human’s. Additionally, as in humans there is a tendency for muscles to degrade and movement to become “sluggish”, with this decline often noted in adult *C. elegans* [21]. Finally, *C. elegans* are easy to work with. Many worms can be used at once and thus data can be collected in mass. Collecting the amount of *C. elegans* needed for experiments is not a hard task, they are extremely prolific and easy to grow, store, and care for. All of these factors allow for a solid model for more complex organisms, in particular humans.

Thus this project endeavors to use a model organism, the *C. elegans*, to examine the effects of aging on the behavior and decision making of organisms by looking at the cognitive and neurological effects of aging. In future work and in other projects it is hoped that work will be done to feature in the effects of various other factors, such as hormones, environment, etc., whether it be in *C. elegans* or in a higher level species so that the process of aging can be even more thoroughly understood. With the findings of this project in particular, it is hoped that future work can be done to see how the changes in the neurons can be prevented or treated so the decline in brain function is no longer an issue and so that treatment of many of the devastating neurological diseases can be developed.

2.2 *C. elegans*

This section introduces *C. elegans*, including their anatomy, differences between young adults and adults, and details concerning their neurological system. Additionally, information pertaining to these animals' research applications, specifically within microfluidic environments, is reviewed in this section.

2.2.1 Anatomy of *C. elegans*

C. elegans are semi-transparent soil nematodes that grow to about 1.5 mm and have a maturation time of 3 days, after which they can produce anywhere from 300-350 progeny. The animals can procreate with a self-inseminating hermaphrodite or a male can inseminate the hermaphrodite. Hermaphrodites are far more common than males and can produce more young but the males are capable of producing more diverse offspring. The average *C. elegans* lives for two to three weeks; see *Figure 2.1* for more details on the life cycle. These simple organisms are also easily cultivated in a laboratory for research purposes if kept at 20 degrees Celsius and supplied with enough food (*E. coli*). They are not anatomically complex, with N2 wild-type *C. elegans* containing only about 1,000 cells. The number of cells is consistent within every animal, though males have slightly more cells than hermaphrodites due to an increase in neuronal cells dedicated to mate searching and mating [24]. All of these factors (their simplicity, consistency, transparency, and life/reproduction cycle) make them suitable laboratory research subjects.

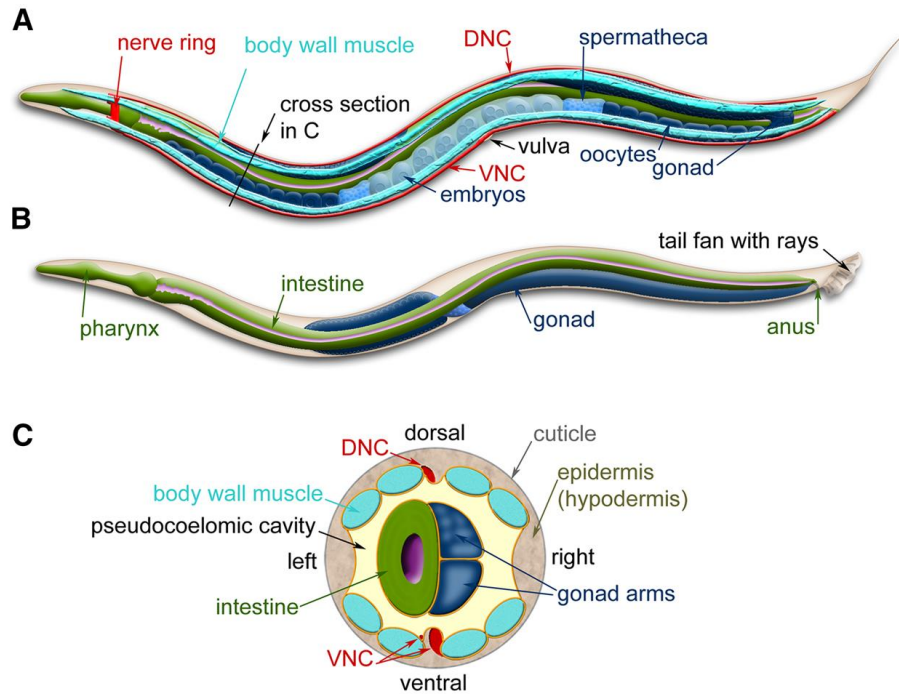


Figure 2.2: *C. elegans* Anatomy - including the ventral nerve cord (VNC) and dorsal nerve cord (DNC) [25]

2.2.1.2 Young Adult versus Adult *C. elegans* Anatomy

Young adult and adult *C. elegans* are physically different. Young adults are 900-940 micrometers long, while adults are generally 1110-1150 micrometers long. The *C. elegans* exists in the young adult stage for about 8 hours, while adults exist in that stage for about 13 days [27]. Additionally, adult *C. elegans* undergo synaptic decline, while young adults still have very active connections [26]. Synaptic decline is theorized to contribute to the overall decrease in functionality of the human brain as it ages.

Electron microscopy and neuronal aging assays were used by Toth *et al* to confirm that adult *C. elegans* experience severe morphological changes in their neurons as they age [26]. The main dendrite for touch neurons develops many more branches after the *C. elegans* passes the reproductive age, and the number of branches increases with age. The soma was also found to develop growths after the animal was past reproductive age. Additionally, at this age, the

neurons became wavy instead of remaining straight. However, it is important to note that no neuron loss or connection breaks were found [26].

The changes that were found in adult *C. elegans* are consistent with what has been observed in the human brain, making the *C. elegans* a great model for aging. Older humans' brains contain deformed dendrites and deteriorated synapses [26]. The similarities between the aging nervous systems suggest that both animals have the same or similar mechanisms that cause these issues. Though the mechanisms are unknown, studying *C. elegans* may help uncover more information that could lead to medical applications allowing the human brain to remain functioning at a higher level for longer.

2.2.2 Neurological System of *C. elegans*

Sydney Brenner was the first researcher to truly recognize the potential modeling abilities of the *C. elegans* nervous system. He wrote code allowing him to use serial-section microscopy electron microscopy to begin building the hermaphrodite *C. elegans* neural wiring system [24]. This neural map, which has been completed, has allowed researchers to study the neurons and neurotransmitters of *C. elegans*, and then expand their research to other animals with more complex systems.

Hermaphrodites contain 302 neurons consisting of 118 categories. 33% of these are motor neurons, 23% are sensory neurons, and the rest are interneurons that enable communication to pass between the central nervous system and the sensory and motor neurons. Males have the same neural layout, except they possess an extra 79 neurons dedicated exclusively to mating applications [31]. The majority of the neurons are located in the anterior section of the animal, along the ventral axis, and in the tail [24].

2.2.2.1 Synaptic Plasticity

Synaptic plasticity refers to the dynamic relationship between a neuron and its target. The connection will be eliminated if it is not used and strengthened if it is. This relationship can be explored through learning paradigms such as associative learning [29].

It has been theorized that sensory synaptic remodeling can in some instances affect how *C. elegans* chemotax to food. For instance the *C. elegans* will arrest and enter the dauer stage if conditions, such as food availability and temperature, do not suit it. The dauer will not chemotax to food, likely due to the weakening of synapses responsible for that action [29]. In this instance, the non-favorable environmental conditions are causing the change.

Learning can also cause synaptic remodeling, whether is it associative or non-associative. Associative learning occurs when a correlation between a behavior or stimulus is made with another stimulus. Non-associative learning occurs when a single stimulus changes the behavior. Habituation, a stimulus that decreases the response, and sensitization, which is a stimulus that increases the response, both fall into non-associative learning [24].

2.2.3 *C. elegans*' Applications in Research

C. elegans are easy to maintain and research, as previously discussed. However, the reason that they are used in research is because they can model humans and human conditions. For example, there are *C. elegans* mutants that can model Alzheimer's disease or autism. These animals are similar to humans and other human models. They share their general body development cycle with flies and mice, both of which are established models for humans.

2.2.3.1 *C. elegans* in Microfluidic Environments

It is important to understand how *C. elegans* will react with the environment they are being tested within. In a microfluidic environment with posts, the force an animal exerts depends on the input from its sensory neurons. The input will greatly depend upon the dimensions and structure of the device, as seen in *Figure 2.3*.

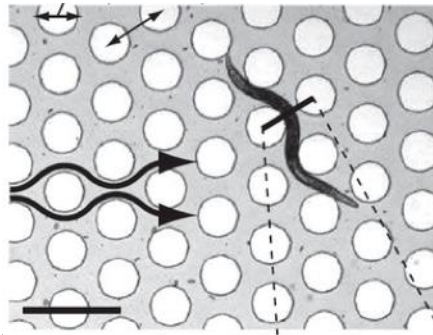


Figure 2.3: Location of Mechanosensory Neurons [8]

The maximum force is exerted at the animals' midsection. For a lattice design with posts 110 microns apart, adult *C. elegans* exerted an average of 14 μ N and a speed of 100 μ m/s. For a hexagonal design with posts 110 microns apart, adult *C. elegans* exerted an average of 21 μ N and a speed of 60 μ m/s. For a lattice design with posts 140 microns apart, adult *C. elegans* exerted an average of 5 μ N and a speed of 275 μ m/s. For a hexagonal design with posts 140 microns apart, adult *C. elegans* exerted an average of 6 μ N and a speed of 140 μ m/s [31].

2.2.4 Light Stimulus of *C. elegans*

Light has been proven to be a stimulant that *C. elegans* recognize and respond to innately [14, 31]. Wavelengths below green-1 light of 500nm have been found to generate the greatest response, with greater sensitivity the lower the wavelength. The most sensitive wavelength has been found to be UV-A at 350 nm [14, 31]. Worms react instinctively to the light with reverse locomotion and pirouettes. With UV-A it has been found the worms move at a locomotive rate of

at least three times greater than that of the basal rate. It was hypothesized that the phototaxis behavior displayed by the worms are the result of a protective mechanism to prevent cell death from harmful wavelengths of light.

Both the degree of damage caused by the light and the level of response has been found to be proportional to the intensity of the light. The lower the wavelength, the less light intensity is required to elicit a similar response. Edwards displayed how $50\mu\text{W}/\text{mm}^2$ of UV-A light generated a response equal to $350\mu\text{W}/\text{mm}^2$ of blue violet light at 441nm and $720\mu\text{W}/\text{mm}^2$ of blue light at 470nm [36]. Edwards experimented with light exposures of both a minute and continuous exposure. It was found that response to the light was rapid, over the course of 5 seconds and peaking at 30 seconds. Afterwards the removal of light elicited a slow decay and return to the basal rate over the course of three minutes. Continuous exposure displayed a reduction of locomotion rate over time, eventually to that below basal rate and eventual worm death over the course of 30 minutes. Wards identified the time and intensities necessary to induce worm paralysis and death, with the $200\mu\text{W}/\text{mm}^2$ of UV-A light inducing death after 13 minutes. Wards has also found that worm reversals began as soon as 1 second after light introduction. In both studies it was agreed that worms exhibited an innate negative reaction to lower wavelengths of light, proportional to intensity.

2.3 Current State of the Art

2.3.1 Agar Plate Standard

Agar plates, petri dishes filled with a flat agar media, are one medium used for studying *C. elegans*. It is an easy to set up an experiment, manage the *C. elegans* and programs being improved to conduct RNAi screens on agar plates [36]. However, agar plates presents a number

of problems which ruins the efficacy of experiments. First the plates are not representative of the worms' natural environment which contains obstacles to movement rather than a flat plane [12]. Second it is difficult to control or introduce experimental parameters such as stimulus solute and odorant concentration [12]. Finally it is difficult to control the worms on an agar plate. However, agar plates do remain an easy way to culture and cultivate *C. elegans*.

Table 2.1: Traditional vs. Microfluidics for *C. elegans* [32]

	Traditional techniques	On-chip manipulation
Functionality	Only one output parameter is monitored per experiment	Multiple parameters are monitored per experiment
Manipulation	Manual (possible damage to the worm while handling)	Automatic (reproducible experimental conditions)
Sensitivity and precision	Imprecise delivery of external stimuli	High sensitivity and high resolution experiments (precise delivery of small amounts of reagents)
Real-time monitoring	Limited	Possible, also for extended lengths of time
Integration	Low	High
Throughput	Low	High
Cost	Expensive (large volumes of reagents) and costly to maintain	Inexpensive, disposable
Personnel	Well trained operators are required	Minimally trained personnel can operate the systems, user-friendliness is possible

2.3.1.2 Microfluidics Standard

The current gold standard for studying *C. elegans* are microfluidic devices [8-12]. The creation of these devices can be simplified into two steps [33, 8, 11]. First the master mold is designed and made through photolithography. Photolithography is a process through which UV light is used on a light sensitive substance shielded in part by a photomask to create a geometric pattern [35]. It is simple to translate design changes into devices through photolithography and as a result one can easily create devices for different purposes and worm sizes. Second, using the master mold the design is imprinted upon an elastomer to create a negative through soft lithography. This can be done by heating and casting the elastomer over the master mold creating

a “positive”. This imprinted elastomer is the device which contains the desired features to be used on the worm experiments.

Microfluidic devices also allow for controllable introduction of stimulants. Fluid flow within the device is laminar which can be utilized for gradient formation and concentration control [8]. *C. elegans* were shown to respond to concentrations of stimulus to the order of 1,000 lower than agar plates [8, 12]. Device design can also control the movements of the *C. elegans*. This allows for easier automated tracking, and data gathering [8, 13]. Devices can also be separated into multiple arenas, with each arena consisting of its own experiment. As a result, a number of novel devices have been created for different *C. elegans* studies. Single worm devices have been fabricated to trap and immobilize worms to study sensory neurons and interneurons [11, 36]. These devices can immobilize mechanically through suction, compression, and microchannel narrowing or through CO₂ gas [36]. Multiple worm immobilization also exists through electrical or gel means [36]. Free roaming worm devices have been made to study response to stimulants [36, 8]. Devices and protocols also exist for lifelong studies on a single population, flushing out eggs laid by adults [13]. A last example of note are devices which can sort *C. elegans* based on size with high throughput and accuracy [35].

2.3.2 Soft Lithography Material - PDMS

The material properties of the microfluidics device must also meet several parameters. For working with *C. elegans* Polydimethylsiloxane (PDMS) is the elastomer of choice [8, 38], [11]. It is transparent, gas permeable, non-toxic, cost effective and easy to manipulate through soft lithography [38]. This conveys the necessary properties of maintaining viable worms that can be visibly tracked. PDMS is also exhibits hydrophobic recovery and forms a seal with conformal contact [38]. Hydrophobic recovery renders experiments necessitating surface

treatment of PDMS difficult as PDMS polymer chains can diffuse through the bulk on to the surface displacing treated functional groups [38]. However this property also allows for easy cleaning of PDMS devices. Device assembly requires the PDMS to be clamped between two fluorinated glass pieces to create an airtight seal, resulting in negligible amounts of stimulus evaporation [8]. Other alternative materials such as glass and silicone can also be used for microfluidic device fabrication. However, PDMS holds the advantage of easy fabrication and allow for rapid prototyping as it does not require special equipment [38]. The major limitation of PDMS with *C. elegans* is its high level of compliance. It has an elastic modulus of 1-3 MPa and shear modulus of 100 kPa-3 MPa, three orders of magnitude lower than glass, with a glass transition temperature of -125°C [36, 37]. This is low enough so that *C. elegans* can elastically deform the PDMS. This applies a constraint to devices needing to be optimized for certain sizes of *C. elegans* so that the worms do not cross intended arena boundaries and render experiments useless.

2.3.3 Microfluidics for Chemotaxis

The current state of the art devices designed and used in Professor Albrecht's laboratory are made from PDMS and optimized for young adults [8]. These devices contain 2 cm x 2 cm 70µm-deep arenas with hexagonally arranged microposts, 200µm in diameter and spaced 300µm apart [8]. The devices served to test for chemotaxis in both temporal and spatial gradient odor patterns. Experiments consisting of gradients requires the worm to physically move to towards or away from the location of highest stimulus concentration to signify attraction or repulsion [8]. Temporal odor patterns signify worm behavior with the type of movement exhibited, categorized by forward, reverse, and pirouette (sharp directional change) [8]. However, as the current device

stands, the design is poorly optimized for the morphologically different adult *C. elegans* making adult chemotaxis experiments difficult.

2.4 Experimental Data Analysis

Data analysis and interpretation of the performed experiments were an important part of the project. The team used two main programs - ArenaWormTracker for tracking the locomotion and behavior of the *C. elegans*. In this section these scripts are introduced.

2.4.1 ArenaWormTracker

MATLAB is a powerful computing tool that is capable of complex data analysis and visualization of the data. Several different MATLAB programs were developed to analyze behavior of *C. elegans* and their locomotion in a microfluidic device. These programs require a simple set up – a camera lens to acquire an input for the program in a video format and a MATLAB compatible computer.

Parallel worm tracker is a MATLAB program that was developed at the Stanford University. The program consists of three main modules – worm tracker, worm analyzer, and video capture. The arena with worms is captured on a video and the video is segmented in the program. The worm tracker is based on the pixel intensity of the arena and the worms. A pixel intensity threshold and approximate worm size are set by the user in order to accurately track the worms on the arena. The movement tracks of the worms are identified and mapped, when there is a movement interference with other worms, the program erases the mapped crawling. The worm analyzer investigates the worm speed, angular speed and the direction of the movement based on the movement tracks [40]. The parallel worm tracker program was used in Ramot *et al.*

study that investigated the average speed and drug induced paralysis in *C. elegans*. The study concluded that the parallel worm tracker is a suitable program for studying the locomotion of *C. elegans* [40].

Arena Worm tracker, script available in Appendix H, was based on the parallel worm tracker program and was developed at the Laboratory of Neural Circuit and Behavior at the Rockefeller University [8]. This program is currently used in the Quantitative Neurotechnology Lab at WPI and is the fundamental tool for data analysis of the behavior of *C. elegans*. The arena worm tracker consists of several sub-programs. The program requires the user to input the arena boundaries and the size of the arena. Also, it allows the user to select the video frame rate and duration. Using the sub-programs, it maps ethograms as seen in *Figures 5.2-5.4*, which are summaries of the exerted behaviors of the *C. elegans* and outputs plots that reflect the speed of *C. elegans* over time. Instantaneous morphological behaviors are also summarized in the output of the program [8].

Chapter 3: Project Strategy

The goal of this project was to create a system in order to study the changes that occur in cognitive function and neurological structure with age. Within this the team had to create a microfluidic device that allows for the tracking and imaging of adult *C. elegans*. This device was meant to add to the understanding of how human learning changes and degrades with age. This was accomplished by studying associative learning in adult *C. elegans*, which serve as models for the human neural system, in the microfluidic device. To accomplish this project, the team had to take into consideration background research on the model organisms, microfluidic laws, and other devices such as a device that reflects light at certain intensity and that could alter the learning pattern of *C. elegans*. Additionally, the team focused on input from the client and validating results from experiments using this microfluidic and light devices. The following objectives and constraints were also used to guide the design process.

3.1 Initial Client Statement

At the beginning of the MQP the possibilities and goals of the upcoming project were discussed with the client, Prof. Albrecht. Based on the provided information the initial project statement was formed. It is as follows:

Design a microfluidic device that allows adult *C. elegans* the ability to move naturally and tests how they respond to different stimuli. Furthermore, explore at least one learning paradigm in adult *C. elegans* that is known for young adult *C. elegans*.

From this client statement the team began forming design requirements for the project as detailed in the following sections.

3.2 Technical Design Requirements

3.2.1 Objectives of the Microfluidic Device

The client statement was used as a resource to identify and prioritize the project design's primary and secondary objectives. Based on the need and group discussions, four primary objectives were identified - safety, device functions, ease of use, and cost effectiveness. Secondary objectives were grouped together under the primary objectives based on their functions. An objectives tree was created using the identified objectives, which is presented in *Figure 3.1*. In the objectives tree, the secondary objectives were listed in descending order of importance. The primary and secondary objectives are further discussed below.

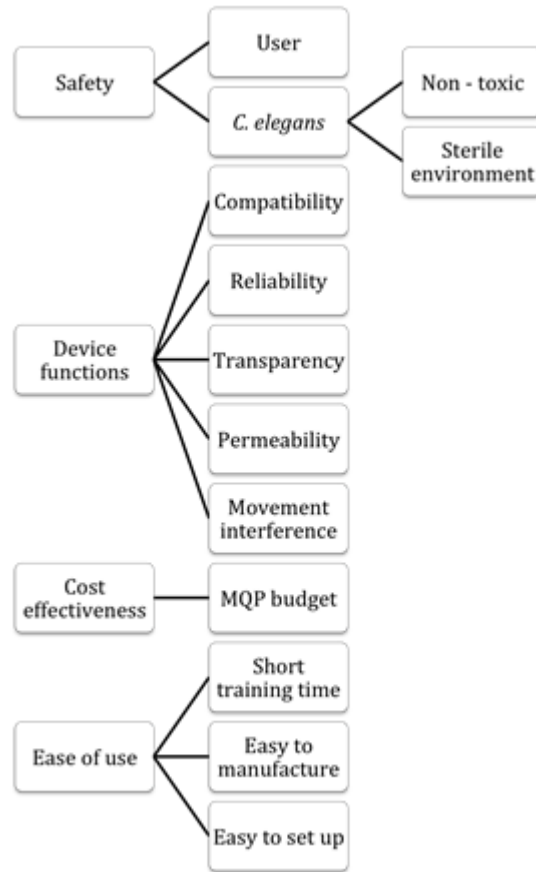


Figure 3.1: Objectives Tree

To determine the significance of the primary objectives, the team developed a Pairwise Comparison Chart (PCC), shown below in *Table 3.1*. The team assigned points to the objectives based on the importance. The objective that got the highest total point was determined to be the main priority. According to the PCC, safety and device functions received the highest scores and were ranked both equally. Safety in laboratory space and experiments is always important, it is always necessary to follow the laboratory protocols and safety guidelines. Device function is equally important because the main goal of the project is to use the device to get an accurate and reproducible data for analysis. Since the project is under a budget, the cost effectiveness was ranked third. Ease of use was determined to be the less important objective compared to safety, device functions, and cost effectiveness.

