MOLBIOL 3M03 Principles of Development Laboratory Manual

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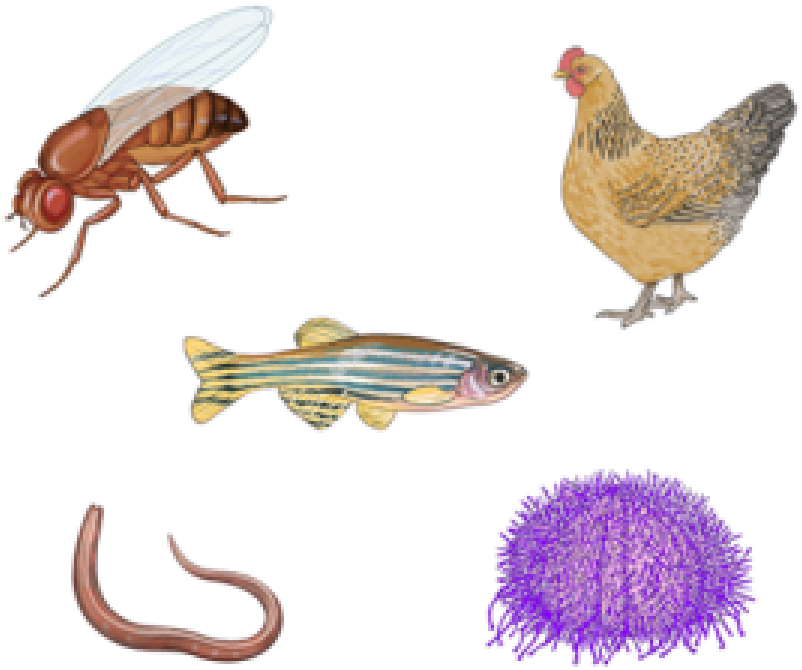
Contents

Acknowledgements



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PART I

LABORATORY #1 MICROSCOPY BASICS

Overview

The first lab consists of several exercises that will familiarize you to the basic concepts required for this developmental biology laboratory. Most importantly, it will be your introduction into the microscopes you will be using for the remainder of the semester. It is important you become comfortable with the proper handling and setting up of both types of microscopes (compound and stereoscope), as we will be using them frequently during our laboratories. The better you understand how to properly set up the microscope, the easier the following labs will be.

Lab Components and Objectives

The lab will have three activities to be finished within a single week.

Activity 1. Familiarize yourself with the **compound microscope**

1. Handle the Zeiss Axiostar*Plus* (or Motic BA310) and use the Köhler illumination method
2. Learn how to prepare a footed coverslip slide

Activity 2. Familiarize yourself with the **dissecting (stereo) microscope**

1. Handle the Wild M3C and observe a water flea

Activity 3. Calibrate each microscope

1. Calibrate the microscopes with both an **ocular** and **slide micrometer**
2. Learn to measure the **field of view** at different magnifications with both microscopes

Key Terms and Concepts

Equipment

Compound microscope: is a high magnification microscope that uses two lenses to multiply (compound) the magnification.

Stereoscope: is a low magnification microscope enabling 3-D depth view through its eyepiece for proper sample investigation and dissection.

Types of Slides

Smear: a suspension of cells or samples is spread on the slide as a film.

Section: a slice of the sample is cut and mounted on a slide.

Whole mount: the entire sample is mounted or embedded in clear mounting medium on the slide.

Basic Microscopy Terms

Field of view (FOV): is the diameter of the visible area of the specimen you are viewing through a microscope.

Aperture: is the hole that controls the amount of light reaching the specimen.

Stage micrometer: is a microscope slide with a scale marked on the surface for measurements.

Ocular micrometer: is a glass disk with a marked scale that is inserted into a microscope's eyepiece.

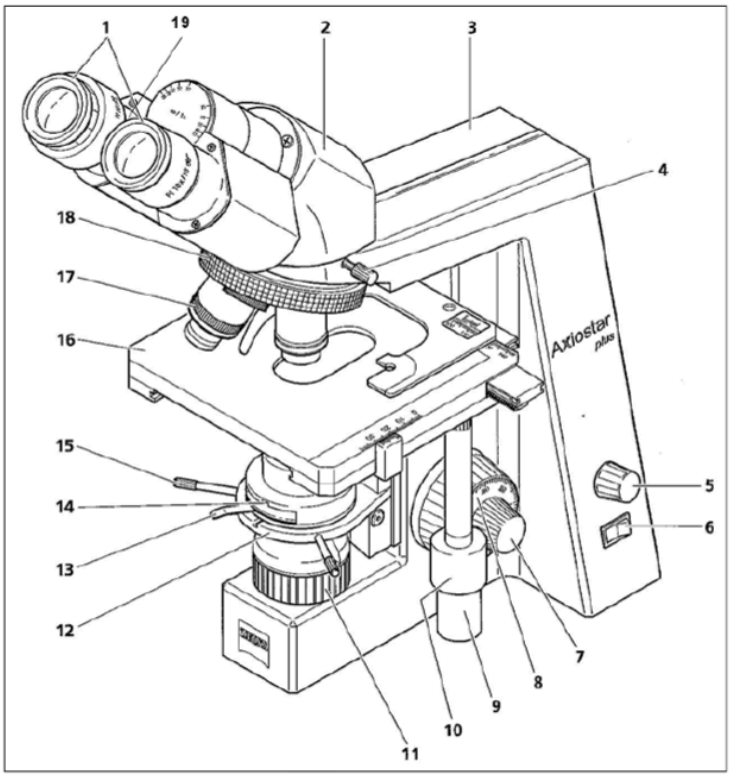
Activity 1: Using the compound microscope

These exercises will familiarize you with the **compound microscope** you will be using in MOLBIOL 3M03 laboratories: the Zeiss Axiostar*Plus* **compound microscope**. For most of your labs, you will be using the transmitted-light brightfield technique, which is ideal for viewing high-contrast or stained specimens (e.g., prepared slides). To maximize resolution, the microscope needs to be set up for the Köhler illumination method, which will enhance the contrast of your microscopy image with even illumination. The Köhler illumination method is carried out by adjusting the luminous field diaphragm, the condenser, and the **aperture** diaphragm. By the end of this lab, you should understand what each component of the **compound microscope** does and how they are adjusted according to the Köhler illumination principle to maximize resolution.

Please refer to the diagram in Figure 1 for understanding each component of the **compound microscope** you will be using throughout the semester. If you need to clean the lenses of the microscope, ***please use the lens paper provided and NOT KimWipes.***

Although KimWipes are useful for cleaning slides and the non-optical components, the optical lenses are delicate enough that the KimWipes may damage them!

Zeiss Axiostar*Plus* Compound Microscope



1	Eye pieces (ocular lenses)
2	Binocular tube
3	Microscope stand (arm)
4	Knurled screw for tube locking
5	Brightness control
6	On/off switch
7	Fine focusing knob
8	Coarse focusing knob
9	Mechanical stage drive (x-plane)
10	Mechanical stage drive (y-plane)
11	Luminous field diaphragm
12	Condenser carrier
13	Lever for aperture diaphragm
14	Condenser
15	Centring screw (for condenser)
16	Mechanical stage
17	Objective lens
18	Nosepiece
19	Diopter ring

Figure 1 | Labeled schematic of a **compound microscope**

Methods: Preparing the microscope

Read through all the steps below before beginning. Bold-face numeral references in parentheses refer to Figure 1 for Zeiss AxioStar compound microscope. You may be using either Zeiss or Motic compound microscopes throughout the course. Please refer to microscope manuals for detailed instructions of how to use each microscope: 1- Zeiss Manual, 2- Motic Manual, 3- Wild M3C Manual

1. Place the microscope on the desk a few inches away from the desk with the arm (**3**) away from you. Turn on the microscope by plugging in the power cord and pressing the on/off switch (**6**).

2. Check to see that all the parts are in working order and the eye pieces (**1**) are clean.
3. Rotate the binocular head (**2**) such that the eyepiece is protruding away from the arm. Lock the binoculars in place by tightening the screw (**4**).
4. Turn the nosepiece (**18**) until the 5× lens is in the optical path.

Always begin microscopy with the lowest magnification!

