

Lab # 11 *Caenorhabditis elegans*

Part I - Anatomy and Behavior (4-10-12)

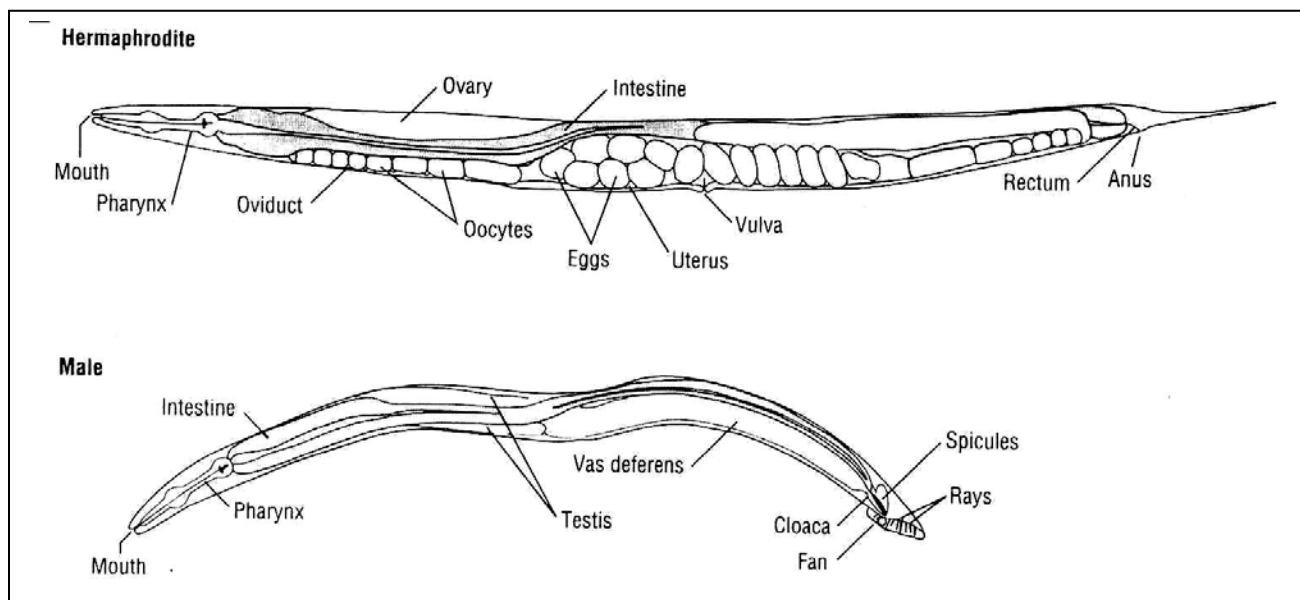
Nematode worms are ubiquitous. There are over 500,000 species of nematodes. Of every 5 animals on earth 4 are nematodes. Some species are human parasites, for example *Wuchereria bancrofti* causes elephantiasis in humans. Also, hookworms, pin worms and *Ascaris* are nematodes. However most, including *Caenorhabditis elegans*, are free-living non-parasitic worms.

Basic Characteristics of *C. elegans*

Small in size, the adult is about 1 mm in length. They can barely be seen by the unaided eye. They are adapted to life in the soil. They are non-parasitic, free-living organisms. In nature it eats practically anything it can fit in its mouth, but prefers bacteria. In the laboratory *C. elegans* is grown on an agar plate seeded with a lawn of *E. coli* (strain OP50).

Anatomy

A tough cuticle covers the outside of the worm. Muscles attached to the cuticle allow for snake-like movement. The gut is a simple tube that originates at the buccal cavity (mouth). Next in line is the pharynx, which contains a “grinder” with tooth-like projections that disrupt bacterial cells and propel them into the intestine. Food is enzymatically digested in the intestine, nutrients are absorbed, and undigested material is expelled out the anus. The nervous system can sense external stimuli and respond with appropriate behavior. *C. elegans* has a sense of touch, smell and taste; also, it can sense differences in temperature. Motor neurons stimulate the contraction of muscles that control motility and eating. The gonads of the hermaphrodite produce both eggs and sperm. The male produces sperm only.