Table 3.1 Pairwise Comparison Chart

	Safety	Device functions	Ease of use	Cost effectiveness	Total
Safety	x	½	1	1	2 ½
Device functions	½	x	1	1	2 ½
Ease of use	0	0	X	0	0
Cost effectiveness	0	0	1	X	1

3.2.1.1 Safety

Safety was always the main priority for the team while working in laboratory. The primary objective ‘safety’ was concerned with two secondary objectives that the team identified – user and *C. elegans*. Both secondary objectives had an equal significance and are discussed below.

Safety for the users was essential because the top priority was to have a safe laboratory environment. The device needed to have minimal or no chemical spills during or after the experiments. This allowed the team to maintain a clean and a safe workplace.

It was also important to consider safety for *C. elegans*. According to the laboratory protocol *C. elegans* cannot be exposed to an open air for a long period of time because there is a high chance that they could get contaminated. Thus, the device needed to provide a sterile setting for *C. elegans*. Additionally, it had to be made out of non-toxic material in order to provide a pleasant ‘natural’ environment for *C. elegans*. This allowed no external interference in the experimental data collection.

3.2.1.2 Cost Effectiveness

The project budget was \$624. Thus, the manufacturing of the microfluidic device and other expenses needed to fall under the budget. Additionally, it was assumed that many of the costs would be absorbed by Professor Albrecht's Lab expenses as they could serve a double purpose as general lab tools and MQP devices.

The estimated cost breakdown is shown in *Table 3.2*.

Table 3.2: Cost Breakdown

Item	Approximate cost
Master mold	\$ 150.00
S. Basal per experiment (~110mL)	\$ 6.25
Butanone (20L)	\$ 544.00
PDMS per device	\$60.00
Glass slide per slide	\$ 35.00
Agar plate per plate	\$ 1.00
Estimated total	\$ 600

3.2.1.4 Ease of Use

The primary objective 'ease of use' was concerned with the user friendliness of the device. For this primary objective, the team identified three secondary objectives - short training time, easy to manufacture, and easy to set up.

Short training time had more emphasis because this objective was closely related to the laboratory safety measurements. As well as in order to have a working device, training was important.

Because the experiments needed several microfluidic devices, the manufacturing process had to be simple to follow.

The device needed to have an easy set up in order to save time for the actual experimental observations and data analysis.

3.2.2 Constraints of the Microfluidic Device

The microfluidic device constructed was constrained by its size and material. The device had to be small enough for the recording camera to capture the areas relevant to the experiments. Both the arena and the worms had to be captured along with the movement and location of the worms able to be identified. Furthermore the device had to be big enough so that a statistically relevant amount of worms can be introduced and not compromise the experiment. If the device was too small, the device would either be at risk of being overcrowded, or being heavily swayed by outliers. The material from which the device was constructed from was also a constraint. Above all, the material has to be able to form the microfluidic channels through soft lithography, gas-permeable, non-toxic, and biocompatible. To this extent PDMS met all the constraints as well as being able to form a seal with encasing glass to significantly reduce fluid evaporation. Furthermore PDMS was relatively transparent and cheap material to use.

The project was also constrained experimentally. The experiment ideally occurred over the course of an hour to minimize the effects of desensitization to the stimulus and environment. Data for the experiments had to be gathered over the course of an hour unless protocol calls for otherwise, i.e. experiments studying the degree of desensitization. Therefore experimental time was a constraint placed on this project. *C. elegans* age and device crowding also served as a constraint for the experiment. The relative age of the *C. elegans* had to be identifiable in order to conduct experiments on the effects of aging and learning. This was done by isolating the *C. elegans* by the notable changes in morphologies between stages in the *C. elegans* life cycle. The quantity of *C. elegans* was also a factor, as denoted by the size of the device. It was critical to

ensure that the amount of *C. elegans* did not flood the device and cause skewed results. That being said it was important to get as many data points as possible and therefore in the designed microfluidic device, 30-50 *C. elegans* was a suitable amount.

3.2.3 Microfluidic Device Functions

The primary objective ‘Device functions’ was broken down into five secondary objectives – compatibility, reliability, transparency, permeability, and movement interference. All of the secondary objectives had an equal emphasis on the device design.

- Compatible with the existing lab set up; it had to be a certain size in order to be used under the microscope and camera lens for observation.
- Reliable in order to get an accurate and reproducible data. It needed to have strong borders within the device because it needed to hold up to 50 actively moving *C. elegans*.
- Transparent to allow clear imaging of the moving *C. elegans* under the camera lens that was used for data collection.
- Permeable to allow oxygen transfer and gas exchange for the *C. elegans* to survive.
- Does not interfere with the natural movement of *C. elegans* in any ways, because it would cause experimental errors in the behavior and locomotion analysis of *C. elegans*.

3.2.4 Microfluidic Device Specifications

In designing the microfluidic device the measurements of the channels and overall measurements of the device was based on the previously existing young adult device as there

was proven laminar flow and proper parameters of movement for the *C. elegans*. Thus for those areas of the microfluidic device not being interacted with by the *C. elegans* the device was to be changed as little as possible. This included everything except for the worm barriers and the arena pegs, the design of which will be detailed in Chapter 4. For the photolithography process there is a tolerance of $1\mu\text{m}$ so variations of this size are possible in the design. However, $0.001\mu\text{m}$ was chosen as the tolerance of the designs as this is the degree of rounding that SolidWorks uses and thus the degree of difference that would be possible in the designing process. This meant that any of these measurements of the existing device had to be transferred over with an accuracy within $0.001\mu\text{m}$. Additionally the arena peg spacing and diameter was based off that of the young adult devices proportions in relation to the young adult *C. elegans*. The *C. elegans* girth diameter is 30% of the Peg diameter and 60% of the Peg gap. Thus similar $0.001\mu\text{m}$ precision was to be maintained in these measurements as well.

The specification for operation mainly involved maintaining laminar flow of fluid and allowing for natural *C. elegans* movement. The flow was tested using Dye Tests, described in detail later in Chapters 5 and 6, in which dye was inputted into the device and qualitative and quantitative analysis was performed on the basis of speed (i.e. time it takes to cross the arena) of dye crossing arenas, noting any pauses of fluid, and noting any swirling (indicating turbulent flow). Slight slowing of fluid when encountering the worm barrier was allowed so long as it stayed within 0.2 seconds. No turbulent flow was tolerated however as this could damage the *C. elegans* if they encountered it.

The specifications for material usage was fairly strict as the materials used were all the same as the previous devices as no improvements to this were required at the time. The material used was PDMS and had to be made according to lab protocol which called for 45g of base to 5g

of binder. Any and all particles or bubbles within the PDMS were unacceptable and immediately lead to creation of a new PDMS device being made.

3.2.5 Objectives of the Light Device

The main purpose of the light device is to expose light to the arenas of the microfluidic device to train the *C. elegans* to “like” or “dislike” the light exposure and to study their behavioral changes when the organisms are exposed to light. Objectives of the light device included reliability, compatibility with the existing laboratory devices, and ability of changing the intensity and wavelength of the light source. The team agreed that all the objectives had equal importance and significance to the project.

3.2.5.1 Reliability

The device needs to be reliable in order to give accurate and repeatable results. The experimental setting such as intensity and angle of light reflection should not alter throughout the experimental time, therefore reliability is important. During the experiments, the device needs to maintain its position, thus the team needs to design a reliable light device that is physically stable and that can be used in the laboratory even after the completion of the MQP.

3.2.5.2 Compatibility

It is important to have a light device that is compatible with the current lab setup and that doesn't interfere with the existing laboratory devices at the QNTL Lab. The device needs to be small sized so it won't cause imaging interference with the camera lens that is used to record the experiments. Additionally, the device needs to expose uniform light on the arenas of the microfluidic device, however the light cannot reflect back to the camera lens, as it may result in

inaccurate camera reading. It is highly desirable if the light device can be connected to a computer for automated switch controlling purposes.

3.2.5.3 Ease of modification

As the experiments may require slight modifications of the intensity and wavelength of the light based on the response of *C. elegans*, it is essential to have a device that is capable of some modification. It should be noted that these modifications should not alter the reliability and the stability of the devices.

3.2.6 Constraints of the Light Device

The most important constraint of the light device is the size. It restricts the positioning of the device in the hood. With a limited space available in the hood, there's a chance that the light device may result in an interference with other devices in the hood. Accessibility of other devices may become an issue in the longer run, as the light device may take up most of the free space. If positioned at a wrong angle, the light may reflect in a non-uniform way, this would cause in inaccurate experimental results and the behavioral changes of *C. elegans* may not be interpretable.

3.2.7 Functions of the Light Device

The light device was the main stimulus being applied to the *C. elegans* as such it needed to perform a series of functions.

- Output a constant intensity and wavelength throughout the experiment
- Ease of modifying the intensity and wavelength
- Turn on and off in response to the valve system already existing in the QNTL

- Produce a light that covers the entirety of the microfluidic device
- Maintain a constant intensity across the entire area the light touches

These functions were kept in mind as the light device was created and greatly influenced how it was built and connected to the existing system.

3.2.8 Light Device Specifications

Specifications for the light device concentrated mainly on the degree of precision and operational ranges of the light outputted. The materials used did not have any prior specifications as the team designed and created the device from separately purchased parts.

Wavelength was the first specification that the team addressed. The wavelength had to remain perfectly constant with no tolerance for deviation during the experiment. Using a purchased LED would allow for this to be achieved but in order to test multiple wavelengths multiple LEDs were purchased in 470 nm, 447.5 nm, 415 nm with 5 nm deviations from the reported wavelength allowed so long as it was a constant wavelength.

Intensity had to have a highly precise measurement across the entire area the light touches. The intensity deviation from center to edge had to be within +/-10% in order for the intensity to be considered even. Additionally, as light intensities will be based upon values determined by previous literature, discussed later in the paper, the intensity must match these levels within $10 \mu\text{W}/\text{mm}^2$.

As any light device using a LED would have a very small area of contact it was proposed from the beginning that some form of lens system would have to be used to expand the area covered. This meant that focal length of these lenses would have to be considered in order for accurate distribution of light to be achieved. The tolerance determined by trial and error

experimentation was determined to be only 0.5 mm as extreme accuracy is involved in optical physics.

Finally the response of the light device to the computer command to turn on or off had to be nearly immediate and the same every time so that the pairing of stimuli would be exact and precise. A response time of 0.2 seconds or faster was determined to be a suitable response time. However this was not predicted to be an issue as previous use of this system with liquid stimuli has proven to be exact and accurate. Furthermore LED's operate at kHz frequencies, which allows a timing set up less than those of the light device.

3.3 Standards of Design

3.3.1 Engineering Standards

3.3.1.1 Microfluidic Device

The engineering standards that the team followed while designing the microfluidic device were based on the standards that are followed at the QNTL at WPI. Since the designed microfluidic device is only used in research, the larger scale standards such as ISO did not necessarily affect the device design. Some of the main guidelines were delivered during the client meetings with Professor Albrecht. The newly designed microfluidic device had to have a resemblance in external shape with the existing microfluidic device as well as it needed to be compatible with the laboratory set up. The ultimate goal of the manufacturing the microfluidic device was to have a 'bubble free' PDMS device that had a consisted fluid flow with no bubbles present in the system.

3.3.1.2 Light Device

The standards for the light device in our experimentation specifically entails the use of controlled stimulus delivery, as detailed by previous literature. As we are experimenting with *C. elegans*, we also had to consider animal experimentation standards. These standards were detailed in international standards such as ISO 10993-2:2006, and ISO 10872:2010, which are described below. However as *C. elegans*, being microscopic nematodes, barely qualify as animals, certain standards could be taken loosely, such as those related to ethical handling. This is of concern due to evidence supporting intense light exposure causing motor deterioration and eventual death of *C. elegans*. As such for both experimental and ethical purposes, we tried to minimize the permanent effects of the light exposure.

3.3.2 Specifications

3.3.2.1 Microfluidic Device

In the usage and fabrication of our microfluidic device, and fluids used for experimentation, we recognized and abided by several engineering standards as listed below

ISO 7712:1985 - For the use of disposable micropipettes

ISO 7550:1985 - For the use of disposable serological pipettes

ISO 10993 - For the use of devices in contact with the body directly or indirectly, including sterile and nonsterile microfluidic devices

ISO 10993-2:2006 - For the use of medical devices on animals experimentation and welfare

ISO 28641:2010 - For the use of organic compounds in experiments

ISO 3696:1987 - For the use of water in analytical experiments

ISO/TC 212 - For laboratory testing

IEC 62047-2:2006 - For micromachining and photolithography

ISO 10872:2010 - For determining acceptable toxicity levels for *C. elegans*

ISO 11553-1:2005 - For the use of laser processing machinery in relation to photolithography

Most of these standards apply to hazardous chemicals and industrial purposes. As we are conducting experiments using minimal quantities of non-hazardous chemicals, we loosely adhered to these standards presented. Of particular importance are the notes for maintaining lab sterility found amongst the different standards. This is to ensure a proper experimental environment with reliable results. This entails proper usage of micro and serological pipette usage. Waste disposal of chemicals must also be considered for environmental factors.

3.3.2.2 Light Device

- **ISO 12609-2:2013** - For the use of eyewear when using light sources for humans and animals

The most relevant IEEE standards for the light device included the “IEEE Recommended Practices for Modulating Current in High-Brightness LED’s for Mitigating Health Risks to Viewers” and “LED Lighting Flicker and Potential Health Concerns: IEEE Standard PAR1789 Update” [40, 41]. However, these two standards were less significant to the light device design as the team intended to design the device in a way that it would not reflect light directly to the person using it. This goal was achieved by using an effective optics system.

3.4 Revised Client Statement

After extensive research into the field and further meetings with Prof. Albrecht a more detailed and focused client statement has been developed. It is as follows.

Design a microfluidic device that accommodates adult *C. elegans*' unique movements, limited mobility, and increased strength. Furthermore, explore associative learning in adult *C. elegans* that is understood in young adult *C. elegans* to compare the development and learning progression with age, through the use of external light device and perform reactivity tests to validate the hypothesis.

Many factors affected the reforming of the client statement including time constraints, previous experimentation on younger *C. elegans*, available research, and the ranked objectives given by the client, Prof. Albrecht. Based on Prof. Albrecht's suggestions and research on learning and training of *C. elegans*, the team agreed that an external device that reflects light on the arenas of the microfluidic device could potentially provide interesting outcomes. Further research proved that light exposure results in a diverse response in *C. elegans*, thus the light device was determined to be one of the most important parts of the client statement. In regards to the microfluidic device, the modifications to the details about the design of the device, as opposed to using the original, was due to the unique problems adult *C. elegans* present. This change was made because of the information gathered about adult *C. elegans* and their abilities and from the concerns presented by the client about the existing devices. The new design includes resizing of the worm barrier pegs to prevent the worms passing through them, increasing the peg size to accommodate worm size and strength, and modifying the spacing between pegs in order to allow more natural movement. In an earlier rendition of the client statement the details of the redesign and needed changes were added but it was cut in the most recent rendition so as to keep the statement succinct and not as restricting to the redesign parameters.

3.5 Management and Scheduling

The management of this project was broken down into sections according to individual expertise in certain areas which lead to a rolling leadership position based on what was the focus at the time. This project can majorly be broken into four sections with different focuses in each. In the first quarter of the project the main focus was on research and project development. It was during this time that the revised client statement was developed and a majority of this paper was written. Also within this section the cultivation of the *C. elegans* began so that experimentation could be conducted later on. Due to her expertise on lab practices Alexa Hiznay assumed a lead role this quarter.

In the second quarter the focus was on the design of the microfluidic device as well as the production of the final product and the beginning of testing of the device. These verification tests will be explained in more detail later in the paper. Due to her expertise in CAD systems Julia LaValley was heading this quarters endeavors.

The third quarter concentrated mainly on development and testing of the light device as well as detailed testing of the microfluidic devices designed in the previous section in order to determine the final device for experimentation. Due to their experience with electrical systems Dulguun Gantulga and Kevin Lou lead the project this quarter.

The final quarter concentrated on validation and experimentation with the entire system as well as completion of the paper and wrapping up of the entire project. The details on experimentation will be detailed later in this paper.

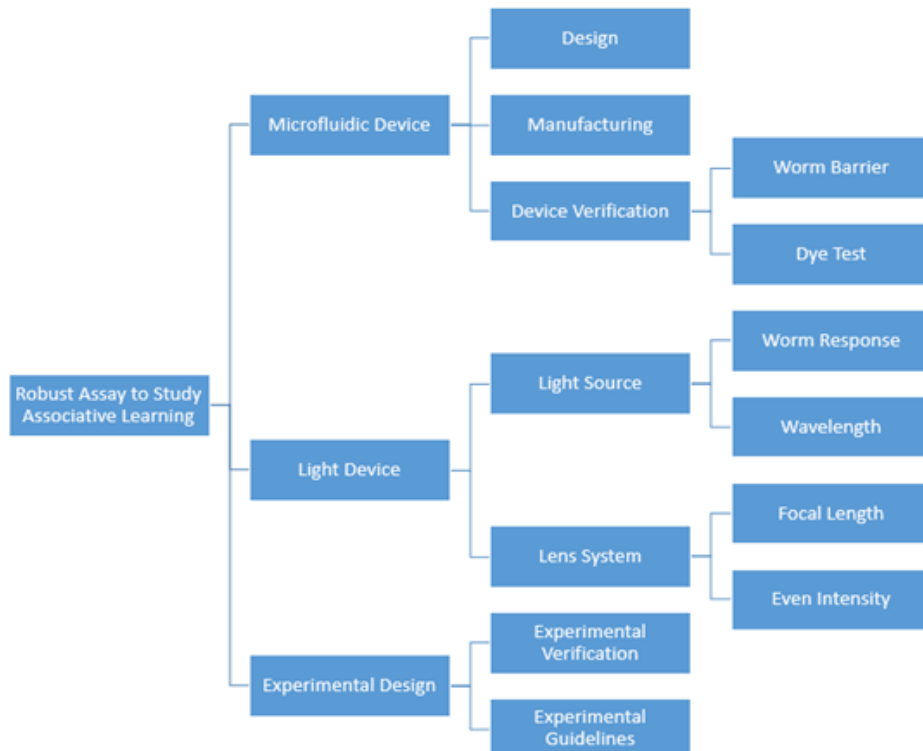


Figure 3.2: Project Breakdown

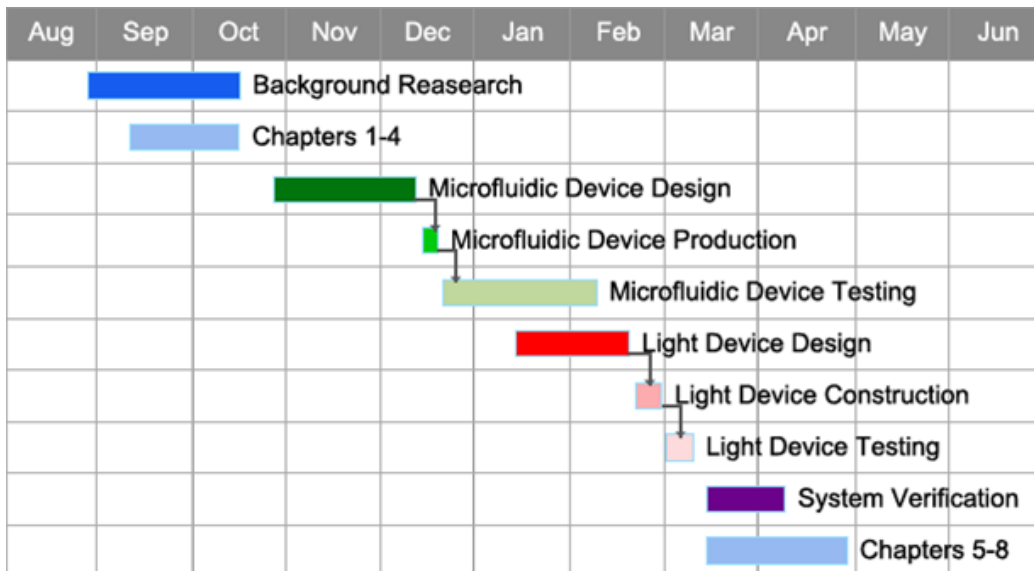


Figure 3.3: Project Timeline

Chapter 4: Alternative Designs

4.1 Light Exposure Device

4.1.1 Needs Analysis

Light presents a novel way to measure associative learning capabilities of *C. elegans* inside microfluidic devices. Light can be utilized as an easily adjustable stimulus that is of a different class than the currently used chemical stimulants.

4.1.1.1 General Functions

The light device needs to project a light of a known wavelength over the entirety of the testing arenas. The light must create a field of equal intensity so that the worms are exposed to the same amount of light regardless of their location in the testing arenas. This intensity must also be a known, adjustable value. Furthermore, the light must also be collimated to allow for easy manipulation of the experimental area.

4.1.1.2 Industry Standards

LEDs are available in various wavelengths, including 505nm, 470nm, 447.5nm, and 400-420 nm. However, they are not true point sources and are difficult to collimate properly. To generate a more accurate point source, optics are used to focus the light. Lenses and diffusers are used for this purpose. Lenses are utilized to collimate and focus the light rays while diffusers are used to spread the light rays to ensure even intensity in the desired area.

4.1.2 Conceptual Designs

Per Professor Albrecht's suggestion, the team purchased "LED Light Housing Kit" from "LEDsupply" for the light device purposes. This kit has previously been used in the Quantitative Neurotechnology Lab for similar purposes. The ideal conceptual design of the light device involved ease of modification of the intensity of the light exposure, digitally controlled control system, and the physical size of the device that would not interfere with the existing imaging system.

4.1.3 Preliminary and Alternative Designs

4.1.3.1 Preliminary Design

The complete experimental setup of the preliminary design of the light device consisted of the following hardware:

- ValveLink 8.2 controller hardware
 - Controlled valves and other potential digital inputs that can be controlled from the computer using a MATLAB script
- DB15 female and male cable breakout board
 - The signal from the MATLAB code was transmitted through the DB15 cable to the ValveLink 8.2 controller
- LED Light Housing Kit
 - Came in with Wired BuckPuck – a small circuit that assists with powering the LED
- Power relay
 - Acted as a switch in the circuit

- Luxeon Rebel Color LED's
 - Single 1-up LED's for 470nm, 447.5nm, and 415nm

The ValveLink 8.2 automated controller can control up to eight valves simultaneously, however in the behavioral experiments, only the first two valves are used – Valve 1 and Valve 2. Thus, the other five valves stay idle during the experiment. Upon a discussion with Professor Albrecht, the team decided to use the output of Valve 3 to control the light device. In order to achieve this, Valve 3 had to be added to the MATLAB script, this would let the team to define the functions of the light device such as duration of the light exposure and the binary on/off switching of the light in Valve Control MATLAB code.

