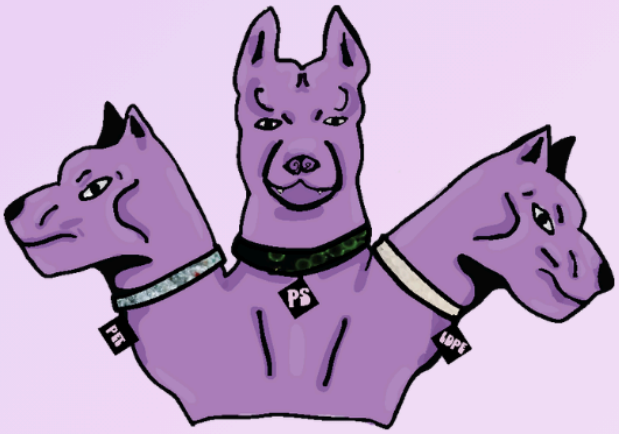
iGEM Athens 2025

Lab Notebook



KERBEROS

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Laboratory 3

Laboratory of Experimental Physiology

Supervising Professor: Christos Consoulas, Associate Professor, School of Medicine, National and Kapodistrian University of Athens (NKUA)

Day 1: Thursday, August 28, 2025

Introduction to the Laboratory and Experimental System

Familiarization with the laboratory space and equipment. Safety protocols were strictly followed throughout all laboratory procedures to ensure the well-being of both the personnel and the *Drosophila* cultures. Personal protective equipment (PPE) including lab coats, gloves, safety goggles, and masks was used when handling chemicals.
Presentation on *Drosophila melanogaster* as a model organism and a literature-based overview of potential microplastic exposure experiments.

Drosophila Handling and Theoretical Training
Theoretical instruction on *Drosophila melanogaster* biology and life cycle. Hands-on training on fly handling, transfer between vials with fresh food, and sex differentiation.

Drosophila Life Cycle Highlights:

- Eggs hatch into 1st instar larvae within ~24 hours.
- Progression through 2nd and 3rd instars over ~5 days.
- 3rd instar larvae (crawling stage) climb vial walls, seeking drier areas.
- Pupation occurs within ~1 day; adult flies emerge ~1 day later.
- Full development from egg to adult: ~9 days.
- Adults mate within ~1 day post-eclosion.
  Total: ~10 days from egg to reproductive adult.

Lifespan:

- At 30°C: ~1 month
- At 25°C: ~2–2.5 months (70–80 days)
  Each female can lay ~40–50 eggs in her lifetime.

Anesthesia & Handling Tips:

- CO_2 used for anesthesia; flies immobilized in vials placed at an angle to prevent contact with food.
- CO_2 is initially cold due to pressure; warming it to room temperature reduces static electricity and avoids fly dispersal during handling.
- Virgin flies with a soft brush to prevent injury due to their underdeveloped exoskeletons.

Sex Differentiation Criteria:

- Males: smaller size, dark pigmented abdomen tip, presence of sex combs on forelegs.
- Females: lighter abdomen, absence of sex combs.
- Virgins: lighter color; presence of meconium (yellowish food spot in abdomen).

For **longevity assays**, all males used must be of the same age. Food vials are to be changed every 2 days to maintain optimal conditions.

Preparation of standard *Drosophila* food medium with and without microplastics, according to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*). After making the food medium, we placed filter paper or a small piece of absorbent paper over the opening of each vial and covered the vials with a plastic bag or protective cover to prevent environmental contamination, for 24h in room temperature.

Day 2: Friday, August 29, 2025

Solidification and Quality Control of Food Vials
The prepared food had solidified inside ~350 vials, which were sealed with foam plugs and stored at the fridge (10^{o}C).
One vial was removed due to contamination by a fly. Upon microscopic inspection, it was determined to be male (no eggs present), and the remaining vials were deemed uncontaminated and retained for use.