5. Set the condenser turret (**14**) to the Brightfield setting: 'H'.

Methods: Observing the specimen – lowest magnification

1. Remove a slide from the slide box on your bench, and if necessary, clean with deionized water and KimWipes.
2. Lower the mechanical stage (**16**) with the coarse adjustment knob (**8**) and place the slide (with the coverslip up) in the slide holder on the mechanical stage.
3. Observing the mechanical stage with your eyes, use the coarse adjustment knob to raise the mechanical stage until it is 1 cm below the 5× lens. Centre the specimen under the objective with the X and Y stage drivers (**9** and **10**).
4. Looking through the eye pieces (**1**), use the fine adjustment knob (**7**) to raise or lower the stage until the specimen is in focus. Adjust the brightness (**5**) as needed. Be careful not to raise the slide into the objective – this can cause the slide to break or worse, the objective to become scratched.
5. Adjust the interpupillary distance by moving the eye pieces closer together or further away from each other until a sharply depicted image is seen with both eyes. **Record below** the reading on the interpupillary scale [55 – 75] for your reference.
6. Look through the non-acuity adjustable eye piece (the one without the diopter ring) and close your other eye. Bring the specimen into focus using the fine adjustment knobs (**7**).
7. Look through the eyepiece with the diopter ring (**19**) and close your other eye. Turn the diopter ring (**19**) until the specimen is brought into focus.

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If you have successfully adjusted the interpupillary distance and the acuity eye piece, you should see a clear image of the specimen under the scope.

Methods: The Köhler Illumination Method

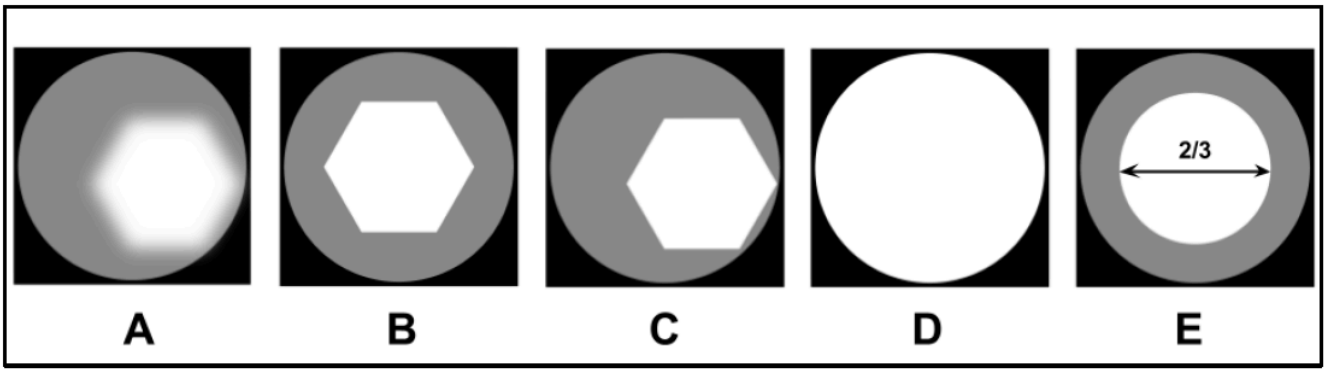


Figure 2 | Alignment steps for Köhler illumination

1. Move the condenser (**14**) to the upper stop position by turning the knob that moves the condenser turret counterclockwise. This knob is on the left side of the microscope (not shown in Figure 1). Move the **aperture** diaphragm lever (**13**) to the centre position.
2. Close the luminous field diaphragm (**11**) by rotating the dial counterclockwise. You should see an outline of the diaphragm in the **field of view**, like Figure 2A. It may not be in focus. Bring the luminous-field diaphragm into focus (Figure 2B) by moving the condenser (**14**) up or down by turning the condenser knob.
3. Turn the centring screws (**15**) to centre the luminous-field diaphragm in the middle of the cross hairs – this is the middle of the **field of view** (Figure 2C).
4. Open the luminous-field diaphragm (**11**) by turning it clockwise until the edge of the diaphragm just disappears from the **field of view** (Figure 2D).
5. To increase contrast, you may close the **aperture** diaphragm lever (**13**) by moving it to the right. The ideal setting for the **aperture** diaphragm lever is approximately 2/3 to 4/5 the diameter of the objective. You can check this by removing one eye piece, looking into the tube with your naked eye, and moving the **aperture** diaphragm lever. Figure 2E shows the lever open at 2/3 of the objective. Replace the eyepiece and view the specimen.
6. Slowly rotate the nosepiece (**18**) to bring the 10× objective into the optical path. If it looks like the objective is going to hit the slide, lower the stage slightly. View the specimen with the 10× objective and bring it into focus.
7. Repeat steps 3-8 for the 10× objective. You may need to adjust the light brightness using the knob (**5**). In general, you will require greater light at higher magnifications.
8. Once you are done, let your partner set up the microscope and repeat the above steps.

Exercise 1: Preparing a footed coverslip slide to observe with the **compound microscope**

1. From your TA, obtain plasticine, a coverslip, an empty microscope slide, and algal water.
2. Take the plasticine and place a small amount of it on each corner of the coverslip.

The elevation from the plasticine prevents the coverslip from damaging the sample!

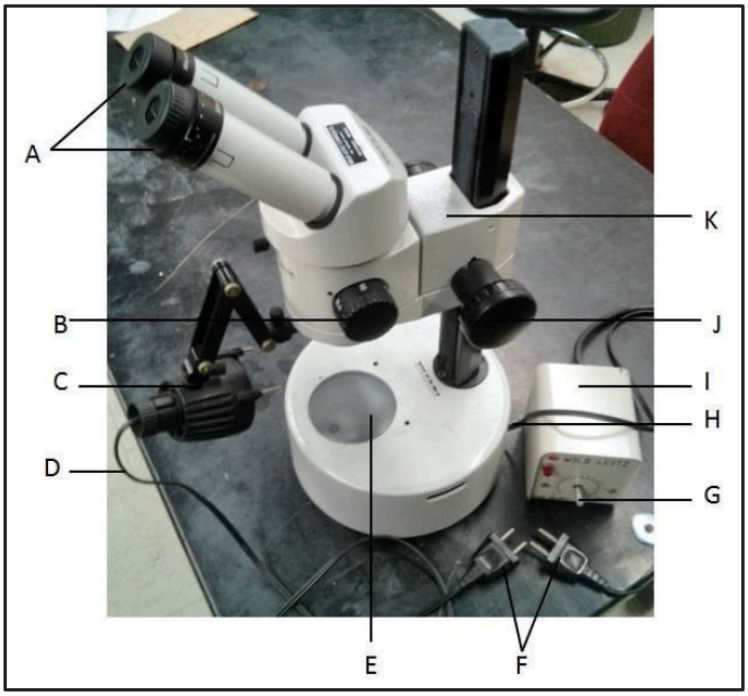
3. Place a small amount of algal water on the microscope slide and gently place your elevated coverslip over it.
4. Observe your sample under the **compound microscope** as you previously practiced.

Activity 2: Using the stereoscopic (dissection) microscope

When we use the **stereoscopic microscope** in our labs, it will mainly be the Wild M3C. This type of microscope uses two optical systems to produce a three-dimensional image. While there is a much lower magnification on **stereoscopic microscopes**, they do have a much greater depth of focus. **Why is this beneficial or preferred?** Often used for dissections, a **stereoscopic microscope** is also referred to as a **dissecting microscope**.

Please refer to the following diagram for understanding each component of the **stereoscopic microscope** you will be using throughout the semester. If you need to clean the microscope, ***please use lens paper provided and NOT KimWipes.***

Wild M3C Stereoscopic Microscope



A	Eye pieces
B	Magnification selector
C	Light source (above specimen)
D	Light source (above) cord (plugs into transformer)
E	Light source (below specimen)
F	Light source plugs (connect to transformer)
G	On/off switch (press in) and light intensity adjustment knob (turn)
H	Light source (below) cord (plugs into transformer)
I	Transformer
J	Focus control
K	Arm (hand grip)

Figure 3 | Labelled schematic of a **stereoscopic microscope**

Read through all the steps below before beginning

Methods: Preparing the microscope

1. Place the microscope a few inches away from the desk edge with the arm (**K**) away from you. Plug in the power cord and push the on/off switch (**G**) to turn on the microscope.
2. Plug the transformer (**I**) into an electrical outlet. Practice changing the light intensity by turning the dial clockwise or counterclockwise from the transformer.