When the script with active Valves 1 – 3 starts running, the signal is sent through the DB15 cable from the computer to the valve controller. Since the DB15 cable breakout board (refer to *Figure 4.2* for the DB15 female and male cable breakout board) is connected in between the computer and valve controller, it breaks the transmitted signal to pins that are easily accessible from the outside. The pin that is transmitting the intended signal for Valve 3 is directly connected to the power relay, which acts like a switch for the light device. With the LED MATLAB to power supply and the relay that was receiving signal from MATLAB, the hypothesis was that MATLAB and the power relay would control the LED.

Based on the above hypothesis, a simple circuit diagram was designed for the assembly of the LED kit, which is presented in *Figure 4.1* (Pin 1 is ground, Pin 5 controlled Valve 3). However, the following circuit design had minimal control over the current flow in the system. The high current limited the voltage in the system to turn on the LED as well as it did not provide enough accessibility to modify the intensity of the LED. Thus, the team had to reconsider the conceptual design of the circuit design.

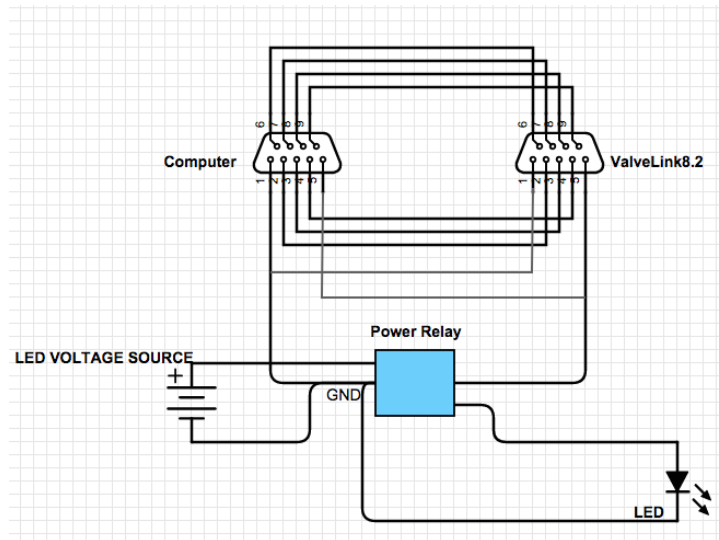


Figure 4.1: Summarized Schematics of the Conceptual Design

LED's were chosen based off wavelengths discussed previously in literature. To collimate the light, we initially intended to use a single lens of focal length $f = 25\text{mm}$. However, the LEDs in our design are not true point sources. There is a notable pattern in the result light intensity in the shape of the LED. Therefore we must adjust our design to ensure proper collimation and light distribution.



Figure 4.2: Physical DB15 female-male pin breakout board

4.1.3.2 Alternative and Final Design

Alternative design of the light device considered the suggested circuit design from the BuckPuck datasheet. The given circuit diagram used a PNP transistor and additional resistors to limit the current flow in the system. The circuit also allowed a dimming option with the presence of a resistor – R, which is highly desirable as the light device required intensity modification. By modifying the resistance value of R, the intensity of the LED was changed. The circuit diagram is presented below in *Figure 4.3*. The overall device setup was similar to the Preliminary Design set up. The signal intended for Valve 3 on the ValveLink 8.2 controlled the light device; this was accomplished when the Digital V_{out} was connected to the pin on the DB15 cable breakout board for Valve 3 with a reference to GND (common ground). In other words, the Matlab script generated signals for three valves, but only the Valve 1 and 2 on the ValveLink 8.2 received signals to switch the valves, and the signal for Valve 3 went to the Digital V_{out} and controlled the binary switching on/off of the light. The V_{in} for the BuckPuck was a 12V voltage source to power the LED. When the digital $V_{out} = 0V$ – the LED was OFF and when the digital $V_{out} = 5V$ – the LED was ON.

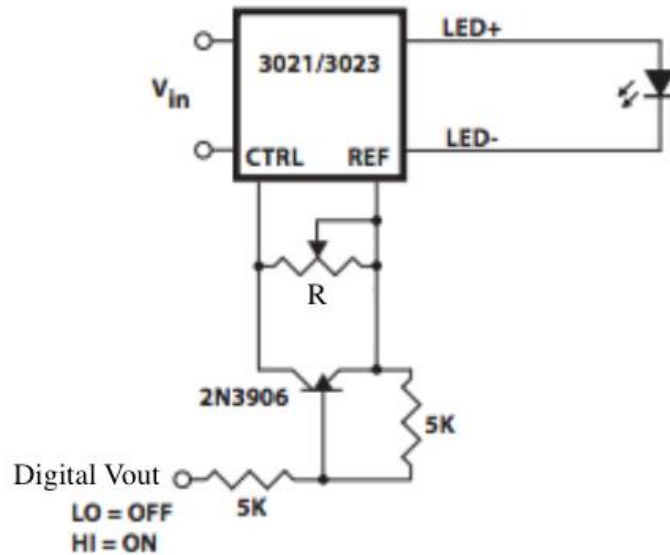


Figure 4.3: Schematics of the Alternative Design [42]

To deliver proper collimation of the light device we utilized a three lenses and diffuser system. We utilized an aspheric lens to roughly collimate the light, which passes through a diffuser lens. The diffuser lens served to jumble the light rays so that the LED pattern that was seen in the output is dissipates. We then used another aspheric lens to focus this light source down to a true point source to be collimated by the plano-convex lens. This can be seen in the rough sketch below in *Figure 4.5*.

4.1.3.3 Modeling

After designing our light system, our first step was to build and construct the LED housing unit. The circuit components were soldered together and placed into the LED holder as pictured below in *Figure 4.4*. The device had four inputs: two inputs were for placing the resistor - R to control the intensity; the third input was for the common ground; fourth was for the Digital V_{out} that acted like a switch. Additionally, the device had an external power input.

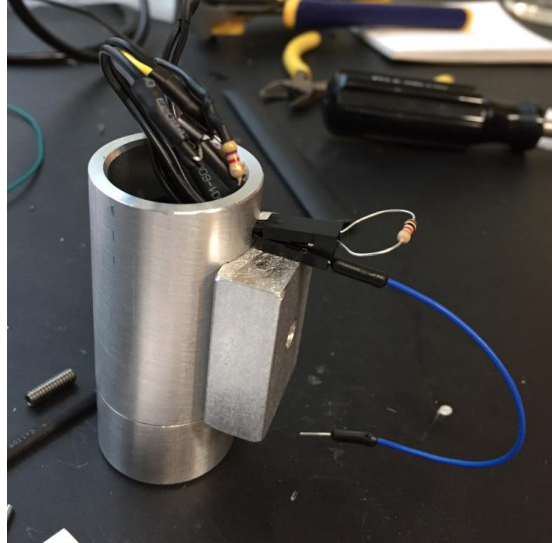


Figure 4.4: Modeling of the Light Device

The optics were designed using Thorlabs lenses. We used two aspheric condenser lenses of $\text{Ø}30\text{mm}$, focal length $f = 26\text{mm}$ with a 600 grit $\text{Ø}50.8\text{mm}$ diffuser lens placed in between. We chose this focal length to ensure the device will fit within the current lab set up. The rationale for the diameters is to ensure that the entirety of the light beam is captured. The three lenses were placed at minimal distances apart, with the first aspheric lens placed at focal length's distance away from the LED. This was done to minimize the size of the device, for compatibility with the lab set up. The three lens system, the two condensers and a diffuser, served to create a more accurate representation of a point source, which we used a plano-convex lens of $\text{Ø}50.8\text{mm}$ $f = 60\text{mm}$ to collimate so that there was a total coverage of the device. This fourth lens was placed at 86mm away from the second aspheric lens, sum of the two focal lengths. (Refer to *Figure 4.5*) This was assembled using Thorlabs LH2 adjustable mounts to hold the lens. The lenses were then fixed to a wooden support at the distances described above.

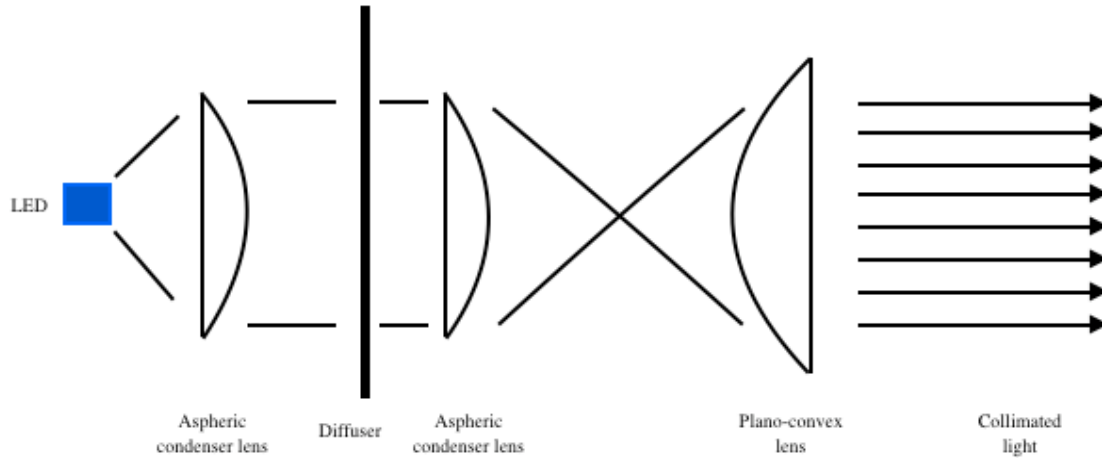


Figure 4.5: Schematics of the Optics System

4.1.3.4 Decisions Made

Performance tests were implemented on both preliminary and alternative designs. The final results from the alternative design met the team’s anticipation and the device setup allowed simple system control. Additionally, the alternative design provided more flexibility when adjusting the intensity of the LED. Thus, the alternative design was determined to be the final design of the light device. The final design of the Light Device with the optical system consisting of three lenses and a diffuser presented below in *Figure 4.6*

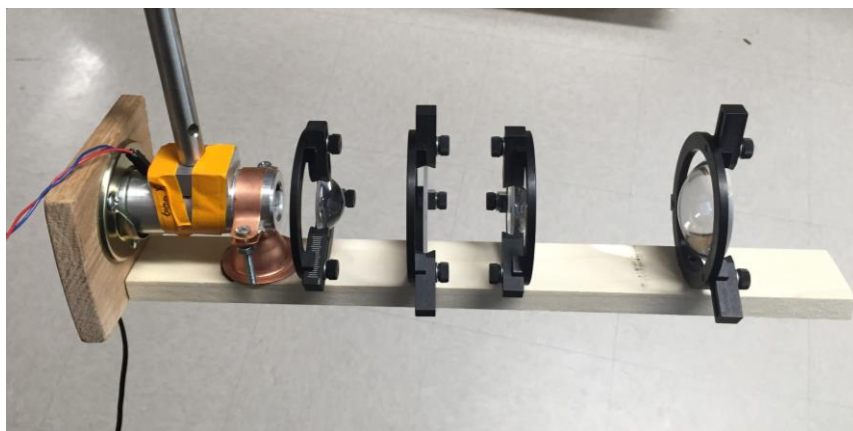


Figure 4.6: Final Design of the Light Device

4.2 Microfluidic Device

4.2.1 Needs Analysis

The team had several needs to meet for the design of the microfluidic device. The worm barriers needed to be resistant enough so that *C. elegans* could not squeeze through or become trapped between the barriers. Additionally, the microfluidic device had to maintain an unimpeded flow rate. To determine the correct spacing, the team designed several conceptual designs (see Section 4.2.3). The final spacing measurements are contained in Section 4.2.4.5. The device also needed to be transparent on the top so the camera could track the *C. elegans*. For this to be accomplished, the sizing and shape had to be compatible with the existing experimental setup. A device with worm barrier of 75 μm , post diameter of 280 μm , and post spacing of 140 μm was determined to be compatible. Additionally, ensuring the design of the device in such a way to allow the natural movements of *C. elegans* was also essential. See *Table 4.1* for a further needs analysis. In this table, priorities were ranked on a scale of 1 to 3, with 1 being the most important.

Table 4.1: Needs Analysis Table

Title	Description	Compliance	Priority
Design	The barrier should be thick enough that <i>C. elegans</i> cannot squeeze through	Observation	1
Compatibility	The device should be transparent on the top and sized correctly so the camera can track the <i>C. elegans</i>	Data	1
Humane	The device should provide a comfortable, natural environment for <i>C. elegans</i>	Research / Testing	2

After the needs of the design were evaluated, the team considered the device functions based on the client need. The project advisor wanted the device to also be compatible with the light-stimulation device. Using other stimulants such as light would allow the team to understand if the results hold true in situations not involving attractant or repellent chemicals.

4.2.1.1 General Functions

A list of functions for the microfluidic device for older adult *C. elegans* was created based on the background research completed by the team and objectives that were determined previously in Chapter 3. The list of general design functions consisted of four main functions that were considered for the final design of the microfluidic device. The functions are further discussed below.

List of General Design Functions:

- Providing an environment that would not restrict nor modify the natural behavior of *C. elegans*

The agar plate like environment within the microfluidic device was achieved by designing the arenas of the device to have an adequate post size and spacing that would not interfere the natural sinusoidal locomotion of *C. elegans*. Furthermore, the worm barriers of the device were redesigned to prevent older adult *C. elegans* for entering the nearby arenas while allowing a laminar flow of fluids through the device

- Maintaining a sterile environment for the worms

The device was fabricated using PDMS which is a highly biocompatible material. Additionally, PDMS can maintain its sterility during the experimentation time.

- Being compatible with the existing laboratory set up

The microfluidic device was compatible with the current laboratory setup, as well as the light device described in Section 4.1. The input, output, and worm loading ports were located at the same location as the previous devices that are used at the QNTL Laboratory at WPI.

Furthermore, the overall microfluidic device dimensions stayed the same.

- Allowing a clear imaging

The PDMS and glass slides allowed the device to have a clear imaging and obtaining of a non-distorted observation of *C. elegans* under the camera lens.

4.2.2 Preliminary Data

4.2.2.1 Original Old Adult Device Tests

The QNTL laboratory at WPI had two semi-successful old adult *C. elegans* gradient device designs, developed - by a PhD candidate, Jeremy Shui. The device designs, both named T01A 125, were designed for 5 day old adults. One design had a post radius of 125 μ m, a post spacing of 125 μ m, and a post height of 90 μ m. The second design had a post radius of 125 μ m, a

post spacing of 125 μm , and a post height of 100 μm . Both designs allowed 5 day old adults to move naturally. However, the worms easily escaped through the worm barrier, skewing the results.

The team decided to test the T01A 125 devices with 5 day old adult *C. elegans* to see if they actually did escape through the worm barrier and to make any relevant observations that would help design the next generation of old adult *C. elegans* devices. The team completed three separate tests with the T01A 125 devices. In these tests, the team observed two separate points that allowed the *C. elegans* to escape: the worm loading port and the worm barrier. Multiple worms were observed getting stuck in and/or pushing their way through the worm barrier. To accomplish this, they had to swim all the way across the arena to reach the barrier. As a result, it took more than five minutes for the worms to arrive at the barrier. Additionally, multiple worms swam back up into the worm loading port. This occurred in less than a minutes after the worm-loading syringe was removed from the loading port. However, it was likely not caused by the pressure change created by removing the syringe because it did not occur directly after the syringe was removed.

4.2.2.2 Concept Device Dye Tests

The original designs' flow capacity and speed were tested using a simple dye test in which red food coloring or blue xylene cyanol for the gradient and pulse devices respectively. Standard experiments were run, as detailed in Appendices A and B, without worms and the dye was substituted for the stimuli. Three experiments for each device were performed and all experiments were recorded and visually analyzed for proper flow and adequate flow speed.

4.2.2.3 Concept Device *C. elegans* Tests

The original designs were then tested with a standard experiment, as detailed in Appendices A and B, in which worms were introduced. Three experiments were conducted for each device and all experiments were recorded. The data collected was analyzed in order to determine how the *C. elegans* interacted with the worm barrier and the environment in general. This was done through both visual observation and worm tracking software.

4.2.3 Conceptual Designs

Conceptual designs of the Worm Barrier with no restrictions are detailed in this section with the restrictions, which narrowed down the designs below that. The peg size and spacing conceptual designs were all carried over into preliminary designs so they are detailed in the next section instead. The overall size of the device remains the same so that has not been detailed anywhere in the following sections.

4.2.3.1 Worm Barrier

The intention of the Worm Barrier redesign was to ensure the *C. elegans* could not cross it to enter the opposite arena or into the microfluidic pathways.

4.2.3.1.1 Alternative Size and Spacing

The initial size and spacing for the young adult device was calculated in relation to one another and the new spacing and width of the Barrier as determined in accordance to the relation. The initial sizing of the worm barrier was 400 μ m long, 60 μ m wide with 25 μ m spacing. This made the spacing ~42% of the width. Thus several different widths were considered in order to minimize spacing and attempt to prevent the *C. elegans* could not force the barriers apart with

their strength. The widths proposed were 70 μ m, 75 μ m, and 80 μ m. As the overall device and arena size was remaining the same the width was kept at 400 μ m.

4.2.3.1.2 Curvature

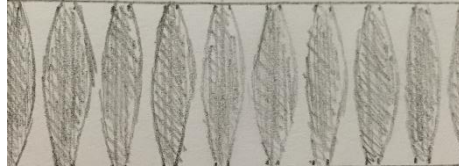


Figure 4.7: Curvature Worm Barrier Design, top view.

This design, pictured in *Figure 4.7*, was intended to create a narrowing passage so that the *C. elegans*, if they encountered it, would feel the narrowing passage and reverse in order to avoid getting trapped. The narrowing channels are at their narrowest the same spacing as in the young adult device, 25 μ m, and widen to 50 μ m. There would be the same amount of posts this way, only the shape would change.

4.2.3.1.4 Triangular Slits

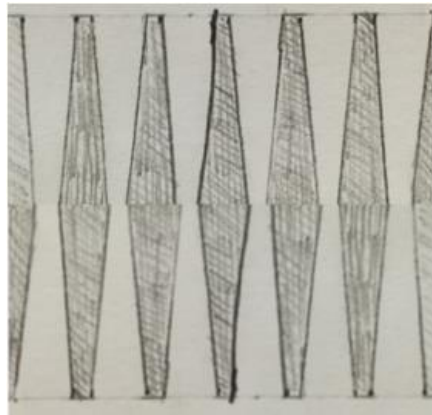


Figure 4.8: Triangular Slits Worm Barrier Design, top view.

This was designed similarly to the above Curvature design but was made so that there was a bit more drastic and angled change. At the spacing this design was $15\mu\text{m}$ and at the widest it was $50\mu\text{m}$. As above the overall number of posts in the Worm Barrier would not change, just the shape. This angular design was meant to give a more easily created form and a steeper narrowing.

4.2.3.1.3 Cut Out

This design, depicted in *Figure 4.9*, has two rows of circular holes going through one, solid worm barrier. The circles are $30\mu\text{m}$ in diameter and are spaced $32\mu\text{m}$, center to center, horizontally and $28\mu\text{m}$, center to center, vertically. This was intended so that no matter what the $80\mu\text{m}$ diameter adult worms could not cross through the worm barrier, even with superior strength.

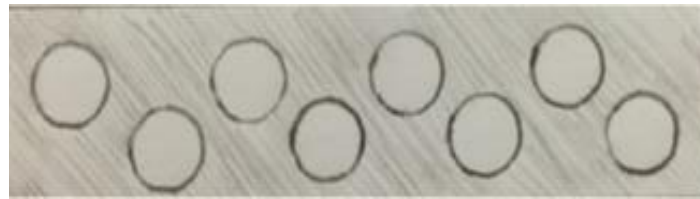


Figure 4.9: Cut Out Worm Barrier Design, side view.

4.2.3.1 Restrictions

Some of the restrictions of the microfluidic device design included laminar flow and the ease of production. These restrictions are further discussed in this section.

4.2.3.1.1 Laminar Flow

The ideal microfluidic device has no turbulent flow. This is due to the fact that turbulent flow is fairly unpredictable and can mix and distribute any stimuli injected into the device in

unpredictable ways, making the worms movement very hard to quantify in turn. Additionally at high levels turbulent flow can have negative effects on worms and cause them to move in erratic ways and even damage or kill them. Thus any design of the device can have no portion, which may promote turbulent flow, only laminar.

4.2.3.1.2 Production

The current method for constructing the final PDMS microfluidic device involves pouring uncured PDMS into a silicon mold and letting it dry. Then the PDMS is peeled from the silicon disc and cut to the appropriate size, details provided in Appendix E. This method of manufacturing allows for very simplistic designs in the sense that it is essentially able to make holes in only the downward direction. If there are any horizontal tunnels or holes or even variability in height of different aspects of the part there would have to be multiple molds. Then the molds would have to be perfectly aligned by hand using a microscope for accuracy purposes. This is a very intensive process that would have to be repeated multiple times and would make each device's success based upon the skill of the assembler. Even with this process any fort of horizontal hole would be impossible. Thus the team decided that any designs requiring this process would be forgone.

4.2.4 Preliminary and Alternative Designs

4.2.4.1 Decisions Made

Taking the various restrictions in mind the conceptual designs were narrowed down into the preliminary designs detailed here. Any multilayered designs or designs where the turbulent flow could be increased were rejected immediately and the remaining designs, the alternative sizing and spacing and the curvature, were presented to the lab team and Prof. Albrecht for

consideration. After getting feedback from them, the team rejected the curvature design as the intended reaction, the *C. elegans* sensing the constriction and backing away, was determined to be unlikely. Additionally the spacing of 70 μ m was determined to be too similar to the 60 μ m and thus the preliminary designs for the Worm Barrier concern the 75 μ m and 80 μ m width Worm Barriers.

The overall decision to keep the same arena and microfluidic design size was based on the extensive trial and error experimentation that has already occurred with the current devices. Additionally no flaws were found with this system and thus there were no apparent improvements needed.

