WORM BOOT CAMP

Laboratory Skill Development

ABSTRACT

This unit is designed to provide lessons and activities related to the use, care, and manipulation of the soil-living nematode *Caenorhabditis elegans* as a model organism for scientific investigation. Upon completion of these lessons and laboratory activities, students will have the skill set necessary to: describe the history and use of *C. elegans* as a model organism, identify different life cycles of the nematode, prepare plates for *C. elegans* propagation, and isolate/transfer individual nematodes for experimental use.

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Author's Note

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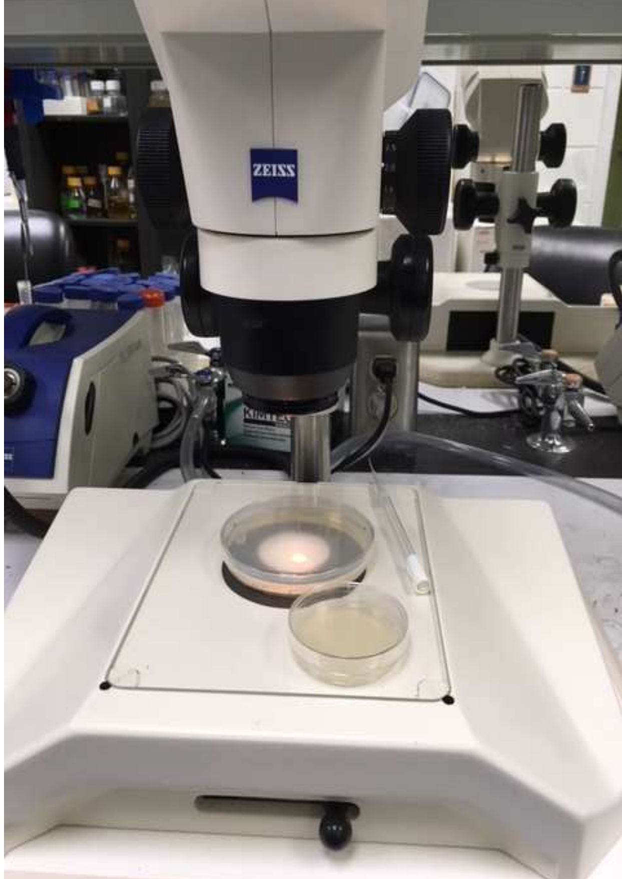
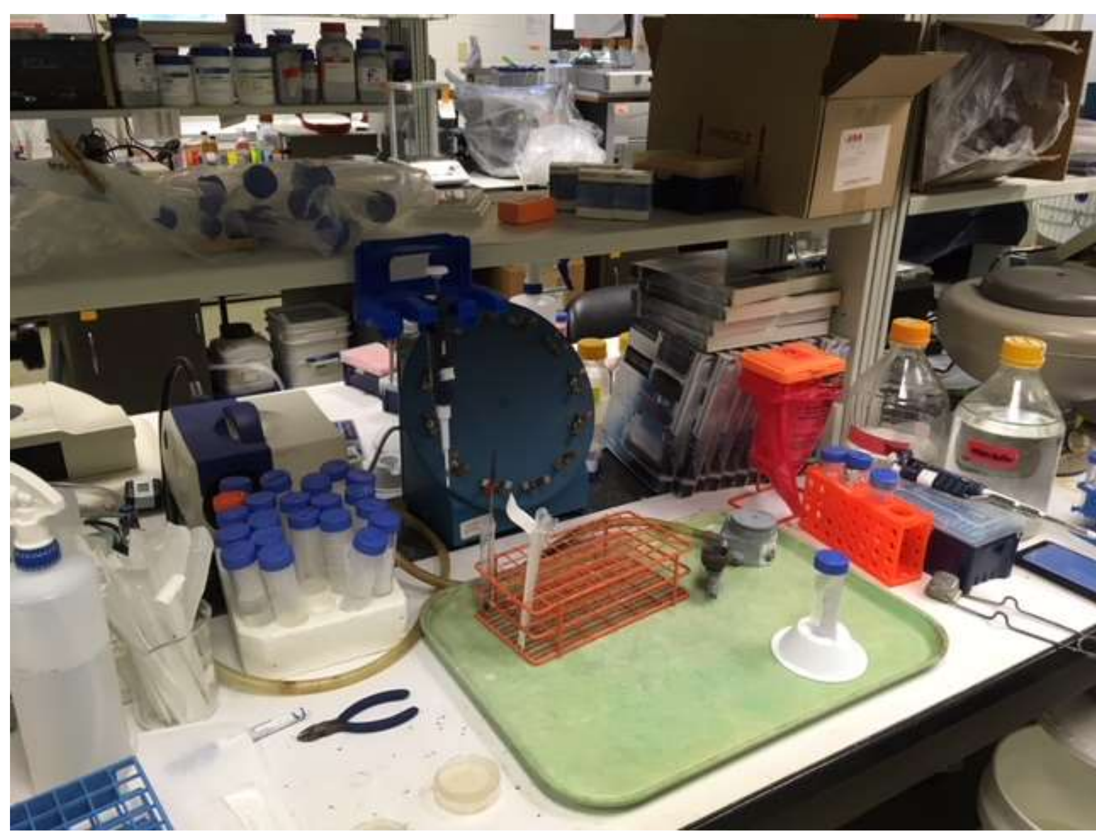
I have used *C. elegans* in the past in my Honors Genetics coursework for students to develop laboratory skills using model organisms. Historically, the activities we have used *C. elegans* in were prescribed "recipe" laboratory activities purchased from a biological supply company that left little to the imagination. I wanted to provide my students with a more thorough introduction to the skills needed to work with a model organism and provide a better understanding of inquiry investigation. Ultimately, the motives for development of this curriculum are two-fold; to increase the skill set of the student and teacher in the many aspects of using *C. elegans* as a model organism for genomic, toxicological, and behavioral studies and to provide my students with the tools necessary to develop and implement their own experimental design using *C. elegans* as a model. As I attempt to challenge my students through independent inquiry and investigation, they will develop a better understanding of the genomic homology between the free-living nematode *Caenorhabditis elegans* and humans.



Introduction

As with all model organisms used in scientific investigation, *Caenorhabditis elegans* is an ideal model for scientific investigation. *C. elegans* came to prominence as a model organism in 1963 by researcher Sydney Brenner in the study of developmental biology and genetics. Since then, scientists have sequenced the entire genome of the free-living nematode, mapped the cell lineage of all 959 cells in the adult worm, and isolated and identified hundreds of mutant strains with specific structural and functional phenotypes.

What makes an organism a good model in science? The best model organisms are small, easy and inexpensive to care for, reproduce quickly and produce many progeny, and have a short life span that allows many generations to be studied in a short period of time. *Caenorhabditis elegans* life cycle is only two weeks and the adult worm can produce upwards of 1,000 eggs each day. *C. elegans* is cultured on agar media plates with *Escherichia coli* bacteria as a food source that is easy to grow and maintain. Healthy cultures can be frozen at -80*C indefinitely to be used for later experimentation. *C. elegans* is transparent throughout its life cycle, making internal observation of development from embryonic through adult life cycles possible. Scientists have learned from the sequenced genome of *C. elegans* the functional homology in human genes, providing a model for human disease study in the nematode.



Tips about Curriculum

Lesson Plan Format: All lessons in this curriculum unit are formatted in the same manner. In each lesson you will find the following components:

KEY QUESTION(S): Identifies key questions the lesson will explore.
OVERALL TIME ESTIMATE: Indicates total amount of time needed for the lesson, including advanced preparation.
LEARNING STYLES: Visual, auditory, and/or kinesthetic.
VOCABULARY: Lists key vocabulary terms used and defined in the lesson. Also collected in master vocabulary list.
LESSON SUMMARY: Provides a 1-2 sentence summary of what the lesson will cover and how this content will be covered. Also collected in one list.
STUDENT LEARNING OBJECTIVES: Focuses on what students will know, feel, or be able to do at the conclusion of the lesson.
STANDARDS: Specific state benchmarks addressed in the lesson. Also collected in one list.
MATERIALS: Items needed to complete the lesson. Number required for different types of grouping formats (Per class, Per group of 3-4 students, Per pair, Per student) is also indicated.
BACKGROUND INFORMATION: Provides accurate, up-to-date information from reliable sources about the lesson topic.
ADVANCE PREPARATION: This section explains what needs to be done to get ready for the lesson.
PROCEDURE WITH TIME ESTIMATES: The procedure details the steps of implementation with suggested time estimates. The times will likely vary depending on the class.
ASSESSMENT SUGGESTIONS: Formative assessment suggestions have been given. Additionally, there is a brief summative assessment (pre/post test) that can be given. Teachers should feel free to create additional formative and summative assessment pieces.
EXTENSIONS: (ACTIVITIES/LITERATURE) There are many activities and reading sources available to augment and enhance the curriculum. They have been included. If you find additional ones that should be added, please let us know.
RESOURCES/REFERENCES: This curriculum is based heavily on primary sources. As resources and references have been used in a lesson, their complete citation is included as well as a web link if available.
STUDENT PAGES: Worksheets and handouts to be copied and distributed to the students.
TEACHER MASTERS: Versions of the student pages with answers or the activity materials for preparation.

Inquiry-based: The lessons in the curriculum invite students to be engaged and ask questions. They work through background information in a guided fashion, but are challenged to think beyond what they have read or done. The teacher serves as the facilitator in these activities, not the deliverer of information.

Content: This unit provides for the development of specific skill sets needed implement laboratory protocols that use *C. elegans* as a model organism for study. The content of these lessons goes above the level of practice provided through the use of pre-packaged laboratory kits provided by science education supply companies.

Implementation notes: This curriculum should be modified and adapted to suit the needs of the teacher and students. To help make implementation easier in this first draft, notes have been included in lessons as needed.

Extensions: There are many opportunities to expand the lessons presented here. My goal is to provide students with the skill set necessary to use *Caenorhabditis elegans* as a model organism in their own scientific inquiry for Science Fair, AP Research, Experimental Science or collegiate-level research. By providing the requisite knowledge for the care and use of *C. elegans* as a model organism, students can develop their own protocol to study toxicology, RNA interference, habituation, and behavior.

Science Subject: Honors Genetics, Research Science

Grade and ability level: 11-12 grade students in upper-level life science classes

Science concepts: use and care of model organism, model systems, microscopy, experimental design

Lesson Summaries

Preparation Homework: Flipped Video Assignment

Students will view a video journal https://www.jove.com/science-education/5110/c-elegans-development-and-reproduction as preparation for Worm Boot Camp and complete an accompanying worksheet. This independent activity allows students the opportunity to watch/rewatch to answer questions and ensure understanding of content.

Lesson One: Introduction to *C. elegans* and Microscopy Skills

Students will engage in whole-group introduction to *Caenorhabditis elegans* as a model organism for scientific inquiry and discovery. This lesson includes basic information about *C. elegans*, its life cycle, and introductory microscopy skills.

Lesson Two: *C. elegans* Culturing

Student will understand the role of *C. elegans* in the food chain and prepare culture media for the growth of *C. elegans* in the laboratory.

Lesson Three: *C. elegans* Life Cycle and Identification

Students will understand and describe the life cycle of C. elegans from egg through adult including the stress-resistant dauer phase.

Lesson Four: Lab Skill – Chunking vs. Picking

Students will determine which testing protocol requires the use of chunking a population of different ages of *C. elegans* onto new culture plates versus selectively picking individual worms and moving to new culture plates based on age or sex. Students will construct their own pick and practice the skills of chunking and picking to establish new culture plates of *C. elegans*.

Lesson Five: Mutant Worms

Students will be introduced to the different mutant strains of *C. elegans* with characteristic movements that can be observed using a microscope. These mutant strains of *C. elegans* are used in research to study a variety of human diseases that have similar genetic profiles to those found in *C. elegans*.

Lesson Six: Skills Practice and Assessment

Students will practice one or more skills learned during Worm Boot Camp. Students will finalize all skill assessments and prepare culture plates for independent inquiry in toxicology.

Lesson Sequencing Guide

Since the classroom teacher knows his or her students best, the sequencing of lessons and the amount of time spent on each should be altered to meet the needs of each individual setting. Below is a suggested pacing guide that can be used when planning to use this curriculum, assuming 45-minute class periods.

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Week 1	Intro Lesson Flipped Homework Assignment Video Journal and Questions	Lesson 1 Introduction to *C. elegans* Skill: Microscopy Practice (45 minutes)	Lesson 2 *C. elegans* Diet Skill: Media Preparation (45 minutes)	Lesson 2 Skill: Bacterial Culture (45 minutes)	Lesson 3 *C. elegans* Life Cycle (45 minutes)
Week 2	Lesson 4 Chunking vs. Picking Skill: Chunking (45 minutes)	Lesson 4 Chunking vs. Picking Skill: Pick Construction and Picking (45 minutes)	Lesson 4 Skill: Picking (45 minutes)	Lesson 5 Mutations of Movement Observations (45 minutes)	Lesson 6 Practice Skills and Assessment (45 minutes)

Extensions:

1) *Introduction to WormBook and WormBank*
   Materials: Computers with Internet access

2) *C. elegans and Toxicology*
   Time: 5 days, includes Introduction and 1 Day for writing Lab Report
   Materials: Dr. K. Choe Toxicology protocol

3) *Project Design and Implementation*
   Time: 3 weeks
   Materials as needed: Chemotaxis, Behavior/Habituation, RNA interference, Environmental toxicology, biological control using other nematode species.

Optional Lesson Sequencing Guide – Omit Lesson 2/Pour Plates, more practice time Lesson 4
Teacher pours plates for Lesson 2

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Week 1	Intro Lesson Flipped Homework Assignment Video Journal and Questions	Lesson 1 Introduction to *C. elegans* Skill: Microscopy Practice (45 minutes)	Lesson 2 Skill: Bacterial Culture (45 minutes)	Lesson 3 *C. elegans* Life Cycle (45 minutes)	Lesson 4 Chunking vs. Picking Skill: Chunking (45 minutes)
Week 2	Lesson 4 Chunking vs. Picking Skill: Pick Construction and Picking (45 minutes)	Lesson 4 Skill: Picking (45 minutes)	Lesson 5 Mutations of Movement Observations (45 minutes)	Lesson 6 Practice Skills and Assessment (45 minutes)	Lesson 6 Practice Skills and Assessment (45 minutes)

Vocabulary

Agar – gelatin substance made from various species of seaweed to provide a nutrient source for the growth and maintenance of microorganisms.

Apoptosis – programmed cell death; *C. elegans* has an invariant developmental process that results in the programmed cell death of exactly 113 cells.

Aseptic technique/transfer – the movement of substances from one location to another using specific techniques to limit the possibility of contamination by unwanted microorganisms.

Autosome – chromosome not responsible for primary sex development.

Auxotrophy - a mutant microorganism that requires a specific additional nutrient that the normal strain does not.

Axenic- a culture that is free from living organisms other than the species required.

Caenorhabditis elegans – (*C.* elegans); free-living soil nematode commonly used as a model organism for scientific investigation.

Chemotaxis - movement of a motile organism in a direction corresponding to an increase or decrease in concentration of a particular substance.

Chunking – movement of a population of nematodes from one plate to another.

Dauer – an arrested developmental variant of *C. elegans* that forms following the first larval stage in response to environmental stress.

Embryonic – developmental stage of growth that promotes differentiation of cells for specific functions.

Escherichia coli OP50 – (*E. coli*); specific bacterial species used as the food source for the culturing of *C. elegans* in a laboratory setting. This strain of *E. coli* (OP50) is auxotrophic as it lacks uracil as one essential nutrient.

Founder cell – a cell that gives rise to tissue by clonal expansion; *C. elegans* has 4 primary founder cells that give rise to its entire cell population.

Genotype – the genetic makeup of a particular organism.

Gravid – pregnant; contains fertilized eggs.

Hermaphrodite – contains both oocytes and sperm.

Homolog - a gene related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation (ortholog) or to the relationship between genes separated by the event of genetic duplication (paralog)

Inoculation – the introduction of a microorganism into a culture medium.
Invariant – unchanging; embryonic development in *C. elegans* has an invariant pattern.

Luria broth – nutrient media used for the culturing of bacteria for growth.

Magnification – process of enlarging the appearance of something; use of a compound light microscope or dissecting stereo microscope to increase the size of a specimen for visualization.

Model organism - a non-human species that is extensively studied to understand particular biological phenomena, with the expectation that discoveries made in the organism model will provide insight into the workings of other organisms.

Mutant – change in a gene sequence that impacts the process of protein synthesis.

NGM – nematode growth media used for the propagation of *C. elegans*.

Ortholog – The relationship between genes separated by speciation.

Petri plate/dish – sterile dish used to store agar for the growth and maintenance of microorganisms.

Phenotype - the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.

Picking – single transfer of specific worm(s) from one plate to another for separation based on observable characteristics such as size, age, or morphology.

Progeny - a descendant or the descendants of a person, animal, or plant; offspring

Next Generation Sunshine State Standards - Science

Science Standards	Lesson						
	Prep	**1**	**2**	**3**	**4**	**5**	**6**
SC.912.N.1.1: Define a problem based on a specific body of knowledge		**X**			**X**	**X**	**X**
SC.912.N.1.4: Identify sources of information and assess their reliability according to the strict standards of scientific investigation.	**X**					**X**	**X**
SC.912.N.1.6: Describe how scientific inferences are drawn from scientific observations and provide examples from the content being studied.		**X**			**X**	**X**	
SC.912.N.1.7: Recognize the role of creativity in constructing scientific questions, methods, and explanations.	**X**	**X**			**X**	**X**	**X**
SC.912.N.2.4: Explain that scientific knowledge is durable, robust, and open to change.	**X**	**X**				**X**	
SC.912.N.3.5: Describe the function of models in science and identify the wide range of models used in science.	**X**	**X**	**X**	**X**	**X**	**X**	**X**
SC.912.L.15.15: Describe how mutation and genetic recombination increase genetic variation.						**X**	
SC.912.L.16.4: Explain how mutations in the DNA sequence may or may not result in phenotypic change. Explain how mutations in gametes may result in phenotypic changes in offspring.						**X**	
SC.912.L.16.7: Describe how viruses and bacteria transfer genetic material between cells and the role of this process in biotechnology.	**X**	**X**	**X**	**X**	**X**	**X**	**X**
SC.912.L.16.9: Explain how and why the genetic code is universal and is common to almost all organisms.	**X**	**X**	**X**		**X**	**X**	**X**
SC.912.L.16.10: Evaluate the impact of biotechnology on the individual, society, and the environment including medical and ethical issues.	**X**	**X**	**X**	**X**	**X**	**X**	**X**

Background information:

Where to start? *Caenorhabditis elegans* has a long and storied history in science. I will attempt to provide concise yet detailed background information for understanding the historical use of this nematode as a model organism for laboratory investigation but I will not do it justice in just a few paragraphs. Please refer to the awesome resources provided at the end of this section, specifically the WormBook. WormBook is an open-access resource distributed under the terms of the Creative Commons Attribution License and has a wealth of information about the use, care, propagation, laboratory techniques, and history of *C. elegans*.

Caenorhabditis elegans is a free-living, non-parasitic, transparent nematode roundworm of the phylum Nematoda. These worms live in nutrient-dense environments, rich in decaying plant material as they consume bacteria as their primary food source. Detailed information about the anatomy and life cycle of *C. elegans* is readily available and immense since scientists began studying the nematode in the 1960's. These worms lack both circulatory and respiratory systems but have a neuromuscular, digestive, excretory, and reproductive systems. Most of the worms are hermaphroditic but males do exist and contain specialized structures that allow them to mate with hermaphrodites. Hermaphrodites lay 300-1,000 eggs; number range is dependent on self-fertilization (300) versus insemination (1,000). Once eggs hatch, larval *C. elegans* progress through four stages of development (L1-L4) to adult worm stage in 2-3 days and live about 3 weeks under laboratory conditions. During environmental stress, food shortage, or temperature fluctuations an alternative larval stage, dauer, can occur. Dauer larvae are resistant to stress, slow moving, and do not consume bacteria. Dauer larvae can survive for months at this stage until conditions improve.

Work with *Caenorhabditis elegans* and related nematodes began in the 1940's but moved to the forefront of research in 1963 in the area of developmental biology by researcher Sydney Brenner. Brenner chose *C. elegans* in part because it has only about 300 neurons out of fewer than 1,000 cells, completes embryogenesis in 12 hours, and is amenable to genetic analysis similar to fruit flies. At the time, fruit flies had already proven very useful for studying genetic control of basic developmental steps, but the insect contains far too many cells (e.g., over 100,000 neurons) for studying many detailed mechanisms of cell development. As stated in my Introduction, simple organisms are essential model systems in scientific investigation for a variety of reasons. Some of the important characteristics of an effective model organism are size, ease of care, short life span, multiple offspring, many generations in a short period of time, and homology to humans. Once introduced by Sydney Brenner, work with *C. elegans* grew exponentially to include over 2,000 research labs worldwide, and we now know more about it than nearly any other animal. *Caenorhabditis elegans* was the first multicellular eukaryote to have whole genome sequencing completed to determine the content of its gene code. Details from embryogenesis to aging, programmed cell death, molecular mechanisms of behavior, cell differentiation, and gene control are just a few areas of discovery that *C. elegans* have provided invaluable insight in understanding human health and disease.