The transformer is required to power the external light source!

Methods: Observing the specimen – lowest magnification

1. Obtain a *Daphnia* (water flea), a microscope slide, and some water from your TA.
2. Place the *Daphnia* on the slide with a small drop of water and place it above the light source (**E**).
3. Observe the slide under the lowest magnification (6.4×).
4. Adjust the interpupillary distance by moving the eye pieces closer together or further away from each other until a sharply depicted image can be seen with both eyes. **Record below** the reading on the inter-pupillary scale [55 – 75] for your future reference.
5. Look through the non-acuity adjustable eye piece and close your other eye. Bring the specimen into focus using the adjustment knobs (**G** – turn).
6. Continue to view the *Daphnia* at higher magnifications.

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Activity 3: Calibrating the microscopes

Activity 3: Calibrating the microscopes

Now that you are familiar with using the **compound** and **dissecting microscope**, you will need to understand how to calibrate them. These calibrations will allow you to assess the magnification of the sample you are observing and will be required for future labs. The instructions for calibration are the same for both microscopes.

You will need to perform two calibrations. You will conduct a **FOV** calculation for both the **dissecting** and **compound microscope** at different magnifications. Additionally, you will calculate the **ocular micrometer** calibration values for the **compound microscope**.

All your measurements from today will be needed for future labs! Record them clearly and make them available to you for the remainder of the course.

Exercise 2: Calculating the **FOV**

As magnification increases, the **FOV** becomes smaller and more focused. It is important to know the diameter of the **FOV** for each magnification so you can make a rough estimate of the size of various specimens. For example, if the **FOV** at 10× magnification is 1 mm, and the specimen occupies ~ ½ the **FOV**, you can estimate that the specimen is ~ 500 µm.

Measuring the FOV: The **FOV** can be measured with a ruler on all magnifications for the **stereoscopic microscope**, and on the **compound microscope** at the lowest magnification (5×). At higher magnifications, a stage micrometre must be used for the **compound microscope**. These are delicate and expensive – handle them with care.

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Exercise 3: Calibrating with the **ocular micrometer**

The second calibration you are required to perform is for the **ocular micrometer** in the **compound microscope**.

This eyepiece has a scale etched into it. This ocular scale needs to be calibrated for each objective lens (5, 10, and 40×), since magnification changes with the objective.

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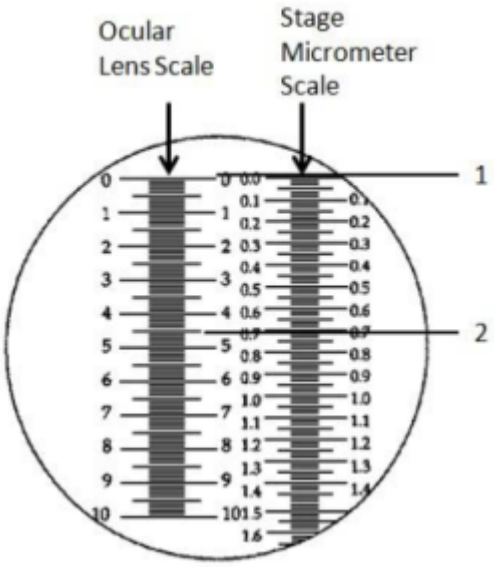
Methods: Ocular Calibration



1. Prepare the **compound microscope** according to (steps 1 – 5).
2. Switch the objective to the 5× and place the **stage micrometer** on the mechanical stage.
3. Focusing on the **stage micrometer**, look through the ocular lens. Using the stage drives (**9** and **10**) rotate the **ocular micrometer** and move the **stage micrometer** until the zero lines on each scale are aligned. Reference the image provided on the right.
4. Determine the exact distance for one ocular unit by superimposing the ocular lens scale over the **stage micrometer** scale. You will need to find a line on the ocular scale that exactly aligns with a line on the micrometer scale.

All done! For the clean up...

1. Clean the microscope slides for future use with deionized water and dry them with a KimWipe.
2. Dispose of the footed coverslip in the glass waste.
3. Turn off the **compound microscope** (**6**) and the **dissecting microscope** (**G**) and unplug the cords.
4. Replace the dust covers on the **compound microscope**.

Useful Equations

The zero scale from the ocular lens and stage micrometer is aligned (line 1). 4.5 ocular units align with 0.7 mm on the stage micrometer (line 2).

$$\frac{4.5\ ocular\ units}{1\ ocular\ unit} = \frac{0.7\ mm}{x\ mm}$$

$$4.5x = 0.7$$
$$x \cong 0.16\ \text{mm}$$

1 ocular unit is 0.16 mm.

Microscope Magnification = Objective magnification × Eyepiece magnification

$$Drawing\ magnification = \frac{\text{Drawing Size (measure what you drew)}}{\text{Actual Size (measured under microscope)}}$$

For example: $\frac{14000\ \mu m}{70 \mu m}$

= 200× drawing magnification.

PART II

LABORATORY #2 SEA URCHIN: FERTILIZATION AND EARLY CLEAVAGE OF EMBRYO

Overview

Sea urchins are a strong model organism to study the early stages of embryogenesis – primarily because they are cheap to maintain, their gametes can be extracted with relative ease, and fertilization can be observed *in vitro*. After finishing an experiment design this week, you will use live sea urchins in next week's laboratory assessments to understand fertilization and early embryogenesis.

Lab Components and Objectives

This lab will have four laboratory activities, with the first activity being split into two weeks. The following three activities will take place in Week 2.

Activity 1. Designing an experiment

1. Familiarize yourself with the literature by reading the selected primary and secondary resources
2. Using the scientific literature as a guide, design and execute an experiment to assess the effects of various drugs as male contraceptives, and to assess the effectiveness of these male contraceptives

Activity 2. Extraction of sea urchin sperm and eggs

1. Learn the morphology of the **gametes** and the changes upon fertilization

Activity 3. *in vitro* fertilization of sea urchin

1. Observe the effects of fertilization on the egg
2. Be familiar with the changes in the fertilized egg during the first 2-3 cell divisions (early cleavage)

Activity 4. Egg activation (parthenogenesis)

1. Understand the role of ion gradients during fertilization and the effects of interfering with key events

Introduction

The eggs and sperm of sea urchins have been widely used as a model for fertilization and embryogenesis. Their **gametes** are easily obtained, are at the same maturation stage when extracted, and fertilization can be observed outside of the adult body. Early studies into the processes governing fertilization, sperm motility, and **gamete** viability were conducted on sea urchin **gametes**. For this series of lab activities, you will be using this same model to observe egg activation, *in vitro* fertilization, and to assess the efficacy of certain chemicals as male contraceptives.

Key Terms and Concepts

Sea Urchin Morphology

Gamete: is a haploid cell (either male or female) that can contribute to the formation of a zygote with a **gamete** of the opposite sex.

Gonophore: is a reproductive structure found in the Hydrozoa class.

Aboral side: is the furthest side from the oral cavity of the organism.

Week One | Activity 1: Experimental design

During the first week of this activity, you will plan an experiment, using sea urchin fertilization as a model system, to assess the effectiveness of your selected chemical as a male contraceptive. The experiment must not take longer than 40 minutes and will be conducted during Week Two. **You must read the assigned reading before beginning your experimental planning.** Make sure you can access the articles during this laboratory (bring your laptop or have them printed).

Resources:

- **Resource 1:** Laboratory on sea urchin fertilization. VD Vacquier. Mol Repr & Dev 78, 553–564, 2011.
- **Resource 2:** Effect of cholinergic agents on human spermatozoa motility. C Dwivedi & NJ Long. Biochem Med and Metab Biol 42, 66-70, 1989.
- **Resource 3:** Inhibition of human sperm motility by inhibitors of choline acetyltransferase. B V Sastry, V E Janson, A K Chaturvedi. J Pharmacol Exp Ther 216(2), 378-84, 1981.
- **Resource 4:** Quantitative evaluation of sperm motility control mechanisms. L Nelson. Biol Reprod. 6(2), 319-24, 1972.