Day 3: Saturday, August 30, 2025

Establishment of Parental Generation for Exposure Assays
Males and females were separated from stock vials to set up mating pairs for microplastic exposure experiments.

Initiation of Microplastic Exposure Treatments
Adult *Drosophila* flies were transferred to fresh food vials containing different microplastic types and concentrations. 7 food conditions were used: control diet (without microplastics) and experimental diet (with PE, LDPE, PET MPs in C=0.01g/mL and C=0.001g/mL, 6 in total).
Each vial contained 15 males and 15 females (~30 flies per vial), all according to the Longevity Assay Protocol *(See Experimental Protocols).*

They were left for 48h to produce the F1 generation.

Day 4: Monday, September 01, 2025

Routine Fly Stock Maintenance (no. 1)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

The F1 generation was ready for our first assay.

In general, assays to follow:

- **Climbing Assay:** according to the Climbing Assay Protocol *(See Experimental Protocols).*
  Each fly is placed in a vial and gently tapped to the bottom.
  Using a foam base and ruler, the climbing distance is measured over a fixed time interval.
- **Crawling Assay:** according to the Crawling Assay Protocol *(See Experimental Protocols).*
  Flies are placed on marked surfaces (e.g., grid paper) within a controlled observation chamber.
  Movement is recorded via video.

Since we had already larvae from the F1 generation we decided to begin with the Crawling Assay, according to the Crawling Assay Protocol *(See Experimental Protocols),* by video recording or 30 seconds each of the 15 larvae form the 7 categories.

CRAWLING VIDEO ANALYSIS GUIDELINES:

1. **Total distance traveled** over 30 seconds.

2. **Continuous movement episodes** (calculate average speed using 2–3 such segments).
3. **Number of direction changes**, defined as shifts over 90° (divide movement into quadrants).
4. **Number of stops** (pauses in movement).
   Notes: It was recommended to search for software tools that was unsuccessful to assist with this analysis, so manual annotation was necessary.

Day 5: Wednesday, September 03, 2025

Routine Fly Stock Maintenance (no. 2)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Day 6: Friday, September 05, 2025

Routine Fly Stock Maintenance (no. 3)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Day 7: Saturday, September 06, 2025

Longevity Assay

The F1 generation has transformed into adult flies after ~10 days, so we began the Longevity Assay.

According to the Longevity Assay Protocol *(See Experimental Protocols)* 7 food conditions were used: control diet (without microplastics) and experimental diet (with PE, LDPE, PET MPs in C=0.01g/mL and C=0.001g/mL, 6 in total).

For each of the seven categories, we separated the males and females of the same age from the F1 generation, because typically in Longevity assays the lifespan is tested individually due to females being more resistant.

In all treatments, 4 replicate male vials and 4 replicate female vials were set up, containing 20 flies each.

A new Excel sheet with proper legend was created for data entry.

Climbing Assay

From the rest of the F1 generation left, a total of 30 adult *Drosophila melanogaster* flies (separately each sex 15 males and 15 females) were placed in food vials for each food condition, for the Climbing Assay. According to the Climbing Assay Protocol (See Experimental Protocols), flies were anesthetized with CO_2 and transferred into the new vials.

Again, 7 food conditions were used: control diet (without microplastics) and experimental diet (with PE, LDPE, PET MPs in C=0.01g/mL and C=0.001g/mL, 6 in total).

So each food condition had 5 vials from each sex with each vial containing 3 flies. (total of 15 from each sex)

Flies were maintained on these diets for 14 days, with the food being replaced every 3 days to ensure freshness and consistent exposure.

P- generation

The parental generation was left to create more F1 generation flies in order to preform Nile Red Staining & Feeding Protocol, ROS Measurement Protocol and Trypan Blue Assay / Dye exclusion test Protocol *(See Experimental Protocols).*

Day 8: Sunday, September 07, 2025

New Food Preparation (no. 1)
Two liters of food were prepared following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*) (sugar, yeast, cornmeal, agar), protected with filter and covered with a plastic bag for 24h in room temperature.