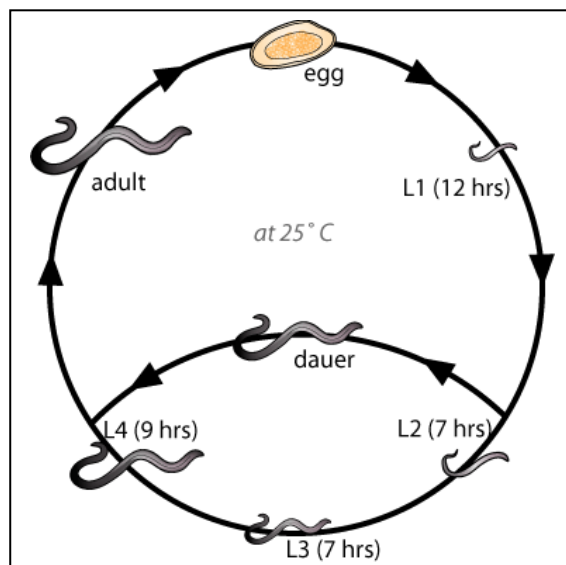


Life cycle

C. elegans has two sexes, hermaphrodites and males. Hermaphrodites are self-fertile, as they can fertilize their own eggs with their own sperm. Males, which produce sperm only, copulate with females whenever possible. During copulation males search for the vulva (entrance to the gonad) with their tail end. Having found it they insert their spicule (penis) and deliver sperm. The male sperm is retained for several days. Although females are self-fertile, they will preferentially use male sperm over their own sperm when fertilizing their eggs. Fertilization of egg with sperm occurs internally within the gonads of the hermaphrodite. Following fertilization, the zygote undergoes cleavage to form many cells. After 13 hours of embryonic development the embryo is shed from the hermaphrodite. The embryo goes through 4 larval stages (L1-L4) before it becomes a fertile adult. Each larval stage is associated with a molt of the cuticle. The time span from zygote to fertile adult capable of shedding its own eggs is about 3-4 days at 25°C. A hermaphrodite produces and sheds its eggs in the first 3-4 days of adult life. After that it lives additional 10-15 days then dies.

Sex Determination in *C. elegans*

The number of X chromosomes determines the sex of the individual. Those individuals that possess two X chromosomes are hermaphrodites. Those individuals that possess only one X chromosome are males. Males are generated by a mistake in meiosis called non-disjunction. In normal meiosis, X chromosomes disjoin from each other during anaphase and move to opposite poles, thus insuring that each daughter cell receives one and only one X chromosome. Because of non-disjunction one daughter cell (haploid gamete) receives 2 X chromosomes while the other receives none. If an egg with no X chromosome is fertilized by a sperm with one X chromosome then the resulting zygote will have only one X chromosome and will be male. Males are highly motile and very aggressive in their mating behavior. One male will mate with several hermaphrodites. Hermaphrodites produce sperm early in adulthood, store the sperm in a spermatheca, and fertilize eggs over the course of a few days. The number of sperm produced within the hermaphrodite limits the brood size.



