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A New Generation of Maze for a *Drosophila* Olfactory Memory Assay

Alyssa Risner[1], Shawn Scialabba[2], Phillip Strickland[3], Alvina Emran[1]
[1]Biology, [2]Physics, and [3]Computing Science
Coastal Carolina University
P.O. Box 261954
Conway, South Carolina, 29528, USA

Faculty Advisors: Varavut Limpasuvan[2] and Fang Ju Lin[1]

Abstract

An olfactory memory assay tests the ability of *Drosophila* to be trained with and recall a conditioned stimulus in the form of an electrical shock paired with an odor. Traditionally, the memory experiments are performed in a T-maze consisting of a training chamber that provides an electrical shock and an elevator to transport the flies to a choice point where two odors are introduced. Learning and memory are measured by the flies' aversion to the conditioned stimulus. However, memory assay set-ups vary greatly since the T-maze is not commercially available, and the associated elevator mechanism tends to kill many flies. These problems potentially affect the measurable behavior and reduce the number of test subjects. To address these drawbacks, a new maze prototype was designed and fabricated. Consisting of multiple parts (unlike the traditional T-maze), this new maze can be easily replicated by others using a 3-D printer at a relatively low cost. The innovative design includes a detached training chamber (with a flexible copper circuit) and allows for ease of assembly and cleaning. Instead of an elevator mechanism, the new design employs compressed air to help transfer the flies from the training chamber to the choice point while minimizing fly casualties. Preliminary trials of this prototype with *Drosophila* were conducted, which suggest an improved way for administering olfactory memory assays.

Keywords: *Drosophila*, Olfactory Memory, Alzheimer's disease

1. Introduction

Olfactory memory assays are used in biological research to understand the development of neurodegenerative diseases like Alzheimer's disease. In humans, a known symptom of Alzheimer's disease is the accumulation of amyloid-β protein plaques in the brain due to a mutation in the amyloid precursor protein (APP) gene[1]. Similar disruptions in the neurons of *Drosophila* can be induced by the integration of the human APP gene into the genome[1]. The accumulation of amyloid-β plaques in the central brain of *Drosophila* has been found to impair the associative learning and memory of odors, which can be tested using an olfactory memory assay[2]. As such, transgenic flies with the mutated human APP gene are primarily used in olfactory memory assays to act as a model to study Alzheimer's disease[3].

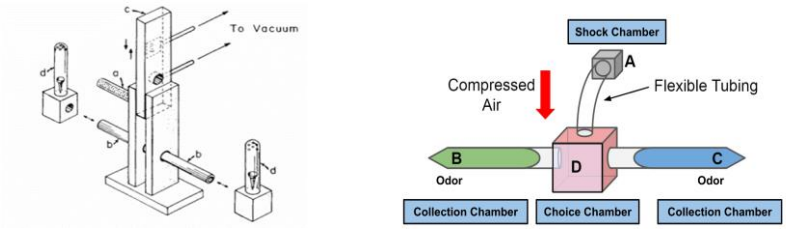

Figure 1. (Left) Traditional T-Maze[6]. (Right) Basic design of the new maze with four main housing parts (A-D).

An olfactory memory assay is traditionally performed in a T-maze[4] (Figure 1, left). The flies are first loaded into a shock chamber (a) for training and are presented with an odor paired with an electrical shock to act as a conditioned stimulus, followed by the presentation of a second odor without an electric shock as an unconditioned stimulus[4]. Once conditioning is complete, the flies are moved by a sliding elevator (c) to the choice points (b), where they are presented with the two stimuli used in training. Learning and memory is measured by an aversion to the conditioned stimulus and preference to the unconditioned stimulus[5].

However, this traditional T-maze has several drawbacks. Primarily, it is not commercially available to experimentalists who wish to perform the assay, and, as a result, each lab must build its own model. This leads to variations in the reported assay results and their interpretation. Additionally, the elevator mechanism used in transferring flies from the training chamber to the choice point kills a number of flies in the process, reducing the number of test subjects[7].

To address these issues, a new maze, along with a control device to administer shocks, was designed to be commercially viable, easily replicated, and to minimize casualties during the memory assay.