4.2.4.2 Worm Barrier Size and Spacing

The final worm barrier widths chosen for testing were the 75 μ m and 80 μ m width designs. To preserve a reasonable flow rate between the arenas, the total space between worm barriers remained the same, however the number of worm barrier posts were adjusted. The width of the worm barrier posts were added together and was determined to be 16.10 mm; same procedure was applied to the spacing between the worm barrier posts and the total length of spacing was 5.6 mm. Equation 4.1 was developed by the team and used to determine the number of worm barrier posts. The calculation was performed twice using the 75 μ m and 80 μ m width designs. The number of individual columns in the worm barrier was counted to be 140 in the case of the 75 μ m width and 131 posts for the 80 μ m width design

Equation 4.1

$$\# \text{ Worm Barrier (WB) Posts} = (\text{Total Width WB Posts} - \text{Total Spacing}) / \text{Individual WB Width}$$

4.2.4.3 Peg Size and Spacing

The Peg sizing and spacing were determined based off that of the young adult devices proportions in relation to the young adult. The *C. elegans* girth diameter is 30% of the Peg diameter and 60% of the Peg gap. As the girth diameter of a fully grown adult is on average



Figure 4.10: Peg and Spacing Visual. [8]

80 μ m, the new spacing was determined to be approximately 136 μ m and the Peg diameter 272 μ m. This was lowered to 135 μ m and 270 μ m for a clean number. Additionally the team decided to add in the measurement of 140 μ m spacing and 280 μ m post diameter in order to account for error and to ensure that the testing was thorough.

4.2.4.5 Final Design

The final design of the older adult device includes a worm barrier of 75 μ m width and 30 μ m spacing, a post diameter of 280 μ m and spacing of 140 μ m. All of these were determined with extensive testing both with and without *C. elegans* and were selected due to superior performance in all aspects.

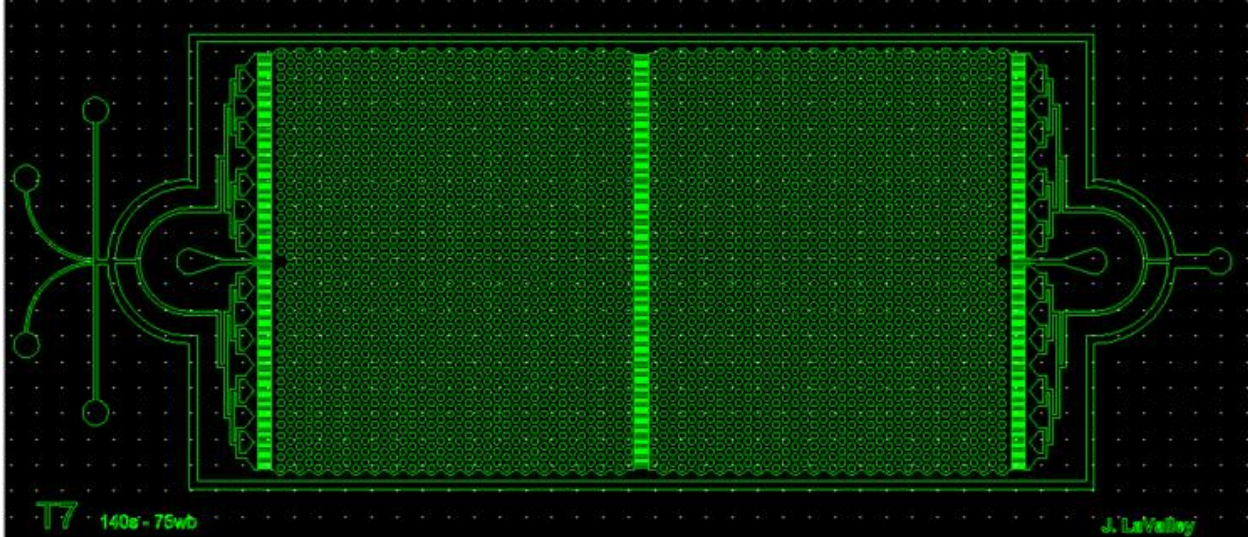


Figure 4.11: Final Design, Pulse Device.

4.3 Experimental Design

The purpose of the project is to design a system to study the effects of aging on *C. elegans* ability to learn and relate it to humans' abilities. The following experiments are proposed guidelines for future experimentation and use of the developed devices as well as the design of the experiment used to perform preliminary validation of the full system. These experiments are designed to be conducted on both young adult and adult *C. elegans* in order to examine the differences between the two age groups. The experimental method detailed below is that of forward classical conditioning in which a slightly negative stimuli, blue light is presented and immediately followed by a positive stimuli - butanone, food odor. Since the team looked at the change due to light response after pairing with butanone, the blue light acted as a Conditioned Stimuli (CS) and butanone was the Unconditioned Stimuli (US). This type of experiment aims to have the *C. elegans* associate the light with the positive stimuli and react favorably to it even

when it is not paired with the food. The unconditioned, and conditioned, reaction is the forward searching motion for food.

4.3.1 Adult Device Functionality

In older microfluidic device designs, the adult *C. elegans* could escape the microfluidic arenas and create challenges in data collection. The first experiments conducted with the new microfluidic device was to test its efficacy and ensure the worms were unable to escape. This were done by creating a bubble free environment with a steady flow of S. Basal. The protocol is similar to the gradient experimentation outlined in *Appendix A*. A dyed solution is used prior to worm introduction to the device to ensure proper gradient formation. Chemoattractants and food was used to stimulate the worm movement. Worm speed, and the effectiveness of worm barriers were recorded by video. This was analyzed and compared with worm behavior in the young adult devices. The device was considered successful when young adults and adults were unable to escape the arenas.

Additionally, worm locomotion was compared to an agar plate control. Of special interest were their sinusoidal movement forward, reverse, omega turns, where the worm sharply turns from head to tail, and pirouette where the worm couples on or more reversals and omega turns [44]. The newly designed adult devices had to minimize any limitations on worm locomotion. PDMS post sizing and distance were adjusted through device iterations in an effort to optimize the design.

The experiment detailed in *Appendix D* was used to analyze the use of the intensity of the light device. This involved exposing an unseeded agar dish of *C. elegans* to the light program for thirty minutes with 60 seconds of light pulsing every five minutes. Visual verification of reaction

to this light was to be performed of the video of the experiments recorded by all members of the team.

The entire system was tested using a basic version of the experiment 4 detailed in Appendix G. In this experiment the standard 60 second pulse followed by four minute latency was used along with the standard butanone mixture. This was done due to a lack of time for the project to complete the entire experimental series. This was meant to verify the ability of *C. elegans* to learn associatively.

4.3.2 Paired Stimuli Testing with Light Elicited Response

In addition to the two experiments described above there were two other proposed experiments. The first was designed to test the ideal stimuli usage, food or butanone. This was done by going through the experimentation of 60 seconds to 4 minute pulse to latency ratio and seeing to which the *C. elegans* responded best. The second was designed to test ideal pulse to latency ratio to create the most robust learning response possible. This would be done simply by changing the ValveBankTiming_DA script to reflect the proper time intervals and analyzing the resulting data. In an ideal experiment these results would have been used to perfect experiment 4, described above and in Appendix G.

Classical conditioning experiments were designed to quantify the differences between adult and young adult *C. elegans* ability to learn. This is done by introducing a stimulus the worms can sense alongside a known attractant. The worms were considered conditioned and to have learned if the innate response to the attractant was elicited in response to the stimulant aka the light. It was important to note that the stimulant to be conditioned should always evoke a less intense innate reaction in comparison to the attractant. This was to ensure that the correct

stimulus was being conditioned. As such the stimulants used was to be as neutral as possible thus the stimulus chosen was that of blue light.

It has been shown that wild type *C. elegans* respond to blue, blue-violet, and UV light as a stimulus [33]. This current experiment was designed to use light to condition the *C. elegans*, providing an easily controllable external stimulus with minimal setup. The minimum light intensity required to elicit such a response is $50\mu\text{W}/\text{mm}^2$ for 440nm, an intensity far above ambient light power [33]. Therefore, the experiment would be conducted and recorded normally within a lab hood. *C. elegans* have a natural disposition to avoid short wavelength light and suffer acute damage and death [14]. The severity of the worm damage is inversely proportional to wavelength, with blue wavelength being the longest wavelength at which worm death is noted. The experiments were designed for short light exposure and conditioning to avoid worm paralysis and the wavelengths selected were those least likely to cause harm.

All the protocols for the proposed experiments described within this section are detailed in Appendices B-G and are described in more detail below.

4.3.2.1 Prime Wavelength and Intensity Response

Preliminary experimentation with the Light device and Adult Microfluidic Device was proposed to involve the testing of sensitivity of the *C. elegans* to the light in the configuration. This process was to confirm and validate the results of the Edwards (2008) study. The experimental procedure involves a 60 second exposure to light with a 4 minute latency period repeated for 30 minutes. The results of each experiment would be analyzed for negative reaction to the light and ensure that there is at least a 75% reversal response. With every iteration the intensity would be lowered until the *C. elegans* no longer react properly then the last suitable intensity and wavelength pair would be used for the ongoing studies.

4.3.2.2 Stimuli Interaction Testing

The stimuli interaction testing is the first time the *C. elegans* are presented with the food or food odor stimuli in conjunction with the light stimuli. This series of testing is to determine the feasibility of the pairing of light and odor in order to get a positive response to the light stimuli by gauging the reaction of the *C. elegans* to the combination of the CS and US. The US will first be Butanone, the food odor, if this fails to counteract the negative response to the light stimuli then the US will be modified to food. If this still fails the worms will enter into the experiment having been starved.

To test this, the *C. elegans* are exposed to 60 seconds of light with the US being presented after X seconds, which is the time it takes for the released stimuli to reach the arena portion of the microfluidic device. After the light exposure, the worms are presented with a four minute latency period. This pulse and latency period is repeated six times, or for thirty minutes, a 15 minute break occurs and the light follows the same pulse and latency pattern as before without the food or butanone. The two stimuli types are then compared for the maximum response.

4.3.2.3 Preliminary Paired Stimuli

This series of testing is to determine the training period necessary for the *C. elegans* to display the trained behavior of positive reaction to the light stimuli. To do this the *C. elegans* are exposed to 60 seconds of exposure to light with the food odor being presented after a set of seconds, this being the time it takes for the released stimuli to reach the arena portion of the microfluidic device. There is then a four minute latency period. This is repeated for thirty minutes. After the thirty minutes, the system is exposed to unpaired stimulation for thirty minutes, in which only the light is presented for 60 seconds with a latency of 4 minutes. This is

repeated five times and analyzed for positive response to the lone light stimulus. If this proves to not elicit the positive reaction to the light stimulus the pulse time and latency time are lowered by 15 seconds and still conducted for 30 minutes, increasing the number of pairings.

4.3.2.4 Comparison Testing

This is the final stage of testing in which all the previously determined values are combined. Arena 1 is filled for the first half of the experiment and the standard training is conducted. After the training period is over, starved worms are introduced to Arena 2 during a 30 minute latency period. The lone light stimuli testing is then presented for 30 minutes to both the trained and untrained *C. elegans* and the results are used as a comparison.

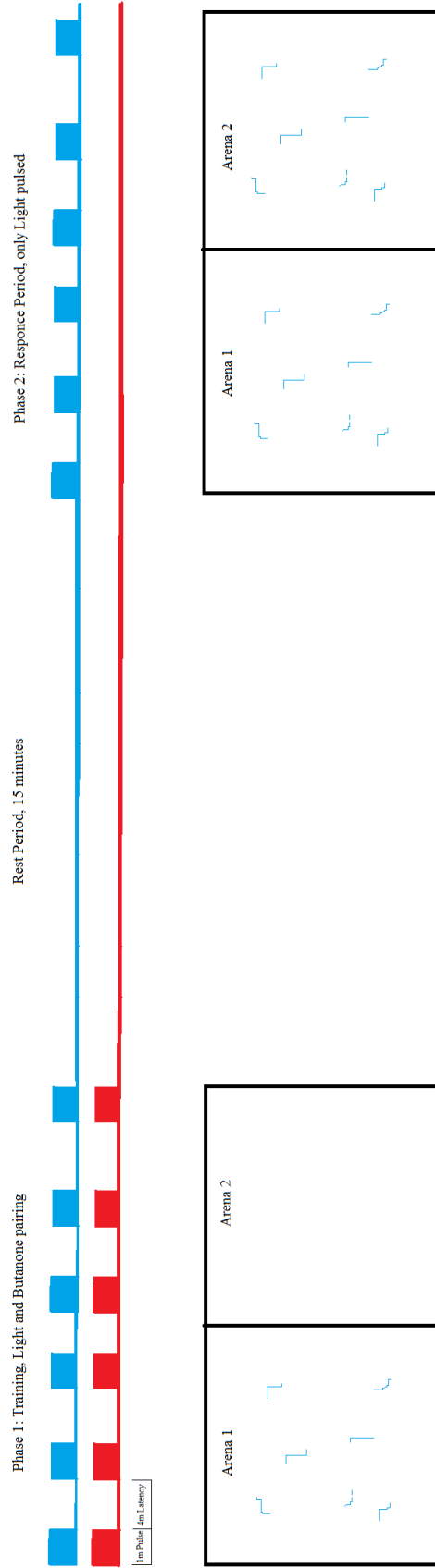


Figure 4.12: Experimental Design

Chapter 5: Design Verification

For this project, there are number of components that needed design testing. This includes the functionality of the both pulse and gradient microfluidics devices, and the light device. Furthermore, data has to be gathered from experiments conducted on *C. elegans* to verify the efficacy of experimental design that the team created.

5.1 Microfluidics Device

Vital to the experimental design is the controlled and well defined introduction of stimulus. The microfluidics device serves to present both a clear experimental area and controlled delivery of chemical stimuli. We conducted tests of the fluid flow profile to satisfy both aspects of the device design. The team conducted three dye tests with food coloring as contrast to determine the flow characteristics for each device. The tests were recorded using a Pixelink camera and the Matlab script gVision. The efficacy of the design was verified visually from the recordings. All gradient and pulse device opacity was noted to be clear. Gradient flow characteristic was noted to be consistent with a higher concentration of dye along the length of center of the device and dissipating near the edge. Pulse device experiments verified the flooding and clearing of both arenas with dye during the flow profile tests. It is important to note that the fluid reservoir set up went under many iterations before determining the correct method.

5.2 Light Device

To utilize light as a novel stimulus, the controlled delivery of light and the ability of *C. elegans* to sense the light must be verified. First, we tested the delivery of the light to ensure it

encapsulates the experimental area and it delivers uniform light intensity. The size of the collimated light beam was determined visually and confirmed to fully encompass the microfluidic device. It was noted the size of the projected beam on the observation area increased with distance between the area and the device, suggestive of imperfect collimation. However, the differences were noted to significant only in differences in the order of meters. Therefore for the experimental setup we proposed, the size of the beam was estimated to be a circle of $\varnothing 75\text{mm}$.

The light intensity was tested using a Thorlabs PM100 intensimeter. The intensity was noted to be holding steady at 3mW across the $\varnothing 9.5\text{mm}$ sensor. This translated to $42.3\mu\text{W}/\text{mm}^2$. We then tested if the resistor did reduce the intensity of the light. This was verified visually, as a $1\text{k}\Omega$ resistor dimmed the light significantly, and no measurements were made. The sensor was also moved to different areas of the beam to ensure equal intensity. The intensity remained within $\pm 5\%$ within the majority of the beam. However, it was shown that an outlier existed in a 12mm^2 area of the beam, at the circumference of the circle, where the light intensity dropped to 40% of other areas as illustrated in *Figure 5.1* This is believed to be due to a slight misalignment but did not effect the training area so was left uncorrected.

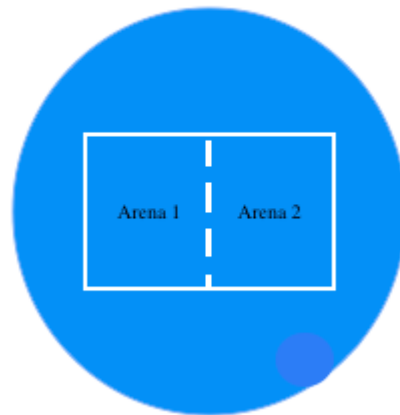


Figure 5.1: Illustration of the collimated light beam

The reaction of the *C. elegans* to the light was tested, as detailed in Appendix D, by exposing the *C. elegans* to the light. This was done in an unseeded agar plate with a ValveBankTiming_DA script pulsing the light for 60 seconds every 5 minutes for half an hour. Video of this was then visually analyzed by all members of the team to track the *C. elegans* response to the light.

Finally, we tested the computer controlled timing. This was done by creating a custom timing script for the control of the binary value for Valve 3 in the ValveLink 8.2 Controller. The light device's BuckPuck driver was then connected by leads to the valve through a 9 pin breakout board. The system was verified visually as the light device turned on/off corresponding to the binary value we set for Valve 3 with our custom timing script.

5.3 Experimental Data

We recorded the responses of *C. elegans* to the light conditioning using a Pixelink camera and the Matlab script gVision. The experiments were conducted using our verified microfluidic and light devices to introduce both light, a negative stimulus, and butanone, a positive stimulus, to the *C. elegans*. We ran five experimental trials. The location and locomotion of each worm was identified and determined by the MATLAB script WormTrackingScript_Pulse, seen in Appendix H. Using the data gathered by this script, worm motion was analyzed by the script uiTimeAnalysis. This analysis produced graphs depicting the time and quantity of forward and backward movement by the *C. elegans*. Below are the results from the uiTimeAnalysis, where grey represents forward movement and red represents reversals. The light device floods the arenas with light for 60 seconds every five minutes, starting at time =

0s and repeating until the experimental session is complete. For the experimental design of this experiment please reference *Figure 4.12*.

Figure 5.2 shows the change in speed differences between the control and the trained *C. elegans* in response to light exposure. The speed change is achieved from taking a measure 10 seconds before and a measure 4 seconds after light exposure. Though there is a visual difference this is not statistically significant ($p= 0.2021$). However the speed change, not shown here, between ten seconds before and four seconds after the light was administered, taken from the combined data of the control and the trained response was statistically significant ($p= 0.028$). This is discussed further in Chapter 7.

Table 5.1: Raw Statistical Data

	Trained (mm/s)	Untrained (mm/s)	Cumulative (mm/s)
1	0.1591	0.0782	---
2	0.1220	0.1869	---
3	0.0109	0.0334	---
4	0.1063	0.1225	---
P Values	0.0853	0.2021	0.028
Mean \pm Std Dev	-0.020 \pm .015	-0.0065 \pm 0.008	.013 \pm 0.013

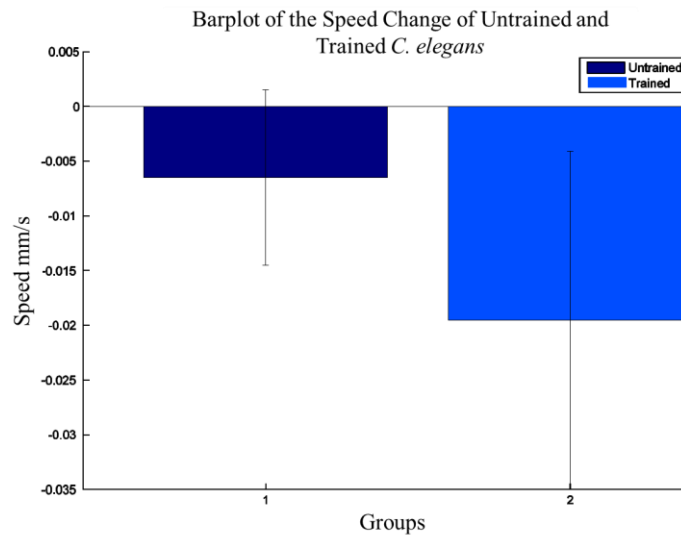


Figure 5.2: Change in Speed in relation to light stimulus between trained and untrained C. elegans