Drawing Worm Anatomy

Procedure

1. Obtain an agar plate with Him-8 *C. elegans* worms. This plate will provide enough worms for your entire group.
2. Observe the plate under the dissecting microscope to confirm the presence of worms.
3. Wash bacteria off the worms in preparation for microscopy and behavioral studies.
 - a. Add 1.4 ml of CTX buffer to the agar plate and swirl to get worms into suspension. Observe in the dissecting microscope to confirm that worms are in suspension.
 - b. Pipet CTX and worms off the plate and into a microcentrifuge tube.
 - c. Allow the worms to settle to the bottom of the tube (3-5 min) and then remove most of the CTX buffer.
 - d. Add 1 ml of fresh CTX buffer to the worms and invert gently several times to mix. Allow worms to settle to the bottom a (3-5 min) and then remove most of the CTX buffer.
 - e. Add another 1 ml of fresh CTX buffer to the worms and invert gently several times to mix. Allow worms to settle to the bottom a (3-5 min) and then remove most of the CTX buffer.
 - f. Add 100 μ l of fresh CTX to the worms. The worms are now free of contaminating bacteria and are ready for use.
4. While washing your worms you should begin making agar slides. Each person in your group will make their own slide and do their own microscopy.
 - a. Prepare a spacer slide as demonstrated by the instructor.
 - b. Pipet 1-2 drops of molten 0.75% agar on a plain microscope slide.
 - c. Place the spacer slide on top of the slide with agar. Try to avoid introducing bubbles.
 - d. Wait a couple of minutes for the agar to solidify. Keep the 2 slides together until you are ready to put worms on the slide.
 - e. Carefully tease the two slides apart in a way that leaves the pad on the slide without tape. Try to work steadily now so that the agar pad does not dry.
5. Resuspend the washed worms and pipet 5-10 μ l of worms on to the center of the agar pad on your slide. You are attempting to get 10-20 worms; adjust the volume until you reach a density close to that. Place a cover slip over the agar pad.
6. View the worms in a compound microscope. Begin focusing with the 4X (low power) objective lens. Increase magnification as you like, however do not attempt to use the 100X objective lens (oil immersion lens). The 100X objective lens would collide with the cover slip due to the height of the agar pad.

Questions:

Briefly note the worm's behavior.

Are they actively moving? Describe their mode of movement.

Questions:

Are there different sizes of worms?

How would you distinguish adult hermaphrodites from juvenile hermaphrodites?

There should be some males in your slide. Identify a male and confirm your find with the instructor. What is the most defining male feature?

7. Produce a fresh agar slide.
8. Pipet 10-20 worms on to the agar pad.
9. Pipet 5 μ l of tetramisole (anesthetic) onto the worms and add a cover slip.
10. Observe the worms in the compound microscope. Are they anesthetized?
11. Make detailed, labeled drawings of an adult hermaphrodite, a juvenile hermaphrodite an adult male and an embryo. To aid in identification of worm structures, go the wormatlas website and use the diagrams and movies to guide your observations in the microscope.
 - Go to the wormatlas home page <http://www.wormatlas.org/index.html>
 - Click on the picture of a hermaphrodite
 - Click on “introduction” to the right of the top drawing
 - Scroll down to figure 1. Use this figure to help you label your drawing of an adult hemaphrodite.
 - Click on the picture in Fig 1 to enlarge. Click on specific parts to enlarge further.
 - Exit this window, and then scroll down the page to view other anatomical parts and movies.
 - To view the male anatomy, scroll down the page to figure 5.
 - Click directly on the picture to magnify. Click on specific parts to enlarge further.
 - Use this figure to help you label the drawing of your worm.
 - Draw worms on the next page.
 - Draw worms as they appear in your microscope, not as they appear in the web site.

Behavioral Studies

Osmotic Avoidance

Osmosis is the net movement of water across a membrane toward a higher solute concentration. Single-celled organisms and small multicellular organisms can be dramatically affected by extreme osmotic environments. Such environments can cause dehydration or over-hydration of the organism. Animals that have a nervous system, such as *C. elegans*, can sense extreme osmotic environments and move to avoid them.