2. Housing Fabrication and Design

The new maze is a variant from the traditional T-Maze. Shown conceptually in Figure 1 (right), its main housing consists of four detachable pieces to allow for ease of set up and cleaning. Part A is the shock chamber where the *Drosophila* are administered odors and trained with an electric shock. Compressed air through a flexible tubing transfers flies from the shock chamber to the choice chamber (Part D). This chamber is inserted into the two collection chambers (Parts B and C), each holding a tube that contains the odors used during the training process.

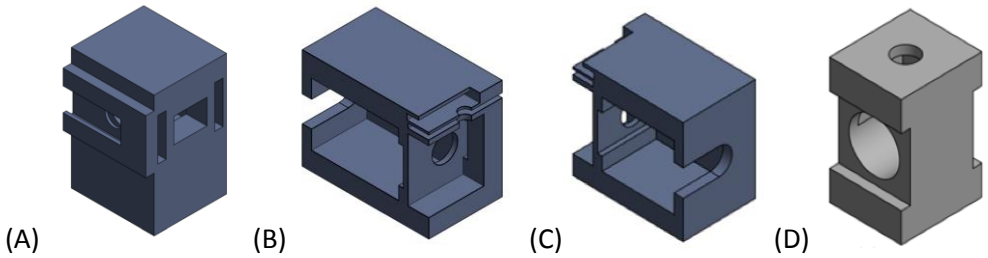

Figure 2. Three-dimensional representation of the housing parts of the new maze.

Figure 2 shows the detailed housing design using Autodesk Inventor 2016 software that allows the user to make and manipulate three-dimensional (3D) drawings[8]. This design was fabricated on a MakerBot 3D printer using polylactic acid (PLA) filaments, a non-toxic and highly durable bioplastic[9]. This printer supports a design with maximum dimensions of 4" x 4" x 4", and the printing cost is 10 cents per gram of filament. The printed pieces were sanded to remove excess printing materials and to ensure a precise fit.

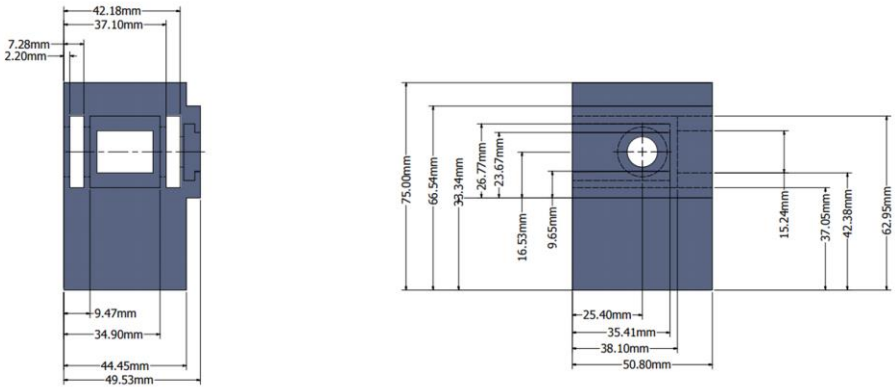

Figure 3. Detailed drawing of the shock chamber (Part A) from the front view (left) and right-side view (right).

The detailed drawing of Part A (the shock chamber) is shown in Figure 3 in metric units. Seen in the front view (left), Part A includes two narrow slots for removable slides to control the movement of flies (discussed below). A square opening (between the slots) accommodates a clamp holding the flexible capacitor for the shocking device. The smaller rectangular opening through the center of the shock chamber allows the leads of the capacitor to connect to the control device. A built-in bracket to attach a flexible tube (used to transfer the flies to the choice chamber) is detailed on the right-side view of Part A (right). Once slid into the bracket, the center of the flexible tubing aligns with the center of the circular opening of Part A.

Figure 4 shows the bottom piece of the aforementioned clamp. Together with a symmetric top piece, this clamp forms a cube with a cylindrical core (with an 18-mm diameter) to hold a rolled flexible capacitor in place. The electrical leads of the capacitor are fed through the side slit of clamp (when put together) and out the back of the shock chamber as discussed above.

