Isolation of Abnormal Feeding Drosophila melanogaster Using Fluorescent Imaging

Submitted By
Sam Kortz

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE IN MECHANICAL ENGINEERING

School of Engineering
Tufts University
Medford, Massachusetts

May 2005

Signature of Author:
Sam Kortz

Certified By:
Professor Chris Rogers
Department of Mechanical Engineering
Tufts University

Committee:
Associate Professor Douglas M. Matson
Department of Mechanical Engineering
Tufts University

Committee:
Alan Kopin, M.D.
Director of Molecular Pharmacology Research
Tufts New England Medical Center

Abstract

The Tufts University Robotics Academy was approached by researchers at the New England Medical Center to expedite a time-intensive experimental procedure that identifies abnormal feeding genes in Drosophila melanogaster, or fruit flies. With over ten thousand mapped genetic lines, a daily throughput of twenty genetic lines called for testing automation. The Robotics Academy team designed a robotic system that tests 16 genetic lines in 35 minutes, compared to the manual 90 minutes, and quantifies the amount of food consumed by larvae.

Due to the constraints of the system, a new assay had to be developed to determine larval food intake. Following much experimentation, a fluorescent dye procedure was selected as a viable solution. Larvae consume this dyed food that is then excited under ultraviolet light. An image of the highlighted digestive tract is captured and processed in LabVIEW to numerically indicate the volume of food eaten. Members of the same genetic line were fed the fluorescent food for 40 and 60 minutes and were imaged. Analysis found the subjects to have highlighted regions that were 11% and 17.5% of volume, respectively. Therefore, fluorescent imaging is a valid structure to identify abnormal feeding within Drosophila melanogaster.

Acknowledgements

I would first like to thank Melissa Pickering for being such an excellent partner. Her relentless working ability and optimistic demeanor is unparalleled by anyone I know; who else flies halfway across the country to build a prototype in her father's woodshop? This is to the innumerous late nights spent down in TUFTL.

To the Tufts New England Medical Team, thank you for your continual help. Kerry, you were willing to drop whatever you were doing to help me with difficulties. Isabelle, you were always optimistic and provided me with invaluable information regarding the inner workings of the fruit fly larvae. Alan, this project would never have happened without your enthusiasm about combining life sciences and engineering.

Chris, thanks for setting the bar so high. Although it was a bumpy road, I came out with a strong understanding of what research entails. Keep playing disc and perhaps I'll stop by for a game down the road.

Table of Contents

List of Figures

I. Introduction

In industry, manufacturing companies implement automated solutions to increase labor and time efficiencies. Combining mechanical and electrical components through a computerized user-interface, a job that previously required 20 tedious labor hours by 20 individuals is reduced to 4 monitoring hours by 2 individuals. In addition to the reduction in time and labor, margin of error and cost are effectively reduced. The benefits of a stand-alone automated assembly system are apparent and can be applied to areas beyond industry, such as the research lab.

Researchers at the Tufts New England Medical Center are using fruit fly larvae to screen for gene descriptions that cause abnormalities in feeding. Current time and labor-intensive research has a throughput of 100 genetic lines per week. With over 10,000 different genetic lines, an automated testing regime would expedite screening of the complete set. The Tufts Robotics Academy Team was approached to design and prototype a robotic system that would expedite this assessment. The Team integrated talent from all fields necessary to design a successful system: mechanical engineers, electrical engineers, and engineering psychologists. Each team member was responsible for designing and testing a subsystem of the entire automated end product.

The integration of subsystems resulted in a full-scale feeding, washing, and image processing robot. Sixteen genetic lines of fruit fly larvae are tested each run during

which they are fed, cleaned, imaged, and categorized as either normal or abnormal feeders.

II. Background

At Tufts New England Medical Center, graduate student Kerry Garrity is conducting research on fruit flies as a model system to probe gene function relevant to mammals. Her research is currently screening mutant Drosophila lines with single gene insertions, thus providing a means to identify genes important in the control of feeding [1]. As so far, Kerry can test 100 different genetic lines each week out of the desired data range of up to 10,000 mutant lines obtained from Bloomington Drosophila Stock Center at Indiana University [2].

Fruit flies are being used because they possess positive attributes for the desired research goal. When studying food intake, it is important to isolate and control variables that could disrupt the results, such as locomotor activity and feeding time. Drosophila larvae in the third instar stage, four days after incubation, are ideal test subjects because they continually eat for the one day duration of the stage. Therefore, feeding time is equivalent to the amount of time spent within the food solution. A linear progression of a dye through the digestive tract can then be monitored.

The easy upkeep and propagation of fruit fly colonies also makes them good candidates for research. After receipt from the stock center, genetic lines are maintained in separate

vials where adult flies lay eggs in a yeast solution at the bottom of the container. Within days, there are hundreds of larvae at the third instar stage eligible for testing. Experimentation can then occur as fast as new genetic lines are ordered and received. Additionally, fruit flies are invertebrates so experiments involving fruit fly test subjects are not as tightly regulated by laws governing animal research.

For the past year, Kerry has been refining her assay and assessing genetic lines (~1000) [2]. Eggs from each genetic line are hatched and allowed to mature until the larvae reach the third instar stage. They are then removed from the standardized yeast food and cleaned. Due to the small size, 3 millimeters, of the larvae (***Figure 1***), the larvae have to be removed by hand using a fine paint brush. This is the most time intensive stage of the experiment and was a main focus in the design of the robotic system.

Figure 1: Size of Larvae (marks are in millimeters)

The washed larvae are then placed in a Drosophila Instant Food base with 10% Brewer's inactive yeast and 0.2% fast green dye. After eating the dyed food for 30 minutes at 25°C, 8 larvae are removed, rinsed, cleaned, inserted into a vial, and crushed into solution. Two vials of 8 larvae are generated to insure accuracy and the tubes are

centrifuged for 20 minutes. An optical density reading of the supernatant is then taken at 625nm, the peak absorption wavelength of the dye (See ***Appendix I*** for a step-by-step guide to the manual procedure). A higher optical density, OD, reading reflects more food consumption for the tested genetic line.

Kerry Garrity verified the accuracy of this assay by feeding larvae the dyed solution for different amounts of time. Larvae fed for longer time periods have a higher OD reading than larvae fed for shorter time periods. ***Figure 2*** indicates a linear response, up to 60 minutes, of food intake to feeding time [2].

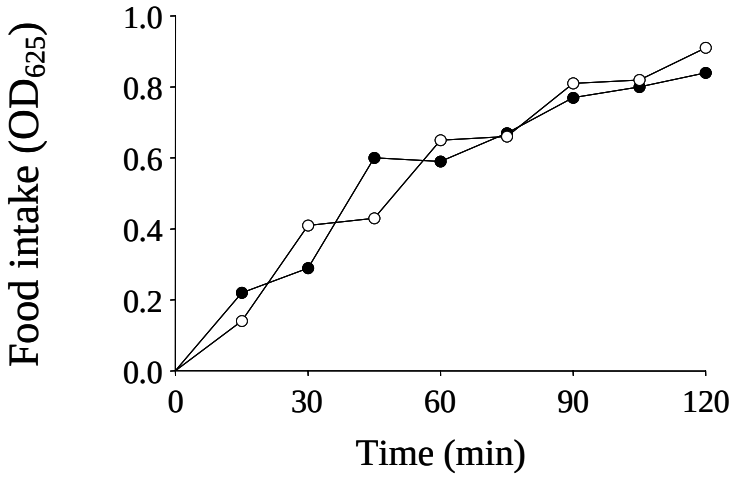


Figure 2: Two Data Sets of Feeding Time vs. Food Intake

Linear results, through 60 minutes, demonstrate that the assay can differentiate the amount consumed by the larvae. Kerry then optimized a constant feeding time to test for abnormal genetic outliers. A 30 minute feeding time the ability to detect both high and low feeders.