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Week Two | Activity 2: Gamete collection

Before conducting your designed experiment, you must first complete Activities 2 – 4. Activity 2 will be performed by the Course lab assistant and demonstrated to the students.

For this activity, you will be separately collecting egg and sperm samples from sea urchins. You will not know the sex of the sea urchin until the **gametes** are released. You will use a 0.5 M KCl solution to stimulate their release. The potassium ions will depolarize a muscle on the reproductive structure of the sea urchins and result in **gamete** secretion. **The eggs and sperm collected in this activity will be used in all this week's exercises.**

It is important that you do not cross-contaminate the egg and sperm suspensions!

Methods: Gamete collection

1. Collect adult sea urchins and identify their oral cavities.
2. Insert a needle with 1-2 mL of 0.5 M KCl into the membrane surrounding the oral cavity and inject the solution.
3. On the side furthest from the urchin's oral cavity (**aboral**), you will observe which **gametes** are released from the urchin's gonopores. Identify the sex of the urchin.
    1. Female: A thick yellow liquid is shed from the **gonophores** (Figure 1)
    2. Male: A creamy white liquid is shed from the **gonophores** (Figure 2)

Figure 1 | Female sea urchin releasing eggs

Figure 2 | Male sea urchin releasing sperm

Methods: *(If female)* Collecting eggs

1. As the sea urchin is releasing eggs, invert it over a beaker containing artificial seawater so the **aboral** surface touches the solution's surface.
2. Once the eggs have fallen to the bottom of the beaker, decant and rinse them with fresh seawater a total of three times. This is to remove any contaminating material that may interfere with fertilization.
3. Place the washed egg suspension at 10°C until it is ready to be used.

Methods: *(If male)* Collecting sperm

1. Collect the sperm suspension by inverting the urchin over a clean petri dish.
2. Transfer the sperm to a microcentrifuge tube and place on ice; this solution is referred to as **dry sperm**.

Exercise 1: Observing the **gametes**

With 'footed' (elevated) coverslips, observe the **gametes** you collected. Prepare 10 for this exercise.

If you get sea water on the microscope, make sure you wipe it off immediately. Use lens paper if it is on the optical parts.

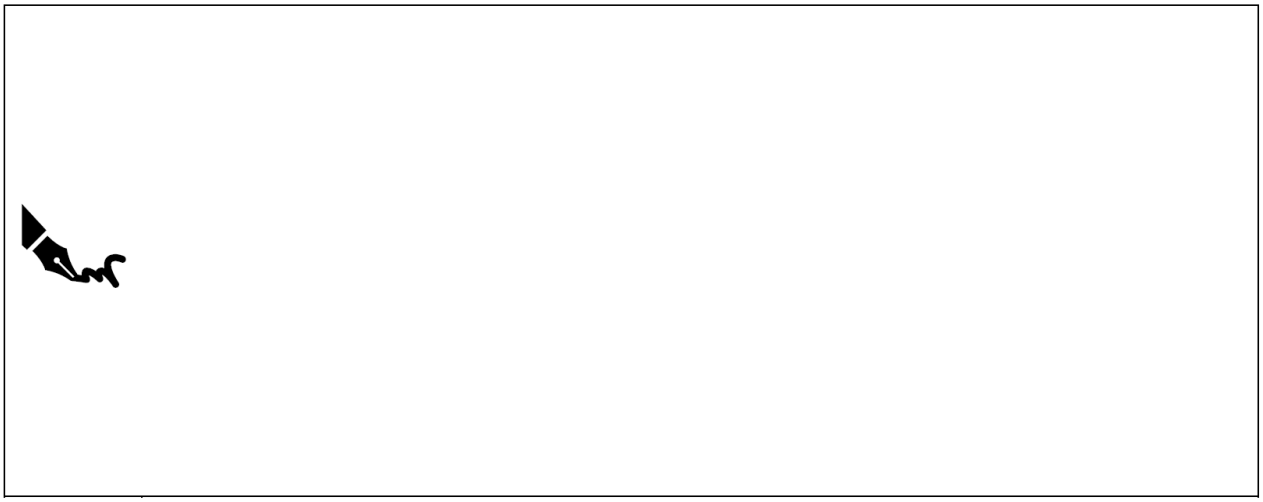
Exercise 1.1 *Observe the unfertilized egg suspensions*

1. Add a few drops of the unfertilized egg suspension to your slide (with the elevated coverslip) and observe under the compound microscope.
2. Make a quick sketch of the egg on the next page. Label the **plasma membrane (PM)** and **vitelline layer (VL).** Append a screenshot of your diagram to the Lab 2 Notes Export file before submitting it to A2L.

Exercise 1.2 *Observing the hydrated egg jelly coat*

Sea urchin eggs have a transparent egg jelly coat that repels the India ink. You will be provided a set slide with India ink to increase result consistency.

1. Add India ink to the unfertilized egg suspension.
2. Add a few drops of the stained eggs to a fresh slide (with the elevated coverslip) and observe under the compound microscope.
3. Make a quick sketch of the egg. Label the **PM, VL, and jelly coat (JC).** Append a screenshot of your diagram to the Lab 2 Notes Export file before submitting it to A2L.



4. **Measure the entire diameter of the egg, including the JC: _____** cm.

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Exercise 1.3 *Observe the sea urchin spermatozoa*

1. From the TA bench, take one of the slides prepared with sea urchin sperm.
2. Using the phase contrast, observe the sperm under the 40× objective. Try to observe the flagellum.
3. **Without using the ocular micrometer,** try to estimate the size of a single spermatozoon: _____ µm.

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Week 2 | Activity 3: in vitro Fertilization

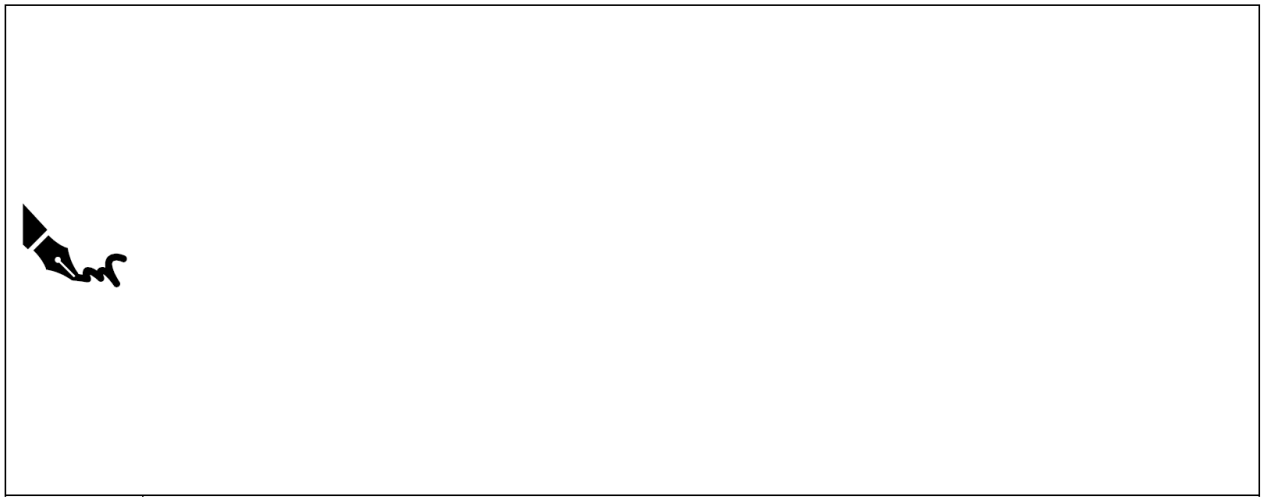
You will use your collected **gametes** and conduct an *in vitro* fertilization experiment. **You must operate quickly after activating the sea urchin sperm.**

Methods: Activating the sperm

1. Add 2- 3 drops of the dry sperm collected from activity 2 to a solution of 100 mL of seawater. **This activated sperm will only be viable for 10 minutes.**

Exercise 2: Observing fertilization

1. To a fresh slide, add a drop of unfertilized egg suspension to your slide with a footed coverslip. Using the 10× objective, focus on the eggs located near the edge of the coverslip.
2. When you are ready, your TA will add a drop of the activated sperm suspension to one side of the coverslip.
3. Make a quick sketch of what happens when the egg becomes fertilized. Append a screenshot of your diagram to the Lab 2 Notes Export file before submitting it to A2L.