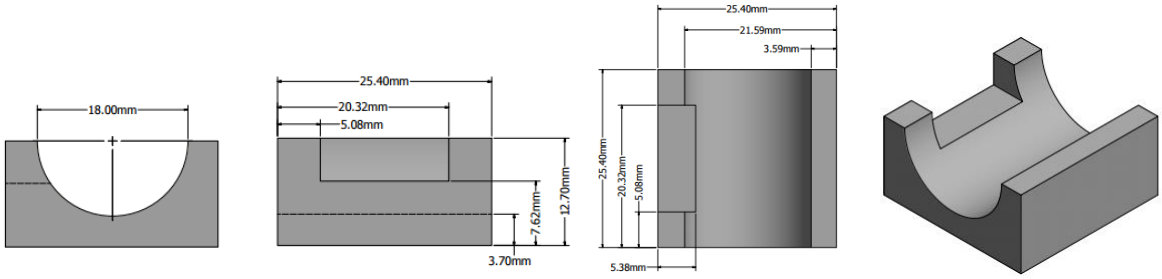

Figure 4. The front, left, top, and 3D view (respectively, from left to right) of the bottom clamp.

The removable slides associated with Part A (Figure 3) are shown in Figure 5. These slides seal the flies in the shock chamber and include a small raised handle to maneuver the slides into place. Inserted into the left narrow slot in Part A, the left slide has two circular holes; the first of which contains a mesh screen to allow for airflow into the chamber and the attachment of a 15-ml Falcon conical tube to administer the odors. The second hole is used to insert the flies into the shock chamber. The right slide shown in Figure 5 is inserted into the right narrow slot in Part A (closest to the bracket), and aids in transferring flies to the choice point via a built-in funnel whose surface is angled 124.6 degrees from the central axis of the circular opening.

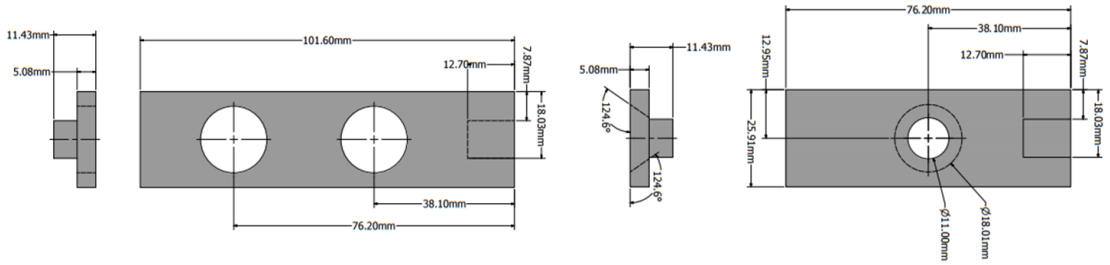

Figure 5. Slides related to the shock chamber (Part A).

Part B of the maze is detailed in Figure 6, and Part C is a symmetric mirror image of this design. These two parts hold the collection tubes into place, which are inserted into the front of the chamber and screwed into place. The slides shown in Figure 7 are used to seal the collection chambers during the memory assays. The slide shown on the left closes off the tube in Part B to safely remove it for counting the collected flies. A second identical slide is inserted in Part C for the same purpose. The slide shown on the right in Figure 7 is inserted into the upper slot of Part B and C to prevent the flies from moving backwards through the maze after their transfer, as well as aid in holding the two parts together

Finally, Part D is the choice chamber. This chamber is inserted into the collection chambers (Parts B and C) due to its I-shape detailed in Figure 8. In this chamber, the *Drosophila* flies are simultaneously exposed to the odors on either side from the collection chambers, and ultimately make a choice to avoid the conditioned stimulus.