Kerry Garrity has been conducting research with this method for the past year and has tabulated 700 out of the 10,000 genetic lines. However, the long term goal of this testing is not to identify abnormal fruit fly lines but rather the genes that cause irregular feeding. The implementation of the robotic system will enable man hours to be spent on gene function analysis first in fruit flies and eventually in mammals. .

III.Project Overview

The goal of this project is to **design and develop of a robotic system to efficiently quantify the amount of food consumed by Drosophila larvae**. The system consists of two subsystems: (1) feeding, cleaning, and separation of specimen, and (2) the collection and analysis of data.

In order to clarify the scope of the automated fruit fly analysis system, each subsystem and their respective components are graphically organized in ***Figure 3***.

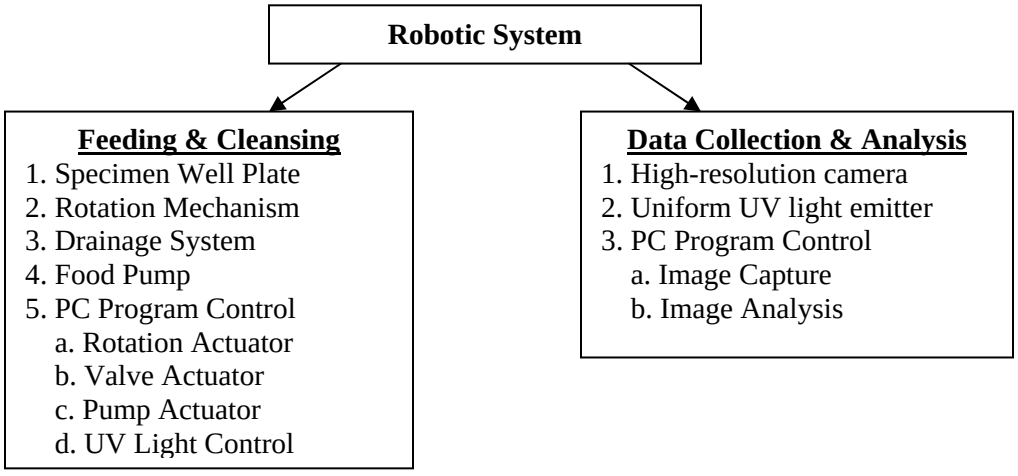


Figure 3: Organization of Automated Fruit Fly Analysis System.

The development of the system was a two stage process. First the automated system would be designed to produce equivalent results to the manual procedure. Once that system was operational, the speed and throughput would be increased to tackle the goal of testing all 10,000 genetic lines.

The design of the system was distributed amongst four team members. Melissa Pickering, a mechanical engineer, acted as the lead system designer and was in charge of washing and feeding the larvae. I designed a new testing procedure involving fluorescent food and compiled image processing software to quantify the amount of food consumed by the larvae. Matthew Magpayo, an electrical engineer, produced a controller board that managed the functions of the rotary table, valves, food pump, and ultraviolet fluorescent tube. Kristen Butler, an engineering psychologist, assisted me in designing the appearance and functionality of the user interface for the control program.

The final system automatically conducts experimentation and analyzes 16 genetic lines in 35 minutes (See ***Apendix II*** for system images). Testing begins by filling each well with a standard yeast food in which eggs can grow into the third instar stage. Parents are enclosed within the wells for 24 hours to lay eggs. This short egg deposition time ensures that all eggs will be at the third instar stage after 4 days. Following the manual loading of the wells, the system begins the automated testing sequence.

The larvae filled well plate is then placed on the rotary table and the system is sealed to external light [3]. The experiment is then initialized by using the computer user interface to record the genetic line numbers of the larvae in each well. When all information has been entered, the system initiates its fully automated testing procedure. All 16 wells are washed of their normal food at two washing stages. Fluorescent dyed food is then pumped into the wells and is eaten by the larvae for 30 minutes. Another washing round occurs and a fluorescent image is recorded under ultraviolet light [4]. Masks and

thresholds are applied to the images so that only the dye affected intestinal region becomes visible. The larger the dye affected region the more food the specimen has consumed. This method of food intake quantification can then be used on all genetic lines and the results can be compared.

This thesis concentrates on the fluorescent imaging and data analysis segment of the project. From the beginning, the goals were deemed to be:

-Differentiate the amount of food consumed between different genetic lines

-Determine ideal feeding time to prevent complete filling of intestinal tract

-Design an image processing program to determine a “food intake” quantity

-Fully automate system testing and data analysis

IV. Definition of a New Assay

The current manual testing procedure is mainly limited by intensive handling of the larvae. First the specimen must be removed from a standard food medium, fed, removed, and cleaned from the dyed medium. Eight equally sized larvae are then ground and centrifuged before an OD reading can be recorded. This transportation of larvae between the feeding and testing chamber drastically increases testing time. Therefore, a new assay had to be designed that achieved similar results while decreasing the necessity for specimen handling. Two trials were then conducted with new food mediums mixed with metallic particles and a fluorescent dye to achieve the desired goal.

Metallic Micro-Particles and Magnetic Attraction

Initial larval testing began with an experimental testing procedure implemented by the NEMC research team. Instead of using food dye as an indicator of consumption, micron sized metallic particles were added to the yeast mixture. The larvae were fed the metallic yeast mixture for a standardized time period and then placed within a magnetic field. Pieces of paper were then stacked until the larvae no longer appeared influenced by this magnetic field. A genetic line was classified by its reaction distance as a high or low eater; the more food consumed the longer the detection distance (***Figure 4***) [2]. The collection of this data was fairly arbitrary because it was based on the tester's judgment of movement.

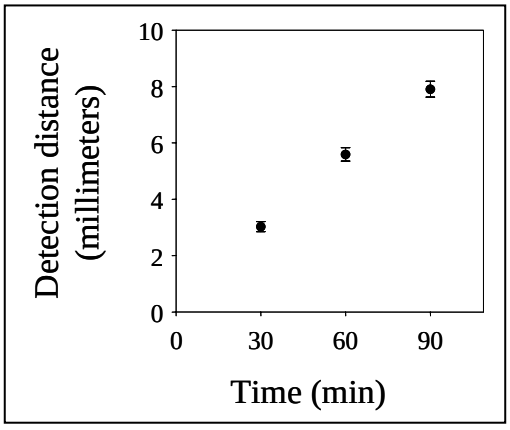


Figure 4: Detection Distance vs. Feeding Time using Metallic Particles

Further exploration was then conducted using movies and image processing to determine the quality of the magnetic food testing procedure. Instead of the paper method, individual larvae were placed within a water bath. As the magnet was gradually moved towards the sample a video camera recorded the critical point when the larva transitioned

from a rest state into motion. The distance between the magnet and the subject was then an indicator of food consumption. Motion monitoring with a computer removed the original subjectivity in the collection of the data.

However, the magnetic assay presented additional problems. After various tests it was found that the orientation of the larva within the solution greatly affected results. The cylindrical nature of the larvae provides two different drag coefficients through the water. Therefore, a low consuming genetic line may register as a high eater if it is oriented perpendicular to the magnetic field. The variable drag coefficient, combined with natural larvae motion, illuminated the difficulties with creating an accurate, reliable testing scheme.

Fluorescent Dye in the Food Medium

Since the optical density test proved too labor intensive and the metallic test too unreliable, a new testing procedure had to be developed. The main constraint that restricts data collection is the small size of the larvae. Any procedure that requires transferring of larvae from one section to another adds unnecessary degrees of difficulty. Image processing is attractive because it eliminates additional movement from the cleaning stage to the testing stage. Images also capture multiple larvae within one area that can then be analyzed individually or as a group. The clear cuticle of the third instar larvae also encourages imaging because the digestive tract can be viewed without any physical alteration of the larvae.