Longevity Assay - Mortality Monitoring (no. 1)
Recording of fly deaths per vial for the longevity assay began.

Day 9: Monday, September 08, 2025

New Food Storage (no. 1)

The prepared food had solidified inside ~350 vials, which were sealed with foam plugs and stored at the fridge (10ºC), following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*).

We sealed the vials containing food and observed that most appeared biphasic. This is likely due to insufficient homogenization of the food mixture prior to distribution into the vials. This issue can be corrected by reheating during the addition of microplastics.

Routine Fly Stock Maintenance (no. 4)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 2)
Continued death count in all longevity vials.

Day 10: Wednesday, September 10, 2025

Routine Fly Stock Maintenance (no. 5)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 3)
Continued death count in all longevity vials.

Day 11: Friday, September 12, 2025

Routine Fly Stock Maintenance (no. 6)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 4)
Continued death count in all longevity vials.

Day 12: Monday, September 15, 2025

New Food Preparation (no. 2)
Two liters of food were prepared following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*) (sugar, yeast, cornmeal, agar), protected with filter and covered with a plastic bag for 24h in room temperature.

Routine Fly Stock Maintenance (no. 7)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 5)
Continued death count in all longevity vials.

Next experiments plans Microplastic samples were transferred from NTUA to the School of Medicine, NKUA. Experimental planning for the upcoming week was carried out.

Day 13: Tuesday, September 16, 2025

New Food Storage (no. 2)

The prepared food had solidified inside ~350 vials, which were sealed with foam plugs and stored at the fridge (10^{o}C), following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*).

Nile Red larval staining setup:
We followed the Nile Red Feeding Protocol *(See Experimental Protocols*).

Larvae (~15 per vial) were transferred into food containing Nile Red for the following groups:

- PET 1
- PET 0.1
- LDPE 1
- LDPE 0.1
- Control

There is no PS category because this staining protocol was unsuccessful in labeling polystyrene (PS) microplastics, indicating limitations in dye affinity or protocol compatibility.

ROS preparation:

Another group of 20 *Drosophila melanogaster* flies were transferred into vials with agar and water instead of food (1g agar for 100ml distilled water) and fed with Paraquat which is a known poison that causes raising of ROS, as a positive control for 1 day before the ROS measurement, according to using the ROS Measurement Protocol *(See Experimental Protocols*).

Day 14: Wednesday, September 17, 2025

Routine Fly Stock Maintenance (no. 8)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 6)
Continued death count in all longevity vials.

Confocal imaging (Nile Red):
Initial confocal microscopy images were acquired after 24h of larvae eating stained MPs and saved on a USB stick. A second round of imaging is planned, this time using adult flies (leaving the larvae to turn into adults, after 7 days).

ROS assay:
Performed ROS detection using the ROS Measurement Protocol *(See Experimental Protocols*) with DCFDA dye, on 20 F1 generation flies that were 14 days old, from each category. After homogenizing them, centrifuging the

mixture and incubating supernatant with DCFDA for 60 minutes, fluorescence was measured using a microplate reader.

Trypan blue preparation:
Flies that were born 6 days ago underwent 16-hour starvation in preparation for Trypan Blue staining. They were placed in vials with agar-water mixture (1g agar in 100 mL water), following the Trypan Blue Assay / Dye exclusion test Protocol *(See Experimental Protocols*), so they will stay properly hydrated.

Flow cytometry experiments (Day 1)

Supervising Professor: Vasiliki Lampropoulou, Assistant Professor, School of Medicine, NKUA

We started our flow cytometry experiments to evaluate the successful surface display of proteins in yeast following the Standard Flow Cytometry Protocol *(See Experimental Protocols*), in our 1rst genetically engineered yeast. The machine was previously set for *S. boulardii* strains.