Procedure:

1. You will do this experiment as a group.
2. Obtain an NGM plate (one that lacks a bacterial lawn). Remove the lid and place a 30 μ l spot of 80% glycerol/bromphenol blue (hyperosmotic solution) on the lid (not the agar).
3. Remove the cap of a fine-tip sharpie permanent marker. Place the open end of the cap on the glycerol drop such that it bisects the drop. Spin the cap to coat the entire circumference of the cap. Lift the cap. If there is a bubble of glycerol then pop it. Place the cap gently on to the center of the agar. Avoid marring the surface of the agar. Allow the glycerol to soak in (3-5 min) and then remove the cap.
4. Gently resuspend your washed worms by bumping the microcentrifuge tube. Pipet 10-20 worms into the center of the osmotic ring. Observe the worms under the dissecting microscope.
5. Remove excess CTX buffer by gently blotting with the tip of a rolled-up Kimwipe. Worms can't crawl until excess buffer is removed.
6. Observe the worms periodically over the next 15-30 minutes and note their response to the high osmotic environment created by the glycerol.

Questions:

Are worms attracted to or repelled by the osmotic ring?

Did any worms cross the osmotic barrier?

Why would worms avoid a region of high osmolarity?

Chemotaxis

C. elegans has a sense of smell and thus can sense the presence of volatile chemical attractants and repellents.

Procedure:

1. You will do this experiment as a group.
2. Obtain an NGM plate (one that lacks a bacterial lawn).
3. Using a permanent marker, mark the bottom of the plate with an X near an outside edge, and then make a second mark 180⁰ from the first.
4. Pipet 4 μ l of isoamyl alcohol/EtOH on the agar above one X and 4 μ l of CuSO₄ at the other X. Allow both spots to dry (3-5 min).
5. Pipet 20-40 worms at the center of the plate, equidistant from the 2 X marks.
6. Observe the worms under the dissecting microscope.
7. Remove excess CTX buffer by gently blotting with the tip of a rolled-up Kimwipe.
8. Observe the worms repeatedly over the course of 20-30 minutes to see which compound is favored by the worms.
9. Obtain another NGM plate. Using a permanent marker, mark the bottom of the plate with an X near an outside edge, and then make a second mark 180⁰ from the first. Draw a line down the center of the plate, perpendicular to the 2 Xs.
10. Pipet 4 μ l of isoamyl alcohol/EtOH on the agar above one of the Xs and 15 μ l of CuSO₄ along the line. Avoid marring the agar with the pipet. Allow both to dry (3-5 min)
11. Pipet 20-40 worms at the second X mark such that the worms are separated from the alcohol by a line of CuSO₄.
12. Remove excess CTX buffer by gently blotting with the tip of a rolled-up Kimwipe.
13. Observe the worms repeatedly over the course of 20-30 minutes to see what happens.

Based on your observations, are worms more attracted to isoamyl alcohol or CuSO₄?

Do worms change their behavior in alcohol?

Are any worms willing to cross the CuSO₄ barrier to get to the alcohol?

Response to Touch

A worm crawling forward on the agar surface of a petri plate can be touched gently near its nose using a sterile probe and the worm will reverse direction. If moving backward and touched on the tail, the worm will again reverse directions and move forward. How many times can this procedure be repeated before the worm learns to ignore this stimulus?

Procedure:

1. Each person will do this experiment on their own.
2. Produce a probe for touching worms.
 - a. Pluck a single eyelash.
 - b. Using nail polish, glue the eyelash to the tip of the toothpick such that the fine end of the lash protrudes well beyond the tip of the toothpick.
 - c. Allow the glue to harden (3-5 min)
 - d. Sterilize the lash by dipping it in alcohol and allowing it to air dry
3. Obtain a plate of him-8 worms. This plate has been seeded with a lawn of OP50 E. coli.
4. Choose an active worm for this experiment. I suggest a large hermaphrodite.
5. Using your eyelash probe, repeatedly touch the worm on the head then tail until the worm fails to respond.
6. Note the number of touches required (add both head and tail touches).
7. Repeat this procedure with 2 more worms.
8. Fill in the table below.