In order to calculate the amount of food consumed using image processing techniques, a contrast must be established between the food and the larvae. A dark or highlighted food would be more visible through the membrane than the neutral colored yeast solution. This color difference clearly demarcates edges of the digestive tract and defines a sharp color transition necessary for image processing. The contrast can be further increased by utilizing fluorescent dyes that emit vibrantly under a narrow light spectrum. An ideal system would capture images of the dyed intestinal region while excluding the remaining larvae components. The size of the resultant area then reflects the amount of food consumed.

V. Prototype I

Possibilities of Fluorescent Imaging

Fluorescent testing began with the setup of a Spectroline Ultraviolet Lamp (365nm), black box, and a Unibrain Fire-I FireWire color camera. Larvae were then fed a medium that contained the fluorescent dye Fluorescein (Uranine; Acid Yellow 73). The dye is nontoxic, has a peak absorption of 380 nm, and a peak emission of 520 nm. Larvae were fed in the dyed yeast solution for 30 minutes, washed, and placed into the fully enclosed testing box. The specimen was then bathed in ultraviolet light while an image was captured. Specimens in picture (a) of ***Figure 5*** were fed a normal yeast solution while larvae in picture (b) were fed a fluorescent dyed food. Both samples were cleaned and imaged under ultraviolet light.

(a) ***(b)***

Figure 5: Normal medium (a) and Fluorescent medium (b)

Test Box with Ultraviolet LEDs

The original Spectroline lamp required warm-up time and did not provide a uniform light intensity over the desired coverage area. To explore the fluorescent technique further, ultraviolet LEDs (395nm) were added to the interior of the black box. Ten LEDs were then mounted on opposing sides of the test box. Side mounting ensured reduced light reflection off the specimen and Petri dish into the camera. For better image quality, the FireWire camera was replaced with an ELMO CCD model SE420 8-bit grayscale linear camera. ***Figure 6*** shows the second prototype testing apparatus. After being fed and washed, larvae were placed into the testing chamber. A LabVIEW program then captured images as the larvae were bathed in ultraviolet light.

Figure 6: Black box prototype with ELMO camera

Each image was masked to remove the Petri dish "glow" (***Figure 8***) from the LEDs. Both the UV LEDs and the Spectroline lamp caused the larvae to react as if influenced by the dye, instead of just the intestinal tracts. ***Figure 5*** and ***Figure 8*** show this phenomenon. This glow can be accounted to light diffraction as the emitted light transfers through the specimen and its cuticle. After applying the threshold, the image consisted only of the affected intestinal region. A blob analysis then calculates the area of the fluorescing region which is directly correlated to the amount of food eaten. The fluorescing region will grow in size as the specimen consumes more food. Therefore, a larger area implies a higher eating genetic line.

VI. Prototype I Results

Fluorescent Imaging vs. Optical Density

To explore the prototype's applicability, simultaneous tests were conducted using both the optical density and fluorescent imaging setups. ***Figure 7*** displays the comparison between these two tests with the blue data representing optical density measurements. The fly lines were ranked according to their optical density reading to see if the fluorescent tests would reveal a similar pattern. Although some fly lines have similarities between the tests, the fluorescent imaging data does not mirror the same rankings as the optical density test.

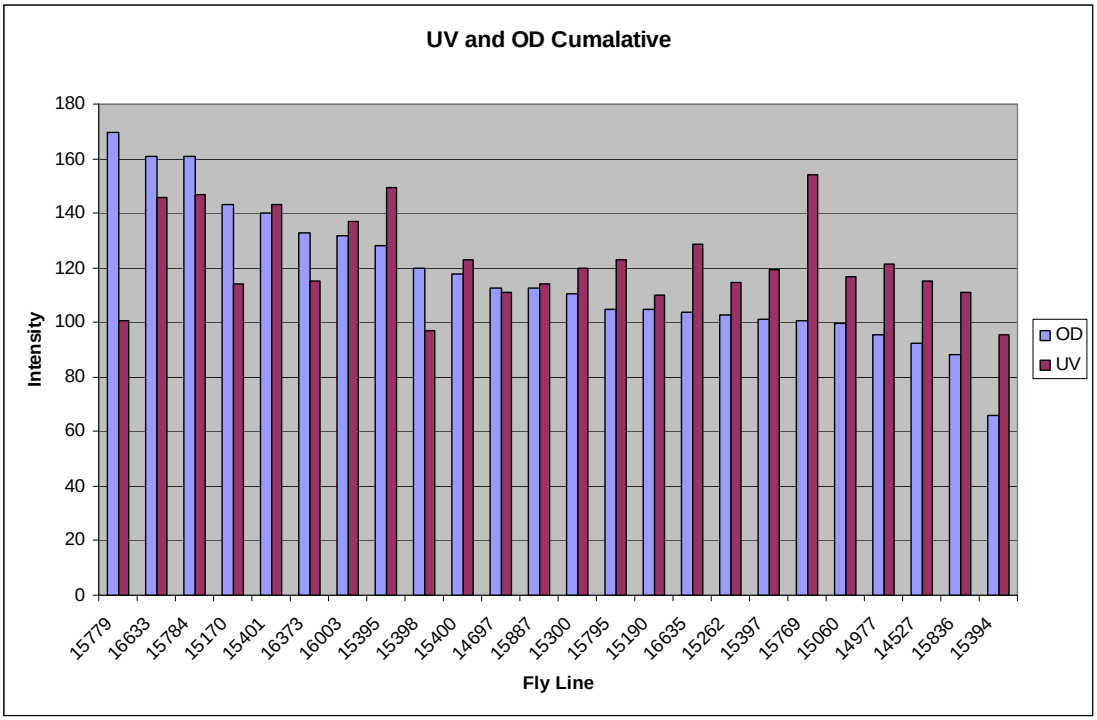


Figure 7: Data comparison between UV and OD tests

Rotational Imaging in Prototype I

Due to the vast differences between the two techniques, it was necessary to redefine the system parameters. First of all, it was speculated that the orientation and high degree of directionality of the LEDs were creating an inconsistent light bath. A plate of larvae were fed and placed within the black box where numerous pictures were taken at different orientations. ***Figure 8*** demonstrates the lighting inconsistencies for different orientations. Not only does the specimen's rotational position matter, but the farthest right larva in the second picture is bending up off of the sample plate. By elevating above the testing plane the larva is receiving more ultraviolet light and therefore triggering as a higher eater.

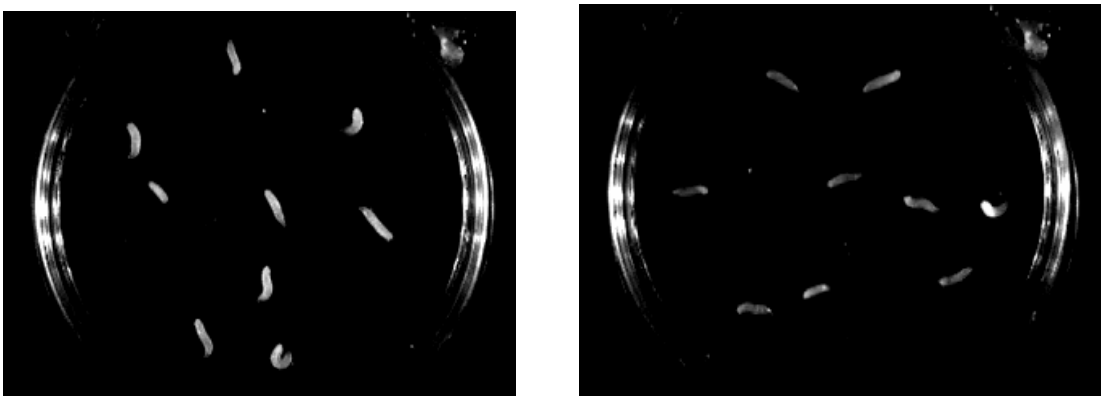


Figure 8: Rotated sample reveals inconsistent lighting

Since the lighting setup creates differences in fluorescence depending on orientation, it was hypothesized that averaging the fluorescence recorded at different orientations would decrease the resultant error. To accomplish this, a rotating plate was constructed in the bottom of the black box. The rotating plate was controlled by a Lego RCX and rotated at 9.25 RPM. Multiple images were captured and averaged as the sample rotated on the plate. Each image was then analyzed and an average fluorescent pixel area over all pictures was calculated. ***Figure 9*** demonstrates the variability of readings as the sample was rotated. The chart confirmed the lighting inconsistencies and favored additional system alterations to eliminate the lighting variability.