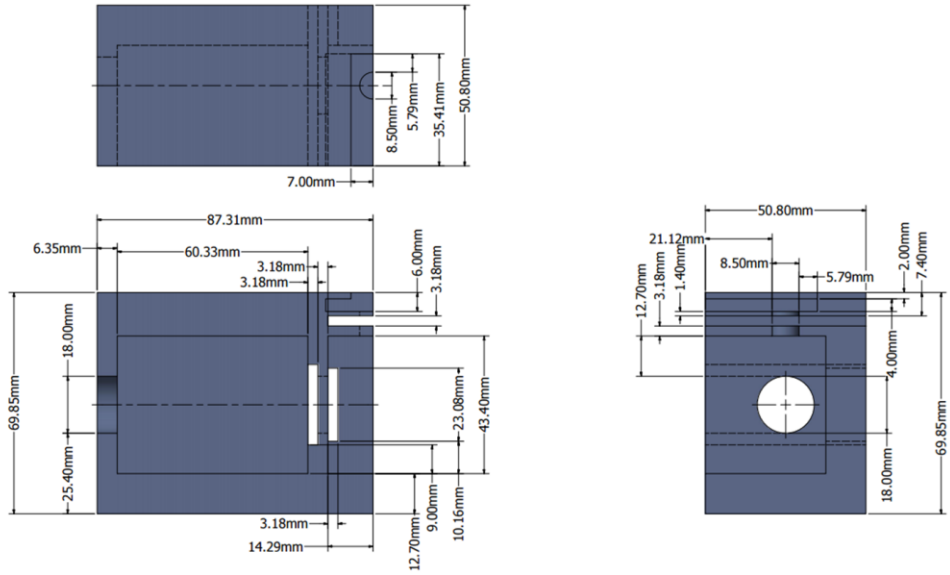

Figure 6. Detailed drawing of Part B from the top (top left), front (bottom left), and right-side view (bottom right).

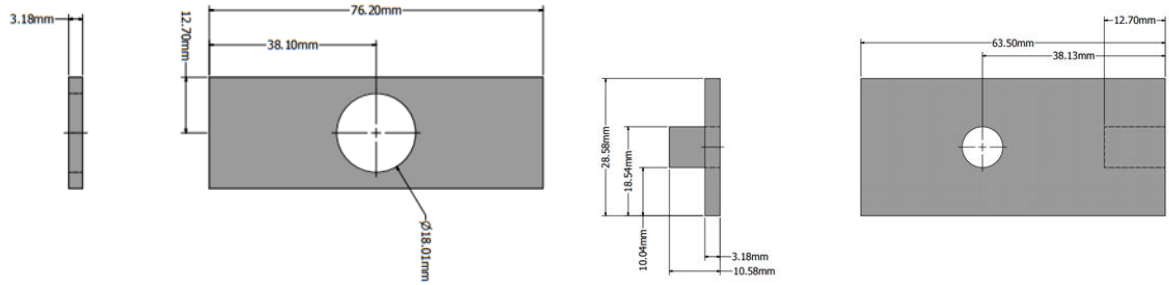

Figure 7. Slides that are inserted into the collection chambers (Parts B and C).

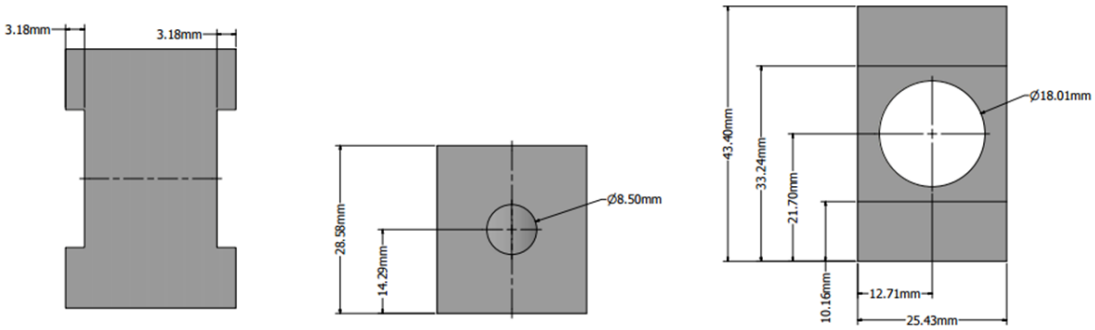

Figure 8. Detailed drawing of Part D from the front (left), top (center), and the right-side view (right).

The choice chamber is associated with two U-shaped slides with handles (Figure 9). The first slide (left) is inserted into the back of the collection chamber through the inner slots detailed in Part B (Figure 6), and is positioned in the grooves of the choice chamber (Part D) while it sits inside the collection chambers. This slide contains two holes on either side of the U-shaped arms, which are used to simultaneously open the choice point to expose the flies to the odors in the collection tubes. The second U-shaped slide (right) is inserted into the grooves of the choice chamber from the front while the slide in the back of the maze is removed. This usage seals the choice chamber for removal from the maze after the assay is complete to count the number of *Drosophila* flies that did not make a choice.