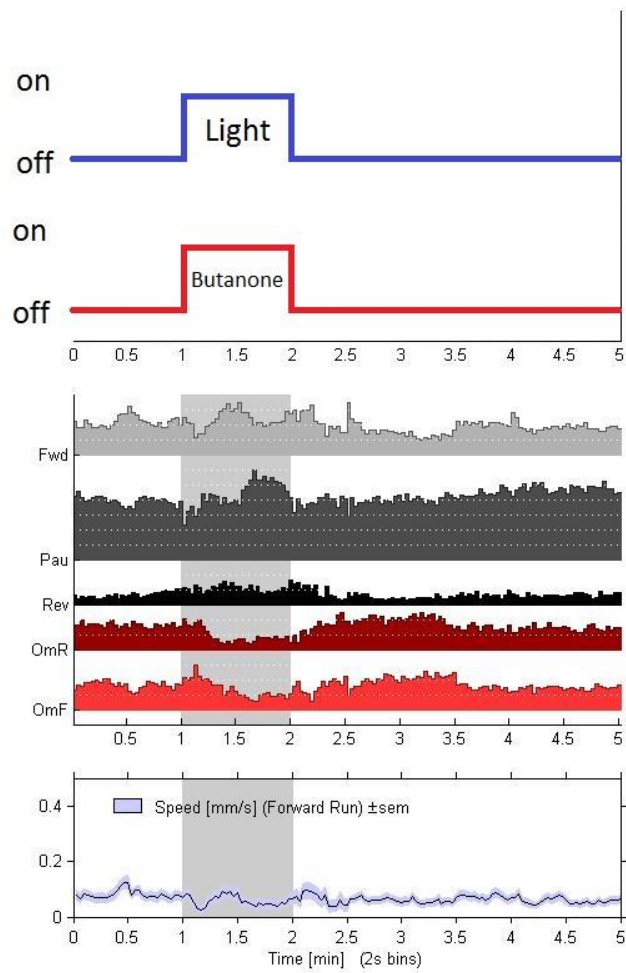


Figure 5.3: Arena 1 Phase 1, Training

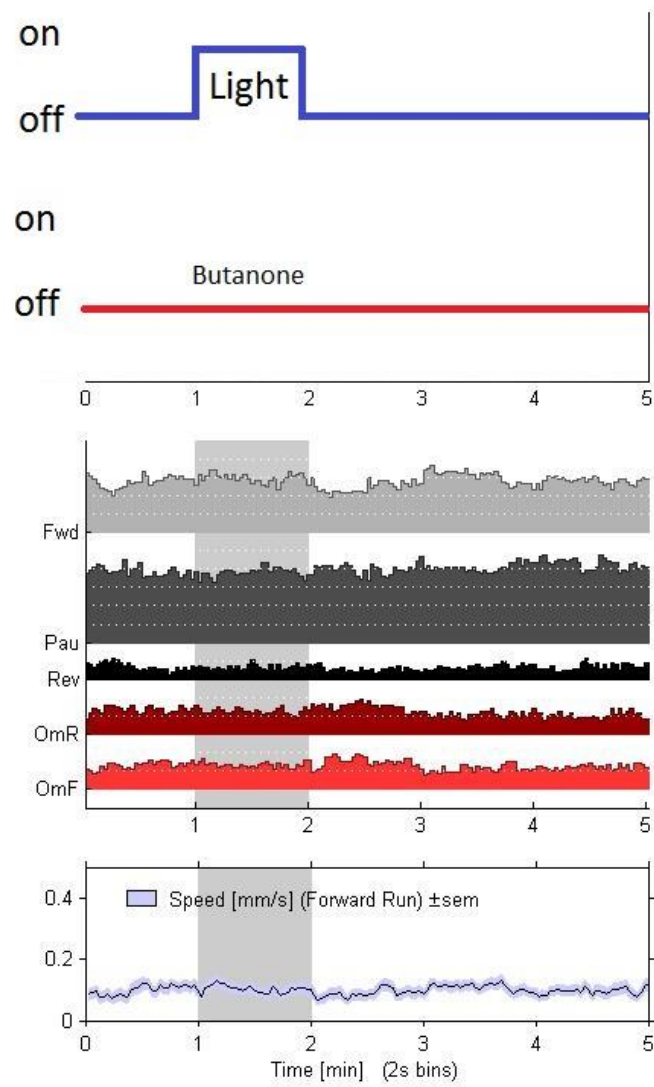


Figure 5.4: Arena 1 Phase 2, Reaction Testing

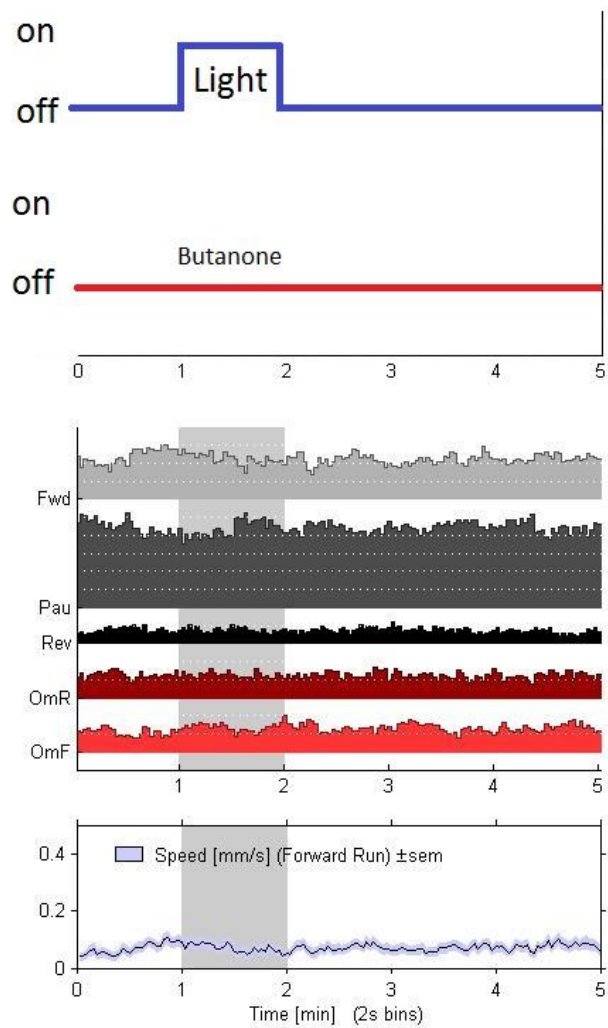


Figure 5.5: Arena 2 Phase 2, Control

Chapter 6: Final Design and Validation

In this section the team briefly discusses the design validation process for the developed devices - microfluidic and light devices.

6.1 Design Validations

In order to validate the final designs of the microfluidic and light devices, an experimental protocol was developed by the team; the protocol was approved by Professor Albrecht and Professor Gegear. The newly developed experimental protocol, was designed to perform a series of validation tests the team used to validate the entire system – microfluidic and light device and their compatibility, as well as the associative learning of older adult *C. elegans* was studied using this experimental protocol. However, preliminary validation testing was performed on each device individually in order to ensure proper function of each portion before integration into the system.

The team investigated the compatibility and cooperation of the light and microfluidic devices using the associative learning experiments. As previously explained in Chapter 4 and seen in *Figure 4.12*, the first half of the experiment required training of older adult *C. elegans* in Arena 1 by exposing them to two different stimuli simultaneously – butanone and light. Butanone – a chemical that flooded through the microfluidic device created a pulse assay and light was reflected from the light device to the arenas of the microfluidic device. During the second half of the experiment, control group of untrained older adult *C. elegans* were added to Arena 2. Furthermore, both arenas were exposed to light during the second half. The associative learning experiments were repeated five times; throughout the experiments the devices were able

to exhibit desired outcomes and minimal hardware malfunction. Further analysis of the collected data showed that the associative learning experiments produced reproducible results, as detailed further in Chapter 7. This success proves the system is capable of operating as designed.

Individual device validation is discussed in the following sections.

6.2 Microfluidic Device Validation

Prior to starting novel experiments, the team investigated the reliability of the microfluidic device using dye tests and worm barrier assessments. The dye tests involved flowing the standard pulse experimental protocol detailed in Appendix I with the stimuli replaced by 1x xylene cyanol. This allowed for qualitative and quantitative visual testing to occur. This included visual examination for any swirls or disruptions indicating turbulent and/or disrupted flow, timing of pulse flow, worm barrier strength, and delay caused by worm barrier. It was essential to have a pulse assay, because with the pulse assay the team intended to study associative learning in *C. elegans*. The dye tests revealed the successful formation of pulse assay within the microfluidic device with a post diameter of 280 μ m and spacing of 140 μ m and unimpeded fluid flow within the channels of the device in all cases. Complete travel of pulse across the device took 5 seconds with the worm barrier impeding the flow for less than 0.2 seconds in all cases. Worm barrier assessments in which *C. elegans* were placed into the device and tracked to ensure they were unable to cross were implemented to ensure the strength of the device borders. The results from the worm barrier tests revealed that the older adult *C. elegans* were not able to force themselves through the worm barriers of diameter 75 μ m width and 31 μ m.

6.3 Light Device Validation

The light device was tested to ensure that there was a constant and even intensity, within +/- 5%, across the entire area of the microfluidic device, and that there could be precise control of the on and off with less than an 0.5 second delay between command and response. The light intensity was tested using a Thorlabs PM100 intensimeter. The intensity was noted to be holding steady at 3mW across the $\varnothing 9.5\text{mm}$ sensor. This translated to $42.3\mu\text{W}/\text{mm}^2$. The functions of the light device were validated using a MATLAB script that allowed precise timing and turning on/off of the device with an immeasurable difference between command time and response time. Additionally, adding resistors of different values to the resistor input port on the light device tested the ease of modification of the intensity. Further validation was accomplished through the associative learning experiments mentioned and detailed above with protocols detailed in Appendices D-G.

6.4 Data Analysis

Dye and worm barrier tests for the microfluidic device required careful observation of the recorded video of the experiments. Based on the observations, the team collectively came to a conclusion that the validations of the final designs were successful. The team members observed the performance and efficacy of the light device.

The associative learning tests required further analysis of the recorded videos. Using a MATLAB script that was developed by Professor Albrecht, the team analyzed the locomotion of *C. elegans* during the experiments. These results were summarized in graphical plots and further interpreted for understanding the associative learning of *C. elegans* and are detailed in later sections.

6.5 Design Considerations

In this section the economic and environmental impacts, societal influence, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability are discussed.

6.5.1 Economics

The team determined that the devices were neither economically advantageous nor disadvantageous. The cost of production was well below the MQP budget and the produced devices had the potential to be reused numerous times if used properly. However, it should be noted that with an automated system of the light device could potentially save time and allow the researcher some flexibility during the experiments.

6.5.2 Environmental Impact

By conducting experiments in a microfluidic device, the use and disposal of chemicals could be reduced. However, the typical wastes such as micropipette and pipette tips, and agar plates are disposed as biohazardous waste. Even though, the mentioned plastic waste does not get recycled, the team expects the negative environmental impacts to be minimal. However, it is important to optimize laboratory protocols and skills to minimize the laboratory disposals.

6.5.3 Societal Influence

C. elegans are used as model organisms to map the human nervous system. The future studies signify on studying mutant *C. elegans* that exhibit the most common human brain and neural diseases. Thus, by studying the *C. elegans* and their learning patterns could potentially

help the ongoing neural research. These outcomes could indirectly influence the society and increase the quality of life of humans in the longer run. However, the team determined that there is no direct and immediate influence on the society due to microfluidic and light devices.

6.5.4 Political Ramifications

The developed devices expect to be used only by researchers, thus the team agreed that there is minimal political ramifications due to our devices. Since the subjects that are studied within this particular device are *C. elegans* – nematode roundworms, which are fairly simple organisms, the cultural controversy on the animal use protocol is expected to be negligible. However, it should be noted that misunderstandings and miscommunication may occur in other cultures when mutant *C. elegans* are used for experiments.

6.5.5 Ethical Concerns

Maintenance of *C. elegans* and experiments involving these organisms are much simpler and are free of ethical concerns. This is because *C. elegans* are considered as invertebrate. *C. elegans* have been extensively used in research; studying these organisms within a microfluidic device does not cause any interference with their natural behavior, thus the team considers the experiments to be ethical. Throughout the experiments, the approved *C. elegans* maintenance and care protocols were strictly followed.

6.5.6 Health and Safety Issues

If the laboratory protocols are followed, the system should not cause any long and short-term health issues to the user. However, even though the minimal amount of chemicals are used during the experiments, the risk of chemical spill is always present, if this situation occurs it

needs to be handled according to the safety protocol. While handling the light device, one should always have dry hands, as there is a chance of electrocution. Thus, it is important to follow the laboratory safety procedures and waste disposal protocols.

6.5.7 Manufacturability

The devices that were created during this MQP can be easily reproduced. Since the system can be easily replicated, it could be potentially commercialized to other research labs. To replicate the microfluidic device, one needs DraftSight schematics that is used on a photomask, and silicon wafers to create the mold of the microfluidic devices. Further steps include pouring PDMS on the mold and drying of the PDMS. Once the PDMS is dried out, it is removed from the mold. To replicate the light device one needs to follow the circuit diagram provided in *Figure 4.1 and 4.3* in Chapter 4, a LED Light Housing Kit (www.ledsupply.com) and a 2N3906 PNP transistor are needed in order to replicate the light device. The optics, as described in 4.1.3.3, were purchased from Thorlabs and can be reordered as necessary.

6.5.8 Sustainability

The developed devices required use of electricity to operate. However, other effects to the sustainability due to the microfluidic system and light device is very minimal. It should be noted that the devices are reusable, if used correctly. Recyclable waste should be recycled whenever it is possible to do so. By reducing the laboratory waste, the system can improve the sustainability. Furthermore, if the laboratory protocols and practices are strictly followed, there is no immediate impact to the environment.

Chapter 7: Discussion

7.1 Interpreting Results

The microfluidic device was tested to ensure that it would serve as a consistent and maintainable environment for the behavioral experiments, as discussed in Chapter 6. The microfluidic device did not leak when assembled properly, which was a required characteristic of the behavioral testing environment. The device met all requirements, including compatibility with the current lab setup, reliability and reproducibility, transparency, gas permeability, and movement interference with the old *C. elegans*.

Once the team confirmed that the microfluidic device was operational, they proceeded to test the associative learning ability of 5 day old *C. elegans*. Part 1 of the experiment refers to the first half hour of the experiment. During this half hour, *C. elegans* are only loaded into Arena 1 and are trained by being exposed to pulses of light (aversive to *C. elegans*) and butanone (attractive to *C. elegans*) for 60 second intervals at a time, with a 4 minute latency period between pulses. Part 2 of the experiment is occurs after more, untrained 5 day old *C. elegans* are loaded into Arena 2. During this half hour, *C. elegans* are exposed to pulses of light without butanone for 60 second intervals at a time, with a 4 minute latency period between pulses.

The team found that the *C. elegans* in Arena 1 did not develop any association between butanone and light. This was concluded from examining the experiment data compiled by the Worm Tracking Pulse MATLAB script (see *Figure 7.1* for a sample of this data, for an average of the data compiled from all experiments).

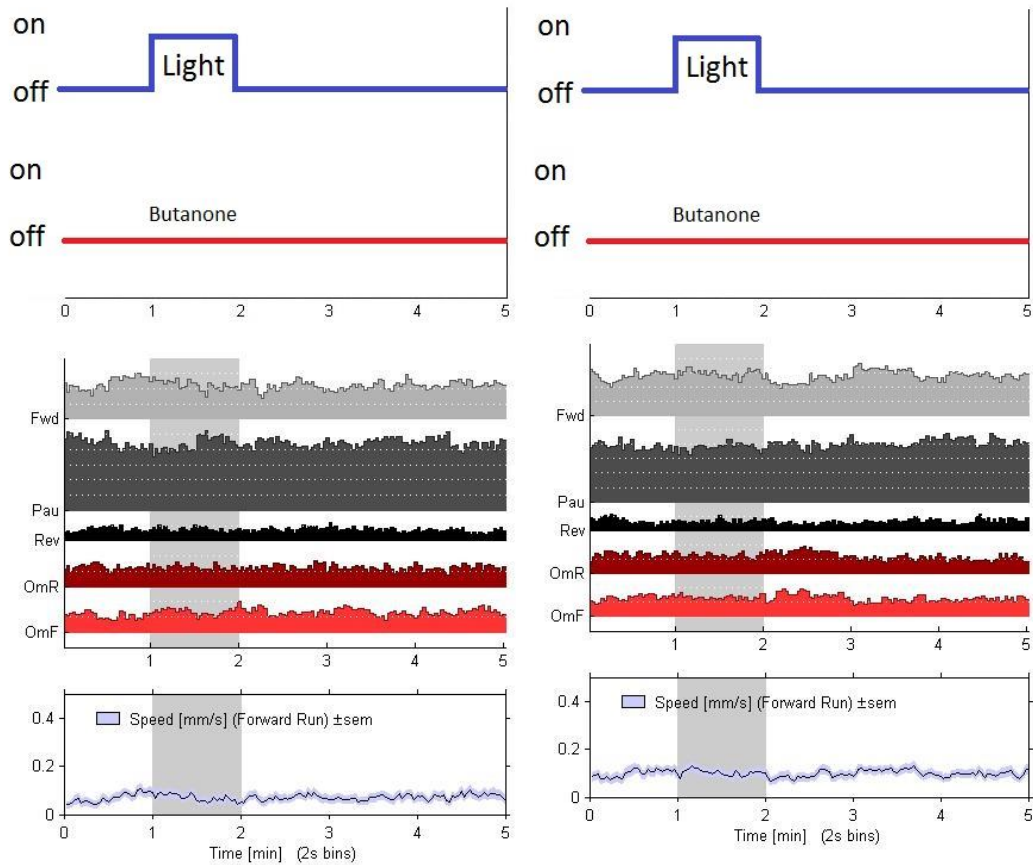


Figure 7.1: Response period average of all experiments
 Arena 1 (untrained) left & Arena 2 (trained) right

C. elegans move forwards when exposed to an attractive stimulus, and reverse when exposed to an aversive stimulus. The experiments did not show any significant differences between the forward or reversal motions of trained versus untrained *C. elegans*. This means that the trained *C. elegans* did not find the light more aversive or attractive than the untrained *C. elegans* did. However, it can be observed that the average forward speed of the trained *C. elegans* dipped as soon as the light stimulus turned on (seen at minute 1 in Figure 7.1), and the forward speed of the untrained *C. elegans* did not display this behavior. This pattern is apparent in the accumulation of the experiments, and means that the trained *C. elegans* did have a slightly

different reaction to the light than the untrained *C. elegans*. However, this reaction occurred in terms of speed and not in terms of a different motion (such as moving forwards or reversing), and therefore cannot be categorized as associative learning.

The trained *C. elegans* in Arena 1 did not demonstrate behavior that indicated the blue light stimulus was not as aversive to them as it was to the untrained *C. elegans* in Arena 2. This indicates that there was no association the *C. elegans* in Arena 1 made between light and butanone that occurred when they were trained with the blue light stimulus/butanone pairing.

However, when analyzing reactions of *C. elegans* to the light alone, the data of which is shown in section 5.3, it was found there was a statistically significant reaction ($p=0.028$) in which the *C. elegans* slowed their movement after the light was presented. However this change in speed did not occur in in the pairing with Butanone. This indicates the *C. elegans* can in fact process and respond to the light at the intensity of $42.3\mu\text{W}/\text{mm}^2$ but the Butanone negates this sensation, most likely flooding the *C. elegans* senses and not allowing them to process the light stimuli. Thus associative learning could not take place. In order to repair this the light intensity will have to be increased to a degree in which the *C. elegans* will still react to the light even when they are also being presented with Butanone.

7.2 Comparing Results

It is difficult to compare this data to previous experiments, as no other currently existing research involves using a light as a stimulus to explore associative learning in *C. elegans*.

However, specific aspects of this research can be compared to previous studies.

The *C. elegans*' positive olfactory chemotaxis response towards chemicals with odors resembling their food (i.e. butanone) is well documented, and is why the team choose butanone

as the positive stimulus in their experiments. Research groups have used butanone, which was also used in this team's experiment, as a positive stimulus in short and long term memory assays [45].

C. elegans' innate aversion to light, as displayed by phototaxis, is a response meant to ensure that they remain within the soil and do not breach the surface. This phototaxis response has recently begun to be studied, and other researchers have noted that *C. elegans* exhibit the same negative phototaxis when exposed to light that this team observed. One research group has even identified the group of sensory neurons most likely to be the photoreceptor cells responsible for the phototaxis [44]. The team's results only show a slight response to light at $42.3\mu\text{W}/\text{mm}^2$, which may be because the light is too dim. Another experiment using a blue light stimulus with $700\mu\text{W}/\text{mm}^2$ found that intensity produces the maximum locomotion for wild type *C. elegans* [48].

Many researchers have explored *C. elegans*' ability to learn through association. The overwhelming consensus of the research community is that *C. elegans* possess associative learning abilities when presented with the proper environment and stimuli. This suggests that the intensity of the blue light stimulus the team used is not conducive to associative learning, and is likely too dim. Previous teams have even observed how associative learning helped adult *C. elegans* avoid pathogenic bacteria [45]. This is especially important to note because the majority of research done with *C. elegans* utilizes young adults, and not the adult aged animals this team used. There are many other examples that demonstrate *C. elegans*' ability to learn through association, including but not limited to salt learning and butanone enhancement learning [49, 50].

When these specific aspects of the team's experiments are compared to prior research, it seems that the team's results were on track. However, increasing the intensity of the light to around $700\mu\text{W}/\text{mm}^2$ would improve the quality of the team's results by resulting in a stronger response from the *C. elegans*. It is important to note that the majority of *C. elegans* focused research uses young adults and agar plates, instead of adults and microfluidic devices as this team did. There have been experiments done that replicate agar-based experiments in microfluidic devices that demonstrate the microfluidic devices allow for more precise stimulus control and behavioral quantification [50].

7.3 Data Limitations

The main limitation associated with this team's experiments and data involved the chemical switches. The chemical switches were manual and were not controlled via the pulse MATLAB script. This is because when the MATLAB script was used, the S. Basal flow profile was not even, and resembled a gradient more than a pulse. The team believes this issue was caused by a pressure differential created when the hardware was supposed to allow the flow, but a malfunction impeded the full flow. The issue was addressed by switching the chemicals on and off manually.

Chapter 8: Conclusions and Recommendations

8.1 Conclusions

This study created an entirely light stimulus system, compatible with existing gold standard systems using chemical stimulation, a new device enabling study of aged *C. elegans*, and provided preliminary testing for the newly created system. This system, designed for associative learning testing opens entirely new windows for possibilities in experimentation with *C. elegans*. This ability to test *C. elegans* with two genres of stimuli allows for more combined stimuli testing without the concern of the, for example, two chemicals combining to form a single stimuli for the *C. elegans*.

The success of the adult microfluidic device means that the experimentation on *C. elegans* can now take place on any age group and developmental level. This allows for the new element of ageing and development to be added into the factors of experimental design. Any experiment that has been conducted on lower developmental stages can be compared to an adult test in order to examine the effect further development has on the brain's processing ability and neural connectedness.

The results of the validation testing, while not statistically significant, indicate the project is heading in the correct direction. Showing that associative learning is possible in *C. elegans* means that the process and skill of an animal's ability to learn can be tracked over the developmental stages of its life. This can give us more details into how neural connections break down or change as animals' age and how various diseases, disorders, and environmental factors can stimulate or prevent these changes. From this the neural and genetic process of learning can be examined and the roots of cognitive development can be explored.

8.2 Recommendations

The first area of research going forward in this project is using the designed experiments, shown in Appendices D-G, to validate the cumulative system in young and aged *C. elegans*. This project showed that there was progress in this area but more time is needed to perfect the process. An increase in light intensity should be explored with all tests taking place in the microfluidic device as there is a discrepancy in reaction of *C. elegans* when exposed to light in agar plates versus in microfluidic devices. Along with this the perfection of stimuli pairing in order to maximize the associative learning capabilities through examination of the ideal ratio of pulses to latency periods.

Additional areas that should be developed include an analysis of the interaction between the pulse system and the large adult microfluidic device. Current theories for the discrepancy between the pulse systems operation and the adult device are that there is a pressure issue, most likely due to the pulse systems output or the limitations in height between the fluid reservoir and microfluidic device. Recommended changes include using the multi valve system rather than the simple one valve system and an examination of hole spacing on the microfluidic device.

After system is fully validated it can be used to evaluate such cognitive comparisons such as age, disease models, gender differences, learning disability models, etc. Any cognitive comparison can be used within this system and thus the neurological and cognitive effect of stimuli can be evaluated successfully. This system help contribute to developing the knowledge base related to aging and learning in humans and all model organisms.