Contemporary studies using *C. elegans* led to discovery of a mechanism of gene regulation called RNA interference (RNAi). RNA (ribonucleic acid) is an integral poly-nucleotide needed to transcribe and translate the gene code into protein products. RNA interference uses two kinds of RNA molecules, microRNA (miRNA) and small interfering RNA (siRNA), to degrade messenger RNA (mRNA) molecules, decreasing the ability to translate specific proteins. The 2006 Nobel Prize in Physiology and Medicine was awarded to Andrew Fire and Craig Mello for their discovery that double-stranded RNA (dsRNA) triggers the suppression of gene activity in *Caenorhabditis elegans*. Since this discovery, RNA

interference has become a ubiquitous method for investigating gene function in many organisms and applications range from treating infectious disease to targeted cancer therapies. Methods for applying RNAi are remarkably simple for *C. elegans*. Although Fire and Mello injected their worms with the double-stranded RNA in their original studies, RNAi can now be introduced by simply feeding worms with strains of *E. coli* that express double-stranded RNA from plasmids. Since the entire genetic code of *C. elegans* is well-annotated and thousands of labs study it, libraries of RNAi feeding bacteria are available so that nearly any gene can be targeted for silencing. This makes the process easy to replicate in a high school or undergraduate level biology or genetics course.

There is a need for science educators to develop teaching tools and laboratory activities that demonstrate the power of modern genetics and help students to better understand the RNAi process. Although *Worm Boot Camp* is designed to develop the skills necessary to work with *C. elegans* as a model organism, the hope is that teachers will provide the time and guidance necessary for students to perform novel research using the skill sets provided in this module. *C. elegans* is an ideal model organism for the undergraduate laboratory because of the simplicity of worm maintenance, its well-studied genetic background, and the fact that it can be employed as a model organism in laboratory environments where vertebrate research is restricted (Anderson, 2008).

Students and academics agree that there is a need to make learning and teaching in the science laboratory more challenging and engaging. There are many published laboratory exercises designed to enthuse and stimulate students through active learning and most teachers agree that we should challenge our students through activities that use questioning and investigation; the Scientific Method. Time is always an issue when trying to provide opportunities for inquiry and students have so many questions and little confidence to design and follow-through with their own experimental design. The hope is that with a solid foundation and skill set and provided the opportunity to learn all of the ways *C. elegans* can be used to study biology, medicine, toxicology, and behavior students will embrace the scientific method and excel in research design.

There are many sources of excellent information about the use of Caenorhabditis elegans as a model organism for scientific investigation. Teachers may wish to view the original sources for images, detailed explanations, and as print resources for their students. The information provided here was excerpted from the following sources:

The Worm Book – The Online Review of *C. elegans* Biology - http://www.wormbook.org/index.html

C. elegans Center (CGC) - https://cgc.umn.edu/

C. elegans Natural Diversity Resource Center - https://www.elegansvariation.org/

Open Worm Science - http://openworm.org/science.html

Cold Harbor Spring Laboratory *C. elegans* Interactive http://www.silencinggenomes.org/

NOVA Labs RNA Guide for Educators http://www.pbs.org/wgbh/nova/labs/about-rna-lab/educator-guide/

Teacher Preparation Tips

The key to success in any laboratory activity is teacher preparation. This is not a lesson that one can do at the last minute or on the fly. Timely and sufficient supply order placement is necessary for success. Here are a few suggestions:

- Allow sufficient time to receive your materials.
  - All strains of the *C. elegans* and the *E. coli* OP50 can be purchased from the CGC (Caenorhabditis Genetics Center, https://cgc.umn.edu/) for $7.00/plate
  - NGM (Nematode Growth Media) and LB (Luria broth) media can be purchased from most biological supplies companies for less than $10.00 a bottle.
    Each bottle will pour plates for 4 - 5 students.
    Instructor will prepare the plates for Lesson 1 ONLY.
    **Lesson 2 is designed to allow students the opportunity to pour their own plates and culture their own *E. coli* OP50 for use in Lessons 4 & 6. Instructor can pour these plates in advance to save time and allow more time to practice skills in Lesson 4.
    Alternative Lesson Planning Guide shown on Page ____.
  - Mini petri dishes (35x10) for use in Lesson 4 are difficult to find. I order them from Amazon as science supply companies no longer have them in stock. You can use small petri dishes (60x15) as a substitute.

- Allow sufficient time for making starter *C. elegans* plates so students can observe all life stages for Lessons 1 and 4.
  - At least three days before Lesson 1 - Pour all plates needed for Lesson 1 (also for Lesson 4).
    Students pour their own plates in Lesson 2 for the remainder of Worm Boot Camp (see ** note above).
    Poured NGM plates can be stored in refrigerator for a few weeks.
    Bring plates to room temperature before culturing *E. coli* or performing worm transfer.
  - Three days before Lesson 1 – Prepare Luria broth tube of *E. coli* OP50
  - Two days before Lesson 1 - Inoculate plates of *E. coli* OP50
  - One day before Lesson 1 - Chunk plates for student use and allow for propagation

- Make sufficient media to prepare starter plates and broth tubes for each section that will be participating in Worm Boot Camp.
  Having enough media helps reduce wait time for students and limits sharing to keep students engaged in activities and observations.
  For example, 2 sections of Honors Genetics, 30 students per section
  - For Lesson 1 – Make
    - 30 NGM media with N2 Wild type *C. elegans* STARTER (60x15) plates, 15 per class
  - For Lesson 2 – Make
    - 30 *E. coli* OP50 LB broth tubes, 15 per class
  - For Optional Lesson 2 – Instructor pours plates – Make
    - 120 mini (35x10) and 240 (60x15) NMG plates, 2 mini and 4 small/student

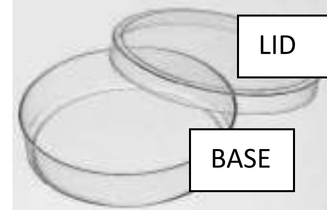
- For Lesson 3 – No media or plate prep
- For Lesson 4 – Use media from lesson 1 and 2
- For Lesson 5 – No media or plate prep
- For Lesson 6 – Use media from previous lessons as needed

- Ensure all microscopes/stereoscopes are in working order prior to start of lesson.
  - Light source
    - Most microscopes have halogen light sources. These light emit heat and kill worms easily. It is simple and inexpensive to change the light source to an LED bulb.
    - If unable to change the light source on your microscope, remind students to turn off microscopes or remove worm plates from stage often to limit death.
  - Clean lenses
  - Sufficient outlets and power strips
  - Electric outlets work
  - May need to remove stage clips for plates to fit on stage

Media and Culture Preparation

To POUR starter plates - Do this at least 3 days before Lesson 1:

1. Follow manufacturer instructions for melting bottled agar.
   I prefer to melt in 1 L beaker of water on hot plate for more even melting and allow to boil sterilize since I lack an incubator. This takes more time but has much better plate results.
2. While agar is melting, open sterile petri plates and place inverted on lab station.
   One bottle of agar will pour 10-15 mini and small plates
   Place plates BASE side down and leave LID in place until ready to pour to prevent contamination.



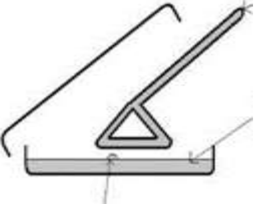
3. Once bottled agar is cool enough to hold in hand without discomfort, pour agar into BASE of plate, about half full
4. Remove 1 lid at a time, pour plate, replace lid, repeat.
5. Allow to cool/set undisturbed for 1 hour.
6. Invert and store refrigerated at 4*C until ready to use.
   Inverted plates will prevent condensation from settling on the agar.

To INOCULATE Luria Broth *E. coli* Culture Tubes – Do this 3 days before Lesson 1: *E. coli* OP50 *can be ordered in plate culture from* Carolina Biological. *You can extend a single plate culture into multiple Luria broth culture tubes for student use.*

1. Obtain Luria Broth glass culture tubes (1 tube/lab station) and *E. coli* OP50 culture plate.
2. Using sterile techniques, transfer loopful of bacteria from plate to Luria broth culture tube
    a. Pass nichrome wire loop through Bunsen burner flame to heat sterilize
    b. Allow to cool
    c. Remove lid from *E. coli* OP50 culture plate and remove loopful for bacteria. Replace lid
    d. Remove lid from Luria broth tube and pass tube through Bunsen burner flame
    e. Place loop of bacteria in broth tube and spin loop to dislodge bacteria.
    f. Flame tube and replace cap.
    g. Flame loop and allow to cool
    h. Repeat steps above for each broth tube
    i. Incubate at 37*C for 24 hours

To INOCULATE starter plates - Do this 2 days before Lesson 1:

1. Remove from refrigeration when ready to use
   Allow plates to come to room temperature before bacterial inoculation.
2. Add 0.05 mL Luria broth culture of *E. coli* OP50 to center of each small petri dish.
3. Dip bacti-spreader in beaker of ethanol and flame sterilize
4. Allow to cool
5. Lay flat surface of bacti-spreader onto seeded agar surface.
   Turn plate and spread bacteria over entire surface
6. Replace lid and allow to set for 10 minutes
7. Repeat steps 8-11 for each starter plate
8. Invert and incubate at 37*C for 24 hours



To CHUNK Wild Type N2 starter plates – Do this 1 day before Lesson 1:

1. Remove *E. coli* OP50 plates from incubator
   Leave INVERTED until ready to chunk
   Make sure condensation on LID does not fall onto agar surface. Wipe with Kim-wipe if necessary
2. Dip spatula into beaker of ethanol
3. Pass through flame to heat sterilize
4. Allow to cool
5. Select a location on the ORIGINAL shipped Wild type/N2 plate received from biological supply company (CGC)
6. Use spatula to cut a ½ inch (1 cm) square from original plate
7. Transfer chunk to new *E. coil* OP50 plate and invert onto new agar surface.
8. Replace lid and use microscope to confirm successful transfer.
9. Repeat steps to make starter plates, 1 starter plate/lab partner

Preparatory Homework – Flipped Video Assignment

KEY QUESTION(S): What are *C. elegans*? What role does *C. elegans* play in scientific discovery?

OVERALL TIME ESTIMATE: 40 minutes

LEARNING STYLES: Visual and auditory

VOCABULARY:
Apoptosis
Embryonic
Founder cell
Genotype
Invariant
Model organism
Nematode
Phenotype

LESSON SUMMARY: Students will view a video journal https://www.jove.com/science-education/5110/c-elegans-development-and-reproduction as preparation for Worm Boot Camp and complete an accompanying worksheet. This independent activity allows students the opportunity to watch/rewatch to answer questions and ensure understanding of content.

STUDENT LEARNING OBJECTIVES:
The student will be able to...

- Describe the use of model organisms in scientific investigation
- Recognize the methods used by scientists that study *C. elegans*
- Identify the food sources used for growth and care of *C. elegans*
- Evaluate the use of bacteria as a means for transmitting genetic information to *C. elegans*
- Explain why bacteria are used to transfer RNA of interest to *C. elegans*
- Identify the discoveries made through the use of C. *elegans* as a model of human disease

FLORIDA NEXT GENERATION SUNSHINE STATE STANDARDS (SCIENCE):
SC.912.N.1.4
SC.912.N.1.7
SC.912.N.2.4
SC.912.N.3.5
SC.912.L.16.7
SC.912.L.16.9
SC.912.L.16.10

MATERIALS:
Copies of *Student Worksheet #1 – Flipped Video Assignment*

BACKGROUND INFORMATION:
Jove is an online video journal and provides detailed visual lessons on a variety of different scientific investigations. The video link provided above and on the top of the *Student Worksheet #1 – Flipped Video Assignment* provide background information on the use and care of *Caenorhabditis elegans* in the laboratory. This assignment is meant as a homework assignment for students to complete individually, which allows them to watch the video as many times as necessary to understand the content and answer questions.

ADVANCE PREPARATION:
Copies of *Student Worksheet #1 – Flipped Video Assignment*, 1 per student

PROCEDURE AND DISCUSSION QUESTIONS WITH TIME ESTIMATES:

40 minutes

1. Provide each student with a copy of *Student Worksheet #1 – Flipped Video Assignment*
2. Navigate to https://www.jove.com/science-education/5110/c-elegans-development-and-reproduction
3. Tell the students to watch the video for homework tonight and answer questions in preparation for Worm Boot Camp
4. Tell students that the worksheet must be completed prior to the next class in order to have the background information needed to successfully work with this model organism.
5. If some student do not complete the assignment, have laptops ready in class the next day for students to complete prior to the basic microscopy lesson.

ASSESSMENT SUGGESTIONS:

- Grade the worksheet for correct answers.

RESOURCES/REFERENCES:
None needed

Student Name ____

Flipped Assignment – *C. elegans* Development and Reproduction

Link to the following Jove Video
https://www.jove.com/science-education/5110/c-elegans-development-and-reproduction
Bookmark this video for future reference.

As you watch, answer the following questions in preparation for next week's lecture and lab activities.

1. *C. elegans* has an *invariant* form of development. Describe the term *invariant* and describe how nematode development differs from the development of more complex organisms, like humans.

2. What is the first major event of embryonic development?

3. Describe the 6 founder cells that result in the formation of the embryonic nematode and what each founder cell eventually becomes.

4. Although the founder cells give rise to the foundation of embryonic development, cell-cell interaction is necessary for the full development of a nematode.

- ABp + P2 =
- ABa + EMS =
- Posterior EMS + P2 =

5. When does the worm lay eggs? When does the egg hatch?

6. What is apoptosis?

7. How many cells are lost to apoptosis during the embryonic stage of worm development? Does this vary?

8. Sketch and describe the typical life cycle of *C. elegans*.

9. What occurs if environmental conditions cause stress to the early larval stages of the nematode?

10. What are the differences between the 2 sexes in both genotype and phenotype? Be specific

Watch the segments *Setting Up and Analyzing a Genetic Cross* and *Applications*.

Flipped Assignment – *C. elegans* Development and Reproduction

Link to the following Jove Video
https://www.jove.com/science-education/5110/c-elegans-development-and-reproduction
Bookmark this video for future reference.

As you watch, answer the following questions in preparation for next week's lecture and lab activities.

1. *C. elegans* has an *invariant* form of development. Describe the term *invariant* and describe how nematode development differs from the development of more complex organisms, like humans.

INVARIANT MEANS IT DOESN'T CHANGE FROM ORGANISM TO ORGANISM; ALL *C. elegans* FOLLOW THE SAME DEVELOPMENT PATHWAY DURING EMBRYONIC DEVELOPMENT. OTHER COMPLEX VERTEBRATE ANIMALS HAVE DIFFERENCE AMONG SPECIES DURING EMBRYONIC DEVELOPMENT.

2. What is the first major event of embryonic development?

ASSYMETRIC CELL DIVISON TO ESTABLISH THE ANTERIOR-POSTERIOR AXIS FOR DEVELOPMENT.

3. Describe the 6 founder cells that result in the formation of the embryonic nematode and what each founder cell eventually becomes.

AB = NEURONS AND PHARYNX

MS = MSUCLE, PHARYNX, AND NEURONS

E = INTESTINAL TISSUE

C = MUSCLES, NEURONS, AND SKIN

D = BODY WALL MUSCLE

P_4 = GERMLINE/SEX CELLS

4. Although the founder cells give rise to the foundation of embryonic development, cell-cell interaction is necessary for the full development of a nematode.

- ABp + P2 = **NEURONS AND EPITHELIAL CELLS**
- ABa + EMS = **PHARYNGEAL CELLS**
- Posterior EMS + P2 = **INTESTINAL CELLS**

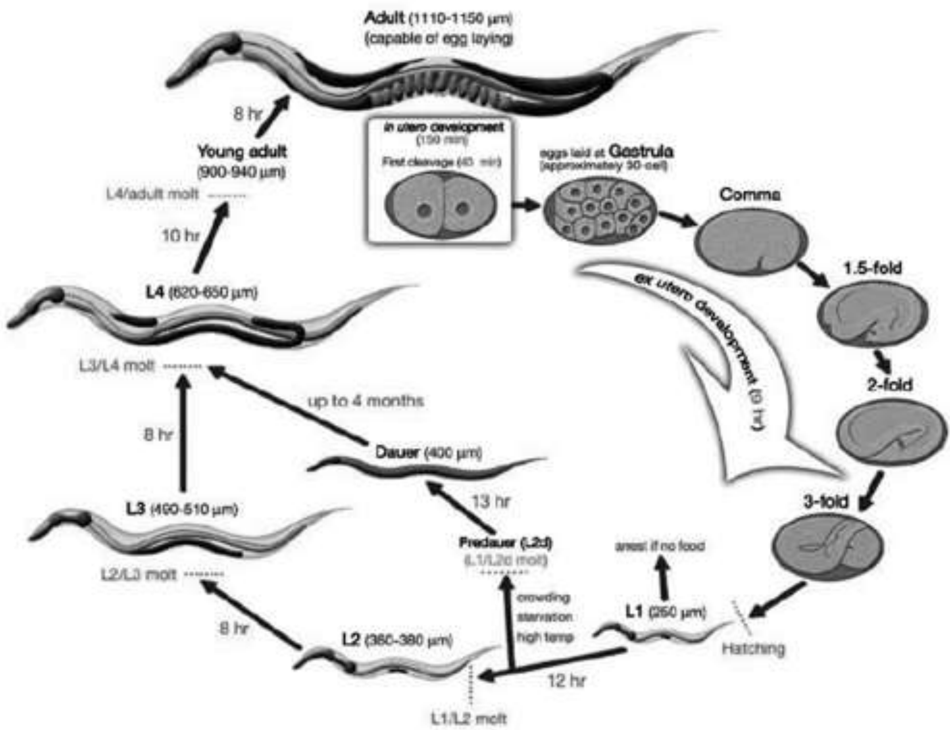
5. When does the worm lay eggs? When does the egg hatch?

AFTER THE 30 CELL STAGE **AFTER FURTHER CELL DIVISIONS THAT LEAD TO INCREASE IN CELL NUMBER AND ORGAN FORMATION**

6. What is apoptosis?
PROGRAMMED CELL DEATH

7. How many cells are lost to apoptosis during the embryonic stage of worm development? Does this vary?
EXACTLY 113 CELLS AND DOES NOT VARY FROM WORM TO WORM

8. Sketch and describe the typical life cycle of *C. elegans*.



9. What occurs if environmental conditions cause stress to the early larval stages of the nematode?
PRODUCTION OF DAUER WORMS THAT ARE RESISTANT TO THESE CHANGES AND CAN LIVE FOR MONTHS UNTIL STRESS IS REMOVED.

10. What are the differences between the 2 sexes in both genotype and phenotype? Be specific

MALES	**HERMAPHRODITES**
SLIM BODY	**LARGER BODY**
DISTINCTIVE TAIL/FAN-LIKE	**SLIM/POINTED TAIL**
PRODUCES ONLY SPERM	**PRODUCES OOCYTES AND SPERM**
GENOTYPE - XO	**GENOTYPE - XX**

Watch the segments *Setting Up and Analyzing a Genetic Cross* and *Applications*.

Lesson 1: Introduction to *C. elegans* and Basic Microscopy

KEY QUESTION(S): What are *C. elegans*? What role does *C. elegans* play in scientific discovery? How can students use a microscope to view *C. elegans*? What do they look like?

OVERALL TIME ESTIMATE: 45 minutes

LEARNING STYLES: Visual, auditory, and kinesthetic

VOCABULARY:
Agar
Chemotaxis
Hermaphrodite
Homolog
Magnification
Ortholog
Progeny
RNAi

LESSON SUMMARY: Students will engage in whole-group introduction to *Caenorhabditis elegans* as a model organism for scientific inquiry and discovery. This lesson includes basic information about *C. elegans*, its life cycle, and introductory microscopy skills.

STUDENT LEARNING OBJECTIVES:
The student will be able to...

- Describe how scientists use C. elegans in developing a scientific investigation
- Provide an example of an inference made by scientists that use C. elegans as a model of disease
- Provide an example of a change in understanding made through the use of C. elegans
- Evaluate the use of bacteria as a means for transmitting genetic information to *C. elegans*
- Explain why bacteria are used to transfer RNA of interest to *C. elegans*
- Identify the discoveries made through the use of C*. elegans* as a model of human disease

FLORIDA NEXT GENERATION SUNSHINE STATE STANDARDS (SCIENCE):
SC.912.N.1.1
SC.912.N.1.6
SC.912.N.1.7
SC.912.N.2.4
SC.912.N.3.5
SC.912.L.16.7
SC.912.L.16.9
SC.912.L.16.10

MATERIALS:
Computer and projection unit
PowerPoint Presentation - *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
Copies of *Basic Microscopy and C. elegans Observations*, Class set
Copies of *Skills Assessment #1 – Basic Microscopy*, 1 per student
Compound light microscope or Dissecting/Stereo microscope
Petri dish of N2/Wild type *C. elegans* culture
Sterile toothpick

BACKGROUND INFORMATION:
It is essential that students have basic microscopy skills to work with this model organism. One obstacle students often experience is the manipulation of light on the microscope. Provide ample practice time for students to practice with light and focus, even extending this lesson to two (2) days if needed for student comfort level.