4. Record the diameter of the fertilized egg: _____ cm.
5. Record, roughly, what percentage of the eggs are fertilized? _____ %
6. How long did it take for the eggs on the slide to fertilize? _____
7. Set this slide aside and prepare a fresh one.
8. With the fresh slide, repeat steps 1 – 3 to observe the fertilization event at a higher (40×) magnification. **Try to locate where the sperm and egg fuse.**

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Week 2 | Activity 4: Egg activation in the absence of sperm (parthenogenesis)

In this activity, you will be using an ionophore to assess the role of Ca2+ in the activation of sea urchin eggs. An ionophore is a compound capable of transporting ions (which are hydrophilic) across membranes (which are hydrophobic). Some ionophores form channels in the membrane and contain a hydrophilic pore, while others act as ion carriers that reversibly bind to an ion and transport it across the hydrophobic membrane**.** You will use the carrier ionophore A23187 which reversibly binds to Ca2+ ions and transports them across cell membranes. Egg activation will be inferred by observing the elevation of the fertilization envelope due to exocytosis of cortical granules (i.e. cortical reaction), without fertilization by sperm. **What do you think will happen to the eggs exposed to A23187 in normal seawater vs. Ca-free seawater?**

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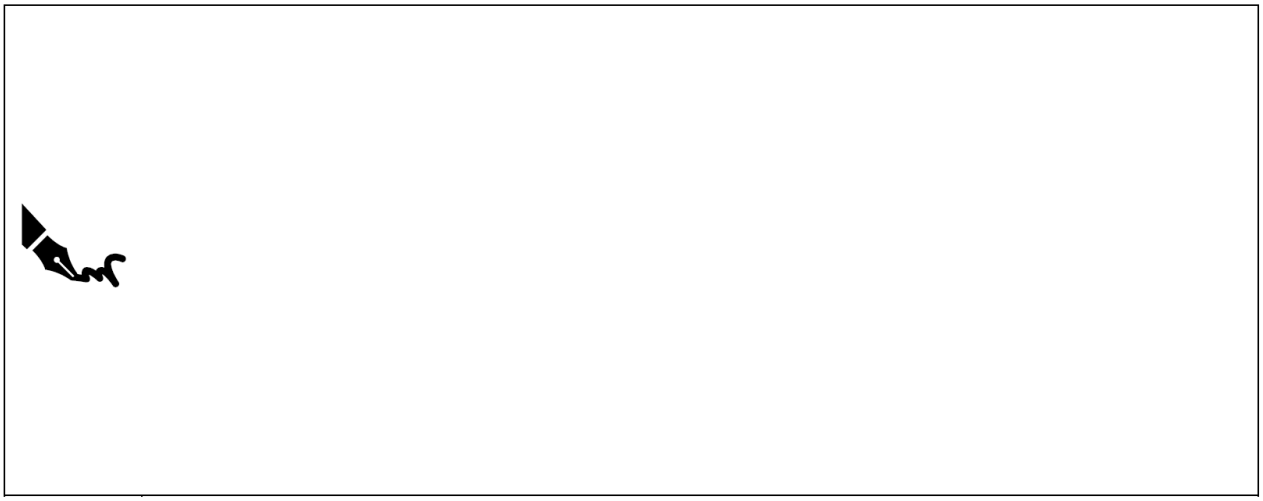
Methods: Egg activation in the absence of sperm (parthenogenesis) using the Ca ionophore A23187

1. Add a few drops of the unfertilized egg suspension to a fresh footed coverslip slide.
2. When you are ready, ask the TA or IA to add some of the Ca ionophore A23187 to your slide.
3. Observe the slide under the compound microscope at 10× magnification.
4. Make a sketch of the activated egg. Append a screenshot of your diagram to the Lab 2 Notes Export file before submitting it to A2L.



5. Record, roughly, what percentage of the eggs show elevation of the fertilization envelope? _____ %
6. Record **how long it took for the eggs on the slide to activate**: _____ min
7. Set the slide aside and observe throughout the remainder of the lab. If you see any cell divisions, compare your observations with what you saw during the *in vitro* fertilization with sperm.

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https://ecampusontario.pressbooks.pub/molbiol3m03/?p=184#h5p-4

Exercise 3: Source of the calcium ions

You will now assess whether the source of the Ca2+ ions is internal or external by observing the effects of adding the Ca ionophore A23187 to unfertilized eggs in Ca-free seawater.

1. Add a few drops of the unfertilized egg suspension **in Ca-free seawater** to a fresh footed coverslip slide.
2. Ask your TA to add some of the Ca ionophore A23187 to your slide.
3. Observe the slide under the compound microscope at 10× magnification.
4. Make a sketch of the activated egg. Append a screenshot of your diagram to the Lab 2 Notes Export file before submitting it to A2L.

5. Record, roughly, what percentage of the eggs show elevation of the fertilization envelope: _____ %
6. Record **how long it took for the eggs on the slide to activate**: _____ min.

An interactive H5P element has been excluded from this version of the text. You can view it online here:
https://ecampusontario.pressbooks.pub/molbiol3m03/?p=184#h5p-4

Keep the slides and observe over the duration of the lab. If you see any cell divisions, compare them with the eggs that were fertilized with sperm or the eggs that were activated in normal seawater.

Week 2 | Activity 1 (continued)

Using the sperm and egg samples collected from Activity 2, conduct the experiment you designed during Week 1. **You will use these observations for your lab report.**

An interactive H5P element has been excluded from this version of the text. You can view it online here:
https://ecampusontario.pressbooks.pub/molbiol3m03/?p=188#h5p-4

Deliverables

At the end of the two-week lab period, you will prepare a mini (at least 11pt font-size, with 1.5 line-spacing for the text, maximum 2.5 pages) lab report based on Lab Activity 1 and have your sketch/diagrams marked for completeness.

SEA URCHIN LAB REPORT (/20)

Introduction: (~1/2 page) Include the hypothesis and background knowledge. Orient the reader on sea urchins as a model, why we care about male contraceptives, and the drug you chose for your experiment. (/4)
Materials and Methods: (~1/2 page) Do not reiterate the procedure above. Explain the experiment that you designed. Include information about positive and negative controls. (/4)
Results: (~1/2 page) Give your raw data and any statistics you have (can be in a table). Provide a graph of this data that includes a written caption. There is no written results section in this report, so make sure your graph has enough information to stand alone. (/5)
Discussion: (~1/2 page) Briefly discuss whether your drug of choice is an effective contraceptive. Make sure your conclusion is biologically relevant. (/5)
References: APA style, with IN-TEXT reference. Include the resource papers from Avenue that you used to design your experiment and any other background you refer to. (/2)

Laboratory 2 Demo Video

One or more interactive elements has been excluded from this version of the text. You can view them online here: https://ecampusontario.pressbooks.pub/molbiol3m03/?p=214

PART III

LABORATORY #3 GALLUS GALLUS : GASTRULATION AND NEURULATION

Overview

The chick embryo is a powerful tool in understanding developmental biology. Many discoveries were supported through the *in vitro* observation of chick embryogenesis, including those made in understanding gastrulation and neurulation. In this laboratory, you will follow the footsteps of Aristotle by observing various stages of chick embryos.

Lab Components and Objectives

This lab will have one activity that takes place within a single week. You will hand in your lab reports at the end of the laboratory session.

Activity 1. Observation of early embryonic development of chick embryogenesis

1. Understand the relationship between **Hensen's node**, **primitive streak**, **Epiblast** and **hypoblast**
2. Visualize early developmental processes, including gastrulation and neurulation

Introduction

The process of embryogenesis was investigated as early as 350 B.C., where Aristotle used the embryo of *Gallus gallus* (domestic chicken) for his observations. Although it may not be the first model organism to come to mind, observation and perturbation of chicken embryogenesis has supported our understanding of vertebrate developmental biology. *G. gallus* is phylogenetically more like mammals than other commonly used model organisms, has a short incubation time, and the embryo is easily accessible during both the *in ovo* and *ex ovo* stages. For this laboratory exercise, you will extract the embryo from chicken eggs that were obtained from Arkell Poultry Research Station at the University of Guelph. These fertilized eggs were incubated at 37°C between 24 and 168 hours (stages 1 – 33). You will use a chick embryo staging tool (**Appendix A**) developed by Hamburger & Hamilton (1951) to stage these embryos, i.e., correlate the structures in the staging tool with your observations.