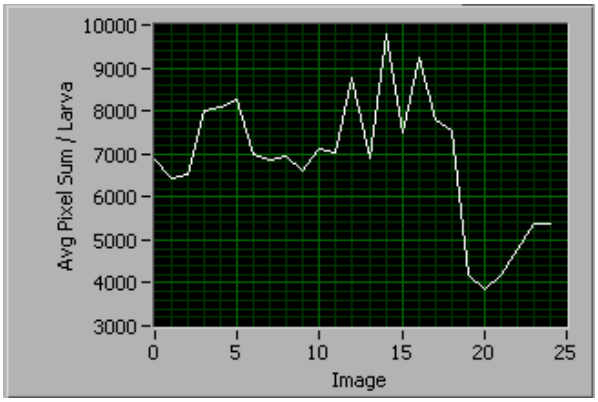


Figure 9: Variation of pixel sums for consecutive rotated images

Top-Oriented LED Design

In order to correct, or at least reduce, the influence of the light positioning, the LEDs were moved onto the top of the box and organized in a ring about the camera hole. The intent was to remove the orientation error by constructing a uniform, circular light bath.

The modifications in the lighting scheme and sample orientation then had to be tested to explore the system's potential. Instead of conducting additional mirror experiments, a variable feeding time procedure was introduced. Differentiating between a high and low eater is essentially the same as analyzing a short and a long time eater; the longer a larva eats the larger the dye affected region.

A single genetic line was chosen and used to experiment with three different feeding times: 10 minutes, 20 minutes, and 30 minutes. After feeding and washing, images were captured as the specimens were rotated. A threshold was applied to each image and an average area in pixels per larva was calculated. ***Figure 10*** displays one image, out of the total 24 taken during rotation, of a 30 minute feeding time. The total area per larva was taken for each picture and averaged over all pictures. ***Figure 11*** shows the progression of food consumption over the 10, 20, and 30 minute feeding times. However, the 30 minute feeding time is inflated above the 10 and 20 minute times. When analyzing the data, it was found that one larva within the 30 minute period was glowing much brighter than the others. The differences in individual larvae feeding habits can be seen in ***Figure 10*** where the observed areas are vastly different. As with any calculation based on averages, the population must be relatively large. Four to five larvae per genetic line were used for

the experiment which does not compose an appropriate sample size. However, this simple test indicates the ability to detect differences between feeding times. As discussed above, detecting variances in food intake by varying feeding times is equivalent to differentiating between normal and abnormal feeding genetic lines.

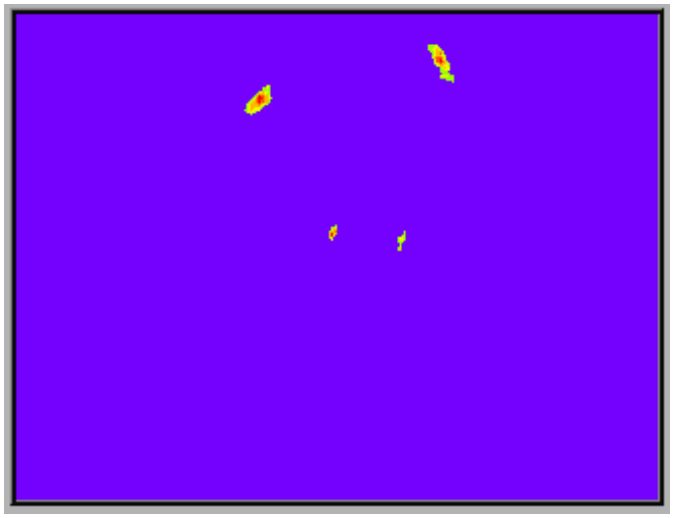


Figure 10: Binary threshold image with rainbow LUT

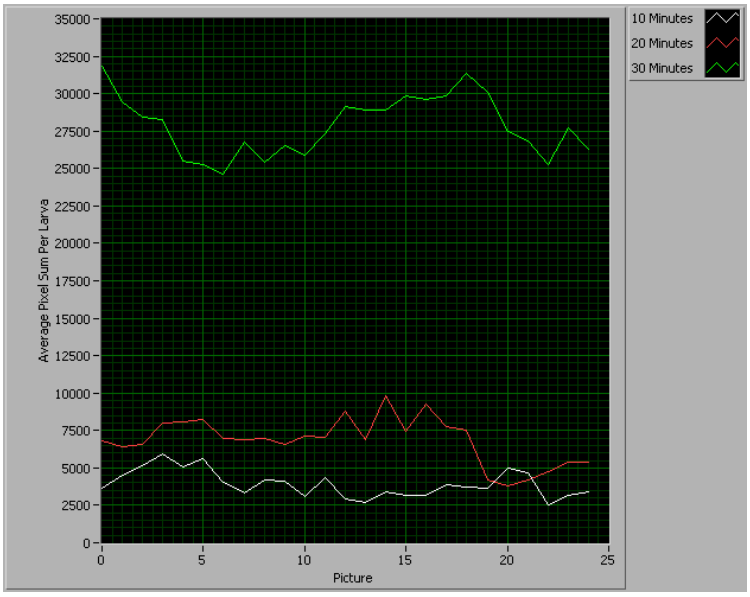


Figure 11: Increasing pixel sum for increasing feeding time

The variability of the recorded values as the sample was rotated indicated that the lighting design was still insufficient to produce accurate results. With the LEDs placed on top of the testing chamber, the larvae and the Petri dish were reflecting the ultraviolet light back into the camera. The large white circles in ***Figure 12*** are reflections of the ultraviolet light off the Petri dish into the camera. The spaces between the LED reflections also verify the uneven coverage of the light over the sample. Therefore, any specimen located within these intensified areas would be triggered as a false positive due to an increased exposure to the excitation light.

Figure 12: UV LED Reflection on testing plate

Prototype II

After analyzing the tests conducted with the LED system it was concluded that a new, more uniform light source had to be implemented. The small angular directionality and the low intensity of the LEDs presented too many problems to be corrected with other design changes. The ten LEDs were then replaced with a 22W FC8T9 Circline UV

Fluorescent Black Light (***Figure 13)*** that emits a stronger, more homogenous ultraviolet light. Unlike the LEDs, the fluorescent tube pulses at a frequency of 60 Hz. Small camera exposure times lead to images taken as the fluorescent tube is transferring between states and therefore a uniform light intensity is not captured over all images. Increasing the camera exposure time removes this error by ensuring multiple light pulses over the image capture time.

Figure 13: FC8T9 Circline UV Fluorescent Tube

Prototype II Results

Rotational Imaging in Prototype II

As with the LEDs, the new circular fluorescent tube had to be tested for its consistency. To check the uniformity of the light, the rotational test was repeated for the circular fluorescent tube. Each picture was then analyzed using the same threshold and pixel sum

algorithm to quantify the amount of food consumed. However, these images were captured with an AxioCam MRc microscope camera instead of the ELMO grayscale camera to incorporate another degree of precision. Higher detailed pictures were captured because the new setup combined magnification along with higher pixel resolution.

Members of the same genetic line were separated into two different feeding groups. Each group consumed the same solution of food, but one was allowed to eat for 20 minutes while the other ate for twice as long. ***Figure 14*** demonstrates both the uniformity provided by the fluorescent lamp along with the hypothesis that the size of the fluorescent marked area is correlated to food consumption. However, the graph also raises additional concerns. It would seem appropriate that the 40 minute feeding time should have an average area double that of the 20 minute feeding time; instead the data indicates a multiplying factor of four. Again, this can be accounted for by a small sample size. Four to five larvae per experiment allows for individual deviations to alter the overall data. A larger quantity of larvae would average out these anomalies and produce more consistent data.