ADVANCE PREPARATION:
Copies of *Basic Microscopy and C. elegans Observations*
Copies of *Skills Assessment #1 – Basic Microscopy*
Review PowerPoint lesson and other materials, as needed, for teacher comfort level
Set up lab stations for partner groups with 1 microscope, 1 N2/Wild Type culture of *C. elegans*, toothpicks

PROCEDURE AND DISCUSSION QUESTIONS WITH TIME ESTIMATES:

45 minutes, 90 minutes for additional microscopy practice

1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Provide each student with a copy of *Basic Microscopy and C. elegans Observations* and *Skills Assessment #1 – Basic Microscopy*
3. Review first 10 slides of PowerPoint presentation with class, reference homework from previous night as additional examples
4. Provide guidance on general microscope use and light manipulation (see notes on Slide 9)
5. Tell the students to follow the directions provided on *Basic Microscopy and C. elegans Observations* worksheet
6. Tell the students to complete the questions and table on *Skills Assessment #1 – Basic Microscopy*
7. Circulate room to address questions and issues with microscopy

ASSESSMENT SUGGESTIONS:

- Grade *Skills Assessment #1 – Basic Microscopy*

RESOURCES/REFERENCES:
The Worm Book has a wealth of information about *C. elegans* and microscopy skills when working with this model organism. http://www.wormbook.org/index.html

PowerPoint Slides

Caenorhabditis elegans: Using Model Organisms as a Tool in Molecular Genetics

Susan Chabot
Honors Genetics & Experimental Science
Lemon Bay High School

Lesson Sequence

Lesson 1 – Introduction and Microscopy practice
Lesson 2 – Media preparation, bacterial cultures
Lesson 3 – Life cycle, larval forms, sex determination
Lesson 4 – Chunking vs Picking plates
Lesson 5 – Mutations of movement/Videos?
Lesson 6 – Plate bleaching and Egg transfer
Lesson 7 – Skills Practice and Assessment

What is *Caenorhabditis elegans*?

- Free-living soil nematode/roundworm
- Not parasitic
- Small – measure about 1mm
- Survives by feeding on bacteria
- Used as a model organism for laboratory study of:
  - Neural development
  - Chemotaxis
  - Memory
  - Development
  - Apoptosis
  - Aging
  - Space flight

Study Samples

Neurobiology
- Model for neuronal development and function.
- Simple yet sophisticated nervous system (302 neurons/959 total cells).
- Responds to chemo attractants/repellants.
- Selective cutting of neurons and electrophysiology studies can be conducted.
- Connectome (complete "wiring" diagram) established.

Aging
- Short life span permits genetic screens for genes associated with longevity.

Human disease studies
- ~75% of human disease genes have potential *C. elegans* homologs.
- ~40-50% have a *C. elegans* ortholog.

Advantages as a Model Organism

- Simple – 959 total cells in adult (hermaphrodite) worm
- Small
- Easy to maintain cultures
- Short life cycle
- Powerful genetic studies can be conducted
- Transparent visualization of internal structures
- Invariant cell lineage
- Fully described anatomy and development
- Completely sequenced genome
- Tools like RNAi knockdowns can be performed
- Stocks can be frozen and preserved
- No expensive animal house costs, CHEAP TO MAINTAIN!
- Present no biohazard
- Large number of progeny

Nobel Prizes

Apoptosis
Nobel Prize for Physiology or Medicine, 2002
H. Robert Horvitz
John Sulston
Sydney Brenner

Gene silencing by RNA interference
Nobel Prize for Physiology or Medicine, 2006
Andrew Fire
Craig Mello

Development and use of Green Fluorescent Protein (GFP)
Nobel Prize for Chemistry, 2008
Martin Chalfie
(with Osamu Shimamura and Roger Tsien)

Basic Anatomy and Microscopy

Pharynx, Intestine (yellow), Gonad (pink), Vulva, Epidermis, Rectum, Anus
head anterior, ~1 mm, tail posterior

Basic Anatomy and Microscopy

- *C. elegans* is transparent
- With good microscopy skills, students should be able to:
  - Differentiate among life stages
  - Differentiate between males and hermaphrodites
  - Visualize internal organs and eggs
- Best viewed at 40X magnification
  - Low-powered objective on compound light microscope
  - High-powered objective on dissection/stereoscope

Microscope Review

Basic Microscopy Instructions

Follow instructions provided on *Basic Microscopy Instructions* and complete the *Skills Assessment #1 – Basic Microscopy*.

Basic Microscopy and *C. elegans* Observations

Objectives: Students will use a light microscope to visualize *C. elegans* on agar plates; students will switch from low-power to high-power objective and observe the movement of the nematode from different magnifications; students will describe the movement of *C. elegans*; students will recognize that all nematodes on their plate are not the same size, therefore not in the same life cycle; students will count the number of nematodes in a given area of the plate and describe the population distribution of worms in terms of relative size and density.

Materials:

Compound light microscope or Dissecting/Stereo microscope
Petri dish of N2/Wild type *C. elegans* culture
Sterile toothpick

As you work through the instructions, complete the *Skills Assessment #1 – Basic Microscopy* Worksheet Instructions below match with question numbers on worksheet.

1. Locate the microscope at your lab station.
Ensure that the microscope is plugged in and review how to change the amount of light that will pass through the specimen. Note that if you have a compound light microscope, the light source is coming from the base. If you are using the stereo microscope/dissecting microscope, there are two light sources, one from the base and the other from above, just behind the objective lens.

2. View the petri dish of worms supplied by your instructor without the microscope. Recall from the video, adult nematodes of this species measure roughly 1mm.

3. Place the nematode culture dish on the stage of the microscope and work to bring the worms into focus. Nematodes are best visualized at a total magnification of 40X (10X ocular x 4X objective). This can be done on either the compound light microscope or the dissecting microscope. Be sure to change the light source for best viewing and use the fine adjustment knob to bring your specimen into focus.

4. Read the table on the *Skills Assessment #1 – Basic Microscopy* Worksheet and record answers in the spaces provided.

5. Clean up

Remove the plate from the stage and secure the lid.
Dispose of the toothpick in the bin at your lab station.
Turn off the microscope and cover with the dust cover.
Wash your hands and the lab station.

TEACHER PAGES - Basic Microscopy and *C. elegans* Observations

Teacher tips are in BOLD/ALL CAPS

Objectives: Students will use a light microscope to visualize *C. elegans* on agar plates; students will switch from low-power to high-power objective and observe the movement of the nematode from different magnifications; students will describe the movement of *C. elegans*; students will recognize that all nematodes on their plate are not the same size, therefore not in the same life cycle; students will count the number of nematodes in a given area of the plate and describe the population distribution of worms in terms of relative size and density.

Materials:

- Compound light microscope or Dissecting/Stereo microscope
- Petri dish of N2/Wild type *C. elegans* culture
- Sterile toothpick

PLEASE READ THROUGH TEACHER PREPARATION TIPS ON PG 15 _ FOR DETAILED INFORMATION ABOUT STARTER PLATE AND OTHER MEDIA PREPARATION.

As you work through the instructions, complete the *Skills Assessment #1 – Basic Microscopy* Worksheet **Instructions below match with question numbers on worksheet.**

1. Locate the microscope at your lab station.

Ensure that the microscope is plugged in and review how to change the amount of light that will pass through the specimen. Note that if you have a compound light microscope, the light source is coming from the base. If you are using the stereo microscope/dissecting microscope, there are two light sources, one from the base and the other from above, just behind the objective lens.

INSTRUCT STUDENTS TO PRACTICE WITH LIGHT TO ICREASE SKILL AND COMFORT WHEN USING A LIGHT MICROSCOPE. RECALL NOTE IN TEACHER PREP INFORMATION ABOUT HALOGEN LIGHT SOURCES AND REMIND STUDENTS TO REMOVE WORMS FROM STAGE IF HALOGEN LIGHTS ARE USED AS THE LIGHT SOURCE FOR YOUR MICROSCOPES. LED LIGHTS CAN BE LEFT ON CONTINUOUSLY AND NOT DAMAGE WORMS.

2. View the petri dish of worms supplied by your instructor without the microscope. Recall from the video, adult nematodes of this species measure roughly 1mm.

INSTRUCT STUDENTS THAT ALTHOUGH THE WORMS ARE TOO SMALL TO SEE, TRACKS AND CLUSTERS OF WORMS ARE VISIBILE WITHOUT A MICROSCOPE.

3. Place the nematode culture dish on the stage of the microscope and work to bring the worms into focus. Nematodes are best visualized at a total magnification of 40X (10X ocular x 4X objective). This can be done on either the compound light microscope or the dissecting microscope. Be sure to change the light source for best viewing and use the fine adjustment knob to bring your specimen into focus.

WALK AROUND LAB STATIONS AND ASSIST STUDENTS WITH MICROSCOPE VISUALIZATION OF WORMS UNDER 40X MAGNIFICATION. POINT OUT THAT WORMS ARE AT DIFFERENT STAGES OF THEIR LIFE CYCLE SO ARE DIFFERENT SIZE AND AGE. SEE IF STUDENTS CAN SEE EGGS. HAVE STUDENTS NOTE MOVEMENT.

4. Read the table on the *Skills Assessment #1 – Basic Microscopy* Worksheet and record answers in the spaces provided.

5. Clean up

Remove the plate from the stage and secure the lid.
Dispose of the toothpick in the bin at your lab station.
Turn off the microscope and cover with the dust cover.
Wash your hands and the lab station.

PROVIDE SPECIFIC INSTRUCTION FOR YOUR CLASS FOR LOCATION OF STARTER/OBSERVATION PLATES. REMIND STUDENTS THAT ALTHOUGH BACTERIA AND WORMS ARE NOT PATHOGENIC, THEY SHOULD WASH HANDS PRIOR TO LEAVING THE LABORATORY.

Student Name ___________________________

Basic Microscopy and *C. elegans* Observations

Objectives: Students will use a light microscope to visualize *C. elegans* on agar plates; students will switch from low-power to high-power objective and observe the movement of the nematode from different magnifications; students will describe the movement of *C. elegans*; students will recognize that all nematodes on their plate are not the same size, therefore not in the same life cycle; students will count the number of nematodes in a given area of the plate and describe the population distribution of worms in terms of relative size and density.

1. Light Microscope Review

    a. What type of microscope is at your station?

    b. List 2 ways to change the light supplied by your microscope.

    c. How confident do you feel in your ability to alter light levels on your microscope?

2. Initial Observations

    a. Can you see the worms without the microscope?

    b. Can you see movement on the plate?

    c. Are there areas of the plate that appear more likely to contain worms than others?

3. Quantitative and Qualitative Observations

    a. Make one QUALITATIVE observation. Be descriptive

    b. Make two QUANTITATIVE observation.

Skills Assessment #1 - Basic Microscopy

Student Name ________________________________

4. Complete the table.

How many different sized worms do you observe?	
Do the worms group together by size? Describe	
Describe the movement of the worms.	
Lift the lid and use the toothpick provided to LIGHLTY touch one worm. What happened?	
Are the worms evenly spread throughout the plate or clustered together? Describe	

Lesson 2: Culturing *C. elegans*

KEY QUESTION(S): How do *C. elegans* fit in a food chain or food web? What do *C. elegans* use as a food source? How do scientists prepare media for *C. elegans* to use for food in a laboratory setting?

OVERALL TIME ESTIMATE: 90 minutes (45 minutes for Optional lesson, See Lesson Guide page 8)

LEARNING STYLES: Visual, auditory, and kinesthetic

VOCABULARY:
Agar
Auxotroph
Bacterial lawn
Culture
Heterotroph
Inoculation

LESSON SUMMARY: Students will understand the role of *C. elegans* in the food chain and prepare culture media for the growth of *C. elegans* in the laboratory

STUDENT LEARNING OBJECTIVES:
The student will be able to...

- Describe the role of *C. elegans* as a heterotroph in a food chain or food web
- Describe the food sources consumed by nematodes in their environment
- Demonstrate the ability to melt and pour prepared media plates for nematode propagation
- Demonstrate the ability to inoculate an agar plate with E. coli bacteria as a food source for E. coli
- Demonstrate good aseptic technique for the growth of a lawn of bacterial food source for nematode propagation

FLORIDA NEXT GENERATION SUNSHINE STATE STANDARDS (SCIENCE):
SC.912.N.3.5
SC.912.L.16.7
SC.910.L.16.9
SC.912.L.16.10

BACKGROUND INFORMATION:
Students must understand the role that nematodes play in the ecosystem as a multicellular, heterotrophic, eukaryotic organism and the nutrient requirements for using C. elegans in the laboratory. Students can practice the skill of making media plates and preparing those plates with bacteria as a food source for nematode culturing and these skills can be used in future science coursework. Detailed information on media preparation can be found in the Teacher Preparation Tips found on page 15.

To save time, instructors may opt to prepare NGM media plates for students ahead of time as plates can be made and stored weeks prior to inoculation with bacteria (see optional lesson). Students should be

offered the chance to inoculate plate with bacteria. By preparing media plates for students, the schedule can then offer more time for skills practice in chunking plates and picking worms.

OPTION 1 – Original Plan
MATERIALS:

Day 1:
Mini (35x10) sterile petri dishes (2 plates/student)
Small (60x15) sterile petri dishes (4 plates/student)
NGM (Nematode Growth Media) Agar - can be commercially purchased or made
1 liter beaker with 500 mL tap water
Hot plate or Magnetic hot plate with stir bar
Sharpie (1 marker/student)

Day 2:
Poured plates from Lesson 2/Day 1
E. coli OP50 in LB broth media (1 tube/lab station)
p100 micropipette and pipette tips
100 – 200 mL ETOH in 400 mL beaker (1 beaker/lab station)
Bunsen burner of alcohol burner (1 burner/lab station)
Bacti-spreader, metal or glass (1 spreader/lab station)
Sharpie (1 marker/student)

ADVANCE PREPARATION:
Copies of *Basic Media Preparation and Bacterial Growth Instructions*, Class set
Copies of *Skills Assessment #2 – Media Prep and Bacterial Growth*, 1 per student
Review PowerPoint lesson and other materials, as needed, for teacher comfort level
Prepare Luria broth cultures of *E. coli* OP50, See Teacher Preparation
Assemble supplies according to Day 1 or Day 2 requirements
Start melting procedure to prepare agar 1 class period before students will pour the plates

PROCEDURE AND DISCUSSION QUESTIONS WITH TIME ESTIMATES:

Option 1 – Original Plan/Students pour plates

Day 1 - 45 minutes
It is ESSENTIAL that media melt begins prior to start of class period to ensure media is ready to pour during class.

1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Provide each student with a copy of *Basic Media Preparation and Bacterial Growth Instructions* and *Skills Assessment #2 – Media Prep and Bacterial Growth*
3. Review slides 11, 12, 13 on *C. elegans* diet
4. Inform students that today they will pour media plates and tomorrow will inoculate media plates for use in Lesson 4.
5. Instruct students to follow instructions for *Day 1– Plate Preparation*
6. Move around room to answer questions about pouring media plates and demonstrate proper pour techniques.

7. Ensure poured plates are solid, inverted, and placed in refrigerator overnight for use in class on Day 2

Day 2 – 45 minutes

1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Remove poured media plates from Day 1 and bring to room temperature
3. Review safety for use of Bunsen burner/alcohol burner
4. Instruct students to follow instructions for *Day 2 - Plate Bacterial Culture Preparation*
5. Move around room to answer questions about inoculation and demonstrate proper technique
6. Ensure inoculated plates are inverted and placed in 37*C incubator for 24 hour growth check and use in Lesson 4.

Option 2 – Instructor Poured Plates
45 minutes
This option allows more time for students to practice skills rather than taking a full day to pour plates. Although this skill is important, instructors may find it more important to offer more time for microscope use or chunking/picking.

1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Provide each student with a copy of *Basic Media Preparation and Bacterial Growth Instructions* and *Skills Assessment #2 – Media Prep and Bacterial Growth*
3. Review slides 11, 12, 13 on *C. elegans* diet
4. Inform students that they will only perform *Day 2 – Plate Bacterial Culture* Preparation
    a. Explain the importance and ingredients of the plates and describe how plates were made for student use.
5. Remove prepared NGM media plates from refrigerated storage and allow to come to room temperature
6. Review safety for use of Bunsen burner/alcohol burner
7. Instruct students to follow instructions for *Day 2 - Plate Bacterial Culture Preparation*
8. Move around room to answer questions about inoculation and demonstrate proper technique
9. Ensure inoculated plates are inverted and placed in 37*C incubator for 24 hour growth check and use in Lesson 4.

ASSESSMENT SUGGESTIONS:

- Grade *Skills Assessment #2 – Media Prep and Bacterial Growth*

RESOURCES/REFERENCES:
The Worm Book has a wealth of information about *C. elegans* and microscopy skills when working with this model organism. http://www.wormbook.org/index.html

Lesson Sequence

Lesson 1 – Introduction and Microscopy practice

***Lesson 2* – Media preparation, bacterial cultures**

Lesson 3 – Life cycle, larval forms, sex determination

Lesson 4 – Chunking vs Picking plates

Lesson 5 – Mutations of movement/Videos?

Lesson 6 – Plate bleaching and Egg transfer

Lesson 7 – Skills Practice and Assessment

Diet of *C. elegans*

- Nematodes are heterotrophs
- Nematodes consume soil-living bacteria
- In the lab, *C. elegans* are fed a diet of *Escherichia coli* OP50.
  - E. coli OP50 has a limited growth ability which is beneficial for culturing nematodes.

Photo – mouth of C. elegans with E. coli food source

Media Preparation

Follow instructions provided on *Media Preparation and Bacterial Growth.*

Refer to *Skills Assessment #2 – Bacterial Growth.* Referencing the rubric will help you understand the skills needed for success.

Media Preparation and Bacterial Growth Instructions

Objectives: Students will demonstrate the ability to mix and pour nematode growth media (NGM) for the care and feeding for *C. elegans*; plates will be transparent, free of clumps, have a smooth/even surface, and be of equal volume in each poured plate. Students will use aseptic technique to create a lawn of bacterial growth in the center of the NGM plates and successfully transfer worms for proliferation on the new poured plates for use in further skill development.

Materials:

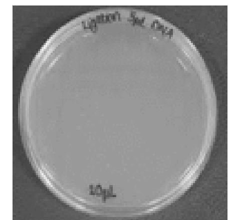
Mini (35x10) sterile petri dishes (2 plates/student)
Small (60x15) sterile petri dishes (4 plates/student)
NGM (Nematode Growth Media) Agar - can be commercially purchased or made
1 liter beaker filled with 500 mL tap water
Hot plate or Magnetic hot plate with stir bar
E. coli OP50 in LB broth media (1 tube/lab station)
p100 micropipette and pipette tips
100 – 200 mL ETOH in 400 mL beaker (1 beaker/lab station)
Bunsen burner of alcohol burner (1 burner/lab station)
Bacti-spreader, metal or glass (1 spreader/lab station)
Sharpie (1 marker/student)

Day 1: Plate Preparation

The Nematode Growth Media (NGM) we are using is purchased through a biological supply company. This media contains the essential nutrients needed by the *Escherichia coli* strain to feed the *C. elegans* nematode, both organic and inorganic compounds. The media comes in either glass or plastic bottles as a solid and then melted to pour into petri dishes. The melting process was started by your instructor to allow enough time for the media to cool to be poured. Media can be melted in the microwave but heating is uneven and incomplete. I prefer to melt media in a 1 liter beaker of water over a hot plate.

1. Complete the melting procedure started by your instructor. Media should be free of clumps and of even liquid consistency. Wear a mitt to remove the bottle from the beaker of water, make sure cap is loose, and set on counter to cool.

2. While bottle is cooling, label petri dishes.



Always label on the PERIMETER of the BASE of the dish.
Include initials, date, and "OP50"

3. When bottle is cool enough to handle, pour plates to half full in base.

Base should be on flat lab surface
DO NOT remove lid until ready to pour each plate
Lift lid, tilt bottle, and slowly pour media into plate
Immediately over poured plate with lid
Fill 4 small plates and 2 mini plates for each student at lab station

4. Do not disturb plates while media is setting.

Once media is solid, INVERT plates to prevent water from accumulating on media surface.
Store in refrigerator INVERTED for 24 hours. This allows media to set and dry.

Day 2: Bacterial Culture Preparation

C. elegans use bacteria as a food source. In particular, the *E. coli* OP50 is the food source preferred by scientists that work with C. elegans. This strain of *E coli* is a uracil auxotroph and growth is limited on NGM plates. A limited bacterial lawn is desirable because it allows for easier observation and better mating of the worms. Your instructor will provide a bacterial culture of *E. coli* OP50 in broth media for transfer to your new plates.

1. Remove plates from refrigerator and place on lab table. Bring to room temperature.

    Leave plates INVERTED to prevent water from accumulating on agar surface

2. Ignite Bunsen burner or alcohol burner.

    The flame will serve two purposes; provide heat as an aseptic environment for the transfer of bacteria from tube to plate, and to flame the broth tube and bacti-spreader for sterilization.