<u>Worm</u>	<u>Number of touches</u>
#1	_____
#2	_____
#3	_____

Observing Mating Behavior

1. Each person will do this experiment on their own.
2. First observe mating behavior on the computer
3. Go to the wormatlas home page <http://www.wormatlas.org/index.html>
4. Click on the drawing of the male.
5. Click on “introduction”
6. Scroll down the page to view pictures of the male. Read the legend below figure 3 and then watch the quicktime movie below figure 3.
7. Now that you know what males look like and what mating behavior is, attempt to observe mating behavior on the worm plate under the dissecting microscope.

Did you observe mating behavior on the plate?

Did males prefer juvenile or adult hemaphrodites?

Did males remain with one hermaphrodite or move quickly between hermaphrodites?

Transferring Worms Between Plates

Most worm experiments require that individual worms be transferred from one plate to another. You will need to master this technique so that your future worm experiments will go well.

Procedure:

1. Each person will do this experiment on their own.
2. Obtain a Him-8 plate and also an NGM plate inoculated with OP50 bacteria.
3. Place the Him-8 plate on the dissecting scope and bring worms into focus under low power (knobs located just below the ocular lenses adjust magnification)
4. Obtain a worm pick (thin platinum wire fused to a glass Pasteur pipet).
5. Sterilize the pick by briefly flaming the platinum wire. As soon as the wire is glowing red it is sterile. Avoid long exposure of the pick to the flame as it will melt the glass.
6. Allow the pick to cool (2 sec).
7. The worms must remain moist during the transfer, thus transfer should happen quickly. Transfer is facilitated by first touching the sterile pick to the bacterial lawn to pick up a glob of sticky bacteria. Then touch the worm you wish to pick with the glob of bacteria and sweep the worm off the plate with a sideways and upwards motion. If done correctly, the worm will adhere to the bacteria and be lifted off the plate. Try to avoid marring the surface of the agar with the pick. Worms will burrow into the agar if its surface is broken.
8. Quickly transfer the worm to the fresh OP50 plate, being careful to avoid smashing the worm into the agar. Again a sideways motion might be best. Sometimes the worm will cling to the pick. In this case keep the tip of the pick on or just above the surface of the agar so the worm can choose to crawl away on its own.
9. Attempt to transfer 3 large hermaphrodites and 3 males to the OP50 plate.
10. Confirm your successful transfer with the instructor.
11. Ask for help when you need it.

Part II - RNAi experiments – Day 1 (4-17-12)

RNA interference (RNAi) is a technique used to transiently inhibit expression of a particular gene in a cell. The mechanism works in the following way: Double stranded RNA (dsRNA), which is homologous to a cellular gene, is introduced into a cell. The RNA functions to inhibit the expression of the homologous gene at the level of transcription and translation, effectively knocking out the gene. This technique is very simple to perform in *C. elegans*. The worms are simply fed bacteria that have been genetically engineered to produce a specific dsRNA. The worms ingest, but do not destroy the dsRNA, which then enters cells and then inhibits expression of the target gene.

JR2522 is a strain of *C. elegans* worms that expresses GFP in its gut cells, but is otherwise wild type. Your group will transfer several JR2522 hermaphrodites to plates inoculated with 3 feeder strains of bacteria that express 3 different dsRNAs. The *unc-22* feeder strain inhibits expression of the *unc* gene and causes genetically normal worms to become uncoordinated. The embryonic lethal feeder strain causes death of embryos. Finally, the anti-GFP feeder strain inhibits the expression of GFP in the gut cells of JR2522 worms.

Transfer of worms to dsRNA plates Day 1 (4-17-12)

Procedure:

1. Each group will perform this experiment.
2. Obtain a plate containing the JR2522 strain of *C. elegans*. This plate contains the normal feeder strain of bacteria (OP50), which does not produce dsRNA
3. Observe the appearance and movement of these worms under the dissecting scope.
4. Obtain plates inoculated with the following dsRNA feeder strains:
 - Unc-22
 - Embryonic Lethal
 - Anti-GFP
5. Also, obtain a plate inoculated with the normal feeder strain, OP50
6. Transfer 3 hermaphrodites to each of the 4 plates.
7. Wrap your plates with parafilm as demonstrated by the instructor.
8. Store the plates at room temperature until the next lab period (next week).