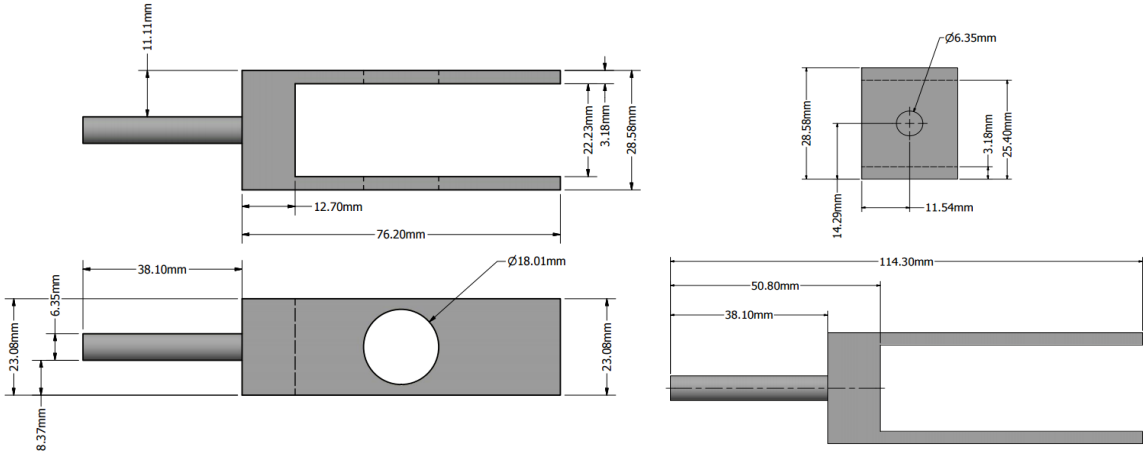

Figure 9. The U-shape slides related to the choice chamber (Part D). The slide that has two holes (left) is inserted in the back of the chamber, while the slide without holes (right) is inserted from the front.

3. Control Unit and Shocking Device

Figure 10 illustrates the schematics for the maze's control unit. It utilizes the household 120-Volt alternating current (VAC) power supply stepped down to 72 VAC by a transformer. The AC power is converted to a direct current (DC) 72-Volt power source consisting of two 36 VDCs connected in series. This power source can be adjusted by a potentiometer. A digital voltmeter displays the resulting DC voltage that charges the shock capacitor rolled into a cylinder and clamped inside the shock chamber (Figures 3 and 4). Following the potentiometer, a manual relay (Black Switch) cuts off the DC supply source when the unit is not in use.

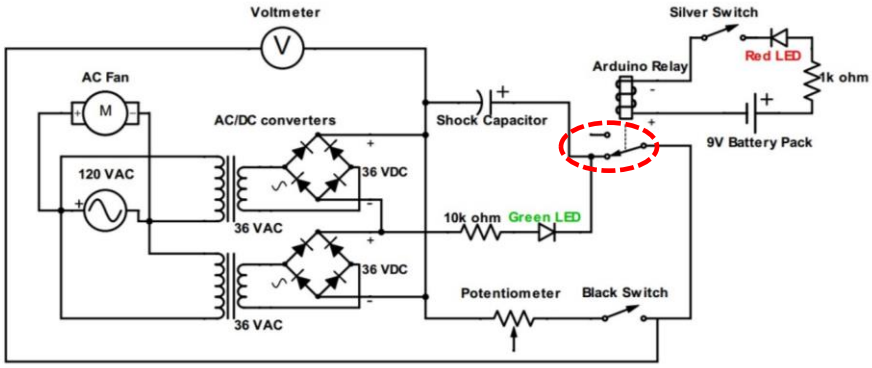

Figure 10. A schematic diagram of the control unit.

```
const int relayPin =8; void setup()
{
pinMode(relayPin, OUTPUT); //initialize relay as an output
}
/*************************************************/
void loop()
{
digitalWrite(relayPin, HIGH); // delay(3750); //open relay, allow no shock
digitalWrite(relayPin, LOW); // delay(1250); //close relay, shock
}
```

Figure 11. Arduino Script to control the power delivery to the shock capacitor. Text in red is given in milliseconds.