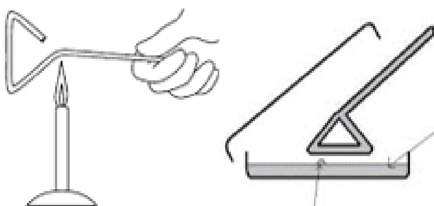
3. Set adjustable micropipette to 50 µm

4. Remove the lid from the broth tube and pass the lip of tube through the flame. This creates a heat barrier and prevents contamination of the bacterial broth tube.

5. Use the micropipette and place 50 µm of broth media to center of each plate. Immediately replace the lid on each petri dish following inoculation.

    No need to change tips between each plate inoculation. Dispose of tip in red biohazard bag when finished.

6. Dip the bacti-spreader in the beaker of ethanol and pass through the flame. This will sterilize the flame to prevent contamination of your bacterial colonies. Allow the bacti-spreader to cool.



7. Lift the lid on one petri dish and place the flat end of the bacti-spreader on the surface of the agar. Move the bacti-spreader in a circular motion over the surface of the agar to spread the bacterial inoculant over the surface.

8. Replace the lid and repeat steps 6 and 7 for each petri dish. When all plates are complete, dip bacti-spreader in the beaker of ethanol and pass through the flame to sterilize.

9. Allow petri plates to sit undisturbed for 10 mins.

10. Invert plates and stack in 37*C incubator to allow for bacterial growth overnight.

11. Clean lab station with spray disinfectant and wash hands.

Student Name ________________________________

Skills Assessment #2 – Media Preparation and Bacterial Growth

Skill	Not Shown 0	Proficient 2	Exemplary 4
Agar	Specks/Precipitate	Cloudy	Transparent
	Many clumps	Few slumps	No clumps
	Uneven surface		Even surface
	Unequal volume		Equal volume
Bacterial Growth	No growth	Isolated colonies	Lawn growth
Worm Transfer	No worms		Successful Transfer

Lesson 3: Life Cycle, Larval Forms, and Sex Determination

KEY QUESTION(S): What are the different stages of development for *C. elegans*? What is the *dauer* phase of the life cycle? What causes *C. elegans* to enter the *dauer* phase? How are the male and hermaphrodite *C. elegans* different? What details are known about the development of *C. elegans* from egg to adult?

OVERALL TIME ESTIMATE: 45 minutes

LEARNING STYLES: Visual and auditory

VOCABULARY:
Autosome
Dauer
Genotype
Phenotype

LESSON SUMMARY: Students will understand and describe the life cycle of C. elegans from egg through adult including the stress-resistant dauer phase.

STUDENT LEARNING OBJECTIVES:
The student will be able to...

- Describe details of the *C. elegans* life cycle.
- Describe different stages of the C. elegans life cycle from egg through adult.
- Evaluate the conditions that impact the development of dauer stage C. elegans.
- Define the developmental embryology of C. elegans as a model for understanding development in other organisms.

FLORIDA NEXT GENERATION SUNSHINE STATE STANDARDS (SCIENCE):
SC.912.N.3.5
SC.912.L.16.7
SC.912.L.16.10

MATERIALS:
Computer and projection unit
PowerPoint Presentation - *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
Copies of *Student Worksheet #2 – C. elegans Life Cycle and Sex Identification*, 1 per student

BACKGROUND INFORMATION:
As part of skill development, this lesson is designed to provide further biological and historical information about *C. elegans* as an experimental model. Much has been learned about C. elegans development that scientists have used to further the use of this organism as a model of development and physiology.

ADVANCE PREPARATION:
Copies of *Student Worksheet #2 – C. elegans Life Cycle and Sex Identification*, 1 per student
Review for further information to add to discussion

http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm
http://wormbook.org/chapters/www_evoldevnematode/evoldevnematode.html

PROCEDURE AND DISCUSSION QUESTIONS WITH TIME ESTIMATES:

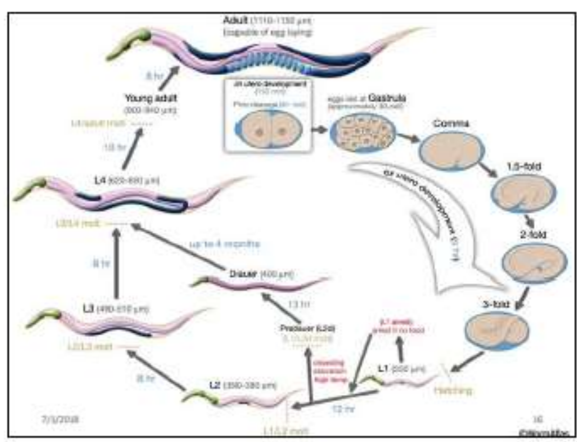
45 minutes

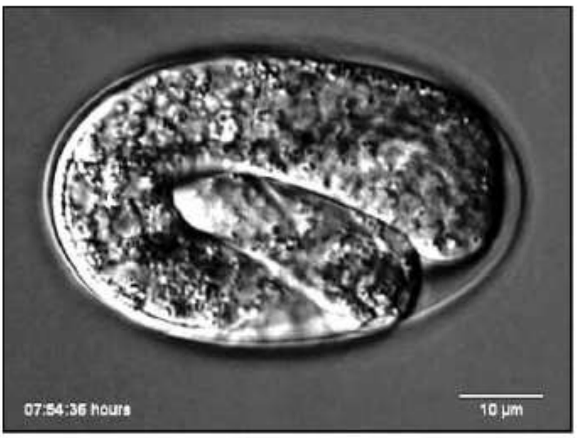
1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Provide each student with a copy of *Student Worksheet #2 – C. elegans Life Cycle and Sex Identification*
3. Review slides 14-25 and refer to homework from beginning of unit for examples.
4. Use PowerPoint slides as guide for class discussion
5. Students complete *Student Worksheet #2 – C. elegans Life Cycle and Sex Identification* as class works through PowerPoint lesson

ASSESSMENT SUGGESTIONS:

- Grade *Student Worksheet #2 – C. elegans Life Cycle and Sex Identification*

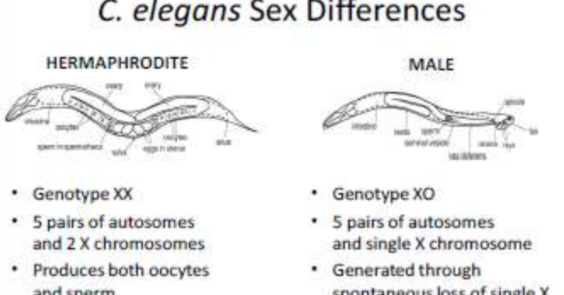
RESOURCES/REFERENCES:
The Worm Book has a wealth of information about *C. elegans* and skills when working with this model organism. http://www.wormbook.org/index.html





The *Dauer* Stage

- *C. elegans* may enter arrested state of life cycle if environmental conditions are not favorable.
- This *dauer* stage occurs at the end of the L2, larval 2 stage, of the life cycle and can last ~ 4 months.
- During the dauer stage
  - No aging
  - Arrested feeding
  - Less movement
- Development to L3 stage will continue once environmental conditions improve.



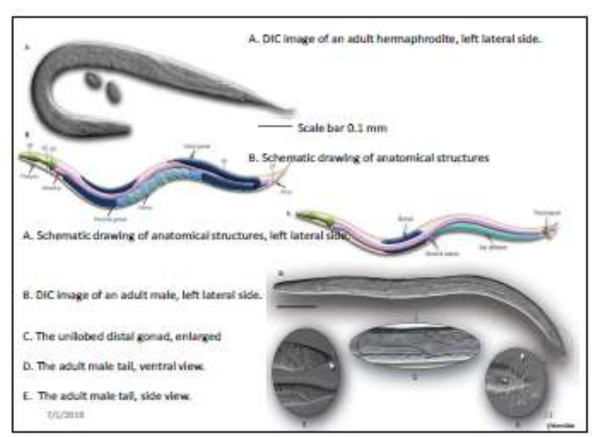
TWO SEXES:
1. Self-fertilizing hermaphrodite (XX)
2. Male (X0)

XX hermaphrodite

ovary, ovary, intestine, oocytes, oocytes, sperm in spermatheca, vulva, eggs in uterus, anus

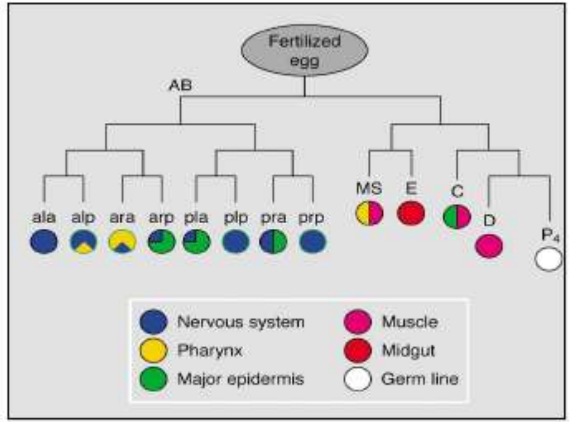
XO male

spicule, intestine, testis, sperm, seminal vesicle, cloaca, rays, fan, vas deferens



Developmental Biology

- Transparent surface allows for thorough understanding of development
- Adults have exactly 959 cells (hermaphrodite) or 1031 (male)
- Observations of programmed cell death – apoptosis
  - 131 lost cells (hermaphrodite)
  - 147 lost cells (male)
- Complete cell lineage, cell pathways, and cell fate



Devo Biology and Discovery

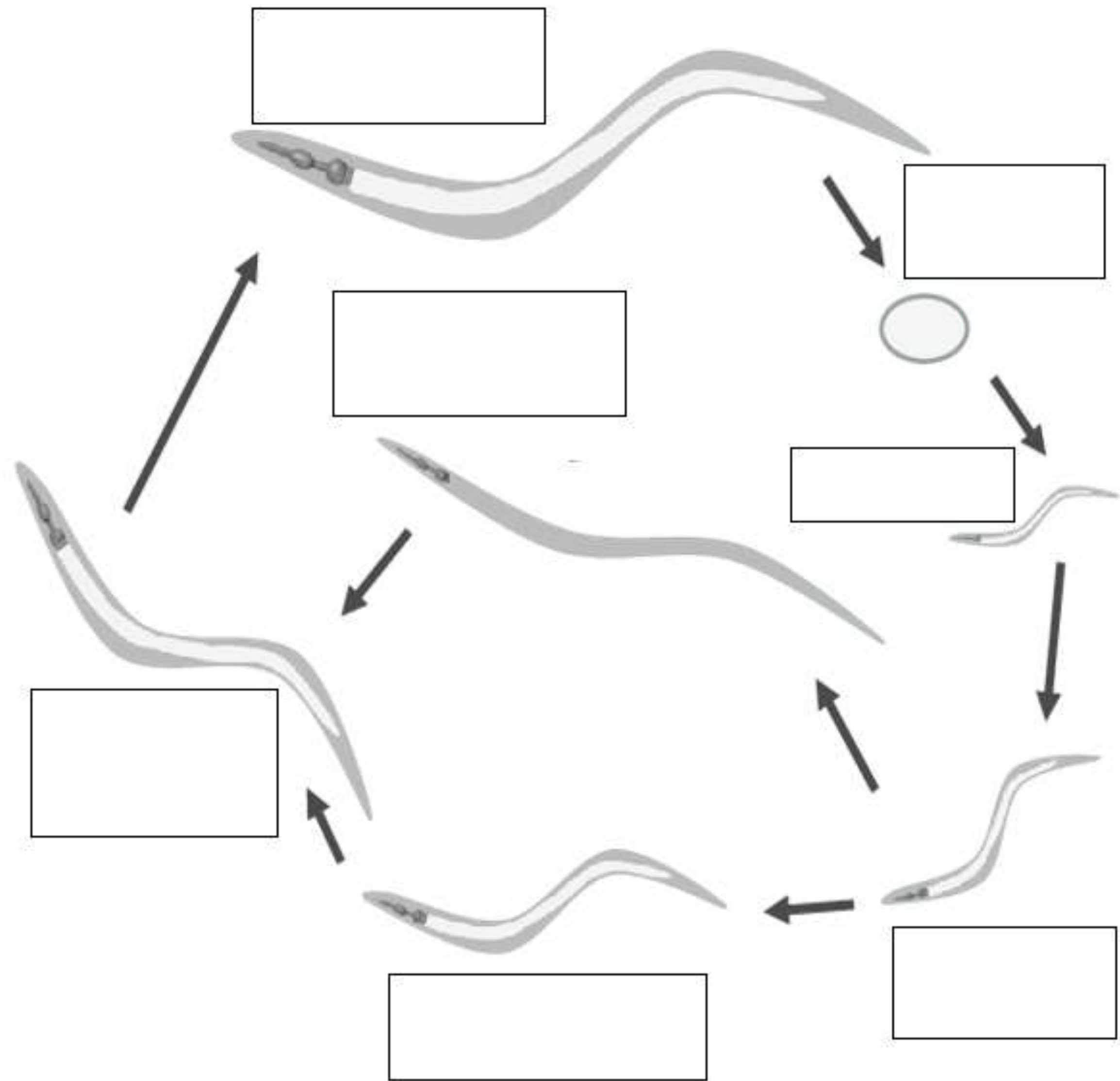
- 1981 - Germline stem cells
- 1983 - Discovery of apoptosis
- 1986 - Complete nervous system cell placement
- 1987 - Axon guidance genes
- 1988 - Distribution of cell components in embryos
- 1990 - Role of RAS signaling in development

All provided invaluable research in developmental biology for all organisms.

Student Name ___________________________________

Student Worksheet #2 – *C. elegans* Life Cycle and Sex Identification

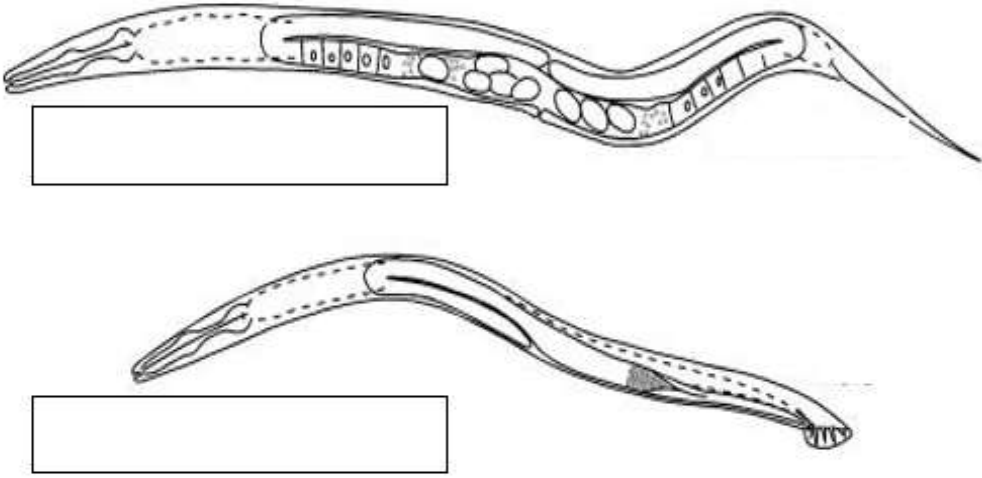
1. Identify each phase of the C. elegans life cycle and the approximate time spent in each phase in the boxes provided.



2. Describe the Dauer stage of the nematode life cycle. What causes the nematode to enter the Dauer stage?

Student Name ___________________________

3. Describe the biological sex differences in the *C. elegans* worm. Identify each below.



4. Describe how the life cycle of *C. elegans* is used in research of other organisms, including humans.

Lesson 4: Chunking versus Picking Plate Transfer

KEY QUESTION(S): Why is it necessary for scientists to move worms from one culture plate to another? What are the techniques used to transfer worms? Why is one technique preferred to another for a worm transfer? What are the materials and skills needed to perform a worm transfer? How do I make my own pick for single worm transfer?

OVERALL TIME ESTIMATE: Three 45 minute class periods, at minimum

LEARNING STYLES: Visual, auditory, and kinesthetic

VOCABULARY:
Aseptic transfer
Chunking
Picking

LESSON SUMMARY: Students will determine which testing protocol requires the use of chunking a population of different ages of *C. elegans* onto new culture plates versus selectively picking individual worms and moving to new culture plates based on age or sex. Students will construct their own pick and practice the skills of chunking and picking to establish new culture plates of *C. elegans*.

STUDENT LEARNING OBJECTIVES:
The student will be able to...

- Differentiate between worm chunking and worm picking.
- Describe the reasons worms need to be moved from one plate to another.
- Perform a successful worm chunk.
- Identify worm life stages to select individual worms for transfer.
- Construct a pick to perform individual worm transfers.
- Perform a successful worm transfer of 6 larval worms to a new NGM OP50 plate.
- Perform a successful worm transfer of a single gravid adult to a new NGM OP50 plate.

FLORIDA NEXT GENERATION SUNSHINE STATE STANDARDS (SCIENCE):
SC.912.N.1.1
SC.912.N.1.6
SC912.N.1.7
SC.912.N.3.5
SC.912.L.16.10

BACKGROUND INFORMATION:
An essential skills when working with nematodes is in chunking worms to new plates for propagation and picking individual worms to new plates for mating or synchronization purposes. The more time you allow to practice these skills, the more confident the students will become when using the worms for their own investigations.

MATERIALS:

Small (60x15) starter plates of N2 Wild Type *C. elegans* (2 plates/student pair)
1 small and 2 mini petri plates of *E. coli* OP50 from Lesson 2
Bunsen burner or alcohol burner (1 burner/lab station)
100-200 mL ETOH in 400 mL beaker (1 beaker/lab station)
Micro spatula (1 spatula/lab station)
Sharpie marker (1 marker/student)
Glass Pasteur pipet, 5 ¾ in long (1 pipet/student)
Roll titanium, platinum, or nichrome wire, 24+ gauge
Needle nose pliers (1 pair/lab station)

ADVANCE PREPARATION:
Copies of *Chunking versus Picking Instructions*, class set
Copies of *Skills Assessment #3 – Chunking vs. Picking,* 1 per student
Assemble materials per lab station

PROCEDURE AND DISCUSSION QUESTIONS WITH TIME ESTIMATES:
The times and schedule set in the Lesson Planning Guide on pages 8-9 are two options that allow for sufficient time to practice these skill sets. Adjust accordingly as you move through this lesson

Day 1 – Chunking Plates
45 minutes

1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Provide each student with a copy of *Chunking versus Picking Instructions*
3. Provide each student with a copy of *Skills Assessment #3 – Chunking vs. Picking*
4. Review slides 26-30 and use slides as guide for class discussion
5. Students perform the Chunking skill and record 0 hour data on *Skills Assessment #3 – Chunking vs. Picking*

Day 2 – Making a Pick and Practice
45 minutes

1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Review slides 26-30 and use slides as guide for class discussion
3. Students make observations and record 24 hour data on Chunk plates on *Skills Assessment #3 – Chunking vs. Picking*
4. Students follow directions for making own pick as described on *Chunking versus Picking Instructions*
5. Students practice skills according to instructions provided on *Chunking versus Picking Instructions* This skill often takes several days to master, especially with students sharing a microscope. Adjust as needed for students to develop mastery
6. When prepared, have students create 2 pick plates, one for larval picks and one for gravid adult
7. Instructor moves around room as needed to observe and provide advice on lab skills

Day 3 – Pick Practice
45 minutes

1. Students can begin practicing their skills as soon as they enter class.
2. Students make observations and record 48 hour data on Chunk plates on *Skills Assessment #3 – Chunking vs. Picking*
3. Students make observations and record 24 hour data on Pick plates on *Skills Assessment #3 – Chunking vs. Picking*
4. Remind students that they should practice for efficient transfer.
5. Remind students to refer to earlier lessons for correct identification of worm stages.
6. Instructor moves around room as needed to answer questions and provide guidance on technique.

ASSESSMENT SUGGESTIONS:

- Grade *Skills Assessment #3 – Chunking vs. Picking*

RESOURCES/REFERENCES:
The Worm Book has a wealth of information about *C. elegans* and skills when working with this model organism. http://www.wormbook.org/index.html

Video tutorials: How to Chunk Worms - https://www.benchfly.com/video/169/how-to-chunk-worms-c-elegans/
How to Pick Worms - https://www.youtube.com/watch?v=Zw1u5TmeHvU
https://www.youtube.com/watch?v=eTQtkQm5hOw

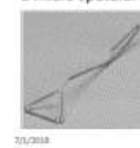
Worm Transfer

- Sometimes it is necessary to move worms from one plate to another.
  - Food scarcity
  - Contamination
  - Synchronize age
  - Mating
- Forms of worm transfer
  - Chunking
  - Picking
  - Bleaching

Chunking vs. Picking Worms

CHUNKING

- Easiest to perform
- Aseptic transfer of an agar chunk from old plate to fresh bacterial culture.
- Requires Bunsen burner and a micro spatula.