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Appendices

Appendix A

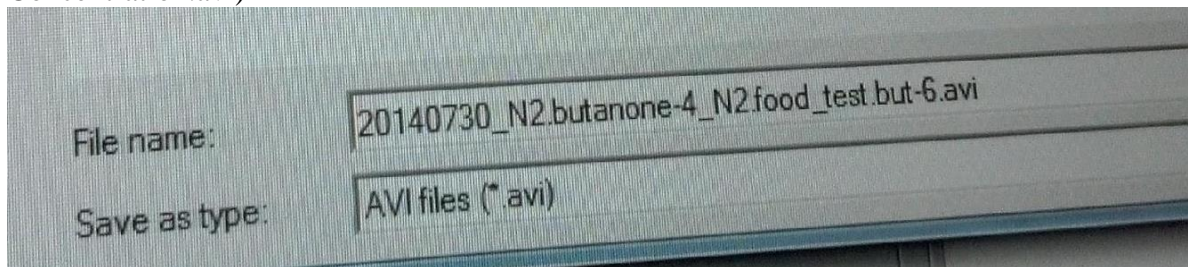
O1 Gradient Device Protocol

Originally written by Karen Tran for QNTL

Revised by the MQP team

Setting up MATLAB

1. Open 64-bit MATLAB version
2. Type gVision and press enter
3. File>Load State>Desktop>gVision States>6.6 MP>1200x720
4. Set ROI: 432 1128 1200 720 and press enter when complete
5. Set gVision Modes: Time Lapse
6. Set Cap Intervals: 0.5
7. Set Frames/Trigger: 1
8. Set Trigger Repeat: 14400 for 2hrs, 7200 for 1hr, 3600 for 30min
9. Set File Frame Rate: 30
10. To save the file, press “Browse” under the file name (normally “temp.avi”)
11. Normally save the file to the D Drive>DATA>Karen
12. If D Drive full, save to Z Drive>Karen>Gradient Assays>Experiments. (Not preferable to save in Z Drive, always try to delete old movie files to increase memory)
13. Create a new folder for the day, following the format YearMonthDay (for example, July 30, 2014 would be 20140730)
14. For the file name, follow the format below
(Date_WormStrain.ArenaOneConditions_WormStrain.ArenaTwoConditions.test Odor Concentration.avi)



For the experiment above, the N2 worms in Arena 1 were pre-exposed to butanone with a concentration of 10^{-4} , and the N2 worms in Arena 2 were pre-exposed to food. The odor concentration used for the testing was butanone with a concentration of 10^{-6} .

Setting up O1 device, Part I

1. Fill O1 device w/ pluronic (F127), and block off the two worm loading ports with metal pins if not done so already as they fill up with pluronic.
2. If air bubbles are visible, let pluronic sit for a while until air bubbles disappear. The bigger the bubble the longer you have to wait.
3. Prepare 30ml syringe reservoirs

- Attach 3-way valve to 30ml syringe
- Attach 3ml syringe to 3-way valve so that it's perpendicular to 30ml syringe
- Attach tubing to last opening on 3-way valve
- Place all 30ml syringe reservoirs onto holder

Making Odors

1. Get tinted glass bottle
2. Follow the recipes below for specific concentrations:
 - Obtain stock solution ($\geq 99\%$) of desired odor to work with.
 - 30ml S. Basal + 3ml stock odor = 10^{-1}
 - 30ml S. Basal + 300ul stock odor = 10^{-2}
 - 30ml S. Basal + 30ul stock odor = 10^{-3}
 - 30ml S. Basal + 3ul stock odor = 10^{-4}

All odor concentrations listed above must be vortexed for 30sec and inverted several times for proper mixing. To make more dilute concentrations, do serial dilutions from one of the above solutions.

- 30ml S. Basal + 300ul of 10^{-3} = 10^{-5}
- 30ml S. Basal + 30ul of 10^{-3} = 10^{-6}
- 30ml S. Basal + 3ul of 10^{-3} = 10^{-7}
- 30ml S. Basal + 3ul of 10^{-4} = 10^{-8}

Odors with concentrations ranging from 10^{-5} to 10^{-8} will only need to be inverted several times for mixing.

3. Add 300ul of 100X xylene cyanol to odor solution that will be used for experiment and invert several times. This solution now has 1X dye.

Setting up O1 device, Part II

1. Check that valve turns off flow to tubing (pointed downwards)
2. Fill 30mL syringe reservoirs with appropriate liquids needed for experiment
3. Flush all air bubbles in reservoir & Tygon tube using the 3ml syringe. NOTE: Turn 3-way valve to allow fluid flow into tube and flick the green leuc lock needle to remove trapped bubbles.
4. Check PDMS device and see if bubbles are gone before making input connections
5. When ready, flow a little more pluronic to create droplet-droplet connection while making the first input (i.e, S.Basal, Odors, etc) connection
6. After 1st input connection is made, you can use that fluid flow for droplet-droplet connections for other inputs
7. Check **EVERYWHERE** for air bubbles and rid of it before moving on to next step
8. Turn on flow for inlet reservoirs and waste outlet then observe gradient formation in device (~5 min).
9. Place waste flask at the same height as green leuc lock needle on 30ml reservoirs
10. After arenas are filled with gradient, then STOP odor reservoir flow and only allow S. Basal to continue flowing.
11. Wait 5 minutes or until the device is filled with S. Basal then stop outlet waste flow.
12. Remove the metal pins plugged into worm loading ports and let S. Basal to fill up before stopping inlet flow.

13. Now go pick worms for loading!

Picking worms

1. Get plate containing worms to be tested and grab unseeded plate
2. Transfer a speck of bacteria from worm containing plate onto unseeded plate for reference point
3. Transfer worms onto unseeded plate
4. When ready to load worms, prep syringes (w/ tubing attached) by filling with worm loading S. Basal and rid of bubbles NOTE: Get rid of bubbles by filling the syringe with S. Basal and inverting it so that the green leuc lock is upwards. Tap the syringe on a table edge so that the bubbles go into the green leuc lock. Put the end of the tube into the worm loading S. Basal tube and eject fluid from the syringe as you continue tapping. Repeat once or twice – until all bubbles are gone. Once this process is complete, double check by tapping on the green leuc lock until no more bubbles float up.
5. Pour S. Basal onto unseeded plate containing worms ~2/3 full
6. Create droplet at end of tubing before immersing into buffer-filled plate to avoid introducing bubbles to syringe. NOTE: can direct worms into a common area as expelling liquid from syringe
7. Aspirate worms with syringe and expel several into a corner of the plate to prevent worms from swimming into the syringe. After gathering all worms into the corner, collect them into worm loading tube. NOTE: keep worms in tubing otherwise they'll get stuck in syringe and makes it harder to flush them out when loading into device
8. Before loading worms, open flow for S. Basal reservoir and remove metal pins in worm loading ports. Allow S. Basal to fill worm loading port to create drop-drop contact with tip of worm loading tube.
9. Insert tubing into worm loading port and slowly push on plunger to load worms. NOTE: keep an eye on worm tubing entrance into device to watch for bubbles that may be traveling through. If many bubbles are present, then stop loading and detach worm tubing. Go back to buffer-filled plate to flush bubbles out and recollect remaining worms. Try loading worms again.
10. When done, expel S. Basal while removing worm loading tube and plug with metal pin.
11. Remove another pin from another port. (Always need 2 ports open when loading because excess liquid need to flow out). Once one arena is loaded and plugged, take out the waste tubing to have 2 ports open– remember, S. Basal must be flowing when you remove tubing.
12. Turn off S. Basal reservoir and prep the next worm loading.
13. Repeat steps 1-13 for each strain loaded into the P6 device.

Food exposure in microfuge tube

1. After worm loading is complete, turn the waste, S. Basal and odor on. Wait until the gradient forms (~2 minutes).
2. Double check the focus by zooming in all the way and focusing the lens, then zoom appropriately (extra room on top, use the side worm barriers as the edge).
3. As soon as the gradient forms, press “Start” in the MATLAB window. Make sure the box above the start button turns green. If not, close out of MATLAB, reopen, and try again. If there are still problems, check the connection to the lens.

Conditions

Naïve= worms are not treated with anything and starved for selected time period (30min, 1hr, 2hrs).

Food only= worms are starved for 1hr first then exposed to OP50 E.coli food for selected time period (30min, 1hr, 2hrs).

1. Get OP50 bottle from 4C fridge and use aseptic techniques when opening and closing.
2. Transfer 1ml OP50 into cuvette then sterilize bottle again before returning to fridge.
3. Get LB bottle from Karen's drawer and use aseptic techniques as before
4. Transfer 1ml LB into cuvette then sterilize bottle before returning to drawer.
5. Go to Multiskan Spectrum to spec optical density (OD) of bacteria
6. Open Multiskan Spectrum software, close first window that pops up.
7. Click on Quick Read
8. Set Wavelength to 600
9. Make sure the Reference and Sample slots in the machine are empty.
10. Calibrate by clicking Zero
11. Place cuvette containing LB into the Reference slot where arrow is aligned with scratch marking on machine
12. Click Zero again
13. Now place cuvette containing OP50 into sample slot aligned in the same direction as LB cuvette
14. Click Read and record OD measurement.
15. Remove cuvettes, transfer OP50 into microfuge tube labeled "OP50"
16. Follow steps 1-7 from protocol "Picking Worms"
17. Transfer worms into a labeled microfuge tube and wait 2 minutes for worms to sink to bottom
18. Use a 200ul pipette set at 100ul volume to remove excess S. Basal from microfuge tube.
NOTE: Be careful not to catch any worms in pipette tip and allow a droplet of S. Basal to prevent worms from drying.
19. Transfer 1ml OP50 into microfuge tube containing worms
20. Place microfuge tube in the Jitterbug machine
21. Press Mix, then press 5 for the mix setting
22. Remember to set a timer for the mixer
23. To clean cuvettes, fill 10% bleach to leave overnight
24. Wash withalconox and flip upside down to dry
25. Once pre-exposed worms have mixed for a set time, remove from Jitterbug machine and allow to sit for 2 minutes to let worms to sink to bottom.
26. Remove excess liquid and transfer worms onto unseeded plate.
27. Follow "Picking Worm" protocol

Butanone only = worms are starved during pre-exposure to butanone 10^{-4} concentration for selected time period (30min, 1hr, 2hrs).

1. Follow "Making Odors" protocol to create butanone 10^{-4} odor.
2. Follow steps 1-7 from protocol "Picking Worms"
3. Transfer worms into a labeled microfuge tube and wait 2 minutes for worms to sink to bottom

4. Use a 200ul pipette set at 100ul volume to remove excess S. Basal from microfuge tube.
NOTE: Be careful not to catch any worms in pipette tip and allow a droplet of S. Basal to prevent worms from drying.
5. Transfer 1ml butanone 10^{-4} into microfuge tube containing worms.
6. Place worms in Jitterbug machine and follow steps 20-23 in “Food only” condition
7. Once pre-exposed worms have mixed for a set time, remove from Jitterbug machine and allow to sit for 2 minutes to let worms to sink to bottom.
8. Remove excess liquid and transfer worms onto unseeded plate.
9. Follow “Picking Worm” protocol to load worms into device

Butanone + food= worms are starved for 1 hr then pre-exposed with both butanone 10^{-4} & food. Follow “Butanone only” and “Food only” protocols but remember to mix odor solution with pelleted bacteria.

Exposing Food and Butanone in Microfluidic Device

1. Use OP50 in LB Media from refrigerator and use aseptic techniques to pipette 10 mL of media to a 15 mL tube.
2. Centrifuge the bacteria for 15 mins at 10,000 rpm. There should be a pellet of food at the bottom of the tube.
3. Remove the excess liquid by pipetting it into a waste container. Be careful not to take up any of the pellet.
4. Pipette 2 mL of S. Basal buffer into the tube and break up the pellet until it is no longer visible.
5. Transfer 1ml S Basal into cuvette
6. Go to Multiskan Spectrum to spec optical density (OD) of bacteria. OD should be no more than ~1.0 or else bacteria will sediment in device. For every 0.5 increment above 1, add 1ml of S. Basal.
7. Open Multiskan Spectrum software, close first window that pops up.
8. Click on Quick Read
9. Set Wavelength to 600
10. Make sure the Reference and Sample slots in the machine are empty.
11. Calibrate by clicking Zero
12. Place cuvette containing S Basal into the Reference slot where arrow is aligned with scratch marking on machine
13. Click Zero again
14. Now place cuvette containing OP50 into sample slot aligned in the same direction as SB cuvette
15. Click Read and record OD measurement.
16. Remove cuvettes, transfer OP50 back into 15 mL tube.
17. Add appropriate volume of butanone to OP50 solution to make desired final butanone concentration. (ex: If total volume is 4mL, pipette 40ul of butanone 10^{-2} into solution and vortex).
18. Transfer 3mL into an open 3 mL syringe reservoir with tubing and pin attached
19. Insert pin into the worm loading port using drop to drop technique. Turn off other reservoirs.

20. Expose worms for 1 hr

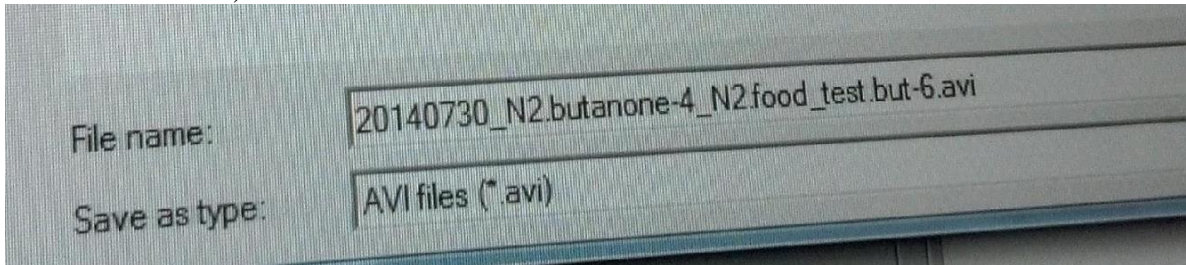
Clean - water and ethanol

Appendix B

Pulse Device Protocol

Setting Up gVision

1. Open 64-bit MATLAB version
2. Type gVision and press enter
3. File>Load State>Desktop>gVision States>6.6 MP>1200x720
4. Set ROI: 432 1128 1200 720 and press enter when complete
5. Set gVision Modes: Time Lapse
6. Set Cap Intervals: 0.5
7. Set Frames/Trigger: 1
8. Set Trigger Repeat: 14400 for 2hrs, 7200 for 1hr, 3600 for 30min
9. Set File Frame Rate: 30
10. To save the file, press “Browse” under the file name (normally “temp.avi”)
11. Normally save the file to the Z Drive>MQP1
12. Create a new folder for the day, following the format YearMonthDay (for example, July 30, 2014 would be 20140730)
13. For the file name, follow the format below
(Date_WormStrain.ArenaOneConditions_WormStrain.ArenaTwoConditions.test Odor Concentration.avi)



For the experiment above, the N2 worms in Arena 1 were pre-exposed to butanone with a concentration of 10^{-4} , and the N2 worms in Arena 2 were pre-exposed to food. The odor concentration used for the testing was butanone with a concentration of 10^{-6} .

Setting Up ValveBankTiming

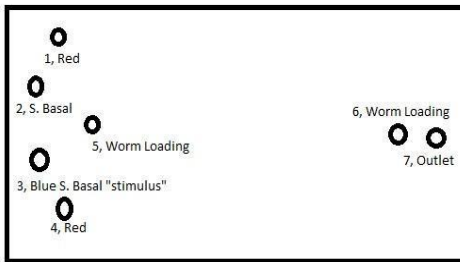
1. In the already open 64-bit MATLAB version, define timing_exp and press enter (see below):

```
timing_exp = [0 1 0; 0 2 1; 0 3 0; 1 1 1; 1 3 1; 60 1 0; 60 3 0; 300 1 1; 300 3 1; 360 1 0; 360 3 0; 600 1 1; 600 3 1; 660 1 0; 660 3 0; 900 1 1; 900 3 1; 960 1 0; 960 3 0; 1200 1 1; 1200 3 1; 1260 1 0; 1260 3 0; 1500 1 1; 1500 3 1; 1560 1 0; 1560 3 0; 2700 3 1; 2760 3 0; 3000 3 1; 3060 3 0; 3300 3 1; 3360 3 0; 3600 3 1; 3660 3 0; 3900 3 1; 3960 3 0; 4200 3 1; 4260 3 0; 4500 2 0];
```

2. Enter output=ValveBankTiming(timing_exp) and press enter

Setting Up the Device

1. Flush device with pluronic and let sit until bubbles are gone
2. The device setup is as follows:



- Holes 1 and 4 are connected to reservoirs containing stimuli (which is controlled/connected to valve 1)
- Holes 2 and 3 are connected to a reservoir with 30 mL of S. Basal
- Holes 5 and 6 are worm ports
- Hole 7 is the outlet
- Hole 1 is connected to Valve 1, Hole 7 is connected to Valve 2
- These are automatic valves, connected to the timing box as pictured below:



Setting Up the Fluid Reservoirs

- Check that the flow to the tubing is off (valve pointed downwards)
- Fill 30mL syringe reservoirs with appropriate liquids needed for experiment
- Flush all air bubbles in reservoir & Tygon tube using the 3ml syringe. NOTE: Turn 3-way valve to allow fluid flow into tube and flick the green leuc lock needle to remove trapped bubbles.
- Check PDMS device and see if bubbles are gone before making input connections
- When ready, flow a little more pluronic to create droplet-droplet connection while making the first input (i.e, S.Basal, Odors, etc) connection
- After 1st input connection is made, you can use that fluid flow for droplet-droplet connections for other inputs
- Check **EVERYWHERE** for air bubbles and rid of it before moving on to next step
- Place waste flask at the same height as green leuc lock needle on 30ml reservoirs
- Test ValveBankTiming Script for experiment - make sure you can see the pulse effect
- Flow S. Basal and wait until the device is filled with S. Basal, then stop outlet waste flow.

11. Remove the metal pins plugged into worm loading ports and let S. Basal to fill up before stopping inlet flow.

Picking and Inserting Worms

1. Transfer worms to unseeded plate
2. Pour S. Basal into plate (fill up the plate almost to the top)
3. Use a worm loading syringe mostly filled with S. Basal to make a drop to drop connection with the plate
4. Insert worms for Arena 1 into worm loading syringe
 - a. When collecting worms, ensure that they are collected all at once
 - b. After worms are in worm loading syringe, take up a small bit of S. Basal to use for the drop to drop connection with the device
5. Load Arena 1 using the drop to drop connection technique
6. When pulling the syringe out after loading, make sure to be steadily pushing out S. Basal
7. Quickly plug the worm loading port with a pin
8. Repeat steps 1-7 for Arena 2
9. Allow the worms to acclimate for 30 min

Appendix C

Video Analysis Protocol

Video Analysis

Analyzing Videos

1. WormTrackingScript_Pulse
2. Select background box
3. Drag box across area that will be analyzed. Anything outside the box will be omitted from analysis
4. Draw box around arena 1, starting from top marker line omitting the first column of posts and ending in bottom marker line omitting last column of posts.
5. The box just drawn is now blue. When clicked again anywhere inside it, the color will change to red and labeled as 1.
6. Do the same for all 4 arenas.
7. After all arenas are marked, click anywhere outside of analyzed region and press ENTER.
8. Then select dimensions of an arena by clicking on the left top & bottom corners of arena 1.
9. “Are all settings made (y/n)?” appears on Command window. Type in “y”

Notes: If error occurs and WormTrackingScript_DA goes back to unfinished jobs, then go to C:\ProgramFiles\MATLAB\R2012a_32bit\work and delete all ‘**inprogress.mat**’ files

Write text file for each experiment for ExpInfo variable to exist in order to combine pre-analyzed data in the next step.