Key Terms and Concepts

Blastodisc : is the blastula found at the top of the yolk in chicken eggs.

Epiblast: is top blastodisc layer, later differentiates into the primary germ layers.

Hypoblast: is bottom blastodisc layer which gives rise to the yolk sac.

Primitive streak: is the linear furrow trailing the node which marks the gastrulation site and initiates germ layer formation.

Hensen's node: the organizer node at the tip of streak; cells of this region release biochemical signals that mediate neural differentiation and drive gastrulation.

Activity 1: Early chick development

You will look at the structures of the developing chick embryo by extracting the **blastodisc** from a fertilized egg. It is important to separate the **blastodisc** from the yolk for young embryos, as the yolk interferes and obscures the structures. During the lab we will look at the developing chick embryos (live tissues) and supplement the experience with prepared slides. **You will generate two drawings** from these observations, of an embryo following 48 hours and 72 hours of development. Methods presented here are based on protocols generated by David Fankhauser.

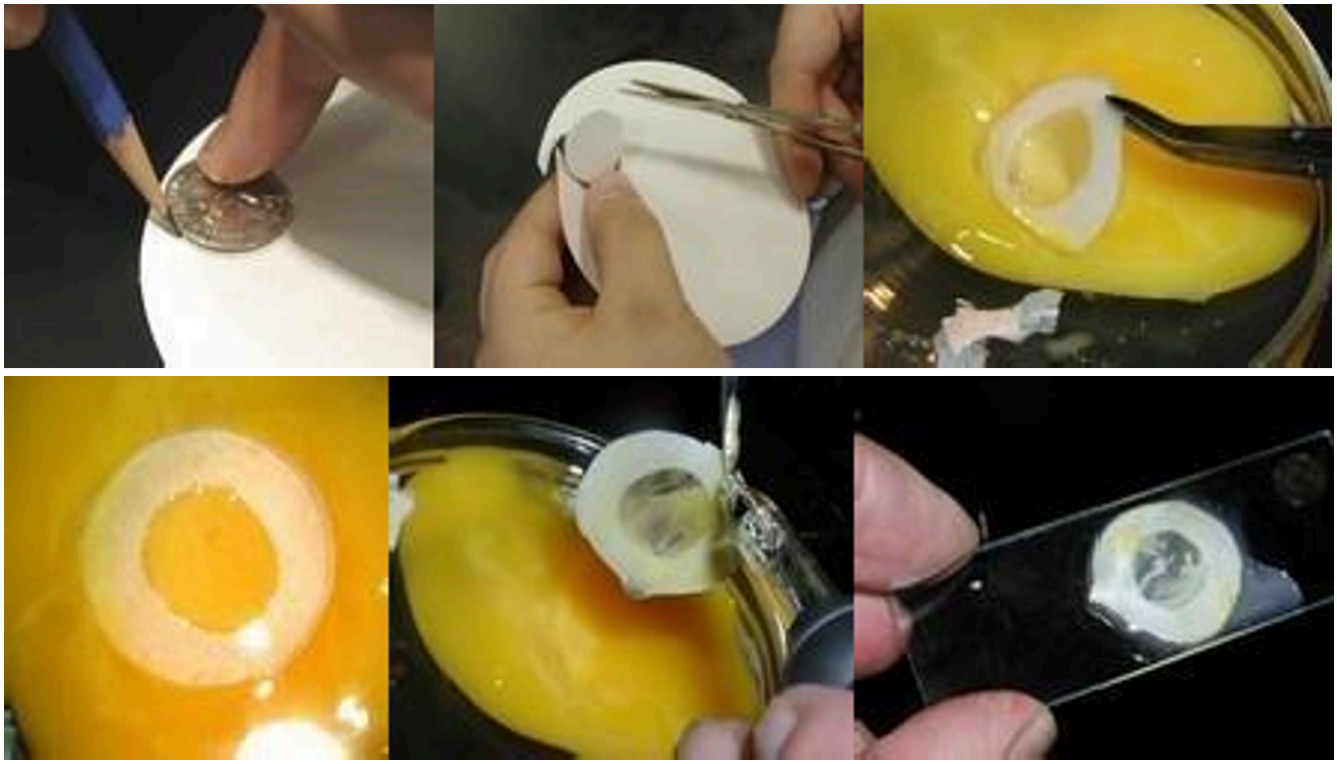


Figure 1 | Schematic of blastoderm preparation for microscopy (reproduced from Dr. Fankhauser's Blog Post | 1980]

Methods: Cracking the egg

1. Cut a filter disk paper in the shape of a doughnut approximately the size of a quarter (Figure 1).
2. Pour warm chick saline solution into a petri dish to a depth of ~ 2 cm.
3. Carefully, and being sure to not break the yolk, crack the egg into the saline solution.
4. The embryo should float to the top of the yolk in the solution.

Keep the egg close to the saline solution and gently crack the shell halfway around the egg before pulling the shell apart!

Methods: Dissecting the **blastodisc** from the yolk surface

1. Place the doughnut shaped filter disk over the embryo such that the embryo rests in the empty centre.
2. Use forceps to grab the edge of the filter paper over the blastoderm.
3. Cut the vitelline (yolk) layer with small rapid snips around the outer edge of the filter disk.
4. After cutting around the **blastodisc**, using the forceps to gently move the filter disk onto a microscope slide.
5. Observe the embryo under a dissecting microscope.

Deliverables

Draw two embryos at ~ 48 and ~72 hours of development (by investigating the live tissue under the microscope if possible). For each drawing, indicate how many hours-old and the developmental stage. Label the following structures in your drawings: neural plate, neural tube, notochord, **primitive streak**, **Hensen's node**, **somites**. These drawings will be due at the end of lab session.

Laboratory 3 Demo Video

One or more interactive elements has been excluded from this version of the text. You can view them online here: https://ecampusontario.pressbooks.pub/molbiol3m03/?p=243

PART IV

LABORATORY #4 DROSOPHILA MELANOGASTER : EVALUATION OF GENE EXPRESSION USING REPORTER CONSTRUCTS

Overview

Drosophila melanogaster is a fundamental model organism for developmental and eukaryotic biology. Fruit flies are inexpensive, propagate quickly, and have many molecular tools designed for their genetics. One of the most powerful tools in *Drosophila* developmental genetics is the use of **transgenic** flies to discover new genes, visualize their expression and even alter gene expression, all to better understand their function in cell physiology. For this laboratory, you will become familiar with the pattern of *tup* expression which encodes for the transcription factor Tailup/Islet1 (Tup).

Lab Components and Objectives

This lab will have three activities spread over the course of two weeks.

Activity 1: *Drosophila* as a model organism for developmental biology

1. Observe various **transgenic** adult fruit flies harbouring developmental mutations

Activity 2: Understanding and visualizing the role of *tup* in fruit fly development

1. Learn how to prepare *Drosophila* embryos for **immunostaining**
2. Conduct an **immunostaining** on prepared embryos with an α-GFP antibody to track Tup-GFP expression during development

Activity 3: Imaging the stained *Drosophila* embryos and assessment of Tup expression

1. Prepare a (at minimum) three panel publication-worthy figure using photographs characterizing the Tup expression
2. Learn how to stage *Drosophila* embryos during their developmental growth
3. Understand the role of *tup* during *Drosophila* development

Introduction

D. melanogaster has been as a model system used throughout the last century to uncover phenomena in developmental biology. This laboratory exercise will take place over two weeks, and its purpose will be to document the role of the transcription factor Tailup/Islet1 (Tup), encoded by *tup*, during the various developmental stages of *D. melanogaster* (Appendix B). You will be using a **transgenic** line of *D. melanogaster* harboring a **P element** transposon encoding the transcriptional fusion reporter *tup-GFP* downstream of the endogenous *tup* regulatory region. Due to this, *tup-GFP* mimics the spatial and temporal expression pattern of wild-type Tup.

You will visualize the expression of Tup-GFP in *Drosophila* embryos through an **immunohistochemistry** staining procedure (Figure 1).