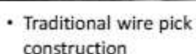
PICKING

- Takes practice to perfect
- Aseptic transfer of single worms from old plate to fresh bacterial culture.
- Requires Bunsen burner and a pick
  - Can be purchased or made
  - Can be wire or hair/filament

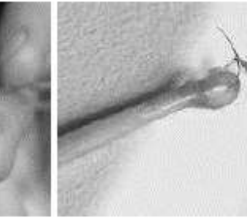
Pick Construction

Procedure

1. Clip a short (< 20 mm) segment of wire.
2. Insert one end of the wire into the narrow opening of the Pasteur pipet using the forceps to hold in place.
3. Melt the glass around the wire using the Bunsen burner.
4. Shape the pick using the forceps and heat from the Bunsen burner, careful not to break the glass.
5. As you practice with the pick, you may heat and melt a few times to get the desired bend in the wire and glass.



- Traditional wire pick construction
- Good for beginners
- Useful for picking adult worms or L4 stage worms



- Eyebrow pick construction
- Useful for L1-L3 stage worms
- CANNOT flame sterilize!

Chunking vs. Picking Instructions

Objectives: Students will differentiate between chunking a plate of *C. elegans* for propagation of worms on a new culture plate versus picking individual worms for propagation on a new culture plate; understand the construction of a "pick" used to select individual worms for transfer; construct a pick for personal use; demonstrate the skills necessary to aseptically transfer an undifferentiated worm population from an old "starved" plate to a new culture plate; demonstrate the ability to recognize worms of different life stages (L1, Dauer, gravid adult, etc); successfully transfer specific population of worms to a new culture plate.

Materials:

Small (60x15) starter plates of N2 Wild Type *C. elegans* (2 plates/student pair)
1 small and 2 mini petri plates of *E. coli* OP50 from previous laboratory activity
Bunsen burner or alcohol burner (1 burner/lab station)
100-200 mL ETOH in 400 mL beaker (1 beaker/lab station)
Micro spatula (1 spatula/lab station)
Sharpie marker (1 marker/student)
Glass Pasteur pipet, 5 ¾ in long (1 pipet/student)
Roll titanium, platinum, or nichrome wire, 24+ gauge
Needle nose pliers (1 pair/lab station)

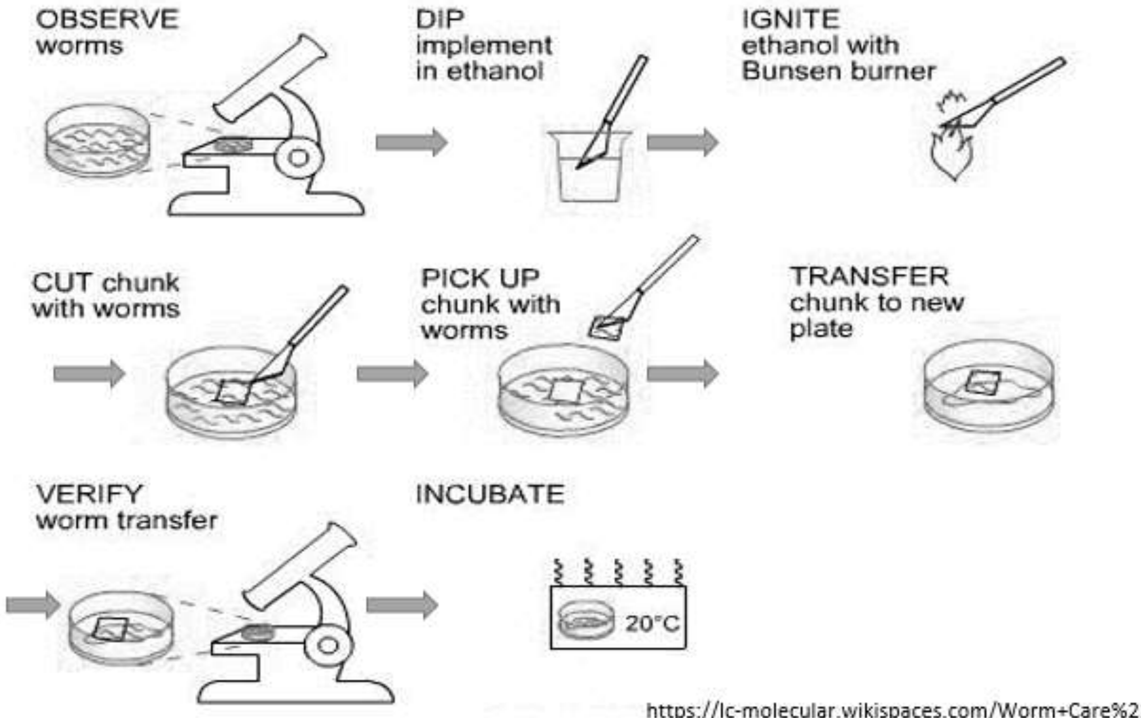
Day 1: Chunking

Recall, chunk transfer of worms allows for C. elegans to be transferred to fresh bacterial cultures for several reasons; a "starved" plate, contamination, or just to have more worms to use for laboratory investigation.

1. View the starter plate of worms provided by your instructor under the microscope to ensure there are living worms for transfer.
2. Label 1 small NGM "OP50" petri dish "Chunk"
   Make sure your initials are clearly visible
3. Ignite the Bunsen burner. The flame does not need to be large.
4. Dip the micro spatula in the beaker of ethanol.
5. Pass the micro spatula through the flame and burn off the ethanol.
6. Allow the spatula to cool
7. Use the spatula to cut a ½-inch (1 cm) square of agar from the old plate.
8. Lift this chunk of agar from the old plate and invert on the edge of a new bacterial culture plate.
9. Dip the micro spatula in the beaker of ethanol and flame to sterilize.
10. Allow plate to set for 5 minutes.
11. View new plate under the microscope to ensure successful worm transfer.
    Record 0 hour observations on worksheet – *Skills Assessment #3 – Chunking vs. Picking*
12. Invert and place new plate in 20*C incubator.
13. Wipe down lab station with disinfectant and wash hands.

**Day 2 and 3 – Record 24 and 48 hour observations on worksheet – *Skills Assessment #3 – Chunking vs. Picking*

Chunk Protocol



https://lc-molecular.wikispaces.com/Worm+Care%21

Chunking vs. Picking Instructions

Day 2: Picking

Picking worms is a delicate procedure that requires practice in addition to the construction of your own "pick" Follow the instructions from the PowerPoint slide (copied below) to construct your own pick for use in class.

Pick Construction

Procedure

1. Clip a short (< 20 mm) segment of wire.
2. Insert one end of the wire into the narrow opening of the Pasteur pipet using the forceps to hold in place.
3. Melt the glass around the wire using the Bunsen burner.
4. Shape the pick using the forceps and heat from the Bunsen burner, careful not to break the glass.
5. As you practice with the pick, you may heat and melt a few times to get the desired bend in the wire and glass.

To ensure your pick is the right shape for personal use, you need to practice using the pick with a mini plate and microscope.

1. Label 1 mini NGM OP50 petri dish "Pick"
   Label the other mini NGM OP50 petri dish "Gravid"
2. View the starter plate of worms provided by your instructor under the microscope to ensure there are living worms for transfer.
3. Use your pick to attempt to pick up a single adult worm. Try a scooping motion and do not gouge the surface of the agar.
4. You may have to reheat your pick and use the forceps or wire cutter and further modify your pick. Once perfected, use a piece of label tape so you can identify your pick. Place the tape on the handle.
5. Continue to practice picking up and moving worms of different sizes from one location of the original plate to another.

When you feel comfortable with your skill level:

1. Transfer SIX L2 or L3 worms from the starter plate to "Pick" plate
2. Transfer ONE gravid adult from the starter plate to "Gravid." Plate
3. Continuously view new plates under the microscope to ensure successful worm transfers.
4. Once transfers are complete record 0 hour observations on worksheet – *Skills Assessment #3 – Chunking vs. Picking*
5. Invert and place new plate in 20*C incubator.
6. Wipe down lab station with disinfectant and wash hands.

**Day 2 and 3 – Record 24 and 48 hour observations on worksheet – *Skills Assessment #3 – Chunking vs. Picking*

Student Name ____

Skills Assessment #3 – Chunking vs. Picking

Follow the directions for Chunking vs. Picking worms for transfer to a new plate.
Answer the questions below and use this worksheet to record results at 0, 24, and 48 hours after your initial transfer of worms.

1. Why is it necessary to transfer worms to a new plate using the chunk protocol?

2. When performing a chunk transfer of worms, what is the purpose of the alcohol and flame?

3. Record QUALITATIVE and QUANTITATIVE observations of your starter plate.
Notice the way the worms are clumping together in masses on the plate.
Do you see any new eggs on the agar surface?
Can you identify any males? Gravid adults?

QUALITATIVE	QUANTITATIVE

Student Name ______________________________________

4. As you work with your new plates over the next few days, record observations in the table

Plate	0 hour	24 hour	48 hour
Small Chunk			
Mini Pick - Larvae			
Mini Pick – Gravid adult			

Skills Rubric

Skill	Not Shown 0	Proficient 2	Exemplary 4
Chunk	No worms present	Chunk too large (too many) or too small (too few)	Chunk is correct size and worms present
Mini Pick - Larvae	No worms present	Fewer than 6 larvae on new plate	All larvae are L2 or L3 stage
Mini Pick – Gravid adult	No worm present	Worm is not gravid adult	Gravid adult present

Lesson 5: Mutant Worms

KEY QUESTION(S): Are all *C. elegans* worms the same? What causes mutations in the worms? Are all mutations in *C. elegans* visible? How do scientists take advantage of these mutations to make discoveries?

OVERALL TIME ESTIMATE: 45 minutes

LEARNING STYLES: Visual and auditory

VOCABULARY:
Mutant

LESSON SUMMARY: Students will be introduced to different mutant strains of *C. elegans* with characteristic appearance or movement that can be observed using a microscope. These mutant strains of *C. elegans* are used in research to study a variety of human diseases that have similar genetic profiles to those found in *C. elegans*.

STUDENT LEARNING OBJECTIVES:
The student will be able to...

- Define mutation/mutant.
- Differentiate between wild type/N2 designations and mutant designations.
- Describe the ways scientists use mutant worms for investigation.

FLORIDA NEXT GENERATION SUNSHINE STATE STANDARDS (SCIENCE):
SC.912.N.1.1
SC.912.N.1.4
SC.912.N.1.6
SC912.N.1.7
SC.912.N.2.4
SC.912.N.3.5
SC.912.L.15.15
SC.912.L.16.4
SC.912.L.16.10

BACKGROUND INFORMATION:
The use of mutant strains of *C. elegans* has become commonplace in the modern research laboratory. So much is known about the *C. elegans* genome, development, and life cycle that the homology of the worm as compared to humans is well understood. An understanding of mutations is a foundational skill in the use of C. elegans as an experimental model in toxicology, genomics, proteomics, development biology, and study of human disease.

MATERIALS:
Copies of *Student Worksheet #3 – Mutant Worms*, 1 per student

ADVANCE PREPARATION:
Copies of *Student Worksheet #3 – Mutant Worms*, 1 per student
Make sure video links work in PowerPoint
Load pdf versions of articles listed on *Student Worksheet #3 – Mutant Worms* to available website or find other appropriate article that provide a link between *C. elegans* and the human conditions listed on the worksheet.

PROCEDURE AND DISCUSSION QUESTIONS WITH TIME ESTIMATES:
45 minutes

1. Load PowerPoint presentation *Caenorhabditis elegans:* Using Model Organisms as a Tool in Molecular Genetics
2. Provide students with copy of *Student Worksheet #3 – Mutant Worms*
3. Review slides 31-38, students complete *Student Worksheet #3 – Mutant Worms* as you progress through the lesson.
4. Provide instructions for completing homework assignment
5. Allow students time to view the video selections provided as links on *Student Worksheet #3 – Mutant Worms* and ask questions for understanding assignment.
    a. This assignment could be done in class the following day to allow for better questions and discussion.

ASSESSMENT SUGGESTIONS:

- Grade *Student Worksheet #3 – Mutant Worms*, 1 per student

RESOURCES/REFERENCES:
The Worm Book has a wealth of information about *C. elegans* and skills when working with this model organism. http://www.wormbook.org/index.html

Student Worksheet #3 – Mutant Worms

View the PowerPoint Presentation section - Mutations of Movement and complete the notes below.

Mutant Worm Identification

1. The CGC (*Caenorhabditis* Genetics Center) developed a naming protocol to streamline publication and discussion of *C. elegans*. Describe this naming protocol.

2. Watch the three videos, two of which demonstrate visible mutant phenotypes. Describe how you could use these differences in a scientific investigation.

3. What term is used to describe genetic similarities among organisms? How can this similarity benefit research science?

4. The ability to silence genes in *C. elegans* is well-documented through the process of RNA interference (RNAi). Based on what you know about transcription and translation of a gene, describe how you think small intervening RNA (siRNA) or micro-RNA (miRNA) interfere with the process of protein synthesis and silence genes.

5. Select ONE of the topic videos below to view for homework.
   Watch the video as many times as needed to understand the use of *C. elegans* to study genes related to human diseases or conditions.
   Read the accompanying journal article to gain more understanding of the research involved in your selected topic.
   Summarize the video and article to provide a better understanding of research using the model organism, *Caenorhabditis elegans*.

   Aging:
   https://www.ted.com/talks/cynthia_kenyon_experiments_that_hint_of_longer_lives/transcript?language=en

   https://www.sciencefriday.com/videos/hotel-nematoda/

   Article: excerpt provided
   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4464094/pdf/tinv-59-059.pdf

   Immune function:
   https://www.pbs.org/newshour/science/glowing-green-worm-can-teach-us-immune-systems

   Article: excerpt provided
   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4248339/

   Neuroscience:
   https://www.salk.edu/news-release/decoding-chemistry-fear/

   Article: excerpt provided
   https://www.sciencedirect.com/science/article/pii/S0149763416308077

Worm Identification

- The CGC has developed a standard naming protocol to identify mutant strains.
- Consist of 3- or 4-letter gene class names followed by a hyphen and a number.
  - Provides info about gene function or biochemical activity
  - Example: dpy-7 = DumPY phenotype, 7th discovered mutation of that phenotype.
  - For comparison purposes, WTN2 = Wild Type Normal worms are used as the control for experimentation or to introduce RNA to knockdown gene function.
- Easy to access in *WormBook* or *WormAtlas*

Easy to Identify Mutant Phenotypes

- For student use, several easy to identify mutant strains are available to study.
- Requires no biochemical testing or genomic sequencing
- Common mutants show variable shape or movement patterns
  - rol = rolling/twist body like corkscrews
  - dpy = shorter body length
  - unc = uncoordinated movement/twitcher
  - sma = small body size

Investigations Using Mutants

- Sequenced genome provides valuable understanding of *C. elegans'* biological mechanisms.
- Mutant worms provide a comparison for function.
  - Loss of Function
  - Gain of Function
  - Lethal alleles
- Homology with human genome provides a basis for understanding human function and disease.
- Homology with other organisms make *C. elegans* useful for environmental studies.

RNA Interference - RNAi

- Introduction of small RNA sequences
  - Disrupt the normal transcription of a gene for protein synthesis.
- Essentially silences a target gene and impacts function.
- Creates a mutant phenotype that scientists use for variety of investigations
  - Toxicity
  - Drug interactions
  - Disease Control
  - Produce Transgenic organisms

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Review article

Habituation is altered in neuropsychiatric disorders—A comprehensive review with recommendations for experimental design and analysis

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ABSTRACT

Abnormalities in the simplest form of learning, habituation, have been reported in a variety of neuropsychiatric disorders as etiologically diverse as Autism Spectrum Disorder, Fragile X syndrome, Schizophrenia, Parkinson's Disease, Huntington's Disease, Attention Deficit Hyperactivity Disorder, Tourette's Syndrome, and Migraine. Here we provide the first comprehensive review of what is known about alterations in this form of non-associative learning in each disorder. Across several disorders, abnormal habituation is predictive of symptom severity, highlighting the clinical significance of habituation and its importance to normal cognitive function. Abnormal habituation is discussed within the greater framework of learning theory and how it may relate to disease phenotype either as a cause, symptom, or therapy. Important considerations for the design and interpretation of habituation experiments are outlined with the hope that these will aid both clinicians and basic researchers investigating how this simple form of learning is altered in disease.

"Together, nonassociative learning and nonassociative gating constitute an intelligent 'firewall' that constantly triages vast amounts of sensory information into actionable and non-actionable categories in order to prioritize. This firewall mechanism shields the mind from the vast amounts of inundating sensory information that constantly compete with one another for attention, and spares it the trouble of having to respond to every tingling except the most salient ones. The triage process not only helps to preserve mental sanity but also conserve physical energy, both of which are important for survival."

Poon and Young (2006)

1. Introduction

Although abnormal habituation has been observed in numerous neurological and neuropsychiatric disorders a comprehensive review of how this form of non-associative learning is altered in each disorder is lacking. Habituation is a non-associative form of learning, defined as a response decrement resulting from repeated stimulation that cannot be explained by sensory adaptation or motor fatigue, and has conserved behavioural characteristics present in all organisms studied (Table 1, adapted from Rankin et al., 2009). In lay terms, habituation may be described as the ability to "ignore the familiar, predictable, and inconsequential," a process almost ubiquitously presumed to be crucial for normal cognitive function. For this reason, habituation is conceptualized as a "building block of cognition," essential to attention, saliency mapping, and more complex forms of learning and memory. This is supported by the observation that there is a correlation between the rate of habituation in infancy and later IQ scores (Kavšek, 2004; McCall and Carriger, 1993).

Despite its ubiquity and importance to normal cognitive function, remarkably little is known about the cellular and molecular processes underlying habituation (Giles and Rankin, 2009; Glanzman, 2009; Ramaswami, 2014; Schmid et al., 2014; Wilson and Linster, 2008). Indeed, several lines of evidence suggest that this elementary form of plasticity is mediated by multiple mechanisms which are recruited by

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Table 1
The ten behavioural characteristics of habituation (Rankin et al., 2009).

The Behavioural Characteristics of Habituation
1. Repeated application of a stimulus results in a progressive decrease in some parameter of a response to an asymptotic level. This change may include decreases in frequency and/or magnitude of the response. In many cases, the decrement is exponential, but it may also be linear; in addition, a response may show facilitation prior to decrementing because of (or presumably derived from) a simultaneous process of sensitization.
2. If the stimulus is withheld after response decrement, the response recovers at least partially over the observation time ("spontaneous recovery").
3. After multiple series of stimulus repetitions and spontaneous recoveries, the response decrement becomes successively more rapid and/or more pronounced ("potentiation of habituation").
4. Other things being equal, more frequent stimulation results in more rapid and/or more pronounced response decrement, and more rapid spontaneous recovery (if the decrement has reached asymptotic levels).
5. Within a stimulus modality, the less intense the stimulus, the more rapid and/or more pronounced the behavioural response decrement. Very intense stimuli may yield no significant observable response decrement.
6. The effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level (which may or may not be zero, or no response). This effect of stimulation beyond asymptotic levels can alter subsequent behaviour (e.g., by delaying the onset of spontaneous recovery).
7. Within the same stimulus modality, the response decrement shows some stimulus specificity. To test for stimulus specificity/stimulus generalization, a second, novel stimulus is presented and a comparison is made between the changes in the responses to the habituated stimulus and the novel stimulus. In many paradigms (e.g., developmental studies of language acquisition) this test has been improperly termed a dishabituation test rather than a stimulus generalization test, its proper name.
8. Presentation of a different stimulus results in an increase of the decremented response to the original stimulus. This phenomenon is termed "dishabituation." It is important to note that the proper test for dishabituation is an increase in response to the original stimulus and not an increase in response to the dishabituating stimulus (see point #7 above). Indeed, the dishabituating stimulus by itself need not even trigger the response on its own.
9. Upon repeated application of the dishabituating stimulus, the amount of dishabituation produced decreases ("habituation of dishabituation").
10. Some stimulus repetition protocols may result in properties of the response decrement (e.g., more rapid rehabituation than baseline, smaller initial responses than baseline, smaller mean responses than baseline, less frequent responses than baseline) that last hours, days or weeks. This persistence of aspects of habituation is termed long-term habituation.

different stimuli and training paradigms (Giles and Rankin, 2009; Rankin and Broster, 1992). Although studies using animal models have revealed that both short- and long-term forms of habituation can be observed (Castellucci et al., 1978), this review will focus on short-term habituation reflecting the focus of the clinical literature to date. While short-term habituation develops within a single training session, long-term habituation persists across training sessions and requires spaced training and protein synthesis for its production and maintenance (Ramaswami, 2014; Rankin et al., 2009). Despite being the simplest form of learning there is very little known about the cellular mechanisms of underlying habituation. Studies using *Aplysia* and rats show that short-term habituation can result from homosynaptic depression of excitatory neurotransmission (Armitage and Siegelbaum, 1998; Castellucci et al., 1970; Castellucci and Kandel, 1974; Farel and Thompson, 1976; Kupfermann et al., 1970; Weber et al., 2002) and studies using *Drosophila* have shown that habituation can also manifest at the network-level by potentiation of inhibitory synapses (Das et al., 2011; Glanzman, 2011).