Scoring Results Day 2 (4-24-12)

Compare the worms on grown on OP50 feeder bacteria to those grown on *unc-22* and Embryonic Lethal RNAi feeder bacteria.

Note your observations below:

Fluorescence Microscopy of RNAi treated JR2522 worms Day 2 (4-24-12)

- ◇ Produce 2 agar-lined microscope slides as demonstrated by the instructor.
- ◇ Label one slide as control and the other as anti-GFP
- ◇ Place a drop of anesthetic on the agar.
- ◇ Sterile the wire pick with the Bunsen burner.
- ◇ Pick up several healthy adult worms from the OP50 plate and transfer the worms to the anesthetic on the control slide. Avoid gouging the agar on the slide.
- ◇ Place a cover slip over the worm.
- ◇ Pick up several healthy adult worms from the Anti-GFP plate and transfer the worms to the anesthetic on the anti-GFP slide.
- ◇ Observe the worms in the fluorescence microscope.

Question

Did anti-GFP dsRNA affect the expression of GFP in JR2522 worms?

Note your observations below:

Briefly explain how RNAi can be used to knock out a gene in *C.elegans*.

Part III - Fluorescence Microscopy of GFP Worms (4-24-12)

In today's experiment we will perform fluorescence microscopy with *C. elegans* worms that express the Green Fluorescence Protein (GFP) in various tissues. You will anesthetize the worms and mount them on a microscope slide. Finally you will observe the worms with a fluorescence microscope and photograph your specimens. Each group should produce 5 slides, one for each GFP strain.

<u>Strain</u>	<u>GFP-expressing Tissue</u>
JR797	neurons
JR2408	pharynx
JR2522	gut
PD7963	body wall muscle
MD701	germline sheath cells

Protocol for Mounting and Anesthetizing Worms

- ◇ Produce an agar-lined microscope slide as demonstrated by the instructor.
 - Place a generous drop of molten 0.75% agar on a plain microscope slide. Put a guide slide, whose surfaces have been raised slightly with two layers of tape, on top of the first slide so that the agar pad has the proper thickness. Wait a couple of minutes for the agar to solidify. Carefully tease the two slides apart. Try to work steadily now so that the agar pad does not dry.
- ◇ Place a drop of Tetramisole anesthetic on the agar.
- ◇ Sterilize the wire pick with the Bunsen burner.
- ◇ Pick up a healthy worm with your wire worm pick.
- ◇ Transfer the worm to the anesthetic on the agar-lined slide. Avoid gouging the agar on the slide.
- ◇ Transfer 3 more worms to the anesthetic. Try to obtain worms in different stages of development (large hermaphrodite with eggs, L1-L4, male if possible)
- ◇ Place a cover slip over the worms.
- ◇ Observe the worms in the fluorescence microscope.
- ◇ Take pictures of the worms as demonstrated by the instructor

Part IV - Genetic Cross (begins 5-1-12)

We will cross two strains of *C. elegans*; him-8 males with CB4384 hermaphrodites. The CB4384 strain is homozygous for the recessive unc mutation, which makes worms so uncoordinated that they move slowly on the plate and respond poorly to touch. The him-8 males are wild type for the unc mutation. The offspring of this cross, the F1 generation, will be heterozygous for the unc mutation and thus should display a wild-type phenotype with regards to coordination and movement. In other words they will look normal but carry one copy of the mutant allele. If the F1 generation is allowed to self cross then we should obtain a 3:1 Mendelian ratio where $\frac{3}{4}$ of the F2 offspring have the wild type phenotype and $\frac{1}{4}$ of the offspring have the mutant, uncoordinated phenotype.