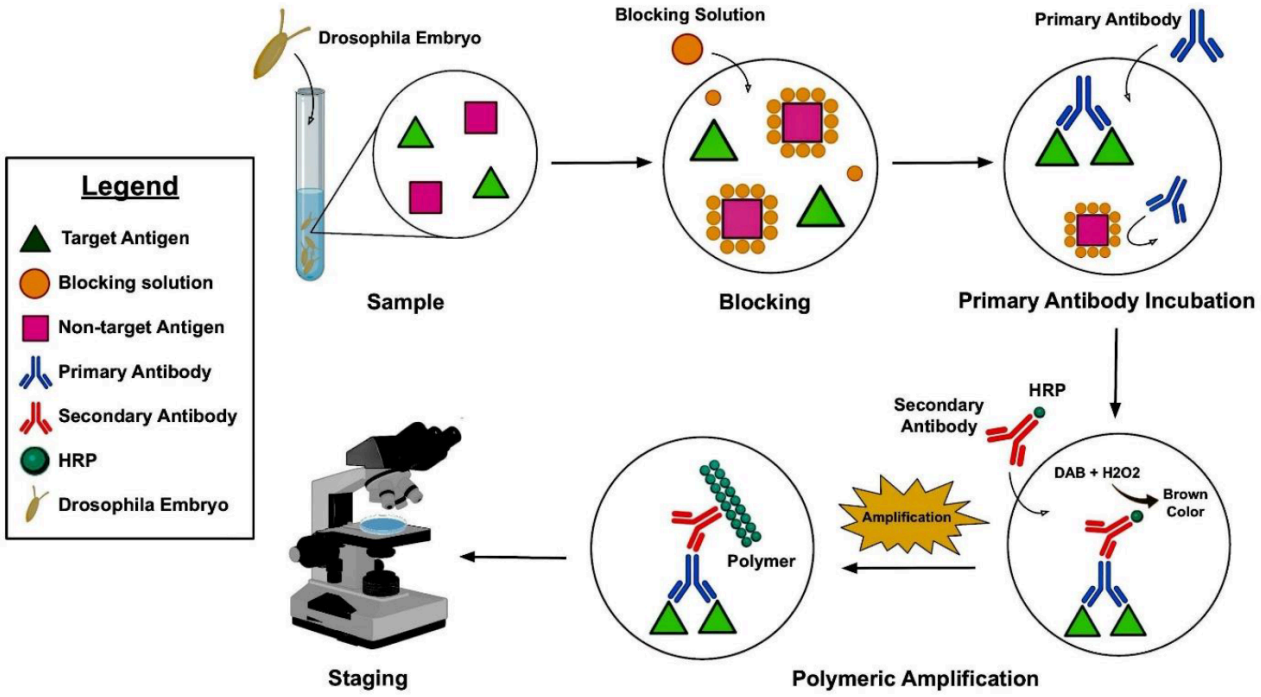


Figure 1 | Flow diagram of a typical dye-based immunostaining procedure.

Key Terms and Concepts

Molecular Genetic Tools

P element : is a transposon originally identified in *Drosophila* that has now been used as a tool in fruit fly molecular biology.

Enhancer trap: is a type of P element which results in the transposition of a reporter gene downstream of known regulatory elements to determine their spatial and temporal expression patterns.

Transgenic: refers to an organism whose genetic make-up has been altered by the introduction of non-endogenous genetic elements.

Experimental Protocols

Immunohistochemistry: is an *in situ* protein staining method which relies on the recognition of specific proteins in the target sample by primary antibodies. This allows for the downstream visualization of detection of these targets with a secondary antibody.

Primary antibody: is an antibody sensitive to a specific epitope target.

Secondary antibody: will recognize the primary antibody and harbors a means of visualization.

Week One | Activity 1: Fruit fly mutants

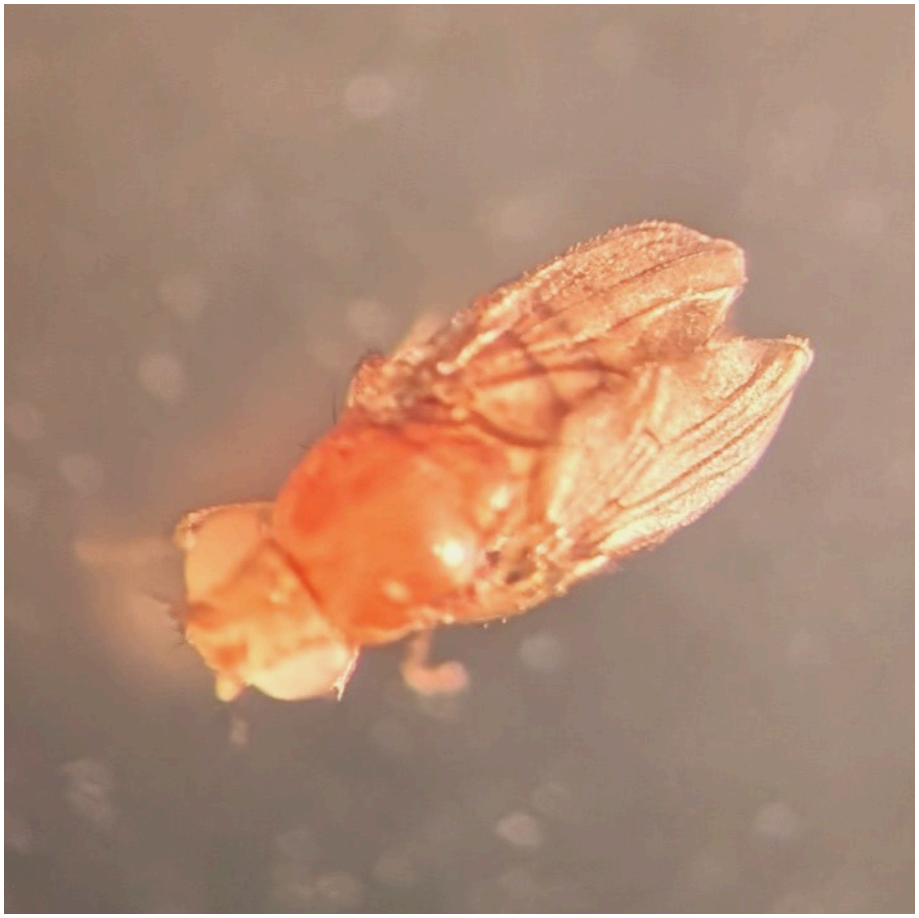
This week, you will begin an **immunostaining** procedure against *Drosophila* embryos and additionally observe a variety of fruit fly mutants.

Your TAs will provide you live fruit fly samples that can be observed. They are harboring various genetic mutations that have affected their development and physiology. Provide a brief description about your mutant flies and attach photos to your Lab 3 Notes export file for submission to Avenue to Learn (A2L).

An interactive H5P element has been excluded from this version of the text. You can view it online here:
https://ecampusontario.pressbooks.pub/molbiol3m03/?p=273#h5p-14

Example mutant flies

Click on the images below to enlarge the descriptions



miniature (m) –

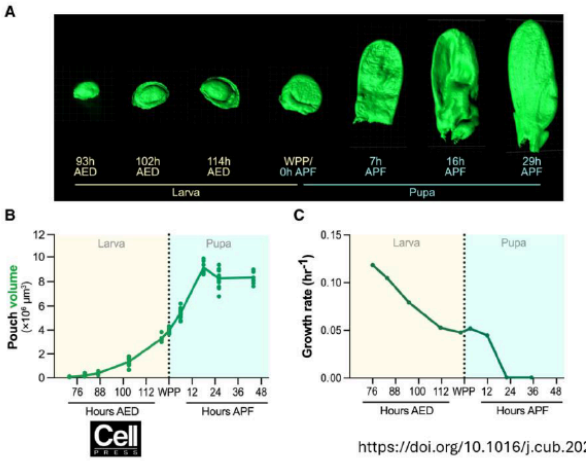
Miniature encodes a ZP-domain containing protein. It is forms a structural "clamp" used to hold cells in place after epithelial cell flattening (to squamous shape from cuboidal) as the final cuticle forms.

We have two loss-of-function alleles:

m[1] **-** moderate hypomorphic mutation.

m[D] **-** Severe, dominant hypomorphic mutation.