Driven by an Arduino UNO R3 microcontroller, a relay module regulates the timing of when the adjusted VDC charges the capacitor. By default, when the microcontroller is off (i.e., the Silver Switch in the open position), DC power is delivered continuously to the shock capacitor. When on, the relay solenoid opens the nearby gate (dashed red circle in Figure 10), stopping power delivery. When linked to a laptop through a universal serial bus (USB) port, the Arduino microcontroller can be programmed to control the time duration of the gate operation to the nearest millisecond with a script (Figure 11) written through the Arduino online tool found on www.arduino.cc. In this example script, the relay sets the gate operation to continually power the shock capacitor for 1.25 seconds and then wait 3.75 seconds before powering it again.

The circuit includes a simple lighting system to communicate with the user. A small green light emitting diode (LED) illuminates when current flows through the relay module to power the shock capacitor. The small red LED lights up when the Arduino microcontroller operates. Whenever the control unit is plugged into the 120 VAC, a red light appears and an AC fan (attached to the main 120 VAC power) starts to cool the circuitry.

When powered, the shock capacitor stores electrical charge. It discharges as the poles simultaneously contact the *Drosophila*'s legs during shocking. It consists of a copper etched grid with the positive and negative poles laid out in a comb pattern (Figure 12) using a freeware called ExpressPCB[10]. The pattern was etched onto a flexible Pyralux copper sheet (of 0.005 inches in thickness) to allow the circuit to be easily rolled into a cylinder.

Figure 12. The shock capacitor design. Each arm of the comb has a width of 0.64 millimeters with a spacing of 0.65 millimeters between each arm.

To initiate the etching process, the pattern was first printed onto toner transfer paper coated in Dextrin using a laser printer. The toner was transferred onto the Pyralux copper sheet using heat via an iron calibrated to melt the Dextrin coating. A high-quality transfer to the Pyralux was produced when heat was applied for 30 seconds, followed by a one-minute cooling period. Heat was then reapplied for three consecutive 10-second intervals with a cooling period of one minute between each interval. After the copper cooled, it was then placed in water to dissolve the Dextrin coating and remove the transfer paper from the copper, leaving the transferred design. Once a successful transfer was completed, the copper was submerged in a ferric chloride solution (38.8% $FeCl_3$, 0.20% $FeCl_2$, and 0.18% HCl) for 35 minutes, which etches away any excess material that was not coated in toner. The capacitor circuit was then rinsed with water to remove the etching solution and cleaned with acetone to remove the toner ink.

4. Laboratory Trial Procedure

4.1 Set-Up

The odorant compounds in the trials were 4-methylcyclohexanol (MCH) and isopentyl acetate (IPA). They were prepared by soaking cotton in 50 µl of each respective compound. The respectively soaked cotton was placed in separate 15-mL Falcon conical tubes. Two sets of MCH and IPA tubes were prepared; one set to act as the collection tubes that attach to the model at the collection chambers and choice point, and the second set to aid in odor delivery. A group of 20 male *Drosophila* (one week in age) were collected one day before the olfactory memory assay was performed using CO_2 and were placed in a holding vial containing Jazz-Mix *Drosophila* food. Only wild type flies were used to test the viability of the design.

4.2 Olfactory Memory Assay

For consistency, the assay was always performed at 2 PM. The *Drosophila* were transferred into the training chamber from the holding tube using a funnel with the right slide of the shock chamber in the closed position and the left slide in the open position. The flies were then sealed in the shock chamber and were given a 90 second rest to become accustomed to the inside of the chamber. After which, the training process began by attaching a tube containing one of the two odors, IPA or MCH. Standard 60-second training commenced, where the first odor was presented while simultaneously administering twelve 60-volt shocks lasting 1.25 seconds with a 3.75 second interval in between each shock[4]. Once shock training was complete, the flies were given a 30 second rest and the odor was removed from the chamber using a vacuum. After which, the second odor was presented for 60 seconds without a shock, again followed by a 30 second rest and removal of the second odor from the training chamber. To transfer the flies, a flexible connector tube was attached to both the training chamber and to the choice chamber of the maze via a bracket, and the chamber lifted and tilted from the base. The slides were set to the open position and an 18-psi compressed air pump was employed to aid in the transfer. The flies were given a 30 second resting period in the choice point, after which the back U-shape slide was placed in the open position. After two minutes, the collection tubes were sealed and the tubes removed from the housing for counting.