Figure 14: Verification of circular fluorescent light uniformity

Cuticle Dyeing

Using the new fluorescent tube system, additional mirror testing against the optical density test were conducted. However, data values were still within close proximity of each other and did not yield a similar ranking hierarchy. It was speculated that the cuticle was possibly being dyed by the fluorescent food and adding noise to the emitted light. A group of deceased larvae were submerged in the fluorescent food solution for 45 minutes, after which they were imaged.

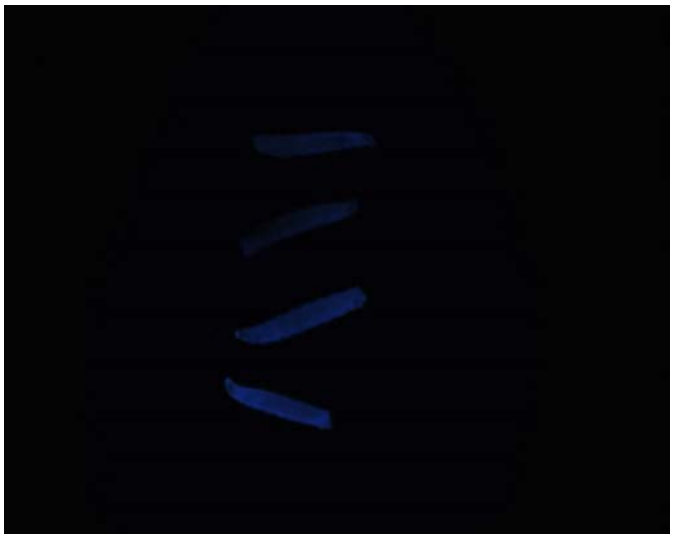


Figure 15: Fluorescein soaked, non-fed larvae

Figure 15 demonstrates that all fluorescent excitation is located within the intestines and not from absorbed particles in the cuticle. The blue color is the reflection of the ultraviolet light and is characteristic of black light fluorescent tubes. Therefore, testing errors could not be attributed to excitation of the dye outside of the digestive tract.

Feeding Time Alterations and Gut Analysis

Although research notes that the intestines of larvae are filled after 90 minutes, a 30 minute feeding time may be too short or too long to create a desirable comparison. Uniform results could be explained by the larval gut being completely filled or essentially empty after 30 minutes. These two extremes would result in uniform results because the intestinal region would be filled the same for all test subjects. With assistance of lab personnel at the NEMC, larvae fed for different time increments were dissected to determine the volume of food in their digestive tract. From these results, an ideal feeding time could be chosen.

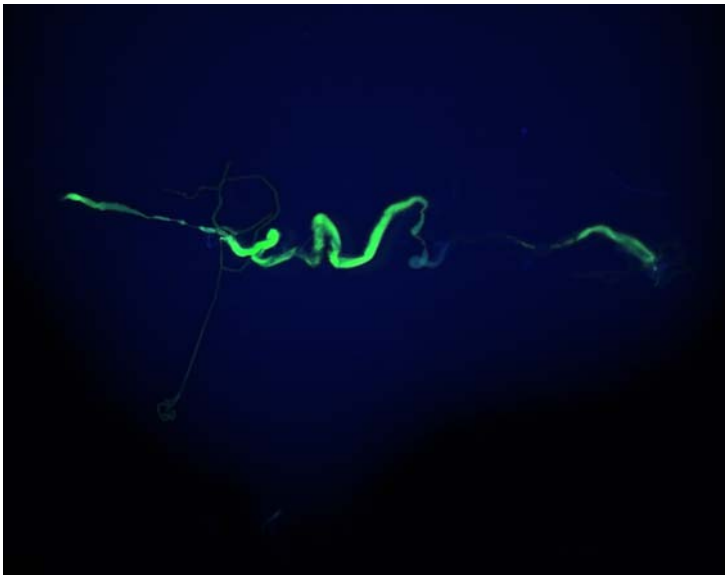


Figure 16: Dissected larval intestines for 75 minute feeding time

Figure 16 is the intestine of a 75 minute fed larva and is oriented with the mouth located at the right side of the picture. First of all, the entire intestinal tract is filled with fluorescent dye. Since researches claimed that the larvae reach full consumption at 90 minutes, it was speculated that the dye was diffusing throughout the digestive system once a small quantity of the food had been consumed. Larvae were fed fluorescent food for 60 minutes and then imaged to produce the white data series (***Figure 17***). The same larvae were then fed normal food for 45 minutes and re-imaged. ***Figure 16*** implies a full digestive system at 75 minutes, so the tested larvae had to excrete dyed food as they consumed standard food. The difference in the two data series indicates that the Fluorescein did not dissolve throughout the intestines. NEMC researchers conducting the dissections also explained that the dye did not dissolve into any other body component. Therefore, any recorded emission was solely due to the intestinal region and not from dye absorption within the body or cuticle.

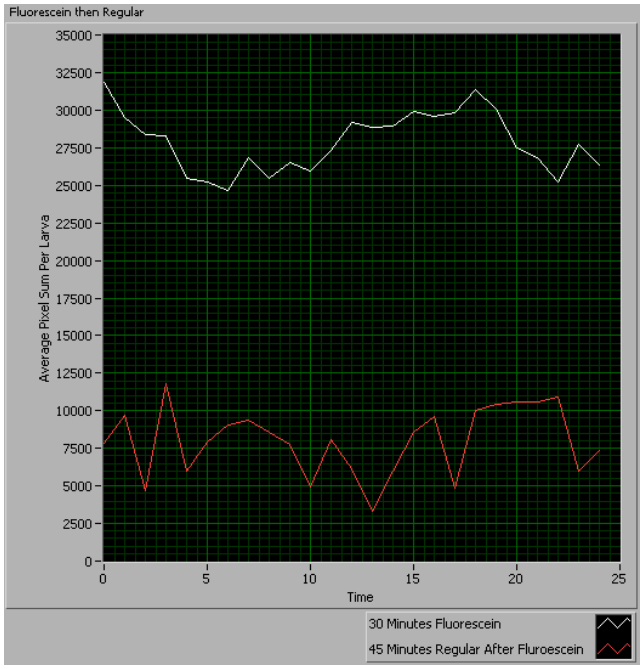
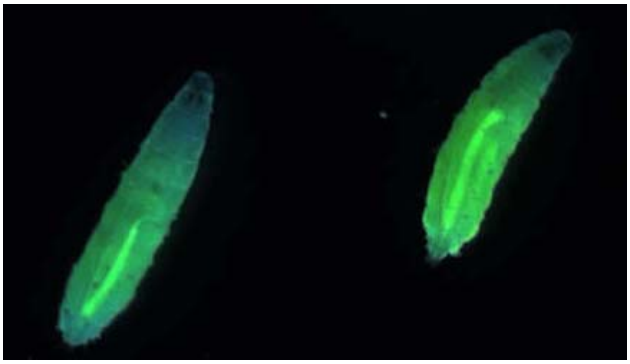


Figure 17: Excretion of dye after 45 minute post-dye feeding session

Further evaluation of the dissected intestine indicates the inability of the dye to fluoresce in the stomach region as indicated by the gap in emission in ***Figure 16***. It was later discovered that Fluorescein does not fluoresce in acidic pH solutions that are characteristic of the larvae stomach. The inability of the dye to fluoresce in the stomach adds error to the system since a volume of food is not accounted for in the overall food consumption. A pH insensitive fluorescent dye, 4-Methyl-Umbelliferone, or Coumarin, was attempted as a replacement for Fluorescein to achieve full intestinal imaging. Unfortunately, the larvae will not feed in a food solution containing Coumarin because of its toxic nature. A final dye has not been selected but the fluorescent imaging market is large enough to provide numerous selections to choose from.