Example=

Genotype, ,N2,VT2392, VT2392, CL2122
Stimulus, 3640/60, 3660/60, Glycerol, 0.5
, 4320/60, 4360/60, Glycerol, 0.5
, 5000/60, 5060/60, Glycerol, 0.5
, 5680/60, 5760/60, Glycerol, 0.5
, 6420/60, 6520/60, Glycerol, 0.5

Analyzing Track Segments

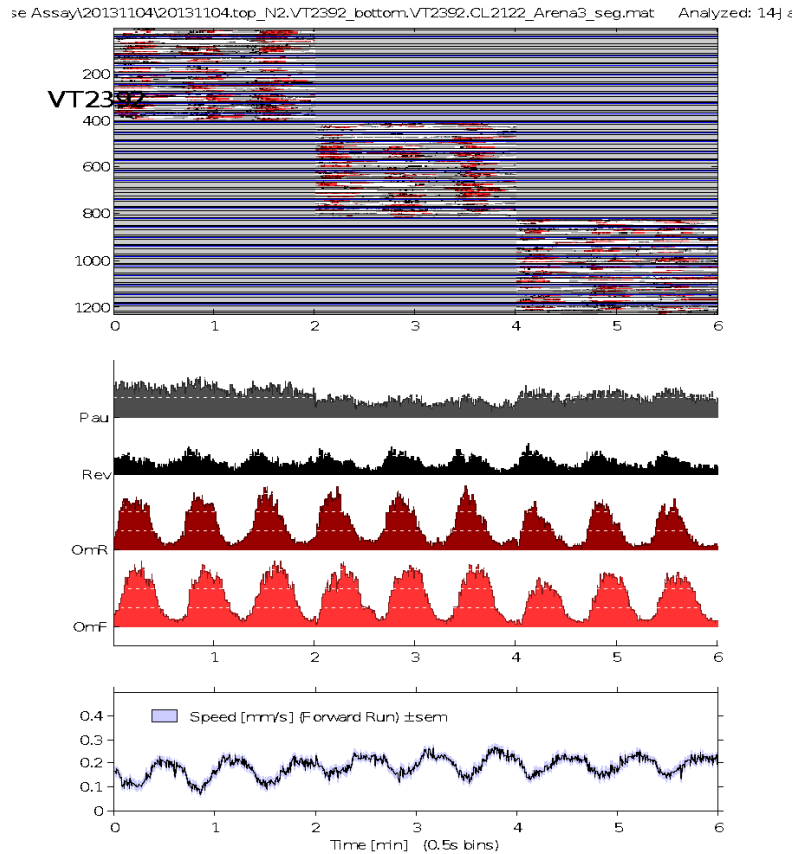
1. uiTimeAnalysis
2. Click Load Segment Data File
3. Analyze Track Segments
4. Save Analyzed Data
5. Clear Data for each one before continuing to load next file
6. After all files are finished, then click Combine Pre-Analyzed Data for files of the same strain and type of experiment
7. Remember to Clear Data after each combined data

TimeShift Analysis (Changes based on experiment)

```
>> ShiftInfo = [0, 4, 1; 5, 9, 0; 9, 14, 0; 14, 19, 0; 19, 24, 0; 24, 29, 0];
TimeShift
Time Shift Info (block start, end, new time) [min]
```

Figure after timeshift function:

*Normally you get all 4 behavior graphs overlapping, so need to replot to separate them.
Click on 'PLOT' tab → 'Split Behavior'



Troubleshooting

Check movie to check if worms are correctly tracked

```
>> PlaySparseMovie
```

*Select .avi file

green= single worm

red= 2 collide

yellow= 3

white= 4 or more collide

blue= unknown

Look at DyeData

Drag and drop .mat file, sometimes file doesn't have .mat extension

```
>>DyeData.Arena( )
```

```
>>plot (DyeData.Time, DyeData.Arena (1).Up)
```

```
>>plot (DyeData.Time, DyeData.Arena (1).Down)
```

*To stack plots on top of each other, do the following

```
>>plot(DyeData.Time, [DyeData.Time, DyeData.Arena (1).Down; DyeData.Arena (1).Up)
```

Figure shows up

Check for Background plot

```
>>plot(DyeData.Time, DyeData.Background)
```

```
>>figure
```

```
>>imshow(background)      *displays actual image of background for device as seen from camera
```

```
>>rectangle ('Position', [x, y, w, h])
```

```
>>rectangle ('Position', [1.1804 e+03 23.8133 10 10]) *coordinates of rectangle for choosing background is retrieved from DyeData
```

If WormTrackingScript continues running past video w/ errors then find inprogress.m file by following path=

```
C:\Program Files\MATLAB\R2012a_32bit\work
```

```
Delete => inprogress
```

*Note - ignore ethnogram error

Appendix D

Prime Pairing and Intensity Response

Prime Intensity Testing

- Total experimental time: 30 minutes

Materials

- S. Basal
- Light Device

Pre-Experiment Preparation

- Picking of *C. elegans* into unseeded plate
- Program ValveBankTiming_DA as detailed in script
- Program gVision as necessary

Protocol

1. Take worms in unseeded plate and position under camera so entire plate in view.
2. Program ValveBankTiming_DA
 - i. `timing_exp = [0 3 0; 1 3 1; 60 3 0; 300 3 1; 360 3 0; 600 3 1; 660 3 0; 900 3 1; 960 3 0; 1200 3 1; 1260 3 0; 1500 3 1; 1560 3 0; 1800 3 0];`
 - ii. Enter `output=ValveBankTiming(timing_exp)` and press enter
3. Activate gVision for 30 minutes and activate ValveBankTiming_DA
4. Analyse visually for reaction to light.

Appendix E

Paired Stimulus Testing

Prime Stimulus Testing

- Total experimental time: 60 minutes

Materials

- Unconditioned Stimulus (UCS):
 - 10^{-6} butanone dyed with 1x cyanol xylene
 - 10^{-6} food dyed with 1x cyanol xylene
- Neutral Stimulus: Light
- S. Basal

Pre-Experiment Preparation

- Standard Pulse set up
- Program ValveBankTiming_DA as detailed in script
- Program gVision as detailed in script

Protocol

1. Set up the pulse device as described in Pulse Device Protocol.
 - a. 10^{-6} butanone dyed with 1x cyanol xylene will be used as the stimulus and will be connected to Hole 3.
2. Setup ValveBankTiming_DA Script with the following:
 - i. `timing_exp = [0 1 0; 0 2 1; 0 3 0; 1 1 1; 1 3 1; 60 1 0; 60 3 0; 300 1 1; 300 3 1; 360 1 0; 360 3 0; 600 1 1; 600 3 1; 660 1 0; 660 3 0; 900 1 1; 900 3 1; 960 1 0; 960 3 0; 1200 1 1; 1200 3 1; 1260 1 0; 1260 3 0; 1500 1 1; 1500 3 1; 1560 1 0; 1560 3 0; 2700 3 1; 2760 3 0; 3000 3 1; 3060 3 0; 3300 3 1; 3360 3 0; 3600 3 1; 3660 3 0; 3900 3 1; 3960 3 0; 4200 3 1; 4260 3 0; 4500 2 0];`
 - ii. Enter `output=ValveBankTiming(timing_exp)` and press enter
 - This will set the light and butanone off for 60 seconds every five minutes, pause for 15 minutes, and resume pulsing light for 30 minutes
3. Load *C. elegans* into both arenas.
4. Start gVision Script.
5. Start ValveBankTiming_DA Script
6. If butanone does not produce proper response switch to food stimuli and repeat experiment

Appendix F

Prime Pairing Time Testing

- Total experimental time: 30 minutes

Materials

- Light
- S. Basal
- Unconditioned Stimulus (UCS): 10^{-6} butanone/food dyed with 1x cyanol xylene

Pre-Experiment Preparation

- Standard Pulse set up
- Program ValveBankTiming_DA as detailed in script

Protocol

1. Set up the pulse device as described in Pulse Device Protocol.
2. Open Valves one and two.
3. Load *C. elegans*.
4. Start gVision Script. Open a new matlab window in order to run both gVision and ValveBankTiming_DA without interference.
5. ValveBankTiming_DA.
6. Using the ValveBankTiming_DA Script immediately turn the light on for 15 seconds. Then, turn it off for 1 minute, then on again for 15 seconds, and repeat for the duration of the experiment.
7. Repeat steps 1-6 using 30 seconds and 2 minutes, then 45 seconds and 3 minutes, and then 60 seconds and 4 minutes for step 6.
8. Analyze to examine which results in maximum amount of associative learning.

Appendix G

Comparison Testing

- Total experimental time: 90 minutes

Materials

- Unconditioned Stimulus (UCS): 10^{-6} butanone dyed with 1x cyanol xylene
- Neutral Stimulus: Light
- S. Basal

Pre-Experiment Preparation


- Standard Pulse set up
- Program ValveBankTiming_DA as detailed in script
- Program gVision as detailed in script
- Place worms for Aena 2 in unseeded plate

Protocol

1. Refer to Pulse Device Protocol to set up the pulse device and gVision on Matlab
 - The UCS is 10^{-6} butanone/food dyed with 1x cyanol xylene and it is connected to Hole 3
2. Set ValveBankTiming_DA script as determined in Experiment 3
3. Load *C. elegans* to Arena 1
4. Start gVision using new matlab window so that ValveBankTiming_DA script and gVision do not interfere.
5. Start ValveBankTiming_DA and gVision
6. During 15 minute break load Arena 2 Worms

Appendix H

WormTracker Script

A screenshot of a MATLAB editor window titled 'Editor - Z:\software\MATLAB files\Active\WormTrackingScript_Pulse.m'. The window shows a script with the following code:

```
1 % Run Tracking script
2 MultiArenaTracker_20130923;
3
4 % Segment & Link Tracks
5 SegFileList = BatchSegmentTracks_20130416fn({File.TrackFile});
6
7 % Make PDF summaries
8
9 for sf = 1:length(SegFileList)
10     Ethogram(SegFileList(sf));
11     WormDensity(SegFileList(sf));
12 end
13
14 % Bin data
15 BinFileList = BatchWormDensityTurn_20100325fn(SegFileList);
16
17 % uiTimeAnalysis
```

```
% Run Tracking script
```

```
MultiArenaTracker_20130923;
```

```
% Segment & Link Tracks
```

```
SegFileList = BatchSegmentTracks_20130416fn({File.TrackFile});
```

```
% Make PDF summaries
```

```
for sf = 1:length(SegFileList)
```

```
    Ethogram(SegFileList(sf));
```

```
    WormDensity(SegFileList(sf));
```

```
end
```

```
% Bin data
```

```
BinFileList = BatchWormDensityTurn_20100325fn(SegFileList);
```

```

% Edge Analysis
for fnum = 1:length(BinFileList)
    EdgeAnalysis20130416fn(char(BinFileList(fnum)));
end

% LocalEdgeAnalysis
for fnum = 1:length(BinFileList)
    Answer = inputdlg({'Genotype','ExcludeBins'}, BinFileList{fnum}, 1, {'N2','[1:60]'},'on');

    Info(fnum).Genotype = char(Answer{1});
    Info(fnum).ExcludeBins = str2num(Answer{2});
    Info(fnum).Odors = [];
    Info(fnum).Pattern = [];
    Info(fnum).OdorPattern = [];
    Info(fnum).Round = [];
    Info(fnum).Date = '20100000';
    Info(fnum).AnimalSize = [];
    Info(fnum).Animals = [];

    Info(fnum).FullName = BinFileList{fnum};
end

ExperimentDatafromXLS;

clear s
LocalStripeEdgeAnalysis_newspd;

```

```

Editor - Z:\software\MATLAB files\Active\MultiArenaTracker_20130923.m
EDITOR PUBLISH VIEW
Save Section Section Bold Italic Hyperlink Bulleted List Preformatted Text
with Title Monospaced Inline LaTeX Numbered List Code
FILE INSERT SECTION INSERT INLINE MARKUP INSERT BLOCK MARKUP PUBLISH
MultiArenaTracker_20130923.m
1 |
2 - keep3('command','blankrect');
3 % if exist('command') keep3 command; else clear; end % clear variables
4 close(findobj('type','figure')); % close all figures
5
6 %-----
7 % Define Constants & Intialize variables
8 %-----
9
10 arena = 16.1; % mm arena height (perpendicular to flow), typically 22 or 16.1 mm
11 MinWormArea = 0.5; % Min area for object to be a valid worm
12 MaxWormArea = 1.7; % Max area for single worm
13 MinDistance = 15; % Min Distance for connecting a new worm to an existing track (in pixels)
14 SizeChangeThreshold = 100; % Max size change between frames (in pixels)
15
16 MinTrackLength = 10; % Min Length of valid track (in frames)
17 PlotFrameRate = 60; % Display tracking results every 'PlotFrameRate' frames
18 Q = 1; % Quit MATLAB afterwards
19 FrameRate = 2; % Framerate (Hz)
20 %TimeStamp = 1; %
21 SaveAll = 1;
22 Gradient = 0;
23 AutoSelectDyeTrack = 1;
24 SaveThresholdMovie = 1;
25
26 TrackDye = 1;
27 TrackWorms = 1;
28 PackMemory = 600;
29
30 bs = 5; % pixels for background and dye levels
31
32 DefaultFragmentLength = 1800; % frames
33 max_processors = 6;
34 num_processors = str2double(getenv('NUMBER_OF_PROCESSORS'));
35 comp_name = getenv('COMPUTERNAME');
36
37 check_status_interval = 30; %seconds
38 MakeLocalCopy = false;
39
40 %-----
41
42 % Set screen info
43 WFigH = findobj('Tag', 'WFIG');
44 if isempty(WFigH)
45 WFigH = figure(20);
46 set(WFigH, 'NumberTitle', 'off', 'Tag', 'WFIG', 'Name', 'ArenaWormTracker');
47 else
48 figure(WFigH);
49 end
50 scrsz = get(0,'ScreenSize');
51 set(WFigH,'Position',[1 scrsz(4)*0.2 scrsz(3)-30 scrsz(4)*0.8]);
52 StatusFigH = findobj('Tag', 'Status');
53 if isempty(StatusFigH)
54 StatusFigH = figure(21);

```

TEXT:

```

keep3('command','blankrect');
% if exist('command') keep3 command; else clear; end % clear variables
close(findobj('type','figure')); % close all figures

%-----
% Define Constants & Intialize variables
%-----

arena = 16.1; % mm arena height (perpendicular to flow), typically 22 or 16.1 mm
MinWormArea = 0.5; % Min area for object to be a valid worm
MaxWormArea = 1.7; % Max area for single worm
MinDistance = 15; % Min Distance for connecting a new worm to an existing track (in pixels)
SizeChangeThreshold = 100; % Max size change between frames (in pixels)

MinTrackLength = 10; % Min Length of valid track (in frames)
PlotFrameRate = 60; % Display tracking results every 'PlotFrameRate' frames
Q = 1; % Quit MATLAB afterwards
FrameRate = 2; % Framerate (Hz)
%TimeStamp = 1; %
SaveAll = 1;
Gradient = 0;
AutoSelectDyeTrack = 1;

```

```

SaveThresholdMovie = 1;

TrackDye = 1;
TrackWorms = 1;
PackMemory = 600;

bs = 5; % pixels for background and dye levels

DefaultFragmentLength = 1800; % frames
max_processors = 6;
num_processors = str2double(getenv('NUMBER_OF_PROCESSORS'));
comp_name = getenv('COMPUTERNAME');

check_status_interval = 30; %seconds
MakeLocalCopy = false;

%-----

% Set screen info
WTFigH = findobj('Tag', 'WTFIG');
if isempty(WTFigH)
    WTFigH = figure(20);
    set(WTFigH, 'NumberTitle', 'off', 'Tag', 'WTFIG', 'Name', 'ArenaWormTracker');
else
    figure(WTFigH);
end
scrsz = get(0, 'ScreenSize');
set(WTFigH, 'Position', [1 scrsz(4)*0.2 scrsz(3)-30 scrsz(4)*0.8]);
StatusFigH = findobj('Tag', 'Status');
if isempty(StatusFigH)
    StatusFigH = figure(21);
    set(StatusFigH, 'NumberTitle', 'off', 'Tag', 'StatFig', 'Name', 'Tracking Status');
else
    figure(StatusFigH);
end

%-----
% Check for incomplete tracking files
%-----
FinishPreviousTrackingSession = 0;

TempFolder = fullfile(matlabroot, 'work');
if exist(TempFolder) == 0 mkdir(TempFolder); end % make TempFolder if it doesn't exist
StatusFile = fullfile(TempFolder, 'inprogress.mat');
delete(fullfile(TempFolder, '_F*')); % temporary files to monitor progress
if exist(StatusFile, 'file') == 2
    load(StatusFile);
    if exist('File', 'var') && ~all([File.CompletedFragments])
        FileNum = length(File);
        FinishPreviousTrackingSession = 1;
        disp('*** Unfinished jobs found. Continuing tracking...');
        for m = 1:length(File); File(m).ClaimedFragments = File(m).CompletedFragments; end % reset claimed
    fragments
end

```

```

end

if ~FinishPreviousTrackingSession

%-----
% Set Loop Parameters
%-----

FileNum = str2double(cell2mat(inputdlg('How many files do you want to track?')));

clear File

Name(FileNum).MovieFile = '';
for MovieNum = 1:FileNum
    UserPrompt = ['Select AVI Files For Analysis ' num2str(MovieNum)];
    [FileName, PathName] = uigetfile('*.avi', UserPrompt);
    cd(PathName);
    Name(MovieNum).MovieFile = [PathName,FileName];
    Name(MovieNum).TrackFile = strrepl(Name(MovieNum).MovieFile,'avi','mat');
end
% for MovieNum = 1:FileNum
%     [pathname,filename,ext] = fileparts(Name(MovieNum).MovieFile);
%     cd(pathname);
%     UserPrompt = ['Save Track Data ',num2str(MovieNum),': ',filename];
%     [FileName,PathName] = uiputfile('*.mat', UserPrompt,[filename,'.mat']);
%     Name(MovieNum).TrackFile = [PathName,FileName];
% end

%-----
% Get User Input for each movie
%-----
% File(FileNum).MovieFile = '';
for MovieNum = 1:FileNum
    MovieName = Name(MovieNum).MovieFile;
    [p,ShortMovieName] = fileparts(MovieName);

    TrackSettingsName = fullfile(p,[ShortMovieName,'_TrackSettings.mat']);
    CollectTrackingSettings = true;
    if exist(TrackSettingsName,'file') == 2
        ButtonName = questdlg('Use existing tracking settings?','Yes', 'No', 'Yes');
        if strcmp(ButtonName,'Yes')
            try
                load(TrackSettingsName);
                File(MovieNum) = FileSettings;

                % check filenames
                [pn1,fn1] = fileparts(Name(MovieNum).MovieFile); % chosen
                [pn2,fn2] = fileparts(File(MovieNum).MovieFile); % from settings file
                if strcmp(fn1,fn2) % if filenames match
                    if ~strcmp(pn1,pn2) % if pathnames don't match
                        File(MovieNum).MovieFile = Name(MovieNum).MovieFile;
                        File(MovieNum).TrackFile = Name(MovieNum).TrackFile;
                    end
                end
                CollectTrackingSettings = false;
            end
        end
    end
end

```



```

else
    disp('Filenames don't match... reselect manually. ');
end

if ~isfield(File,'ClaimedFragments') || isempty(File(MovieFile).ClaimedFragments)
    File(MovieNum).ClaimedFragments = File(MovieNum).CompletedFragments;
end
catch
    disp('Error loading settings. Select manually...');
end
end
end
end

```

```

MovieObj = VideoReader(MovieName);

```

```

if CollectTrackingSettings

```

```

    % show first frame
    Mov = read(MovieObj, 1);
    Mov = Mov(:,:,3);
    MovSize = size(Mov);
    figure(WTFigH); clf; imshow(imadjust(Mov,[],[],2));
    set(gcf,'MenuBar','none');
    set(gcf,'ToolBar','none');
    set(gcf,'Position',[1 1 1200 1200]);

    %-----get information-----
    buttony = 10;
    %uicontrol('Style','text','Position',[20 buttony 90 20],'String','Timer Location: ');
    %h1 = uicontrol('Style','popup','Position',[110 buttony 90 20],'String','Upper Right|Lower Right|Upper
Left|Lower Left|None');

    FrameNum = MovieObj.NumberOfFrames;
    FrameStart = 1;
    NumFragments = max(round(FrameNum/DefaultFragmentLength),1);
    if NumFragments == min(max_processors,num_processors)+1 NumFragments = NumFragments - 1; end

    uicontrol('Style','text','Position',[230 buttony 40 20],'String','Frames');
    h2a = uicontrol('style','edit','Position',[270 buttony 50 20],'String',num2str(FrameStart));
    h2 = uicontrol('style','edit','Position',[320 buttony 50 20],'String',num2str(FrameNum));
    h2b = uicontrol('style','edit','Position',[380 buttony 20 20],'String',num2str(NumFragments));

    %h3 = uicontrol('style','checkbox','Position',[420 buttony 60 20],'String','Gradient');
    h3 = uicontrol('style','checkbox','Position',[420 buttony 60 20],'String','Track Dye','Value',TrackDye);

    uicontrol('Style','text','Position',[500 buttony 60 20],'String','Arena(mm)');
    h4 = uicontrol('style','edit','Position',[560 buttony 40 20],'String',num2str(arena));

    uicontrol('Style','text','Position',[640 buttony 60 20],'String','FrameRate');
    h5 = uicontrol('style','edit','Position',[700 buttony 30 20],'String',num2str(FrameRate));

    uicontrol('Style','text','Position',[770 buttony 50 20],'String','min/max');
    h6a = uicontrol('style','edit','Position',[820 buttony 30 20],'String',num2str(MinWormArea));
    h6b = uicontrol('style','edit','Position',[850 buttony 30 20],'String',num2str(MaxWormArea));

```

```

%-----

set(WTFigH,'Name',[ 'Movie ',num2str(MovieNum),': ',ShortMovieName]);
txt = 'Click background (PDMS)'; title(txt);
label = text(MovSize(2)/2,MovSize(1)*0.4,txt,'FontSize',18,'HorizontalAlignment','center');
[X,Y] = ginput(1); File(MovieNum).BgBox = [X-bs Y-bs 2*bs 2*bs];
rectangle('Position',File(MovieNum).BgBox); text(X+10,Y,'bg');

txt = 'SELECT Tracking Area: Regions outside the box will be blanked.'; title(txt);
title(txt); set(label,'String',txt);
box = getrect(WTFigH); box = box+(box==0); h = rectangle('Position',box); set(h,'EdgeColor',[0,0,0]);
File(MovieNum).MaskBox = box+(box==0);

ar = 0;
% get tracking regions
txt = 'SELECT ARENA(S): click inside to confirm, outside to redo, Enter to end.';
title(txt); set(label,'String',txt);
success = 0; while success == 0
    box = getrect(WTFigH); box = box+(box==0); h = rectangle('Position',box); set(h,'EdgeColor',[1,0,0]);
    [X,Y,button] = ginput(1);
    if length(button) == 0
        success = true;
    elseif button == 1 & (X-box(1) >= 0 & X-box(1) <= box(3) & Y-box(2) >= 0 & Y-box(2) <= box(4))
        ar = ar+1; % arena #
        File(MovieNum).Arena(ar).TrackBox = box+(box==0);
        set(h,'EdgeColor',[0,0,1]);
        ht = text(box(1)+box(3)/2,box(2)+box(4)/2,num2str(ar)); set(ht,'FontSize',36,'Color',[0 0 1]);
    end
end

File(MovieNum).NumArenas = ar;

TrackDye = get(h3,'Value');
% get dye information
for a = 1:ar
    if TrackDye
        if AutoSelectDyeTrack
            box = File(MovieNum).Arena(a).TrackBox;
            File(MovieNum).Arena(a).UpBox = [box(1)-15-bs box(2)+box(4)/2-2*bs 2*bs 4*bs];
            rectangle('Position',File(MovieNum).Arena(a).UpBox); text(X+10,Y,[num2str(a),'up']);
            File(MovieNum).Arena(a).DnBox = [box(1)+box(3)+15-bs box(2)+box(4)/2-2*bs 2*bs 4*bs];
            rectangle('Position',File(MovieNum).Arena(a).DnBox); text(X+10,Y,[num2str(a),'dn']);
        else
            txt = ['Pick upstream and downstream points for Arena: ',num2str(a)];
            title(txt); set(label,'String',txt);
            [X,Y] = ginput(1); File(MovieNum).Arena(a).UpBox = [X-bs Y-2*bs 2*bs 4*bs];
            rectangle('Position',File(MovieNum).Arena(a).UpBox); text(X+10,Y,[num2str(a),'up']);
            [X,Y] = ginput(1); File(MovieNum).Arena(a).DnBox = [X-bs Y-2*bs 2*bs 4*bs];
            rectangle('Position',File(MovieNum).Arena(a).DnBox); text(X+10,Y,[num2str(a),'dn']);
        end
    end
    File(MovieNum).Arena(a).UpBox = [1 1 1 1];
    File(MovieNum).Arena(a).DownBox = [1 1 1 1];
end
end