Consistent with the ubiquity, adaptive importance, and diversity of underlying mechanisms of habituation, habituation abnormalities have been implicated in numerous etiologically diverse neuropsychiatric disorders. The purpose of this review is to bring together accounts of habituation and neurological/neuropsychiatric disorders with the hope that this will lead to insights about both habituation, and the neuropsychiatric disorders in which habituation is altered. It is our hope that this review will serve as a resource for both clinicians and basic researchers investigating habituation and disease. Understanding the habituation deficits in one disorder may serve as a catalyst for studies of another disorder. An additional goal of this review is to provide experimental design and interpretation guidelines that will allow for more consistent observations across studies. An accurate understanding of how habituation is altered in a disorder will facilitate the use of habituation as a tool for differential diagnosis and will allow for more accurate animal models to investigate the cellular and molecular mechanisms underlying these learning impairments.

2. Neuropsychiatric disorder inclusion criteria

Not all neuropsychiatric disorders show abnormal habituation, however a surprising number do. To generate a list of the most prevalent neuropsychiatric conditions for which there is also a substantive literature investigating habituation we queried PubMed for each of the disorder categories listed in DSM-V (American Psychiatric Association, 2013) and the 12 disorders listed as the most common neurological disorders according to Hirtz et al. (2007). Only disorders with more than five empirical research articles comparing habituation in a clinical population to habituation in one or more control groups were included in this review. The disorders that met this criterion were: Autism Spectrum disorder, Fragile X syndrome, Schizophrenia, Parkinson's disease, Huntington's disease, Attention Deficit Hyperactivity Disorder, Tourette's syndrome, and Migraine. Despite the diverse etiology of these disorders the degree of habituation alteration correlates with symptom severity in most of the disorders suggesting that understanding the alterations in habituation might lead to new approaches to understanding, diagnosing, and treating these disorders. To our knowledge, the neuropsychiatric disorders reviewed here represent all disorders for which there are five or more studies examining habituation in human patient populations.

3. Study selection criteria

This work heavily focuses on studies investigating non-associative learning alterations by comparing differences in response plasticity to repeated stimulation in two or more groups. To delimit the scope this review and provide a cohesive narrative, we excluded studies of habituation to drugs in addiction research. For disorders with pre-existing reviews examining habituation alterations (e.g., ASD, Schizophrenia, Migraine), the reviews are briefly summarized and work published since the most recent review are covered in detail. For disorders without a pre-existing review focused on alterations in habituation all studies are reviewed.

We have included only articles whose authors explicitly stated they were investigating altered habituation in a neuropsychiatric disorder group compared to one or more control groups. However, it is important to note that any response change due to repeated non-associative stimulation is the sum of putatively independent underlying sensitization (incremental) and habituation (decremental) processes which are integrated to produce the final behavioural response (Groves and Thompson, 1970). Therefore, the observed changes in habituation discussed here could in principle reflect changes in sensitization.

4. Methods for studying habituation in humans

The training paradigms and methods used to study habituation in humans are as diverse as the diseases and disorders they have been used to study. In order to facilitate accessibility to a broader scientific audience we have provided a description of the common methods used to study habituation in adult humans: acoustic startle, event-related potentials, electrodermal activity, and functional magnetic resonance imaging (Table 2). The methods described in Table 2 are not exhaustive, but rather represent the most common methods that researchers build upon when designing more complex habituation

Table 2
A brief description of common methods for the analysis of habituation in humans.

Common paradigm titles	Brief description of method	Commonly used eliciting stimuli	Response metrics	Detailed description of method	Studies using this method to detect abnormal habituation
Startle reflex habituation or Acoustic Startle Reflex (ASR) habituation	Intense stimuli are presented to induce a startle response: eye-lid closure (blink) and a contraction of facial, neck, and skeletal muscles. Habituation is observed as a decrease in startle response magnitude and/or probability with repeated stimulation.	Usually brief intense auditory stimuli (loud tones presented through headphones). Somatosensory and visual stimuli may also be used to elicit a startle response.	Behavioural output (most commonly the probability of blinking). Electromyographic (EMG) recording of the orbicularis oculi muscle responsible for the blink reflex is common.[a] Recording ERPs (described above) following startle-inducing stimuli is also common.	Davis (1984), Koch (1999)	Geyer and Braff (1982), Braff et al. (1992), Meincke et al. (2004)
Event-related potential (ERP) habituation or Cortical evoked potential habituation	Stimuli are presented to participants while event-related changes in voltage are measured from several electrodes placed across the scalp (i.e. Electroencephalography). Habituation is observed as a decrease in the latency or amplitude of various components of the ERP wave with repeated stimulation.	Simple discrete auditory, visual, and somatosensory stimuli, as well as various nociceptive stimuli. Complex visual stimuli (e.g., checkerboard patterns)	Voltage (i.e., microvolts) is measured from several different areas across the scalp (e.g., frontal, parietal, etc.). Various components of the ERP may be isolated and analyzed individually. For example the negative deflection that occurs ~ 100 ms after the stimulus (N100) or the positive deflection that occurs ~ 300 ms after the stimulus (P300)[b]	Luck (2012)	Coppola et al. (2013), de Tommaso et al. (2014)
Electrodermal activity (EDA) habituation, Event-related skin conductance response (ER-SCR) habituation, or Skin conductance orienting response (SCOR) habituation[c]	Stimuli (usually weak auditory tones) are presented to participants in a standard laboratory or clinical setting. Stimulus-evoked changes in skin conductance are measured. Habituation is observed as a decrease in the probability or magnitude of skin conductance response with repeated stimulation.	Simple discrete auditory, visual, somatosensory stimuli, as well as various nociceptive stimuli	Changes in skin conductance (i.e., micro Siemens) generated by eccrine sweat glands is measured using two electrodes placed on the skin of the hands or fingers	Dawson et al. (2007), Boucsein (2012)	Schoen et al. (2008)
Functional magnetic resonance imaging (fMRI), Blood oxygen level dependent (BOLD) contrast habituation, or Specific brain region habituation (e.g., amygdala habituation)[d]	Stimuli are presented to participants inside the MRI scanner via computer screens and speakers. Stimulus-evoked BOLD contrast responses are recorded throughout stimulus repetitions. Habituation is observed as a decrease in evoked BOLD contrast with repeated stimulation	Simple discrete auditory and visual stimuli (e.g., tones and shapes), complex visual stimuli (e.g., emotive faces)	BOLD contrast	Breiter et al. (1996), Plichta et al. (2014)	Holt et al. (2005), Kleinhans et al. (2009)

We have also provided references where a detailed description of the method can be found as well as examples from this review where the method has been used to detect abnormal habituation in the context of a disease or disorder.

[a] The EMG blink response induced by weak stimuli that do not induce a startle response (e.g. supraorbital electrical stimulation) is also common (see Penders and Delwaide, 1971). The R2 component of the blink response EMG is most often studied because it shows the greatest amount of habituation.

[b] Magnetoencephalography (MEG) is often used to measure event related field (ERF) habituation in order to gain greater spatial resolution compared to EEG.

[c] Galvanic Skin Response is an antiquated term referring to electrodermal activity, the term is no longer used for reasons discussed in Boucsein (2012).

[d] Also referred to as fMRI adaptation, repetition suppression, and repetition attenuation.

paradigms.

5. Impaired habituation is an endophenotype of Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by fixated interests, inflexible routines, stereotyped behaviours, as well as difficulties with communication and social interactions. Despite the large phenotypic variation present within the autism spectrum, improper processing of sensory stimuli appears to be a shared phenomenon that has recently been added to the list of diagnostic criteria (American Psychiatric Association, 2013; Sinclair et al., 2017). Over 96% of individuals with ASD report hypo- or hypersensitivity to stimuli across sensory modalities (Crane et al., 2009; Leekam et al., 2007; Minshew et al., 2002; Sinclair et al., 2017). Indeed, hypersensitivity to sensory stimuli is so common in ASD it has inspired the influential "altered salience" and "intense world" theories of ASD (Markram et al., 2007; Ramachandran and Oberman, 2006). These and other "hyperarousal" or similar "decreased inhibition" theories suggest 1) children with ASD are more easily aroused by sensory stimuli and/or 2) that they show reduced habituation to repeated stimuli compared to other children (Ramaswami, 2014; Rogers and Ozonoff, 2005). In support of these theories, a variety of studies employing different stimulation paradigms and modalities suggest that habituation deficits arise early in the development of ASD and persist into adulthood.

Face-processing deficits are among the earliest reported social deficits in ASD. When repeatedly presented images of faces typically-developing children will habituate to the images by decreasing the amount of time spent looking at each stimulus. Assessing the duration of face-directed gaze, Webb et al. (2010) showed that 18–30 month-old children with ASD, and their siblings, take longer to habituate to images of faces than age-matched controls. These findings provide evidence for decreased habituation at the earliest age that ASD can be reliably diagnosed, but deficits in habituation may be present even earlier in development. An EEG study showed that 9-month-old infants at high risk for ASD (i.e., possessing an older sibling diagnosed with ASD) displayed decreased habituation of auditory evoked potentials when compared to age matched low-risk controls (Guiraud et al., 2011).

Behavioural and electrophysiological studies of habituation in adolescents and adults with ASD have provided less consistent results due to increased heterogeneity of age in the patient population and the diversity of stimulus presentation paradigms employed. In an attempt to address this, an electrodermal study first separated adolescents with ASD into high-arousal or low-arousal subgroups based on their resting skin conductance before administering several blocks of stimuli across all sensory modalities. This study revealed a trend where the high arousal ASD subgroup showed reduced habituation while the low arousal ASD subgroup displayed enhanced habituation, as compared to controls (Schoen et al., 2008). Importantly, this study demonstrates the large differences that can exist for individual habituation patterns within the heterogeneous ASD group. Further investigation of habituation in ASD has come from recent studies primarily focused on measures of sensorimotor gating (e.g., pre-pulse inhibition or P50 suppression). Many of these studies use an acoustic startle paradigm where acoustic tones are delivered to participants through headphones and the subsequent eye-blink response is recorded directly by observation or by electromyography (EMG). A range of results have been obtained from subjecting ASD individuals to this procedure, including reduced habituation (Perry et al., 2007), increased sensitization (Madsen et al., 2014), as well as increased baseline response followed by normal habituation (Kohl et al., 2014; Takahashi et al., 2016). It is likely that the conflicting findings arise from a combination of the heterogeneity of the patient populations and differences in stimulus presentation paradigms used (Sinclair et al., 2017). Despite contradictory findings, recent behavioural and electrophysiological studies provide support for the notion that habituation deficits arise early in the development of ASD and persist into adulthood.

The newest approach for investigating abnormal habituation in ASD comes from a series of fMRI studies assessing the functional neural correlates of face processing deficits. Hypotheses describing the neural circuitry underlying abnormal face processing in ASD have centered on hyperexcitability of the amygdala to social stimuli. However, studies examining amygdala activity in response to social stimuli in ASD patients have found both increased activity (Dalton et al., 2005; Nacewicz et al., 2006) and decreased activity (Ashwin et al., 2007; Critchley et al., 2000). In a seminal paper, Kleinhans et al. (2009) hypothesized that the amygdala response to social stimuli in ASD patients could be best characterized not by simple over- or under-activation, but instead by decreased neural habituation. In other words, while the amygdala response to faces habituates in healthy controls (Fischer et al., 2003), they expected this response to exhibit little plasticity in ASD individuals. To test this, Kleinhans et al. (2009) employed a paradigm where adult ASD patients and controls were shown two sets of neutral faces in two sets of fMRI scanning runs. In the first scanning run both groups showed similar levels of amygdala activation while in the second the authors observed significantly greater activation in the amygdala of ASD patients. Importantly, the authors show that the amygdala activation in the ASD group never exceeded the activation in the control group. Instead, while amygdala activity in the control group decreased with repeated stimulation, activity in the ASD group did not. Intriguingly, post-hoc analysis demonstrated that the habituation impairment correlated with the level of social dysfunction in the ASD individuals (Kleinhans et al., 2009). This study illustrates a recurring issue between brain activity measures (i.e. ERPs, BOLD, EMG) and habituation. Many brain activity measures rely on averaging multiple scans to get a detailed picture of activity in different brain areas. However, processing the data this way lends itself to simple interpretations of hyper- or hypo-activity and may mask underlying abnormalities in plasticity such as habituation.

This finding of reduced amygdala habituation to faces in ASD has provoked several follow-up studies investigating the extent and nature of this deficit. Swartz et al. (2013) found deficits in amygdala habituation in youth with ASD using shorter presentation times as well as a correlation between reduced habituation and social dysfunction. Further, Swartz et al. (2013) found that decreased amygdala habituation in the ASD group correlated with decreased activation in the ventromedial Prefrontal Cortex (vmPFC) compared to controls, suggesting a possible role for reduced vmPFC activity in the habituation deficits observed. Indeed, the vmPFC has been implicated in top-down inhibition of the amygdala during habituation (Hare et al., 2008). Another study from the same group investigated the relationship between face habituation in ASD patients and serotonin transporter genotype (Wiggins et al., 2014). They found that individuals with ASD and low-expressing serotonin transporter genotypes displayed greater deficits in amygdala habituation to sad faces than ASD individuals with high-expressing serotonin transporter genotypes. While these findings offer some mechanistic insight into the cause of decreased habituation in ASD, they are more broadly impactful as they illustrate that the endophenotype of habituation deficits in ASD may be more amenable to genetic analysis than the heterogeneous ASD diagnosis itself. Green et al. (2015) also showed decreased amygdala habituation in ASD patients that coincided with decreased functional connectivity between the vmPFC and the amygdala. Intriguingly, the severity of decreased habituation correlated with increased sensory overresponsivity (Green et al., 2015). Finally, in a recent study Kleinhans et al. (2016) replicated their initial finding of reduced amygdala habituation in response to emotional faces and showed that this deficit was specific to face processing as ASD individuals showed normal habituation to images of houses.

It is interesting to note that recent studies strongly support the notion of decreased habituation in ASD, while older reports produced conflicting results (Rogers and Ozonoff, 2005). One possibility is that

the diagnostic criteria for ASD have shifted the phenotypes of the patient population. Indeed, sensory overresponsivity has only recently been added to the DSM-V criteria for ASD (American Psychiatric Association, 2013; Lai et al., 2013). Since patients with ASD do not all necessarily display decreased habituation, further analysis into the cause of individual differences for this deficit is needed. Habituation measurements could become an additional tool of differential diagnostics for subtypes within the ASD group. Given the evidence for decreased habituation as a tractable endophenotype of this disorder (Guiraud et al., 2011; Webb et al., 2010; Wiggins et al., 2014) further molecular and systems level examinations into how this simple form of learning and sensory filtering is disrupted in ASD are warranted.

In summary, habituation impairments have been observed in ASD throughout development and in adulthood. Several recent fMRI studies have demonstrated impaired amygdala habituation to emotive faces in ASD and made positive correlations between with habituation impairment and social dysfunction. This is intriguing as it suggests that the habituation impairment is not limited to simple stimuli (e.g., auditory tones) but extends to complex social stimuli (i.e., faces) and may have social repercussions. Further, these studies have begun to elucidate the neural circuit and genetic abnormalities that lead to impaired amygdala habituation in ASD. Given the prevalence of drugs used to treat certain aspects of ASD (e.g., antipsychotics used to treat irritability), it will be interesting to see if these drugs also affect habituation.

6. Habituation deficits in Fragile X Syndrome

Fragile X syndrome (FXS) is the most common monogenic cause of intellectual disability (Hagerman et al., 2009; O'Donnell and Warren, 2002; Sinclair et al., 2017). FXS is caused by expansion and hypermethylation of CGC repeats in the promoter region of the Fragile X mental retardation 1 (*FMR1*) gene which results in reduced or abolished FMRP expression. Decreased *FMR1* expression has been shown to alter protein synthesis dependent synaptic plasticity and dendritic spine growth, processes critical to early neurodevelopment (Greenough et al., 2001; Irwin et al., 2001).

FXS is also the leading monogenic cause of Autism Spectrum Disorder (ASD) with approximately 15–33% of individuals with FXS meeting the diagnostic criteria for ASD and 5% of ASD cases being attributed to FXS (Bailey et al., 1998; Cohen et al., 2005). Additionally, individuals with FXS alone (i.e., not comorbid with ASD) often show characteristics reminiscent of ASD, including deficits in social behaviour, delays in language development, sensory over-responsivity, and decreased habituation (Barnes et al., 2009; Berry-Kravis et al., 2007; Hagerman et al., 1986, 1991; Roberts et al., 2007; Rotschafer and Razak, 2014).

In a preliminary study, the electrodermal responses to repeatedly presented olfactory, auditory, visual, tactile, and vestibular stimuli were compared between individuals with FXS and normal controls. Consistent with the habituation phenotype of ASD, individuals with FXS displayed both enhanced electrodermal responses at baseline and decreased habituation compared to controls. Intriguingly, this habituation phenotype was observed across stimulus modalities and was shown to correlate with the level of FMRP expression (Miller et al., 1999).

Studies using electroencephalogram recordings (EEG) have also found reduced habituation in FXS. Castrén et al. (2003) reported increased magnitude and decreased habituation of N100 event-related potentials in response to trains of auditory tones in individuals with FXS. This finding was subsequently supported by three additional EEG studies that also found reduced N100 habituation in response to repeatedly presented auditory tones (Ethridge et al., 2016; Schneider et al., 2013; Van der Molen et al., 2012). Reduced N1 habituation was also associated with parent reports of heightened sensory sensitivities and social communication deficits (Ethridge et al., 2016). The present findings should be interpreted cautiously, however, as other groups have failed to dishabituate N100 response decrements (Barry et al., 1992). Interestingly, treatment with the broad spectrum antibiotic minocycline has recently been shown to increase global functioning, improve performance on tests of anxiety and mood-related behaviours, as well as ameliorate EEG-recorded sensory abnormalities and reduced habituation in individuals with FXS (Leigh et al., 2013; Schneider et al., 2013). Using a mouse model of FXS, Bilousova et al. (2009) demonstrated that minocycline promotes dendritic spine maturation and improves behavioural deficits by inhibiting the enzyme MMP-9 (matrix metalloproteinase 9). This has led to the suggestion that a similar mechanism may underlie the clinical improvements that occur following minocycline treatment of FXS (Schneider et al., 2013). Indeed, a recent study reported that *FMR1* knock-out mice displayed decreased auditory N1 habituation that was rescued by additional knock-out of *MMP9* (Lovelace et al., 2016).

Another FXS deficit that has been studied in the context of habituation is the propensity to avoid eye contact (Cohen et al., 1989; Farzin et al., 2009). A recent study by Bruno et al. (2014) compared individuals with FXS to individuals with idiopathic developmental delay, intellectual disability, or learning disability but no known genetic or neurological disorder, on an fMRI habituation task. Participants were repeatedly presented 4 different neutral faces in two orientations such that half of the images were looking at the participant while the other half were looking away. In individuals with FXS the BOLD response in the cingulate gyrus, fusiform gyrus, and frontal cortex exhibited significantly reduced habituation (and significant sensitization) to all faces as compared to controls. So, despite the observation that individuals with FXS avoid eye contact, they showed reduced habituation to faces regardless of whether the images are looking at the participant or not (Bruno et al., 2014). It is also interesting to note that, similar to Miller et al. (1999), Bruno et al. also found a correlation between increased FMRP expression and decreased habituation.

Taken together, studies of habituation and FXS point to habituation deficits that are present across sensory modalities, and like individuals with ASD, occur along with basic sensory abnormalities as well as more complex deficits in social processing of face stimuli. To date, there have been no studies assessing habituation abnormalities throughout development in FXS. Recent studies have shown that minocycline treatment induced marked improvements in symptom severity that correlate with improved habituation in individuals with FXS. These studies are promising as they suggest the neurodevelopmental abnormalities that result in reduced plasticity in FXS are reversible with an acute pharmacological intervention. Moving forward, it will be interesting to determine whether other monogenic disorders associated with altered neurodevelopment and autistic symptoms (e.g. Rett syndrome, Cowden syndrome, etc.) display impaired habituation as well.

7. Habituation deficits in Schizophrenia

Impaired attention and information processing are among the core cognitive deficits of schizophrenia. Indeed, a reduced ability to filter out irrelevant stimuli has been implicated in schizophrenia for over a century (Bleuler, 1950) and remains a prominent research interest today. Much of the modern interest in this field has been fueled by the influential clinical reports of McGhie and Chapman (1961), who proposed that people must be able to filter out information in order to maintain "perceptual constancy" in a world of otherwise chaotic overstimulation (Geyer and Braff, 1987). They further suggested that impaired inhibitory processing in schizophrenia leads to "sensory overload" and subsequent "cognitive fragmentation." Research into the cause of impaired inhibitory processing in schizophrenia has focused on cataloguing and understanding impairments in two related yet distinct phenomena: sensorimotor gating and habituation. Sensorimotor gating is the ability of a neural system to selectively filter intero- and exteroceptive stimuli that drive a motor reflex. It is often operationally defined as pre-pulse inhibition (PPI), i.e., the ability of a weak prestimulus (pre-pulse) to transiently inhibit the response to a closely

following strong stimulus (test pulse) (Braff et al., 1992). Habituation, in contrast, is a decrement in response following repeated presentations of the same stimulus. While sensorimotor gating and habituation are both forms of inhibition, the former is often considered to be pre-attentive and un-learned whereas the latter is an established form of learning with specific behavioural characteristics (Braff et al., 2001, 1992; Swerdlow et al., 2008; although see Quednow et al., 2006a who suggest that PPI may be subject to learning; Rankin et al., 2009 and Table 1).