Verification of the Fluorescent Imaging Assay

Even though the current dye does not fluoresce throughout the entire intestine, the assay was still able to produce desirable results. ***Figure 18*** demonstrates the hypothesized differences between feeding times. After a binary threshold to the image, the 40 minute larva's affected intestinal region is 11% of volume while the 60 minute larva's is 17.5% of volume.



(a) ***(b)***

Figure 18: 40 minute feeding (a) and 60 minute feeding (b)

Error Reduction by Averaging an Increased Sample Size

The above two larvae present the ideal fluorescent image by having a well defined dyed section. In actuality, the intestinal tract is nonlinear and folds upon itself throughout the lower intestines (***Figure 19)***. These folds present problems because an overlap can not be detected through image processing; folds stacked perpendicular to the testing plane would only be calculated as one point within the larvae. The failure to capture the entirety of the food within the intestines leads to skewed food consumption data. However, the magnitude of this error can be diminished by increasing the sample size. An increase in

the number of larvae tested per genetic line increases the average which would then be a better representation of the amount of food consumed by that genetic line.

Figure 19: Intestinal Overlap

One must also understand that the extent of the experiment is to do follow-up studies on genetic outliers. Kerry Garrity's current research pursues genetic lines for additional testing if values fall outside two standard deviations from the mean. Small errors that appear during initial testing will be corrected during the course of follow-up studies.

Final Design

Once individual larvae testing had proven to be successful, the fluorescent imaging technique was then integrated into the robotic system to analyze a higher experimental throughput. The testing procedure consists of placing eggs of one genetic line into the 16 wells on the testing plate. Their food medium is a normal solution that is approximately one part yeast and two parts water. After four days of incubation, the well plate populated by third instar larvae is placed into the testing chamber (***Appendix II***). A LabVIEW

program then sends serial commands to wash each well of the normal food and pump fluorescent food into the wells. The larvae eat the fluorescent medium for a predetermined time after which they are washed and imaged under ultraviolet light. Images are snapped with a Q-Color 3 camera that captures 2048x1536 resolution color images and is intended for low-light, fluorescent imaging. A high resolution camera was chosen to mimic the results obtained with the AxioCam microscope camera used in earlier tests.

Final Design Results

Incomplete Removal of the Food Medium

Figure 20 is an example of an image snapped during a trial run of the system. First and foremost, there is still fluorescent food that did not wash through the mesh. These leftover particles make isolation of individual larvae for image analysis almost impossible because the entire test section is fluorescing. This issue can be handled by changing to a food medium that is completely soluble. In doing so, the food would wash through the mesh but also ensure a uniform dye distribution throughout the food. ***Figure 20*** shows how not all of the yeast particles are fluorescing. Larvae in this medium are then consuming the same amount of food but not necessarily the same quantity of dye. Full scaled system testing can not occur until the food is evenly dyed and washed completely from the testing wells.

Figure 20: Automated washed and imaged sample

Fluorescent Glue

The highlighted blue region in ***Figure 20*** is a two-part epoxy glue that was used to fasten the vials to the plate; it was not realized until afterwards that the glue had fluorescent properties. Fortunately, this interference can be removed through image processing techniques. First of all, the well pictures are taken at the same rotary position each time. The center of the well within the picture is then known and a circular mask can be applied so that only the meshed area, and not the glued rim, is analyzed. Programming can also be written to remove only the major color plane that composes the selected segment. The green plane can be extracted from the image which would highlight only the fed larvae and minimize the glue's blue fluorescence interference.

Final Design Problems

Besides the aforementioned image processing issues, other concerns were raised during testing. The current testing apparatus has the camera mounted 6.5 well steps away from the second washing station. It was installed at this location to protect it from leaking water coming out of the valves. However, this gap introduces wait time until the washed wells are imaged because additional wells are being washed behind them. Once removed from a food source, the larvae immediately begin crawling in search of a new food supply. This migration has no affect on image capturing unless the larvae begin scaling the tube walls. These larvae will not be captured in the image and therefore results will not be obtained. This error can be reduced by decreasing the time between food removal and image capturing.

The program currently washes all wells consecutively. The first well then experiences wait time as the previous wells behind it are washed. However, once a well is completely washed the rotary table can move directly to the imaging station. The table would then rotate backwards to wash the second well. Although this increases total testing time by introducing additional rotary table action, the wait time between washing and imaging would be reduced from approximately 35 seconds to 10 seconds.

User Interface

User Input

The final automated system is controlled through an interface written in LabVIEW. This overarching program receives input from the lab technician and runs a fully automated testing round. The user interface was designed with the intent of having individuals conduct the experiment without any previous knowledge of the system. Emphasis was placed on minimizing inputs required from the user. This entailed designing a smart system that found and corrected errors automatically throughout the testing run.

To initiate testing, the loaded well plate is placed within the testing apparatus. The plate rests on four support pins of which one is color marked that aligns with a color marked hole on the well plate. This assures that Well 1 is in the same home position for each test so that the genetic line numbers can be attached to the corresponding wells correctly. After inserting the well plate, the user closes all doors and activates the program.

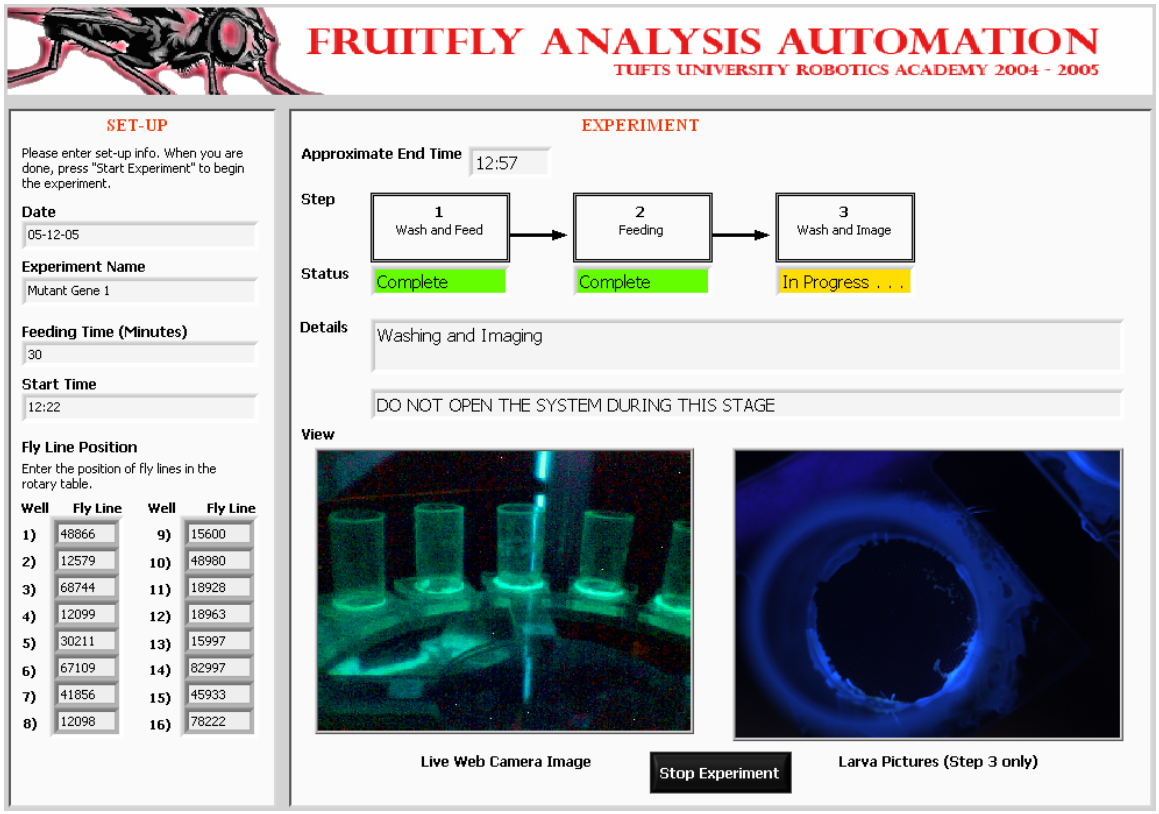


Figure 21: User Interface

To create a constant data storage structure, the Date and Start Time fields are pulled from the system computer (Refer to ***Figure 21*** for references made to the user interface). Combined with the Experiment Name, defined by the user, the data is saved with a filename constructed from the Date and Start Time. Therefore, data will be saved automatically and remain organized without any external effort. This defined structure also enables files to be easily called for image processing analysis. A suggested Feeding Time of 30 minutes is recommended to the user but this value can be altered to accommodate a wide variety of tests. The user then fills in the five digit genetic codes that correspond to each of the 16 wells. This array is carried through the program and is expanded to two dimensions once the quantitative measure of food intake is calculated.