```

```

end

% get scale info
txt = ['GET SCALING: pick points on top and bottom arena edges = ',get(h4,'String'),'mm'];
title(txt); set(label,'String',txt);
[X,Y] = ginput(2);
arenapix = abs(Y(2)-Y(1));

% extra rectangular region for exclusion
if exist('blankrect')
    txt = 'SELECT EXTRA EXCLUSION REGION';
    title(txt); set(label,'String',txt);
    box = getrect(WTFigH); box = box+(box==0);
    blankrect = round(max(box,1));
end

while input('All settings made? (y/n)','s') ~= 'y';
end

File(MovieNum).MovieFile = Name(MovieNum).MovieFile;
File(MovieNum).TrackFile = Name(MovieNum).TrackFile;

%File(MovieNum).TimeStamp = get(h1,'Value');
File(MovieNum).TrackFrames = str2num(get(h2,'String'));
File(MovieNum).StartFrame = str2num(get(h2a,'String'));
%File(MovieNum).Gradient = get(h3,'Value');
File(MovieNum).DyeTracked = get(h3,'Value');
File(MovieNum).ArenaSize = str2num(get(h4,'String'));
File(MovieNum).PixelSize = arenapix / File(MovieNum).ArenaSize;
File(MovieNum).ImageSize = MovSize;
File(MovieNum).FrameRate = str2num(get(h5,'String'));
File(MovieNum).Fragments = str2num(get(h2b,'String'));
File(MovieNum).MinMax = [str2num(get(h6a,'String')), str2num(get(h6b,'String'))];
File(MovieNum).StartFragment = 1;
File(MovieNum).CompletedFragments = zeros(1,File(MovieNum).Fragments);
File(MovieNum).ClaimedFragments = zeros(1,File(MovieNum).Fragments);
File(MovieNum).Stitched = 0;

hi = File(MovieNum).TrackFrames;
lo = File(MovieNum).StartFrame;
NumFragments = File(MovieNum).Fragments;
perfrag = round((hi-lo)/NumFragments/60)*60;
frfr = [lo+(0:(NumFragments-1))*perfrag];
frfr = [frfr, [frfr(2:NumFragments)-1; hi]];

File(MovieNum).FragmentFrames = frfr;

disp([datestr(now),' Image Data Collected for Movie ',num2str(MovieNum)]);
FileSettings = File(MovieNum);
save(TrackSettingsName,'FileSettings');
end

end

```

```

save(StatusFile,'File');

end

%-----
% Start Tracker
%-----
for MovieNum = find(~[File.Stitched]);

    % Gradient = File(MovieNum).Gradient;
    FrameNum = File(MovieNum).TrackFrames;
    Start = File(MovieNum).StartFrame;
    % TimeStamp = File(MovieNum).TimeStamp;
    ImageSize = File(MovieNum).ImageSize;
    FrameRate = File(MovieNum).FrameRate;
    PixelSize = File(MovieNum).PixelSize;
    FragmentFrames = File(MovieNum).FragmentFrames;
    MinWormArea = File(MovieNum).MinMax(1);
    MaxWormArea = File(MovieNum).MinMax(2);
    NumArenas = File(MovieNum).NumArenas;
    TrackDye = File(MovieNum).DyeTracked;
    NumFragments = File(MovieNum).Fragments;

    if NumArenas >= 2
        xdiv = mean([sum(File(MovieNum).Arena(1).TrackBox([1 3])),File(MovieNum).Arena(2).TrackBox(1))]; % x
        separation between arenas
    end
    if NumArenas == 4
        ydiv = mean([sum(File(MovieNum).Arena(1).TrackBox([2 4])),File(MovieNum).Arena(3).TrackBox(2))]; % y
        separation between arenas
    end

    if MakeLocalCopy
        TrackName = LocalDirectory(File(MovieNum).TrackFile);
        MovieName = LocalDirectory(File(MovieNum).MovieFile);

        % copy movie file if not already in local directory
        if ~strcmp(File(MovieNum).MovieFile,MovieName)
            p = fileparts(MovieName); if exist(p) == 0 mkdir(p); end % make local copy folder if needed
            disp([datestr(now),': Copying moviefile to local directory']);
            [s,mess,messid] = copyfile(File(MovieNum).MovieFile,MovieName);
            disp([datestr(now),': Complete... ',mess]);
        end
    else
        TrackName = File(MovieNum).TrackFile;
        MovieName = File(MovieNum).MovieFile;
    end

    [pathname,filename,ext] = fileparts(TrackName);
    partsfolder = '\trackfile parts\';
    FragmentSaveNames = {};
    for i = 1:File(MovieNum).Fragments;
        FragmentSaveNames = [FragmentSaveNames; {[pathname,partsfolder,filename,'_part',num2str(i),ext]};];
    end
end

```

```

if exist([pathname,partsfolder]) == 0 mkdir([pathname,partsfolder]); end;

[p,ShortMovieName] = fileparts(MovieName);

% FileInfo = aviinfo(MovieName);
% FileInfo2 = aviinfo(MovieName,'Robust');
Tracks = [];
AllData = [];
DyeData = [];
DyeData.Time = []; %DyeData.Up = []; DyeData.Down = [];
DyeData.Background = [];
ExpData = [];

%Video Mask: remove bright or dim pixels (deals with timer)
MovieObj = VideoReader(MovieName);
Mov = read(MovieObj, MovieObj.NumberOfFrames);
MovData = Mov(:,:,3);
MaskPix = 10; MaskBorder = 30;
% Mask = (MovData <= MaskPix | MovData >= 255-MaskPix);
% Mask = imdilate(Mask,strel('square',3));
Mask = ones(size(MovData));

% added 20110504
% Mask(MaskBorder:ImageSize(1), MaskBorder:ImageSize(2)-MaskBorder) = 0;
% Mask(MaskBorder:ImageSize(1)-MaskBorder, MaskBorder:ImageSize(2)-MaskBorder) = 0;
% for a=1:File(MovieNum).NumArenas
%     arenatrackbox = File(MovieNum).Arena(a).TrackBox;
%     Mask(round(arenatrackbox(2)+(0:arenatrackbox(4))),MaskBorder:ImageSize(2)-MaskBorder) = 0;
% end
if isfield(File,'MaskBox') % may need error handling here
    Mask(round(File(MovieNum).MaskBox(2)+(0:File(MovieNum).MaskBox(4))), ...
        round(File(MovieNum).MaskBox(1)+(0:File(MovieNum).MaskBox(3)))) = 0;
end

if exist('blankrect')
    Mask(blankrect(2)+(0:blankrect(4)), blankrect(1)+(0:blankrect(3))) = 1;
end

tic;

%-----
% ANALYZE FRAGMENTS
%-----

claimed = File(MovieNum).ClaimedFragments;
completed = File(MovieNum).CompletedFragments;

while ~all(claimed) % if any unclaimed fragments left...
    fragments_left = find(~claimed);
    next_fragment = min(fragments_left);

    processors_used = sum(claimed & ~completed);
    if processors_used < min(num_processors, max_processors)

```

```

claimed(next_fragment) = 1;
File(MovieNum).ClaimedFragments = claimed;
save(StatusFile,'File');

% SPAWN new FragmentTracker
Fragment = next_fragment;
disp([datestr(now), ' spawning for fragment ',int2str(Fragment),'/',int2str(NumFragments)]);
disp([' Fragments completed: ',sprintf('%1d ',completed)]);
disp([' Fragments analyzing: ',sprintf('%1d ',claimed & ~completed)]);
fprintf('\n\n');

SpawnSetting.Save = 1; % save master workspace; slaves will load
SpawnSetting.Exit = 0; % don't exit from master tracker
SpawnSetting.MatlabFlags = '-noFigureWindows -nodisplay -automation';
SpawnSetting.Startup = ['Fragment=',int2str(Fragment),';FragmentTracker_20100129;'];
spawn;

end

pause(check_status_interval);

% parse fragment status files
StatusD = dir(fullfile(TempFolder, '_F*')); StatusLine = [StatusD.name];
StatusM = zeros(1,NumFragments);
a = strfind(StatusLine, '!'); b = [strfind(StatusLine, '_'),length(StatusLine)+1]; b = circshift(b,[0 -1]); aF =
strfind(StatusLine, 'F');
for c = 1:length(a); StatusM(str2num(StatusLine(aF(c)+1:a(c)-1))) = str2num(StatusLine(a(c)+1:b(c)-1)); end
% get percent progress from each fragment tracker

% show status window
figure(StatusFigH);
bar(StatusM); set(gca,'YLim',[0 100]);
ylabel('Completed (%)'); xlabel('Fragment Number');
title([datestr(now), ' ',mat2str(StatusM)],[sprintf(['%d/%d]
',MovieNum,length(File)),File(MovieNum).TrackFile]','Interpreter','none');

% update current status
loadsuccess = 0;
while ~loadsuccess
    try
        load(StatusFile,'File');
        loadsuccess = 1;
    catch
        disp('Error loading status file. Retrying...');
        pause(5);
    end
end
claimed = File(MovieNum).ClaimedFragments;
completed = File(MovieNum).CompletedFragments;

end % END for file fragments

disp('all fragments seeded. waiting for slaves to finish computation...')

```

```

% Check that all fragments are completed
while length(dir([pathname,partsfolder,filename,'*'])) < NumFragments
    % disp([datestr(now),'found
,int2str(length(dir([pathname,partsfolder,filename,'*'])),')',int2str(NumFragments),' completed fragments.' ])
    % dir([pathname,partsfolder,filename,'*'])
    pause(check_status_interval)

    % parse fragment status files
    StatusD = dir(fullfile(TempFolder,'_F*')); StatusLine = [StatusD.name];
    StatusM = zeros(1,NumFragments);
    a = strfind(StatusLine,' '); b = [strfind(StatusLine,'_'),length(StatusLine)+1]; b = circshift(b,[0 -1]); aF =
strfind(StatusLine,'F');
    for c = 1:length(a); StatusM(str2num(StatusLine(aF(c)+1:a(c)-1))) = str2num(StatusLine(a(c)+1:b(c)-1)); end
% get percent progress from each fragment tracker

    % show status window
    figure(WTFigH);
    bar(StatusM); set(gca,'YLim',[0 100]);
    ylabel('Completed (%)'); xlabel('Fragment Number');
    title([datestr(now),' ',mat2str(StatusM)],sprintf(['%d/%d
',MovieNum,length(File)],File(MovieNum).TrackFile]'),'Interpreter','none');
end

disp('found all files. start stitching.')

% Save Combined File
StitchTracks(FragmentSaveNames,TrackName);

% update current status
loadsuccess = 0;
while ~loadsuccess
    try
        load(StatusFile,'File');
        loadsuccess = 1;
    catch
        disp('Error loading status file. Retrying...');
        pause(5);
    end
end
File(MovieNum).Stitched = 1;
save(StatusFile,'File');

% Copy files back to original location
disp([datestr(now),' Copying tracks file from local directory']);
[s,mess,messid] = copyfile(TrackName,File(MovieNum).TrackFile);
disp([datestr(now),' Complete... ',mess]);

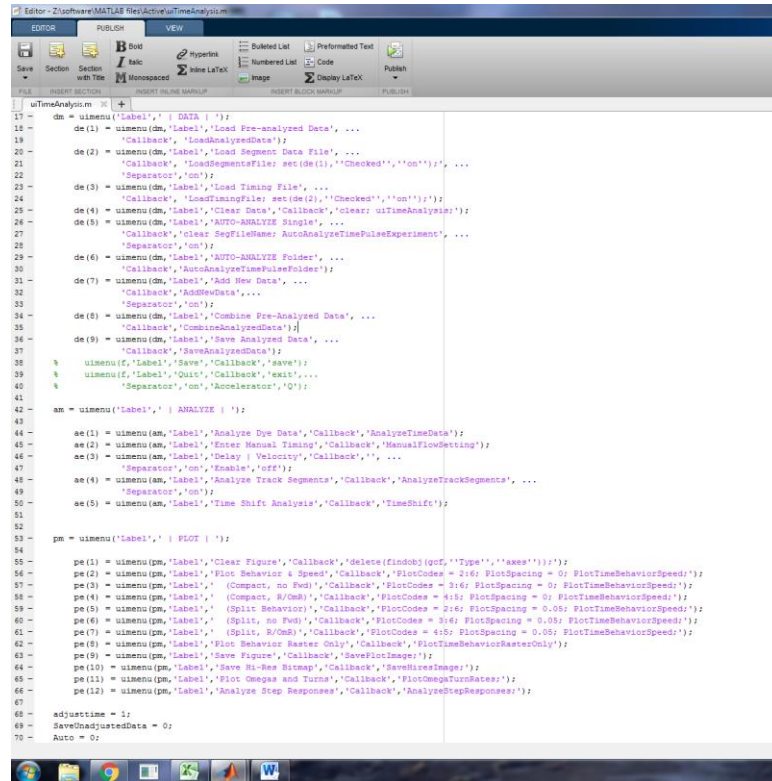
disp([datestr(now),' Copying avi.mat file(s) from local directory']);
[s,mess,messid] = copyfile([MovieName,'*.mat'],fileparts(File(MovieNum).TrackFile));
disp([datestr(now),' Complete... ',mess]);
end % END for i = 1:FileNameSize(1)

```

```
%if all([File.CompletedFragments])
```

```
% if Q  
% quit  
% end
```

uiTimeAnalysis



```
17 dm = uimenu('Label',' | DATA | ');  
18 de(1) = uimenu(dm,'Label','Load Pre-analyzed Data', ...  
19 'Callback','LoadAnalyzedData');  
20 de(2) = uimenu(dm,'Label','Load Segment Data File', ...  
21 'Callback','LoadSegmentsFile; set(de(1),''Checked'',''on'');', ...  
22 'Separator','on');  
23 de(3) = uimenu(dm,'Label','Load Timing File', ...  
24 'Callback','LoadTimingFile; set(de(2),''Checked'',''on'');');  
25 de(4) = uimenu(dm,'Label','Clear Data', 'Callback','clear: uiTimeAnalysis');  
26 de(5) = uimenu(dm,'Label','AUTO-ANALYZE Single', ...  
27 'Callback','clear: SegFileName: AutoAnalyzeTimePulseExperiment', ...  
28 'Separator','on');  
29 de(6) = uimenu(dm,'Label','AUTO-ANALYZE Folder', ...  
30 'Callback','AutoAnalyzeTimePulseFolder');  
31 de(7) = uimenu(dm,'Label','Add New Data', ...  
32 'Callback','AddNewData',...  
33 'Separator','on');  
34 de(8) = uimenu(dm,'Label','Combine Pre-Analyzed Data', ...  
35 'Callback','CombineAnalyzedData');  
36 de(9) = uimenu(dm,'Label','Save Analyzed Data', ...  
37 'Callback','SaveAnalyzedData');  
38 % uimenu(f,'Label','Save','Callback','save');  
39 % uimenu(f,'Label','Quit','Callback','exit',...  
40 'Separator','on','Accelerator','Q');  
41 %  
42 am = uimenu('Label',' | ANALYZE | ');  
43 %  
44 ae(1) = uimenu(am,'Label','Analyze Dye Data','Callback','AnalyzeTimeData');  
45 ae(2) = uimenu(am,'Label','Enter Manual Timing','Callback','ManualFlowSetting');  
46 ae(3) = uimenu(am,'Label','Delay | Velocity','Callback','', ...  
47 'Separator','on','Rmbd','off');  
48 ae(4) = uimenu(am,'Label','Analyze Track Segments','Callback','AnalyzeTrackSegments', ...  
49 'Separator','on');  
50 ae(5) = uimenu(am,'Label','Time Shift Analysis','Callback','TimeShift');  
51 %  
52 %  
53 pm = uimenu('Label',' | PLOT | ');  
54 %  
55 pe(1) = uimenu(pm,'Label','Clear Figure','Callback','delete(findobj(gcf,''Type'',''axes''))');  
56 pe(2) = uimenu(pm,'Label','Plot Behavior & Speed','Callback','PlotCodes = 216; PlotSpacing = 0; PlotTimeBehaviorSpeed');  
57 pe(3) = uimenu(pm,'Label',' (Compact, no Fwd)','Callback','PlotCodes = 316; PlotSpacing = 0; PlotTimeBehaviorSpeed');  
58 pe(4) = uimenu(pm,'Label',' (Compact, R/OM)','Callback','PlotCodes = 416; PlotSpacing = 0; PlotTimeBehaviorSpeed');  
59 pe(5) = uimenu(pm,'Label',' (Split Behavior)','Callback','PlotCodes = 516; PlotSpacing = 0.05; PlotTimeBehaviorSpeed');  
60 pe(6) = uimenu(pm,'Label',' (Split, no Fwd)','Callback','PlotCodes = 316; PlotSpacing = 0.05; PlotTimeBehaviorSpeed');  
61 pe(7) = uimenu(pm,'Label',' (Split, R/OM)','Callback','PlotCodes = 416; PlotSpacing = 0.05; PlotTimeBehaviorSpeed');  
62 pe(8) = uimenu(pm,'Label','Plot Behavior Rates Only','Callback','PlotTimeBehaviorRatesOnly');  
63 pe(9) = uimenu(pm,'Label','Save Figure','Callback','SavePlotImage');  
64 pe(10) = uimenu(pm,'Label','Save Hi-Res Bitmap','Callback','SaveHiResImage');  
65 pe(11) = uimenu(pm,'Label','Plot Omegas and Turns','Callback','PlotOmegaTurnRates');  
66 pe(12) = uimenu(pm,'Label','Analyze Step Responses','Callback','AnalyzeStepResponses');  
67 %  
68 adjusttime = 1;  
69 SaveUnadjustedData = 0;  
70 Auto = 0;
```

```
% Check MATLAB path  
if isempty(strfind(matlabpath,'uiTimeAnalysis'))  
AddPathBelow('CHOICE ASSAY');  
end
```

```
FigH = figure(1);  
clf;
```

```
ExptsLoaded = 0;  
StartDelay = 0;  
Velocity = 0;  
CombineCycles = 0;
```

```
%-----[ Set Menu Items ]-----
```

```
bl = uimenu('Label',' ');  
dm = uimenu('Label',' | DATA | ');  
de(1) = uimenu(dm,'Label','Load Pre-analyzed Data', ...  
'Callback',' LoadAnalyzedData');  
de(2) = uimenu(dm,'Label','Load Segment Data File', ...  
'Callback',' LoadSegmentsFile; set(de(1),''Checked'',''on'');', ...
```



```

    'Separator','on');
de(3) = uimenu(dm,'Label','Load Timing File', ...
    'Callback','LoadTimingFile; set(de(2),"Checked","on");');
de(4) = uimenu(dm,'Label','Clear Data','Callback','clear; uiTimeAnalysis;');
de(5) = uimenu(dm,'Label','AUTO-ANALYZE Single', ...
    'Callback','clear SegFileName; AutoAnalyzeTimePulseExperiment', ...
    'Separator','on');
de(6) = uimenu(dm,'Label','AUTO-ANALYZE Folder', ...
    'Callback','AutoAnalyzeTimePulseFolder');
de(7) = uimenu(dm,'Label','Add New Data', ...
    'Callback','AddNewData',...
    'Separator','on');
de(8) = uimenu(dm,'Label','Combine Pre-Analyzed Data', ...
    'Callback','CombineAnalyzedData');
de(9) = uimenu(dm,'Label','Save Analyzed Data', ...
    'Callback','SaveAnalyzedData');
% uimenu(f,'Label','Save','Callback','save');
% uimenu(f,'Label','Quit','Callback','exit',...
%     'Separator','on','Accelerator','Q');

am = uimenu('Label',' | ANALYZE | ');

ae(1) = uimenu(am,'Label','Analyze Dye Data','Callback','AnalyzeTimeData');
ae(2) = uimenu(am,'Label','Enter Manual Timing','Callback','ManualFlowSetting');
ae(3) = uimenu(am,'Label','Delay | Velocity','Callback','', ...
    'Separator','on','Enable','off');
ae(4) = uimenu(am,'Label','Analyze Track Segments','Callback','AnalyzeTrackSegments', ...
    'Separator','on');
ae(5) = uimenu(am,'Label','Time Shift Analysis','Callback','TimeShift');

pm = uimenu('Label',' | PLOT | ');

pe(1) = uimenu(pm,'Label','Clear Figure','Callback','delete(findobj(gcf,"Type","axes"));');
pe(2) = uimenu(pm,'Label','Plot Behavior & Speed','Callback','PlotCodes = 2:6; PlotSpacing = 0;
PlotTimeBehaviorSpeed;');
pe(3) = uimenu(pm,'Label',' (Compact, no Fwd)','Callback','PlotCodes = 3:6; PlotSpacing = 0;
PlotTimeBehaviorSpeed;');
pe(4) = uimenu(pm,'Label',' (Compact, R/OmR)','Callback','PlotCodes = 4:5; PlotSpacing = 0;
PlotTimeBehaviorSpeed;');
pe(5) = uimenu(pm,'Label',' (Split Behavior)','Callback','PlotCodes = 2:6; PlotSpacing = 0.05;
PlotTimeBehaviorSpeed;');
pe(6) = uimenu(pm,'Label',' (Split, no Fwd)','Callback','PlotCodes = 3:6; PlotSpacing = 0.05;
PlotTimeBehaviorSpeed;');
pe(7) = uimenu(pm,'Label',' (Split, R/OmR)','Callback','PlotCodes = 4:5; PlotSpacing = 0.05;
PlotTimeBehaviorSpeed;');
pe(8) = uimenu(pm,'Label','Plot Behavior Raster Only','Callback','PlotTimeBehaviorRasterOnly');
pe(9) = uimenu(pm,'Label','Save Figure','Callback','SavePlotImage;');
pe(10) = uimenu(pm,'Label','Save Hi-Res Bitmap','Callback','SaveHiresImage;');
pe(11) = uimenu(pm,'Label','Plot Omegas and Turns','Callback','PlotOmegaTurnRates;');
pe(12) = uimenu(pm,'Label','Analyze Step Responses','Callback','AnalyzeStepResponses;');

adjusttime = 1;
SaveUnadjustedData = 0;
Auto = 0;

```