Control trials without odors or shocks were performed to observe the behavior of the flies inside the maze during the transfer process and to see if the maze had any impact of measurable behavior. The *Drosophila* were held in the shock chamber for a total of 3 minutes to simulate the amount of time they would be in the chamber during training, and then transferred to the choice chamber with compressed air. After a 30 second rest, the flies moved through the maze for 2 minutes, 5 minutes and 7 minutes to test the effects of the length of time in the collection chambers. A second set of control trials was performed where the flies were presented with each of the odors, but no shocks were administered. This was to observe if either odor acted as an attractant or repellent during the assay. After the control trials, complete olfactory memory assays with administered shocks were also performed. IPA was paired with the shock during the first set of trials, and MCH was paired with the shock in the second set of trials to eliminate the possibility that the odors used impacted the training of the observed behavior.

5. Results

5.1 Final Construction of the New Maze and Control Unit

Figure 13 shows the completed new maze. A plywood base for the maze was fabricated to stabilize the pieces while being manipulated during the experiments. The base has a U-shaped track made with wooden trim to guide and align the pieces together. Detailed videos explaining how the maze is assembled and used in a memory assay are posted online for reference[11, 12].

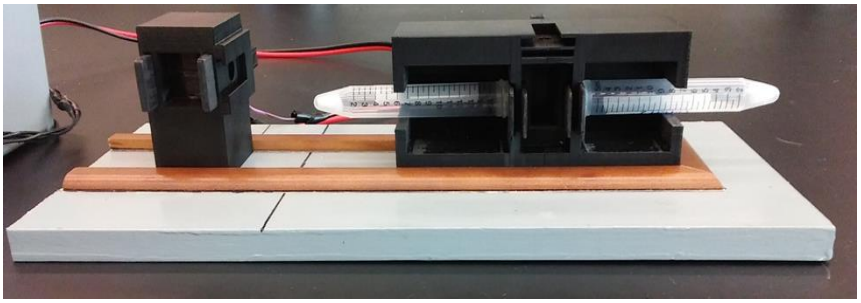

Figure 13. Front view of the new maze completely assembled, as it would be used for an olfactory memory assay with the collection tubes, slides and shock capacitor in place. Attachable flexible tubing is not shown.

Figure 14. The fabricated control unit (Left). View of the wiring inside the control unit (Center). Clamps holding the shock capacitor in the shape of a cylinder (Right).

The finalized control unit (Figure 14) is housed in a 10” x 10” electrical box (left). The manual power switch, the digital voltage display, and the light indicators are visible in front of the box. The manual toggle switch to turn on the Arduino microcontroller and the potentiometer dial are located on the right side of the box. The AC cooling fan is located on the left side of the electric box, and the battery pack and USB cord for the Arduino are positioned on the back of the electric box (not shown). The inner wiring of the control device is shown in Figure 14 (center). Lastly, the final etched capacitor is shown in Figure 14 (right) as it rests in the clamps (see Figure 4). These clamps hold the rolled the capacitor into a cylinder shape into which the flies are inserted and trained with a shock. Utilizing a multi-meter, the potential difference measured at the capacitor is equal to the voltage displayed by the digital voltage display to within 0.15 percent error.

The total cost for the fabrication of both the maze and the control device was $207, which is manageable for a laboratory that wishes to reproduce the model. A detailed expense chart is shown in Table 1.

Table 1. Expenses for the new maze and control unit.

Materials	Cost	Purpose
PCB Toner Transfer Paper (10 sheets)	$22	Etching Process
Flex PCB Pyralux (2 6"x6" sheets)	$14	
MG Chemical Ferric Chloride Solution (1Qt)	$15	
Electrical Box (10"x10")	$15	Control Unit
Electrical shrink wrap and tape	$10	
36V AC-DC converters (2)	$20	
9V battery pack	$5	
Arduino Uno R3	$26	
Relay switch compatible with Arduino	$5	
Cable	$3	
LED lights (2)	$2	
Digital voltage reader	$10	
10KOhm potentiometer	$20	
PLA filaments (400 grams)	$40	3D Printing
Total	**$207**	

5.2 Preliminary Trials with New Maze

During the control trials with neither odors nor shocks, all of the flies stayed in the choice chamber after being transferred. Little to no movement toward either collection chamber was observed during a 2-minute, 5-minute, or 7-minute time period. In the control trials in which the flies were exposed to both odors but not trained with a shock, movement toward the collection chambers increased due to the presence of a stimulus, but the majority remained in the choice chamber.