Automation begins once the user presses the Start Experiment button and checks are made to see if the Experiment Name and all Fly Line input boxes were filled. If not, a warning box informs the user that the program can not continue until all fields are filled. Currently the program is designed to analyze all 16 wells but future programming adjustments can be made to accommodate any desired number of wells.

Live Update

Once the Start Experiment button is pressed, the system initiates a fully automated sequence that does not require any additional user input. The Experiment tab is a live updated section that provides the tester with information regarding the experiment. An approximate end time is displayed and future work will introduce a countdown timer. The system begins by washing the standard food from the larvae and feeds them the Fluorescein dyed food. The Wash and Feed status box is yellow during this stage and indicates that the step is "In Progress . . .". As the stage is completed, the status box transitions from yellow to green and displays "Completed" while the proceeding stage displays a yellow "In Progress . . ." icon (***Figure 22***).

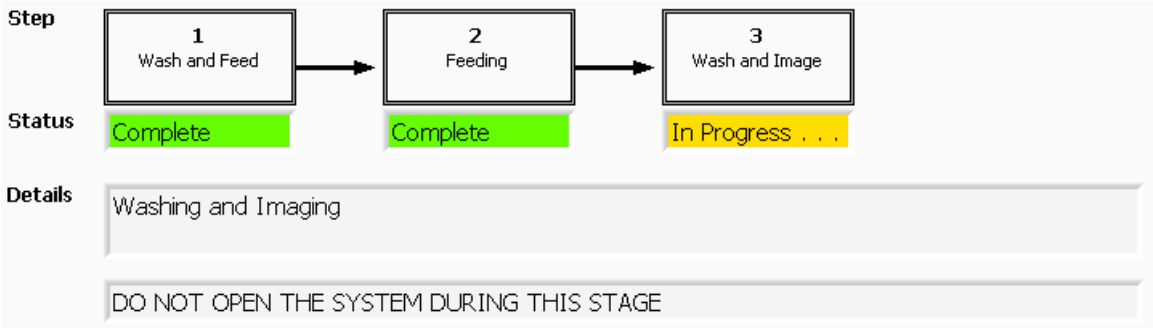


Figure 22: Status Update for the User Interface

The Details information box informs the user of which action is currently being executed. As of now, it displays the same text at the numbered Step boxes, such as Feeding, but additional work can be done to provide precise information about which well is being washed or imaged. The second tab displays warnings or errors to the user. For instance, the system must be closed to all external light during fluorescent image processing and displays the warning “DO NOT OPEN THE SYSTEM DURING THIS STAGE”.

The bottom section of the Experiment tab (***Figure 23***) has two image boxes: one for live updating and one for fluorescent images. Once the program starts running, a webcam located just above the well plate provides a live view of the system progress. It is positioned to observe the washing and feeding of the wells and acts as the main error trap during this stage. Any overflow or misalignment can then be seen by the researcher and appropriate actions can be taken. Additional automatic error traps should be installed for the washing and feeding but have yet to be implemented.

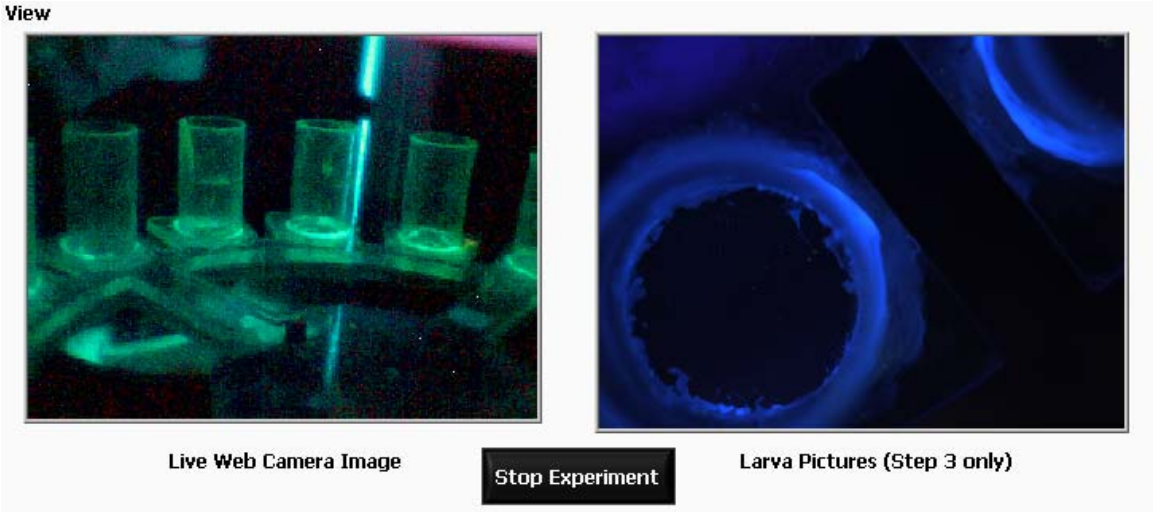


Figure 23: Image Display Boxes for the User Interface

The second image box displays the most recent fluorescent image. These are quick, pre-processed thumbnails that inform the user about the status of the fluorescent imaging. ***Figure 23*** is an example from a trial run without any food or larvae. The current program has this thumbnail replaced as new pictures are acquired but it would be more beneficial to the user to view previous images as new ones were captured.

Data Analysis and Image Processing

The current system program saves multiple fluorescent pictures of the 16 wells with the predetermined filenames and folder structures. The final program will compute a number that signifies food consumption. This data will then be loaded by the analysis program where a list of all data sets is automatically populated. The selected data files are loaded into the interface and can be sorted either by Fly Line number or their food intake numerical value (***Figure 24***). A histogram of the food intake values is compiled and any genetic outliers beyond two standard deviations from the mean are displayed on the user interface.

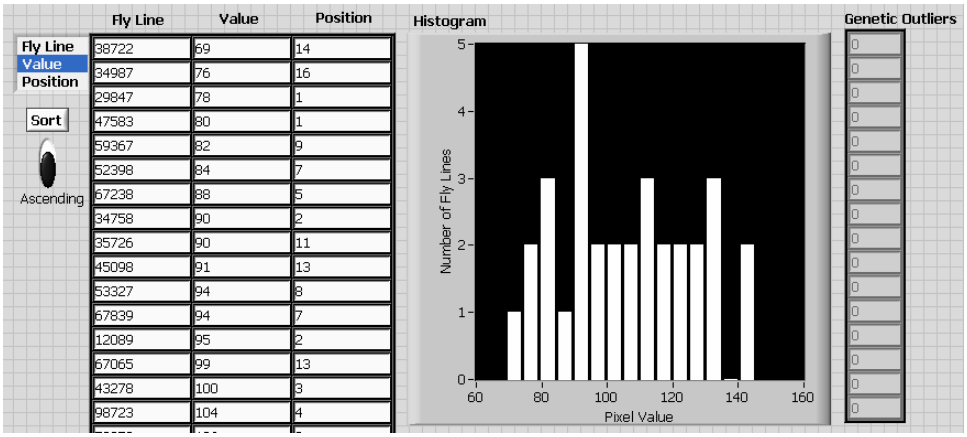


Figure 24: Data Analysis User Interface (Simulated Data)

Genetic outliers are determined based on the average of all previous tests. Therefore, the data analysis program needs to be altered to calculate this universal average instead of obtaining the average from only the selected data series. The program must locate and load all data sets and average values from similar genetic lines. If the genetic line 62871 is repeated throughout multiple tests it must be averaged first before determining the overall consumption average across all the genetic lines. The final software package will still allow the user to load a handful of data sets to be ranked individually but they will be measured against the universal average when isolating any genetic outliers. This program therefore reduces overall time spent doing genetic testing because all data will be processed automatically. Researchers can then take the genetic outlier list to conduct further testing.