We measured optical density or cell concentration using a spectrophotometer by transporting them in a glass tube (which has to be measured firstly alone):

- Blank = 0.5 MCF
- Sample = 6.3 MCF

Based on the lab's previous calibration (0.5 MCF = 1 × 10^8 CFU/mL), and the protocol target (1-10 × 10^7 cells/mL which correspond to 0.5 MCF in our case), perform a 1:12 dilution by mixing 300 µL of cell suspension with 3.3 mL PBS/BSA and re-measuring:

Final concentration = 0.7 MCF → 1.4 × 10^8 CFU/mL

<u>Day 15: Thursday, September 18, 2025</u>

Trypan Blue assay:
Flies were exposed for 2 hours via filter paper soaked in 7% glucose in water and 10% trypan blue solution (2g trypan blue in 20ml distilled water), placed in vials with agar-water mixture (1g agar in 100 mL water), following the Trypan Blue Assay / Dye exclusion test Protocol *(See Experimental Protocols*). Following staining, flies were dissected using the Gut Dissection Protocol *(See Experimental Protocols*), and observed under a stereomicroscope. No visible differences were noted between control and microplastic-exposed groups. The experiment will be repeated due to the use of an overly

concentrated 10% trypan blue solution, which affected the accuracy of the viability assessment. A more appropriate dilution will be used in subsequent trials.

Fixation:
Samples were fixed using 4% paraformaldehyde (in PBS) for later analysis under a stereoscope with camera. Remaining flies were transferred to clean food to remove excess dye for 6 days.

Flow cytometry experiments (Day 2)

Supervising Professor: Vasiliki Lampropoulou, Assistant Professor, School of Medicine, NKUA

The experiments were repeated and done with all 3 categories of genetically engineered yeasts (each one with a different CBM) and with non-modified yeast cells to see the differences, following the Standard Flow Cytometry Protocol *(See Experimental Protocols*).

Day 16: Friday, September 19, 2025

Routine Fly Stock Maintenance (no. 9)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 7)
Continued death count in all longevity vials.

Day 17: Sunday, September 21, 2025

Climbing Assay

The F1 generation of flies have been fed for 14 days and from each category they were transported to flies were gently transferred into 9 cm plastic empty tubes with a ruler beside them. The top of the tube was sealed with a foam plug. The tube was gently tapped 1-2 times on the bench to bring all flies to the bottom simultaneously and they were recorded for 30 sec, following the Climbing Assay Protocol *(See Experimental Protocols).*

Day 18: Monday, September 22, 2025

New Food Preparation (no. 3)
Two liters of food were prepared following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*) (sugar, yeast, cornmeal, agar), protected with filter and covered with a plastic bag for 24h in room temperature.

Routine Fly Stock Maintenance (no. 10)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 8)
Continued death count in all longevity vials.

Day 19: Tuesday, September 23, 2025

New Food Storage (no. 3)

The prepared food had solidified inside ~350 vials, which were sealed with foam plugs and stored at the fridge (10^{o}C), following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*).

Day 20: Wednesday, September 24, 2025

Routine Fly Stock Maintenance (no. 11)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days) However, due to high activity and good condition, several flies escaped. In one case, a vial was not sealed in time, resulting in complete loss of that sample (*PS treatment*), thus reducing the sample size for that group.

Longevity Assay - Mortality Monitoring (no. 9)
Continued death count in all longevity vials.

Trypan Blue remaining flies Dissection and staining:
Surviving flies that were left 6 days in normal food to remove excess dye were dissected, using the Trypan Blue Assay / Dye exclusion test Protocol and the Gut Dissection Protocol *(See Experimental Protocols*).

- Blue staining (Trypan Blue) was observed under a light microscope, possibly indicating microplastic presence in specific organs

Nile red observation in flies (previous larvae left for 7 days in Nile Red MPs & food). Specific tissues were isolated: gut, thoracic regions, and one ovary per fly, using the Gut Dissection Protocol *(See Experimental Protocols*).