Since the pioneering work of Geyer and Braff (1982), most studies of habituation and schizophrenia have used the acoustic startle response as a paradigm to understand habituation. Using this paradigm, Geyer and Braff (1982) demonstrated that schizophrenic patients habituate more slowly to acoustic startle as compared to normal controls. This study represents an unfortunately rare example of the use of consistently spaced identical stimuli to directly compare habituation between patients, patient controls, and healthy controls without any intervening irregular stimuli or changes in stimulus intensity.

Despite the clarity of this initial finding, results of subsequent studies have been inconsistent. Several groups replicated Geyer and Braff's original finding of significant habituation deficits in schizophrenia patients (Akdag et al., 2003; Bolino et al., 1994, 1992; Greenwood et al., 2011; Hokyo et al., 2010; Ludewig et al., 2003; Moriwaki et al., 2009; Parwani et al., 2000; Takahashi et al., 2008); Several more found a non-significant trend towards decreased habituation (Braff et al., 1992; Ludewig et al., 2002a, 2002b; Perry et al., 2002, 2001), while others did not detect habituation deficits in schizophrenia (Braff et al., 1999; Cadenhead et al., 2000; Csomor et al., 2009; Hasenkamp et al., 2011; Kumari et al., 2002, 2000; Quednow et al., 2008, 2006b; Wang et al., 2013; Xue et al., 2012).

Two key factors have been suggested to account for this discrepancy (Meincke et al., 2004). First, the majority of these studies were optimized to assess PPI rather than habituation. In the most common design the average response to a block of identical pulses at the beginning of the experiment is compared to that elicited by a second block of pulses at the end of the experiment in order to assess habituation (Fig. 1). In between these two habituation blocks, PPI is assessed by delivering weak pre-pulses followed by test pulses that are identical to the pulses delivered in the habituation blocks. During this phase the interstimulus interval is varied and test pulses can occur with or without pre-pulses, and the intensity of prepulse can vary. In this protocol habituation is usually assessed by comparing the average response in Block 1 (Baseline Response) with the average response in Block 3 (Habituated Response). While this protocol has become standard practice for assessing habituation it is problematic because altering the interstimulus interval and stimulus intensity within the same period (as in the middle pre-pulse block) are known to affect habituation (e.g., see Broster and Rankin, 1994; Meincke et al., 2004; Rankin et al., 2009). A further concern is that condensing habituation trials into blocks fails to take into account any potential initial sensitization to the startle-eliciting stimuli. The possibility of an initial period of sensitization prior to habituation is well-documented (Groves and Thompson, 1970; Rankin et al., 2009). Indeed, Meincke et al. (2004) found habituation deficits in schizophrenic patients but only when the effects of sensitization were removed from analysis. Therefore, intermixed pre-pulse and habituation stimuli, masking due to sensitization, and bias due to focus on PPI are issues that may underlie the inconsistencies in the literature. Finally, using blocks of responses to assess habituation can mask a difference if habituation is very rapid (often the biggest response decrement is over the first 2–3 responses) and if the early responses are averaged with decremented responses the actual decrement is minimized or lost (i.e., Fig. 1, Block 1).

Unfortunately, the majority of studies of habituation phenomena in schizophrenia over the last decade have been carried out without regard for these issues in their experimental design. There have been more than 30 studies focused on PPI of the acoustic startle reflex in schizophrenia published in the last ten years that continue to use pulse-alone trials mixed with pre-pulse trials to assess habituation. It is perhaps not surprising then that the results of these studies remain inconsistent, with several finding impaired habituation, and others failing to find significant differences between patients and controls. To properly assess habituation it is important to design studies in a way that optimizes the proper assessment of habituation. One potential alteration to the standard paradigm would be to increase the number of pulse-alone stimuli delivered within the first block and assess response plasticity within that block before pre-pulse pairings are delivered (Lane et al., 2013). This would allow for a more direct analysis of short-term habituation and mitigate the confounding effects of pre-pulse stimuli.

Despite these issues, some of the recent PPI-intermixed startle habituation studies have found deficits in habituation and have made intriguing correlations between habituation abnormalities and a variety of genetic variants implicated in schizophrenia. Of note, a relatively large study (81 schizophrenic patients and 71 controls) investigated the relationship between decreased habituation and seven "top-ranked" gene variants implicated in the etiology of schizophrenia (Hokyo et al., 2010; Allen et al., 2008). There was a significant interaction, with patients homozygous for the schizophrenia-associated rs1019385 (T200G) variant in the *GRIN2B* gene showing significantly decreased habituation compared to controls. There were no interactions between the other schizophrenia-associated gene variants and habituation (Hokyo et al., 2010). Another large study by Greenwood et al. (2011) assessed 94 candidate genes associated with schizophrenia and evaluated whether each gene associated with both the qualitative diagnosis of schizophrenia as well as impaired habituation. Using a sample of 203 patients with schizophrenia and 119 controls, impaired habituation was found to be associated with mutations in the genes *GRIN2B, NRG1, HTR2A, COMT, NOS1AP, SLC6A1, GRID2, GRM2,* and *NEUROG1*. Intriguingly, many of the genes analyzed in this study have been shown to interact at the molecular level and several displayed pleiotropy, with several single gene mutations showing associations for multiple schizophrenia phenotypes (Greenwood et al., 2011). Overall, these studies support the large body of research implicating dopaminergic and glutamatergic signaling abnormalities in the cognitive deficits of schizophrenia as well as a role for these neurotransmitter systems in impaired habituation in schizophrenia.

Although the acoustic startle paradigm has been the most commonly used paradigm to study habituation phenomena in schizophrenia, several groups have also investigated habituation using other behavioural and physiological responses. Perry et al. (2009) documented decreased habituation of locomotor activity in an open-field paradigm using an automated human behavioural pattern monitor. An early series of studies examined differences in the electrodermal skin conductance orienting response between schizophrenic patients and controls. The most reliable finding from these studies is that a large portion of schizophrenic patients do not respond to the series of repeatedly presented stimuli during a habituation task (Bernstein et al., 1982; Dawson et al., 2007). More central to this review is the relatively understudied finding that the schizophrenic patients who did respond to the repeated tones displayed abnormal skin conductance habituation (Dawson et al., 2007; Gruzelier and Venables, 1972; Schiffer et al., 1996). However, some electrodermal studies found reduced rates of habituation while others found more rapid rates of habituation. This discrepancy may have been caused by a lack of controlling for differences in baseline responsiveness and differences in scoring methods between groups (Bernstein et al., 1982; Dawson et al., 2007; Depue and Fowles, 1973; Spohn and Patterson, 1979). Still, it is worth noting that the degree to which habituation was reduced in these patients was correlated with poor performance on several neuropsychological tests, including the Wisconsin Card Sorting Task and Trail Making tests (Bartfai et al., 1987; Schiffer et al., 1996).

Holt et al. (2005) were the first to use fMRI to examine whether the

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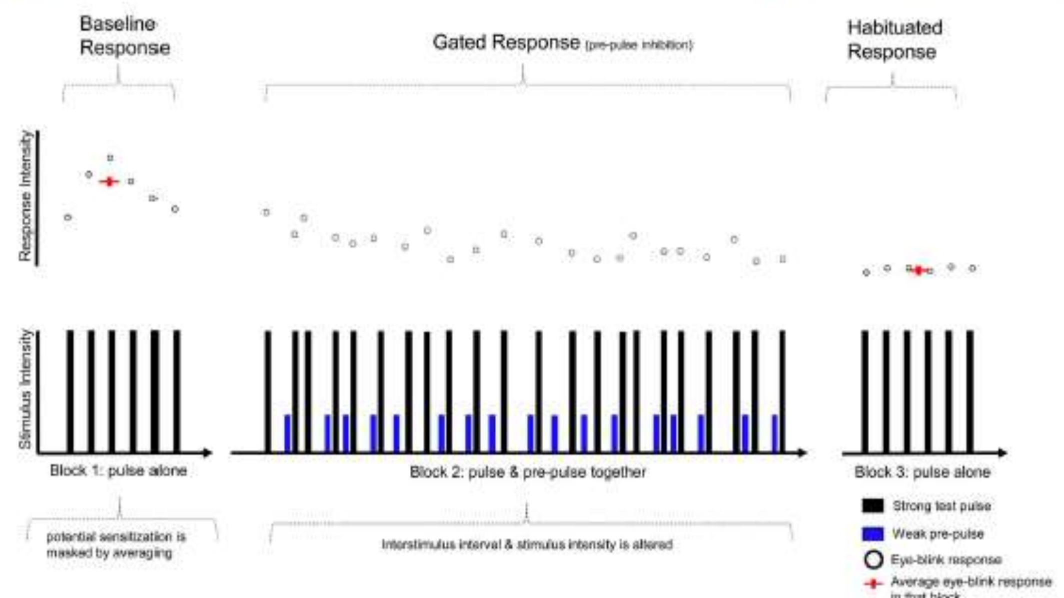

Fig. 1. Typical protocol for assessing habituation and sensorimotor gating within the same time period. The mean eye-blink response intensity to a block of identical test pulses (usually auditory) at the beginning of the experiment is compared to that elicited by a final block of identical test pulses at the end of the experiment in order to assess habituation. In between these blocks are the prepulse inhibition trials containing test pulses at various interstimulus intervals, some preceded by weaker pre-pulses, some not. Additionally, some protocols also vary the intensity of pre-pulses (not shown in figure). This design is problematic given that it a) may mask potential sensitization that occurs initially (see response to block 1) and b) incorporates a pre-pulse inhibition block which means that both the interstimulus interval and stimulus intensity are changed throughout the experiment. Such changes in the manner of stimulus presentation are known to significantly affect habituation profiles (Meincke et al., 2004; Rankin et al., 2009). Of note, both the interstimulus interval and stimulus intensity may vary drastically from study to study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreased habituation found in schizophrenia would also manifest at the level of activity in specific brain regions. In particular, they examined the hemodynamic response of the medial temporal lobe – including the amygdala, hippocampus, and parahippocampal gyrus to repeated presentations of fearful faces in male schizophrenic patients and controls. As hypothesized, patients with schizophrenia failed to display temporal lobe habituation in any area, in response to fearful faces whereas control subjects displayed neural habituation in the anterior right hippocampus (Holt et al., 2005). Based on the large body of research in schizophrenia demonstrating sensorimotor gating deficits (Braff et al., 1992; Light and Braff, 2005) and the evidence for perturbed processing of faces (Javitt, 2009), Williams et al. (2013) hypothesized that neural habituation in response to neutral faces may also be reduced in the primary visual cortex and the fusiform face area (FFA). Interestingly, patients with schizophrenia displayed reduced habituation of both the hippocampus and primary visual cortex but not the FFA. These habituation deficits were specific to faces, as they were not observed when participants viewed objects (Williams et al., 2013). In a direct follow-up study by the same group, Blackford et al. (2015) showed that the same sample of schizophrenic patients also displayed selectively impaired habituation to opposite-gender faces in the amygdala, visual cortex, and hippocampus, providing evidence for abnormal processing of more complex social information such as gender-based out-group bias. Importantly, all of these studies correlated decreased neural habituation with diminished performance on behavioural tests of either visual memory, verbal memory, or social impairments (Blackford et al., 2015; Holt et al., 2005; Williams et al., 2013). Taken together, these findings suggest that habituation in different brain areas may be mediated by different mechanisms, some but not all of which are disrupted in schizophrenia. It is also interesting to note that, similar to ASD, the most consistent recent evidence for decreased habituation in schizophrenia comes from fMRI studies examining the functional neural correlates of face processing. Indeed, abnormal neural habituation to social stimuli may represent a transdiagnostic biomarker for social impairment (Blackford et al., 2015).

In summary, there have been more studies focused on habituation abnormalities in schizophrenia than in any other neurological disease. Despite this extensive research program, the acoustic startle habituation studies that constitute the majority of the work in this field remain inconsistent. The current standard of assessing acoustic startle habituation with intermixed pre-pulse stimuli has confounding variables that precludes unambiguous interpretation of the results. More studies focused directly on habituation to repeated stimuli are needed. Further, comparing habituation responses across other sensory modalities will allow for a more holistic picture of how this elementary form of learning is disrupted in schizophrenia. Recent fMRI studies measuring neural habituation to complex face stimuli provide more evidence for decreased habituation in schizophrenia. A more complete depiction of how habituation is disrupted in schizophrenia will greatly facilitate the utility of this learning deficit as a phenotype for basic research into the underlying cause of the disorder.

8. Enhanced habituation may underlie perturbed attention in Attention Deficit Hyperactivity Disorder

Attention-deficit hyperactivity disorder (ADHD) is characterized by deficits in executive function not limited to attention, memory, and response inhibition. Few ADHD studies exist which are optimally designed to assess habituation phenomena. Of the studies which do report habituation phenomena in ADHD, several find enhanced habituation (Iaboni et al., 1997; Shibagaki et al., 1993; Zahn and Kruesi, 1993) others deficient habituation (Jansiewicz et al., 2004; Massa and O'Desky, 2012), whereas several other studies report no habituation abnormalities (Conzelmann et al., 2010; Feifel et al., 2009; Holstein

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Insights from the worm: The *C. elegans* model for innate immunity

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Abstract

The nematode worm *Caenorhabditis elegans* comprises an ancestral immune system. *C. elegans* recognizes and responds to viral, bacterial, and fungal infections. Components of the RNA interference machinery respond to viral infection, while highly conserved MAPK signaling pathways activate the innate immune response to bacterial infection. *C. elegans* has been particularly important for exploring the role of innate immunity in organismal stress resistance and the regulation of longevity. Also functions of neuronal sensing of infectious bacteria have recently been uncovered. Studies on nematode immunity can be instructive in exploring innate immune signaling in the absence of specialized immune cells and adaptive immunity.

1. Introduction: The *C. elegans* model for studying immunity

C. elegans is a small nematode that can be found in the soil, rotting fruit, compost heaps, and snails [1]. The nematode worm has been employed since nearly half a century in the laboratory where it can be maintained in a cost effective manner on agar plates typically containing *E. coli* OP50 strain as a food source [2]. The worm's development from embryo to adulthood takes 3 days at a standard temperature of 20°C; within following 3 days of adulthood a single hermaphrodite worm produces approximately 300 genetically identical progeny facilitating the establishment of large animal populations. Males can easily be obtained in order to perform genetic crossings. The average life span of *C. elegans* at 20°C is approximately 2-3 weeks. The genome of the nematode is sequenced and loss-of-function mutants for the majority of genes are available from public sources. Also gene knock-downs can easily be established by feeding worms on bacteria that contain vectors expressing respective gene-specific dsRNAs. All these factors have to date made *C. elegans* a model of choice for many genetic studies that produced major breakthroughs such as understanding of cell fate determination during development, the process of apoptosis, mechanisms of RNA interference, the genetic regulation of life span, to only name a few. With the discovery of inducible anti-microbial and anti-viral innate immune responses in the nematode over the past fifteen years, *C. elegans* has emerged as intriguing model also in the field of immunity.

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Several microbes that are known human pathogens also infect the intestine of the nematode. Therefore, their virulence and other aspects of host-pathogen interaction can be studied in the simple metazoan. From an evolutionary perspective, the immune system of *C. elegans* precedes the highly complex immunity of vertebrates as it contains only some of the most ancestral signaling networks. The reduced complexity can be experimentally advantageous in terms of detailed characterization of the immune signaling cascades. Also systemic interactions between innate immunity and other signaling systems such as stress responses and mechanisms involved in the regulation of longevity have been established by using *C. elegans*. Recently, a novel function of innate immunity in integrating cell autonomous hazards such as germline DNA damage with systemic stress resistance has been uncovered. Another topic of considerable attention and controversy -the regulation of innate immunity by the nervous system- has also gained some experimental proof from studies in *C. elegans*. All together, in addition to offering a highly suitable model for classical studies of immunity, the relatively low complexity of the nematode system also provides extended possibilities for dissecting non-conventional aspects of innate immune responses.

2. Anti-viral immune defense in *C. elegans*

The anti-viral response of the nematode relies on the intracellular RNA interference (RNAi) machinery (Figure 1). Anti-viral surveillance through the RNAi machinery is an ancestral defense mechanism that was first described and characterized in plants [3]. The attempts to dissect the anti-viral immune functions of *C. elegans* were for a long time complicated by the absence of a natural virus that would infect the nematode. However, studies based on infecting isolated worm cells with the mammalian Vesicular stomatitis virus (VSV) [4] and on transgenic expression of the Flock house virus (FHV) replicon in the worm [5], immediately suggested the key role of the RNAi machinery in the process of antiviral defense with factors like the argonaute protein RDE-1 and the dsRNA binding protein RDE-4 being essential for antiviral silencing.

Only recently natural sampling experiments led to the discovery of the first natural viral pathogen of *C.elegans*: the RNA+ Orsay virus that belongs to the *Nodaviridae* family [6] and causes easily detectable morphological abnormalities in the intestine of the worm. It was demonstrated that infection load upon treatment with the Orsay virus was elevated in mutants lacking such important RNAi factors as RDE-1, RDE-4, the RNaseD MUT-7 and the dicer-related helicase DRH-1, which also carries a RIG-I-like domain [6-8]. Also the presence of small viral RNAs (both primary sense and secondary effector anti-sense) was detected upon infection. Interestingly, the susceptibility to the viral infection varied between different wild-type *C. elegans* isolates suggesting natural variation in somatic RNAi efficiency [6].

The opportunity of using *bona fide* viral infection in combination with previously reported functions of specific RNAi factors significantly extended our mechanistic view of anti-viral surveillance in *C. elegans*. It is currently believed that upon infection with an RNA virus, the double stranded RNA intermediates produced during replication of the viral genome comprise the initial step of the RNAi response. The intermediates are first sequestered by the dsRNA binding complex RDE-1/RDE-4 whose proposed function is detection and retaining

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of exogenous dsRNAs [9]. The dsRNA is then passed to the DExD box RNA helicase DRH-1 that interacts with RDE-1/4 [9], unwinds the RNA molecule and facilitates its acquisition by the worm dicing complex [7]. The primary small RNAs produced through processing of the double stranded replication intermediate by the *C. elegans* Dicer homolog DCR-1 serve as template for RNA-directed RNA polymerases (RdRP) resulting in accumulation of secondary anti-sense viral small RNAs that mediate degradation of the target full length viral RNA in a manner dependent on the Rnase MUT-7 [10] and Argonaut proteins.

Similar mechanisms are also utilized for the silencing of transposons and repetitive elements, such as multiple copy transgenes, in the germline of *C. elegans*. Thus far, no apparent natural integration of exogenous elements was found in the *C. elegans* germline, as no vertical transfer of the virus was detected in case of Orsay infection [6]. The germline silencing pathway might be evolutionary derived form the CRISPR mechanisms - an ancestral prokaryotic "immune system" conferring RNAi-like surveillance against exogenous genetic elements (such as phage genomes) integrated into bacterial DNA [11]. In case of germline silencing pathway the initial step that triggers an RNAi response is random "read through" transcription of the DNA region containing repetitive elements. Upon transcription double stranded RNA structures are formed via intramolecular cross-hybridization of repetitive sequences [12]. Such structures are putatively recognized by the DExD box RNA helicase MUT-14 that is proposed to unwind dsRNA and pass it to DCR-1 for processing similar to the somatic DRH-1 helicase [10]. It has been clearly demonstrated that both DCR-1 and MUT-14 are essential for transposon silencing in the germline with MUT-14 playing a role only in the germline and not during somatic RNAi. The final steps of the germline transposon repression pathway are proposed to be the same as the ones described for the somatic anti-viral RNAi.

Different mammalian viruses are known to have developed ways of avoiding their detection by the immune system. For instance, several viruses are capable of blocking MHC class I antigen processing and presentation, thereby escaping recognition by T-killer cells [13]. The most prominent example how viral proteins escape the RNAi surveillance through inhibiting the RNAi machinery in *C. elegans* is the Flock house virus protein B2 that significantly downregulates exogenous RNAi and enhances susceptibility of worms to the Orsay virus infection without having any effect on the endogenous physiological micro-RNA pathway [14]. Another important connection between *C. elegans* and mammalian anti-viral mechanisms is the homology between DRH-1 and the RIG-I (Retinoic acid inducible gene I) RNA helicase – a key cytoplasmic sensor of foreign dsRNAs in mammals [7,8]. The strongest homology between the two proteins resides within DExD/H-box helicase domain responsible for RNA binding, while domains involved in protein-protein interactions are different consistent with the fact that DRH-1 targets dsRNAs for Dicer processing whereas RIG-I activates a classical systemic anti-viral immune response mediated by interferones and pro-inflammatory cytokines [15]. It seems probable that the two molecules as well as the two responses to the presence of viral RNA are evolutionary connected with currently existing differences having evolved according to particular needs of respective organismal systems: while cell-autonomous anti-viral surveillance is sufficient in *C. elegans* a more

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complex mammalian physiology raises a requirement for a systemic alert and response. It is also worth mentioning that DRH-1 is dispensable for RNAi that targets cellular transcripts [7] making this protein a specific factor in foreign sequence RNAi surveillance.