After the control trials, complete olfactory memory assays with the incorporation of the shocks were performed to test the viability of the capacitor and the control unit. A positive indication that the flies were being affected by the charged capacitor was the "jumping" behaviors normally observed in response to the presence of a shock[7]. As the relay module cycled on and off, the digital voltage indicator fluctuated roughly two volts, which was accounted for with the powering on and off of the green LED as the voltmeter does not measure the voltage across this device when the LED is off.

During the complete olfactory memory assay with 60 volts supplied to the shock capacitor at 1.25 seconds on and 3.75 seconds off for an experimental time of one minute, zero flies were found in the collection chamber containing the odor that acted as the conditioned stimulus, while activity increased in the chamber containing the odor that acted as the unconditioned stimulus. However, it was found that the flies were not as responsive to training with IPA as they were to training with MCH. Also, a majority of the flies still remained in the choice chamber during both sets of trials. An average of 4.5 flies were lost from the start of the assay to the end due to an error in handling the model, but no fly deaths were recorded during any of the trials.

6. Conclusion and Discussion

The main goal of this study was to design a new maze that (1) minimizes the casualties seen in the traditional experiment and (2) is easily reproducible to those who wish to perform an olfactory memory assay. As with the traditional T-maze, the newly designed maze trains *Drosophila* with a conditioned stimulus and is used to track olfactory learning. However, the transport system to move the flies from the shock chamber to the choice chamber has been improved upon by replacing the traditional elevator mechanism with a compressed air system and flexible tubing. Easy to clean and maneuverable, the detachable pieces of the new model were fabricated at low cost using a 3D printer, which increases the reproducibility of the design for other laboratories using the detailed drawings provided above.

Depending on the 3D printer, the fabrication process may not be precise and may lead to an accumulation of PLA filament. For this study using the MakerBot 3D printer, finished pieces needed to be sanded down to ensure an appropriate fit. A bowing of the filament inward in the slots for the slides in Part A was also an observed problem. However, this issue is unique to the chosen printer, and was not the result of a design flaw.

Results from the preliminary trials suggested that the choice chamber (Part D) needs to be modified since the flies preferred to remain in the chamber without making a choice during both the control trials and the complete olfactory memory assay. The amount of time spent in the maze did not impact behavior and it is likely the maze itself that is causing these behaviors. In its current design, the choice chamber may be too large, and during training the flies may choose to remain in the choice chamber to avoid the conditioned stimulus instead of moving toward the unconditioned stimulus and away from the conditioned stimulus. A possible change to the model to remedy this problem would be to constrict Part D to force the flies to make a choice outward from the choice chamber to the collection chambers.

Another area that can be explored and modified is the odor delivery system. Since training with IPA was not as successful as it was for MCH, it must be replaced with another odorant compound. Also, in the trials conducted, the odor may have not been successfully delivered and paired with the shock to create a conditioned stimulus because the odors were administered relying solely on diffusion. As a result, training may not have occurred due to an inadequate concentration of the odors in the chambers, which could also explain the low activity seen in the memory assay trials. The incorporation of an air pump to transfer the odor inside the chamber must be explored. Furthermore, the odors used may have also been too dilute, and further testing must be completed to determine the appropriate concentration.

The control device used in the preliminary trials performed as needed for the experiment. Having to manually displace the toggle switch added a small amount of error in the assay experiment due to human reaction time. The addition of a timer in the circuit would limit this as a factor in the experiment.

After the assembly of the maze and preliminary trials, the new maze meets the goals originally set for the design of minimizing casualties and of being reproducible and cost effective. There were no observed casualties from the transferring mechanism using compressed air or from the slides used, which is an improvement from traditional T-maze designs. While there was an average of 4.5 flies that were lost during the assay, this loss can be rectified by increasing the sample size of flies. Even though the choice chamber needs slight modification, the new design suggests an improved way of performing *Drosophila* olfactory memory assays.

7. Acknowledgements

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