- Isolated tissues were further examined using **confocal microscopy** for fluorescent microplastics

Additional setup, Yeast Feeding assay (Day 1):
Filter papers with yeast paste and 7% glucose were placed in vials containing agar and water for feeding behavior studies., according to the Genetically Engineered Yeast Feeding Protocol *(See Experimental Protocols*).

Flow cytometry experiments (Day 3)

Supervising Professor: Vasiliki Lampropoulou, Assistant Professor, School of Medicine, NKUA

Processing samples from bioreactor (*See Laboratory 4 section*) (D1: donor 1). Samples: D1 18/09 t=0h, D1 18/09 t=24h, D1 23/09 t=0h, D1 23/09 t=24h. They were thawed at room temperature for ~15 minutes. The process was accomplished following the Flow Cytometry after Bioreactor Protocol *(See Experimental Protocols*).

Cell concentration was very low. This may be due to strong autofluorescence from native microbes interfering with detection, and possibly poor yeast survival or protein expression due to absence of lactose in the growth medium.

Day 21: Thursday, September 25, 2025

Trypan blue repeated: Flies were transferred to Trypan Blue solution (0.4% more diluted this time) with 7% glucose and incubated for 2 hours. Following

this, flies were dissected, fixed in 4% paraformaldehyde, based on the Trypan Blue Assay / Dye exclusion test Protocol and the Gut Dissection Protocol *(See Experimental Protocols*). They were washed twice with PBS, and stored on glass slides in the refrigerator,

Yeast Feeding assay (Day 2): Yeast-glucose filter papers were refreshed.

Day 21: Friday, September 26, 2025

Routine Fly Stock Maintenance (no. 12)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 10)
Continued death count in all longevity vials.

Yeast Feeding assay (Day 3): Yeast-glucose filter papers were refreshed.

Day 22: Monday, September 29, 2025

New Food Preparation (no. 4)
Two liters of food were prepared following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*) (sugar, yeast, cornmeal, agar), protected with filter and covered with a plastic bag for 24h in room temperature.

Routine Fly Stock Maintenance (no. 13)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 11)
Continued death count in all longevity vials.

Yeast Feeding assay (Day 4 - with MPs): The filter papers supplemented with yeast were refreshed. Fluorescent PET microplastics were incorporated

into the food, and flies previously maintained on yeast-based substrates (CBM2 strain, known for its high PET-binding affinity) were transferred accordingly.

Images of Trypan Blue-stained flies were acquired.

Day 23: Tuesday, September 30, 2025

New Food Storage (no. 4)

The prepared food had solidified inside ~350 vials, which were sealed with foam plugs and stored at the fridge (10^{o}C), following the standard protocol to the Drosophila melanogaster Food Preparation Protocol *(See Experimental Protocols*).

Day 24: Wednesday, October 01, 2025

Routine Fly Stock Maintenance (no. 14)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 12)
Continued death count in all longevity vials.

Day 25: Thursday, October 02, 2025

Flow cytometry experiments (Day 4)

Supervising Professor: Vasiliki Lampropoulou, Assistant Professor, School of Medicine, NKUA

The final flow cytometry was to determine the viability of the yeast cells, following the Flow Cytometry Staining with Propidium Iodide (PI)*(See Experimental Protocols*). **Staining Setup**

Notes: Only the 19/09 bioreactor and non-recombinant (S. boulardii) samples were stained with PI.

Day 26: Friday, October 03, 2025

Routine Fly Stock Maintenance (no. 15)
Regular monitoring was performed for dead flies, mold growth, or food degradation.
Flies were transferred to fresh food vials to maintain healthy rearing conditions. (every 2-3 days)

Longevity Assay - Mortality Monitoring (no. 13)
Continued death count in all longevity vials.