The functionality of the data analysis program will be added to the system control program. Therefore, researchers can load a well plate and discover if there are any genetic outliers in the sample after 40 minutes. The secondary data analysis program will

still be used to rank genetic lines across multiple tests and to produce histograms but its algorithm will be nested in the main control program to provide immediate results.

Conclusion

Through an intensive design process and collaboration with the NEMC team, the Robotics Academy reached the desired goal of a functional automated fruit fly analysis prototype. The system is capable of screening 16 genetic lines for feeding abnormalities in 35 minutes. A new assay was developed to differentiate and quantify the amount of food consumed by fruit fly larvae. Ingestion of fluorescent dye in the larval medium enables the amount of food to be calculated through image processing rather than using an optical density scanner. These fluorescent images can be captured in the testing section which removes the time-intensive handling aspect of the manual procedure.

The major system design accomplishments include the following:

(1) Verification of data collection with a fluorescent dye.

(2) Creation of an easy-to-use control program user interface.

(3) Automation of data collection and analysis.

Once some image processing issues have been resolved, the system will be ready for mirror testing against the optical density technique.

References

[1] Edgecomb, R.S., Harth, C.E. & Schniederman, A.M. Regulation of feeding behavior in adult Drosophila melanogaster varies with feeding regime and nutritional state. *J Exp Biol* 197, 215-235 (1994).

[2] Kopin, A. S. A Bioengineering Approach to Gene Discovery. Pending NSF grant proposal (2005).

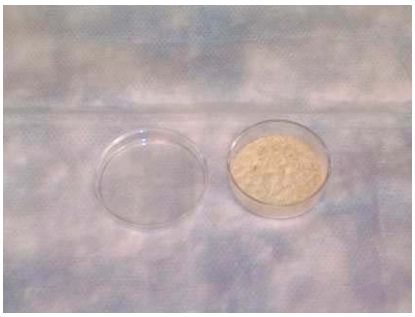
[3] Pickering, M. Design of an Automated Device to Prepare Drosophila Melanogaster for Image Analysis. Tufts University Undergraduate Thesis (2005).

[4] Copetta, J. Rogers, C. Dual emission laser induced fluorescence for direct planar scalar behavior measurements. *Experiments in Fluids* 25, 1-15 (1998).

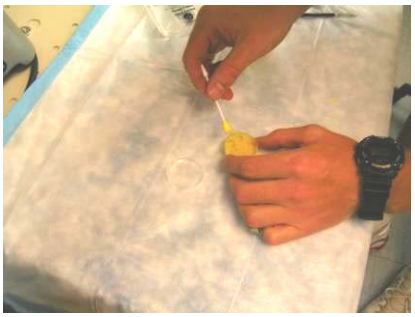
Appendix I: Manual Testing Procedure

Step 1: Eggs hatch & grow to 3rd instar larvae stage in vials w/ media.

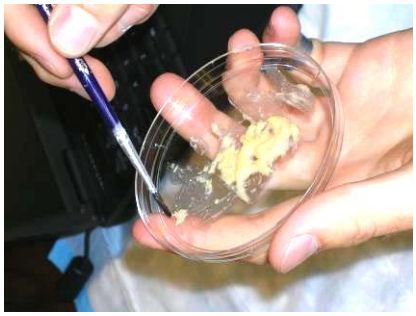
Step 2: Fill Petri dish with new media



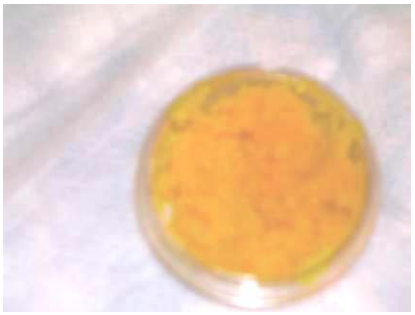
Step 3: Add Fluorescent die to new media & mix.



Step 4: Remove larvae from old media in vial.



Step 5: Set larvae in new media for 30 min. to allow feeding.



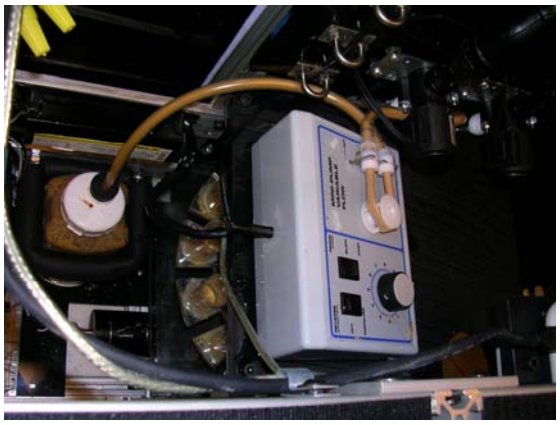
Step 6: Remove fed larvae from media.

Step 7: Carefully clean each larvae of exterior dye & place in new dish.

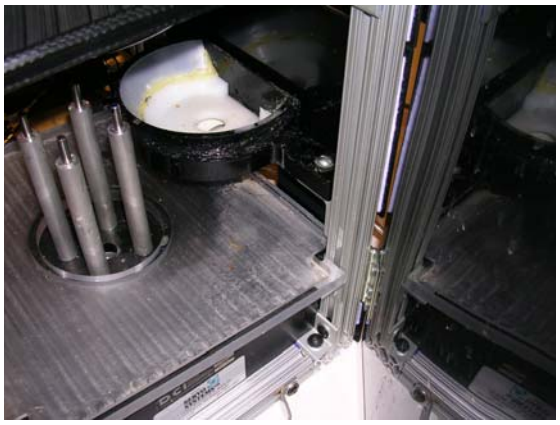
Step 8: Conduct appropriate OD or fluorescent test.

Appendix II: Final Design Images

Washing Valves

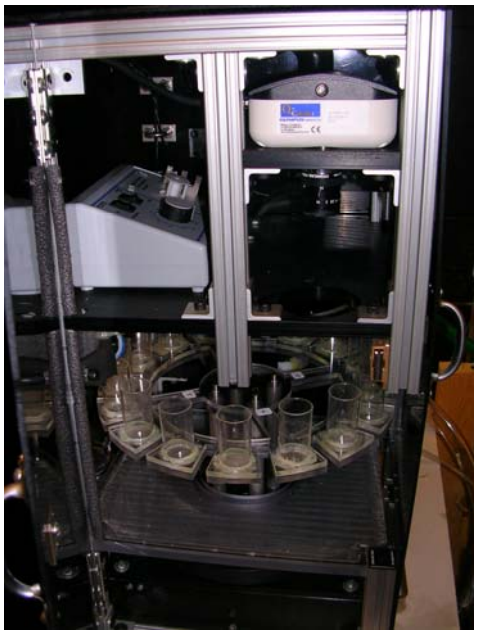


Pump and Food Reservoir



Drainage System

Well Plate



Front View and Camera