3. Anti-microbial responses in *C. elegans*

C. elegans is naturally exposed to numerous microbes, some of which can be benign food sources others dangerous pathogens for the worm. The close interaction throughout millions of years of nematode and bacterial evolution led microbes to evolve ways of keeping nematodes at large and worms to shape means of avoiding bacterial infections and toxicity. Most bacterial species that are known to be pathogenic for *C. elegans* cause deleterious phenotypes by infecting the intestine of the worm. Interestingly, bacterial gut colonization as such is not necessarily detrimental as demonstrated by feeding worms with *Enterococcus faecium* which propagates in the intestine to large titers without causing significant death of the animals [16]. Microbes only become pathogenic to the worms when they produce active virulence factors. The bacteria causing intestinal infections in *C. elegans* belong to both Gram-positive (*Enterococcus faecalis, Staphylococcus aureus* [16,17]) and Gram-negative (*Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens*, [18-20]) classes. Many of the *C. elegans* intestinal pathogens are also pathogenic to humans under all or restricted conditions with a number of virulence factors targeting nematodes and mammals, making *C. elegans* a powerful model system for virulence factor screens [18,21,22].

The most studied *C. elegans* intestinal pathogens are *P. aeruginosa* and *S. typhimurium*. *P. aeruginosa* is a common soil and water bacterium and an opportunistic human pathogen that causes disease in immune compromised individuals or upon extensive wounding and trauma. Under laboratory conditions the clinical isolate of *P. aeruginosa* strain PA14 has been demonstrated to kill *C. elegans* and inflict infection in mice [18,21]. Two types of nematode killing were recognized for *P. aeruginosa*: the so called "fast killing" occurring within several hours and mediated by bacteria grown on high-osmolarity medium and the "slow-killing" by bacteria cultured on low-nutrient media which takes several days [18,21]. The mechanisms of the two killing processes are distinct: the fast killing is mediated by toxins such as phenazines and does not require living bacteria while slow killing is a *bona fide* infectious process that involves living microbes. A screen based on using random transposon insertions in PA14 strain led to identification of several virulence factors required for maximum killing of the worms during slow infection [18]. Three of the identified genes - *lasR, gacA* and *lemA*- have already been identified as virulence factors in other model systems. *GacA* and *lemA* (also known as *GacS*) are components of *GacA/GacS* regulatory system that is responsible for bacterial biofilm formation while *lasR* encodes an essential transcriptional activator. *S. typhimurium*, a serotype of *Salmonella enterica*, is lethal to mice by causing typhoid-like disease; *C. elegans* worms fed with *S. typhimurium* from adulthood also exhibit reduced life span [19]. Interestingly, infection with *Salmonella* is accompanied by an increase of programmed cell death in the germline of *C. elegans* [23] that involves the core apoptotic factors the Apafl homolog *ced-4* and the caspase *ced-3*. Mutant worms lacking apoptotic induction are more susceptible to *S. typhimurium* indicative of a protective role of germline apoptosis during pathogen defense. Bacterial mutants of the *phoP/phoQ* virulence-signaling pathway and genes involved in lipopolysaccharide synthesis

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result in reduced killing and lack of programmed cell death during *S. typhimurium* infection suggesting a link between pathogenicity, germ cell apoptosis, and antimicrobial defense.

Not only the intestine but also the worm's cuticle can be target of infections. The most well studied cuticle distortion is the one caused by *Microbacterium nematophilum*. *M. nematophilum* is a Gram-positive organism that is able to colonize the *C. elegans* rectum, which is lined with cuticle, as well as the small peri-anal region of the exterior cuticle. During infection the anal region becomes swollen producing a phenotype known as Dar (deformed anal region) [24]. Despite being non-lethal, *Microbacterium* infection induces an immune response in the nematode [25]. *Yersinia pseudotuberculosis* a close relative of a bubonic plague pathogen *Y. pestis*, produces polysaccharide rich biofilms that attach predominantly to the cuticle of the head region of *C. elegans* preventing the worm from feeding and inhibiting growth [26]. This phenomenon is similar to how *Y. pestis* distorts the feeding of the flea promoting its transmission to mammals. Feeding blockage in both *C. elegans* and flea require genes involved in polysaccharide biosynthesis [27].

The mechanisms involved in recognition of microbes by *C. elegans* are incompletely understood. In mammals and *Drosophila,* Toll like receptors and Imd/TNF signaling are major driving mechanisms of anti-microbial response. The nematode's genome encodes a single Toll like receptor homolog TOL-1 that is, however, not implicated in anti-microbial defense unlike its *Drosophila* and mammalian counterparts [28]; neither are the nematode orthologs of TNF receptor associated factor-1 (TRF-1), Pelle and IL-1R-associated kinases (PIK-1) and inhibitor of NF-κB (IKB-1). Other key components of known anti-microbial recognition cascades such as TLR adaptor MYD88 and NF-κB family of transcription factors have to date not been found in the *C. elegans* genome. In addition to TOL-1, another gene product containing Toll/IL-1R (TIR) protein-protein interaction domain - a scaffold protein TIR-1, was identified in the nematode. Genetic analysis uncovered that both knock-down and mutations of *tir-1* result in severe hypersensitivity to a variety of established *C. elegans* pathogens implicating TIR-1 in anti-microbial defense [29-31]. However, the receptor(s) that activates TIR-1 in response to microbial presence still remains to be identified.

In parallel to genomics-based approach, genetic screens for mutants showing enhanced killing by *P. aeruginosa* identified a p38 MAP kinase-related signaling pathway as an essential player in anti-microbial defense (Figure 2). The cascade consists of neuronal symmetry family member 1 (*nsy-1*), SAPK/ERK kinase 1 (*sek-1*) and p38 MAPK family member 1 (*pmk-1*) [32,33]. NSY-1, SEK-1, and PMK-1 seem to function in a pathogen-activated linear phosphorylation cascade, where PMK-1 is phosphorylated by SEK-1 and SEK-1 by NSY-1. This pathway is orthologous to mammalian apoptosis signal-regulating kinase 1 (ASK1) - MAPK kinase 3/6 (MKK3/6) - p38 MAPK cascade, which is also involved in triggering innate immune responses. Knock-down of *tir-1* blocks activation of PMK-1 upon infection suggesting TIR-1 as candidate for the most upstream member of the cascade [29]. The phosphorylation state of PMK-1 is also regulated by heavy metal stress activated MAPK kinase MEK-1 and MAP kinase phosphatase VHP-1 [33].

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Using *C. elegans* for aging research

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Over a century ago, the zoologist Emile Maupas first identified the nematode, *Rhabditis elegans*, in the soil in Algiers. Subsequent work and phylogenic studies renamed the species *Caenorhabditis elegans* or more commonly referred to as *C. elegans*; (*Caeno* meaning recent; *rhabditis* meaning rod; *elegans* meaning nice). However, it was not until 1963, when Sydney Brenner, already successful from his work on DNA, RNA, and the genetic code, suggested the future of biological research lay in model organisms. Brenner believed that biological research required a model system that could grow in vast quantities in the lab, were cheap to maintain and had a simple body plan, and he chose the nematode *C. elegans* to fulfill such a role. Since that time, *C. elegans* has emerged as one of the premiere model systems for aging research. This paper reviews some initial identification of mutants with altered lifespan with a focus on genetics and then discusses advantages and disadvantages for using *C. elegans* as a model system to understand human aging. This review focuses on molecular genetics aspects of this model organism.

Keywords: *C. elegans*; longevity; aging; insulin/IGF-1; dauer

Introduction

In 1974, a little more than a decade after his first thoughts about working on a model organism, Brenner published four manuscripts, including one entitled 'The genetics of *Caenorhabditis elegans*'(Brenner 1974) and a new field began. In this influential paper (Brenner 1974), Brenner outlined methodology for isolation, complementation, and mapping of worm mutants. Importantly, the publication also included the successful isolation of several hundred mutants affecting behavior and morphology, a discussion of the number of defined genes, and an estimation of mutation frequency. Since that time, many discoveries including dissection of programmed cell death (Coulson et al. 1986; Ellis et al. 1991), the systematic cloning of the genome (Coulson et al. 1986; Crawford 2001), the deciphering of the entire DNA sequence (Consortium 1998), microRNAs (Lee et al. 1993; Reinhart et al. 2000), RNA interference (Fire et al. 1998), and the use of GFP (Chalfie et al. 1994) have been done in *C. elegans* which has led to an expansion in the number of researchers working with *C. elegans*.

C. elegans for aging research

For research on aging, early studies in *C. elegans* focused on the feasibility of measuring lifespan and the use of 5-Fluoro-2'-deoxyuridine (FUDR) to maintain synchronous cultures of aged animals (Hosono 1978a, 1978b). In 1977, Klass (1977) published that *C. elegans* was a good system for aging studies as he established a method to consistently measure lifespan, and he concluded that this could lead to future detailed analysis combining genetics and biochemistry. In these early studies, Klass found that altering either temperature or the amount of food resulted in a change in lifespan. In addition, only small effects on lifespan were observed based on parental age or parental lifespan. Klass performed a clonal genetic screen for mutants with altered lifespan and identified five mutants (Klass 1983). Interestingly, later genetic work on these mutants in the laboratory of Tom Johnson, mapped all of them to a single genetic locus, named *age-1* (Friedman & Johnson 1988). This was the first breakthrough in aging research for studies based on *C. elegans* as this study revealed that it was possible to identify mutants that altered lifespan and more importantly, individual genes could modulate lifespan.

From the initial characterization of mutants that altered lifespan, the words lifespan and aging have often been used interchangeably. However, lifespan is a single measureable parameter that defines the amount of time an organism is alive but does not give any indication for how an animal is actually aging. Lifespan as a measurement gives little detail about the health of the animal. For this reason, healthspan, defined as the time that an individual is active, productive and free from age-associated disease, is starting to become the focus of aging research (reviewed in (Tissenbaum 2012)).

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Equally important for aging research is the use of the term regulation. Is aging regulated? Is lifespan regulated? A regulated process should indicate that this is a trait that would be selected for over time. However, fitness competitions between wild type and *daf-2* mutants, show that after four generations, none of the *daf-2* mutants remained primarily because of the early fertility defects in the *daf-2* mutants (Jenkins et al. 2004). Therefore, similar to other studies with long-lived mutants and consistent with the antagonistic pleiotropy theory of aging (Lakowski & Hekimi 1996; Gems et al. 1998; Chen, Pan et al. 2007; Chen, Senturk et al. 2007; Curran & Ruvkun 2007; Anderson et al. 2011), *daf-2* mutants exhibit a heavy fitness cost with lifespan extension (Jenkins et al. 2004). Taken together, lifespan and aging should not be used interchangeably and the use of the word regulation should be monitored (reviewed in (Lithgow 2006; Tissenbaum 2012)).

Several years after the *age-1* gene was identified, another gene was shown to modulate lifespan. Similar to mutation in *age-1*, *daf-2* mutants showed adult lifespan extension (Kenyon et al. 1993). Interestingly, previously, both *daf-2* and *age-1* had showed similarity based on a different phenotype. Under favorable growth conditions, *C. elegans* develop from an egg through four larval stages (L1-L4) each separated by a molt, and then a final molt into a reproductive self-fertilizing adult hermaphrodite. In response to unfavorable growth conditions, in particular, high levels of a secreted pheromone (i.e. crowding, low food), worms can enter an alternative developmental mode (at the L3 stage) forming dauer larvae (Riddle & Albert 1997). Dauer (German for enduring) larvae (alternate L3) maximize survival until conditions become more favorable, whereupon they will molt and form a reproductive adult. Genetic screens identified mutants affecting the ability to enter this dauer program. These mutants were named *daf* mutants indicating the dauer formation phenotype. Both *daf-2* and *age-1* were initially isolated in this type of screen because both *daf-2* and *age-1* (originally identified as *daf-23*) mutants show a dauer constitutive (*daf-c*) phenotype such that even under good growth conditions, mutants will enter the dauer stage (Albert et al. 1981). Genetic epistasis analysis placed these two genes in a similar genetic epistasis pathway for dauer formation that was distinct from the other *daf-c* mutants (Vowels & Thomas 1992). These studies also revealed that both *daf-2* and *age-1* mutants could be suppressed by a mutation in the *daf-16* gene (Albert et al. 1981; Riddle 1988; Vowels & Thomas 1992; Riddle & Albert 1997). *daf-16* (also known as *daf-17*) was also isolated in these early dauer formation genetic screens because *daf-16* mutants show a dauer defective (*daf-d*) phenotype such that even under poor growth conditions, mutants will not enter the dauer stage (Albert et al. 1981; Riddle 1988; Vowels & Thomas 1992; Riddle & Albert 1997).

Subsequent molecular cloning beginning in 1996, explained why these genes were separate and distinct from other pathways. The genes encoded for members of an insulin/IGF-1 signaling (IIS) pathway where *daf-2* encoded for an IIS receptor, *age-1* encoded for the catalytic subunit of the PI 3-kinase, and *daf-16* encoded for a forkhead box O (FOXO) transcription factor downstream of the PI 3-kinase signaling cascade. Since then, studies have shown that the IIS pathway is evolutionarily conserved such that mutations in this pathway in flies and mice are also linked to lifespan extension (Barbieri et al. 2003; Yen et al. 2011).

Molecular and genetic studies in *Drosophila* and *C. elegans* have identified FOXO as a central regulator of lifespan (Lin et al. 1997; Ogg et al. 1997; Giannakou et al. 2004; Hwangbo et al. 2004). Modulation of *Drosophila* FOXO (dFoxo) and *C. elegans* FOXO (*daf-16*) dosage can either decrease or increase the lifespan of the organism (Lin et al. 1997; Ogg et al. 1997; Giannakou et al. 2004; Hwangbo et al. 2004). Importantly, advances in genomic research have led to new findings in the area of genome-wide association studies in humans. Multiple human population studies have found an association between single nucleotide polymorphisms (SNPs) in human FOXO3 and human lifespan extension (Lunetta et al. 2007; Willcox et al. 2008; Anselmi et al. 2009; Flachsbart et al. 2009; Li et al. 2009; Soerensen et al. 2010; Zeng et al. 2010; Banasik et al. 2011; Malovini et al. 2011), and the strength of the association appears to increase with age (Flachsbart et al. 2009). Therefore, FOXO3 has emerged as a candidate longevity gene in humans. Taken together, just over a decade from the molecular identification of DAF-16 in *C. elegans*, multiple studies have linked SNPs associated with human DAF-16/FOXO3 and human lifespan extension.

Dauer and longevity connections

Early studies on dauer larvae showed that dauers were 'ageless'; namely once a dauer recovers and develops into a reproductive hermaphrodite, the subsequent adult lifespan (post-dauer) is independent from the time spent as a dauer (Klass & Hirsh 1976). Therefore, it was thought that *daf-2* and *age-1* were long lived merely due to activation of part of the dauer program manifested in the adult. However, (Kenyon et al. 1993) addressed these concerns by performing lifespan analyses on several other *daf-c* mutants (later shown to be part of a TGF-β signaling cascade) and found that these mutants did not affect lifespan and the issue seemed resolved. However, approaches including genome- wide microarrays and unbiased LC/MS proteomics have shown that the profiles

of adult long-lived *daf-2* mutants are most similar to wild-type dauer larvae (McElwee et al. 2004; McElwee et al. 2006; Depuydt et al. 2014). Moreover, recent studies (Shaw et al. 2007) re-examined the TGF-β *daf-c* mutants and found in contrast to earlier studies, these mutants showed lifespan extension. Similarly, recent genetic data revealed that the connections between the IIS pathway and the TGF-β signaling pathway are intertwined to modulate both lifespan and dauer formation (Narasimhan et al. 2011). Taken together, multiple studies suggest that the longevity of *daf-2* mutants is due to activation of the dauer program in the adult. Despite the fact that a dauer program, an alternative hibernation state to delay reproduction until growth conditions are favorable, seems worm specific, the signaling pathways that were identified to regulate dauer formation modulate longevity from worms to mice, and are associated with human longevity.

Advantages of worms

Why has *C. elegans* been used so successfully for aging research? What would make an organism suitable for aging research? As suggested by Sydney Brenner in 1963, the ability to easily and cheaply grow large quantities of worms in the lab is very helpful for aging research, especially when identifying long-lived mutants. *C. elegans* also have a relatively short lifespan (average approximately 17 days at 20 °C), and the lifespan is largely invariant. The latter allows for identification of mutants that shorten or lengthen average lifespan by a little as 10–15% and still be of statistical significance. Additional benefits of using *C. elegans* include that the entire genome is sequenced and annotated, the availability of an RNAi library comprising approx. 80% of the genes in the genome, the ease of generating transgenic strains and the recent development of gene-targeting approaches. This has allowed for extensive forward and reverse genetic screens for genes that modulate lifespan. The RNAi library allows RNAi to be done by feeding worms bacteria that produce the desired dsRNA and then either the worm or their progeny are scored for a longevity phenotype (Ahringer 2006). Using genome-wide RNAi feeding libraries, the importance of the mitochondria, signal transduction, the response to stress, protein translation, gene expression, and metabolism were found to modulate lifespan (Dillin et al., 2002; Lee et al., 2003; Hamilton et al. 2005; Hansen, Hsu et al. 2005; Hansen, Taubert et al., 2007). Another advantage working with *C. elegans* for studying the aging process is that the lifespan assay is straightforward, which allows for large numbers of worms to be assayed in a single experiment. Therefore, statistical significance can be tested in addition to the analysis of mortality rates. Together, these techniques allow one to comprehensively survey the worm genome for genes that modulate lifespan. This has led to the identification of more than 200 genes and regimens that modulate lifespan in *C. elegans* and revealed evolutionarily conserved pathways that modulate lifespan. Therefore, the combination of the short, invariant lifespan, ease of assays, ample genetic, molecular and genomic tools, and evolutionary conservation has allowed *C. elegans* to develop into a premiere model system for aging research.

Disadvantages of worms

Despite all the excellent advantages of working with *C. elegans* for aging research, there are also several disadvantages for *C. elegans* as a model for human aging. First, *C. elegans* have a simple body plan, and lack many defined organs/tissues including a brain, blood, a defined fat cell, internal organs, and is evolutionarily distant from humans. Second, *C. elegans* are also only 1 mm in length which makes biochemistry more difficult. Typically, all biochemistry, microarray, immunoprecipitation, and chromatin immunoprecipitation is performed on whole worm extracts of either mixed-stage animals or animals at a similar growth stage. This may lead to limited understanding of any tissue-specific signaling such as whether a gene is expressed in the hypodermis or the intestine. Finally, *C. elegans* cell culture is limited with no system equivalent to *Drosophila* S2 cells.

Conclusion

C. elegans has proved to be an invaluable animal for aging research. Thus far, research has focused on the use of lifespan as a measurement of the aging process. These studies have led to the identification of hundreds of genes and regimens that modulate lifespan. Although the initial studies identified genes that altered lifespan and affected dauer diapause, these signaling pathways have nonetheless identified longevity-associated pathways across phylogeny. However, to truly use *C. elegans* for aging research, future studies should focus on understanding the connection between longevity and how an animal ages, with a focus on health. Aging is much more than a lifespan measurement. Aging involves the coordination of multiple systems in an organism and how they change as a function of time. We should strive to use model systems to reveal this systemic coordination on a molecular and genetic level, and how this leads to healthy aging rather than simply lifespan extension.

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Lesson 6: Skills Assessment and Practice

KEY QUESTION(S): Are all *C. elegans* worms the same? What causes mutations in the worms? Are all mutations in *C. elegans* visible? How do scientists take advantage of these mutations to make discoveries?

OVERALL TIME ESTIMATE: 45 minutes

LEARNING STYLES: Visual, auditory, and kinesthetic

LESSON SUMMARY: Students will practice one or more skills learned during Worm Boot Camp. Students will finalize all skill assessments and prepare culture plates for independent inquiry in toxicology.

STUDENT LEARNING OBJECTIVES:
The student will...

- Reflect on the laboratory skills practiced in Lessons 1, 2, and 4.
- Practice and perfect the laboratory skills learned in Lessons 1, 2, and 4.
- Describe the ways scientists use mutant worms for investigation.

MATERIALS:
Copies of *Student Final Assessment*, 1 per student
Other materials will depend on needs of student.
Make all materials for chunking and picking, extra plates

ADVANCE PREPARATION:
Copies of *Student Final Assessment*, 1 per student
Ensure there are additional NGM OP50 plates available for practice
Ensure there are additional worm starter plates available for practice

PROCEDURE AND DISCUSSION QUESTIONS WITH TIME ESTIMATES:
45 minutes

1. Provide students with copy of *Student Final Assessment*
2. Provide instructions that this assessment is a combination of skills and questions
3. Students should attempt to improve skills assessment scores by demonstrating these skills again for a re-grade.
4. Complete questions and ensure all worksheets/skills assessments have been turned in for grading/re-grading.

ASSESSMENT SUGGESTIONS:

- Grade *Student Final Assessment*, 1 per student

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