The overall timeline of this cross is the following:

- Day 1 (tues, 5-3-11) Cross him-8 males with CB4384 hermaphrodites.
- Day 2 (wed, 5-4-11) Transfer the mated hermaphrodites to new plates.
The mated hermaphrodites will produce F1 heterozygotes.
- Day 3 (fri, 5-6-11) Observe plates to see if F1 generation appears wild type as predicted.
Then move 3 F1 hermaphrodites to a new plate
- Day 4 (tues 5-10-11) Observe the F2 generation and score mutations.

Genetic Cross

Day 1 (Tues, 5-1-12)

1. Each person will perform this experiment
2. You will be given a plate with CB4384 worms and a plate with him-8 worms, and a plate on which to do the cross.
3. Observe him-8 worms under the dissecting scope. Be sure that you can tell the difference between males and hermaphrodites.
4. Observe a plate of CB4384 worms under the dissecting scope. Try to distinguish L4 hermaphrodites from adult hermaphrodites. L4 hermaphrodites are almost as long as adults, but are much thinner and lack embryos and eggs. Also, L4s have a **white crescent with a small black dot** in the middle of the animal. This corresponds to the developing vulva, the structure used to lay eggs. In contrast, adult hermaphrodites lack this crescent and eventually fill with eggs. We will want to use L4s for the cross because they are still virgins and have not yet self-crossed.
5. Obtain a small agar plate seeded with OP50 bacteria. Label the plate as “Cross-day1” and with your initials and the date. Write small and on the edges of the plate so as not to limit visualization of results.
6. Transfer three CB4384 L4 hermaphrodites to the small plate.
7. Transfer three him-8 males to the small plate. Smaller males are better than larger males because they have not yet spent most of their sperm.
8. You must avoid transferring him-8 hermaphrodites or embryos on to the mating plate. If this happens then you must remove them.
9. Incubate the mated worms at room temperature for 1 day.

Caution: Fungal contamination is a significant issue. You should minimize the time that plates are left open to the environment with their lids off.

Genetic Cross

Day 2 (wed 5-2-12)

Obtain a new plate and label it as “cross–day 2” and with your initials and the date
Transfer the mated hermaphrodites to new plates. Leave the males behind on the old plate.
The mated hermaphrodites should produce F1 heterozygotes.

Genetic Cross

Day 3 (fri 5-4-12)

Obtain a new plate and label it as “cross–day 3” and with your initials and the date.
Observe the day 2 plate to see if the F1 generation appears wild type or uncoordinated.
Identify 3 F1 hermaphrodites that express the wild type phenotype (normal, coordinated movement and good response to touch) and transfer them to the new plate.
Incubate this plate at room temperature until Tuesday.

Question:

Does the F1 generation on the day 2 plate appear to have wild-type morphology and movement or do they continue to move in an uncoordinated manner?

Genetic Cross Day 4 (tues 5-8-12)

Observation of Individual Mutant Phenotypes

1. Obtain your mated worms.
2. Observe the worms under the dissecting microscope.
3. Tally the number of worms having either a wild type phenotype or an unc phenotype and fill in the table below.

Phenotype	Number of worms
Wild-type	_____
unc	_____

Questions:

1. How are male nematodes chromosomally different from hermaphrodite nematodes?
2. Hermaphrodites are self fertile, yet when we cross males with hermaphrodites the vast majority of the offspring will be products of the cross rather than products of self-fertilization. Why is this so?
3. When crossing males with hermaphrodites do you expect to get any males in the offspring? If so, at what frequency?
4. The CB4384 hermaphrodites are homozygous for the recessive *unc* mutation. This mutation is expressed phenotypically, resulting in a very dysfunctional worm. However, the F1 offspring from our cross will look phenotypically normal. Explain why.
5. Assume you have on a plate a single hermaphrodite that is heterozygous for the *Unc-22* mutation. This hermaphrodite fertilizes itself and produces 400 offspring. How many offspring are predicted to have an uncoordinated phenotype? Explain your logic.