Notice that the wings look like small "wild-type" wings and they look darker. They are not more pigmented, just have thicker cells with reduced surface area.



Loss of function mutations in *miniature* fail to maintain the final cell shape change. Specifically, the final "flattening" from cuboidal cell shape to squamous cell shape. As such they have the same number of cells, but the cells have a reduced surface area (but same volume).

https://doi.org/10.1016/j.cub.2025.07.020

DROSOPHILA MELANOGASTER SAMPLE MUTANTS

scalloped (*sd*) –

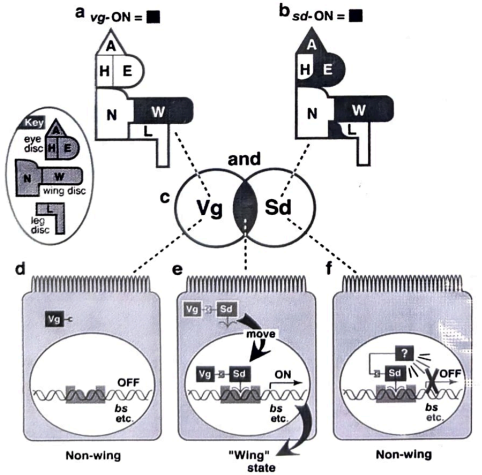
Encodes a transcription factor that is one of the key players in determining wing identity (along with vestigial). LOF Mutations in *sd* cause loss of wing identity, reduction in cell proliferation and increase in apoptosis in the wing imaginal disc.

Please compare the phenotypic severity for two *scalloped* mutants provided:

sd[Etx4] - a moderate hypo-morphic allele (with considerable variation in phenotypic expressivity). All individuals are homozygous (hemizygous in males), but some show almost wild-type wing morphology, while others have reduced wings.

sd[58d] - a severe hypomorphic allele, where everyone has extremely reduced wings (just little "nubs" of wings). **Minimal heterogeneity in phenotypic expressivity.**

Both of the *scalloped* mutations are regulatory (i.e. no coding sequence changes).



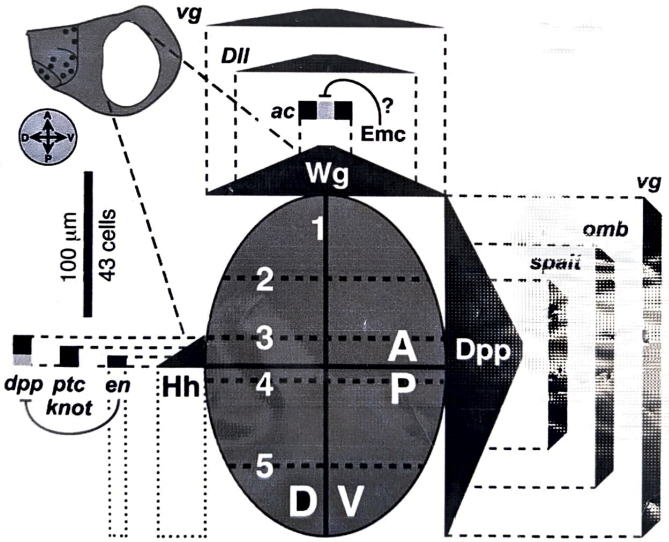
A = Antenna
E = Eye
W = Wing
L = Leg
N = Notum
H = Head

https://www.sdbonline.org/sites/fly/lewheld/id610.htm

Optomotor blind (*omb*) –

Encodes a transcription factor which is downstream target of the **Dpp (Drosophila BMP-homolog) gradient** in the wing disc.

The mutation we have ***omb[md653]***, is caused by an enhancer trap insertion of a synthetic transposable element containing Gal4, which leads to a modest reduction in gene expression. This strain also contains a balancer (as the mutation has low viability). The **balancer** is called Fm7, and you will see **females** with **kidney bean shaped eyes** instead of oval eyes. Wing phenotype should be present.



https://www.sdbonline.org/sites/fly/lewheld/id63.htm

Week One | Activity 2: Immunostaining Drosophila embryos

For this portion of the lab, you will conduct the *Drosophila* embryo **immunostaining** procedure. The entire experimental protocol takes two days. **You and your lab mates will only be conducting the protocol of day one, and the course lab assistant will complete day two.** It is nevertheless important that you familiarize yourselves with the protocol of both days to understand how **immunohistochemistry** works.

Methods: Preparing *Drosophila* embryos for **immunostaining**

1. On your bench, you will find *Drosophila* embryo dishes. Remove the lids. Using a Kimwipe, try to remove as much yeast as possible from the embryo dishes.
2. Add the 50% bleach solution into the embryo dishes such that the surface of the dish is covered, and the embryos are immersed.
3. Leave the embryos in the bleach solution for five minutes and swirl the plate gently every two minutes.

The 50% bleach solution strips the chorion layer of the embryos.

4. **Fume hood:** Prepare the fixation solution: In the fume hood, add 5 mL of heptane to the scintillation vial containing 4.5 mL of 1× PBS (phosphate buffer saline pH 7.4) and 0.5 mL of 37% formaldehyde–fix solution. Save this solution for step 10. This solution may have already been prepared for you.
5. Grab the glass vial placed over the nylon sieve.
6. After the embryos have incubated with the 50% bleach solution for five minutes, pour the contents of the embryo dish into the glass vial.
7. Wash the embryo dish to dislodge any remaining embryos. Add $^{dd}H_2O$ into the embryo dish and gently brush off the embryos into the solution with a paintbrush. Pour this mixture over the same sieve as step 6.
8. Repeat the step two more times.
9. Blot the bottom of the sieve with a paper towel and let it dry.
10. **Fume hood:** Take the paper towel and the sieve and bring into the fume hood. Using tweezers, dunk the sieve (embryo side in) into the scintillation vial with fixation solution.
    1. Collect more embryos from the side of the glass vial. Use a paintbrush and go over the walls of the funnel gently. Dip the brush into the fixation mix to dislodge the embryos.
11. Place the scintillation vial on a rotator for 20 minutes and allow the embryos to fix. You will get two layers of liquids with embryos between the top layer (heptane) and the bottom layer (PBS and formaldehyde).

The heptane is an organic molecule that produces holes in the vitelline membrane that allow the formaldehyde to access the plasma membrane and cross links proteins.

The PBS in the fix solution mimics physiological salt and pH levels. This provides osmotic balance.

12. During this wait period, rinse the sieve and glass vial. Leave them on a paper towel to dry.
13. **Fume hood:** Once the embryos are fixed, you will extract **the bottom aqueous layer** (PBS + Fix) with a glass Pasteur pipette and discard the solution in the waste container found in fume hood. You want to keep the organic later in the scintillation vial.
14. Add ~ 2 full Pasteur pipettes of methanol (MeOH) and **forcefully** shake the vial for 20 seconds.
15. **Fume hood:** The embryos will have broken open from their vitelline membrane, which will remain in the top layer (heptane) while the **embryos fall to the bottom** (MeOH). Discard the top layer lacking the embryos into the waste container in the fume hood.
16. **Fume hood:** With a plastic transfer pipette transfer the embryos to a clean test tube.
17. Wash the collected embryos with MeOH by removing the as much of the liquid in the tube as possible without disturbing the embryos, and refilling it new MeOH.
18. Repeat this wash step for a total of 3 times.

Methods: Immunostaining the *Drosophila* embryos

Day One:

1. Replace the MeOH with PBT (1× PBS with 0.3% Triton-X).
2. Wash the embryos 3 times with PBT and place on rotator for 20 minutes.
3. Remove the PBT from the test tube (until ~ 40 mL remains) and add 100 mL of PBT with 10 mL of normal goat serum (NGS). Place the test tube on an orbital shaker for 30 minutes.

NGS contains antibodies that will bind to nonspecific sites in the embryo, allowing the primary antibody to bind specifically to its target. This step is referred to as blocking.

4. 1 µL of **primary antibody** solution (Goat α–GFP) and incubate the mixture in an orbital shaker at 4°C overnight.

Day Two: (Steps to be completed by Course Lab Assistant)

1. To remove background staining, wash the embryos. Remove the blocking and primary solution and wash with PBT. Place the test tube on a rotator for an hour. Repeat this a total of four times.
2. Remove as much PBT as possible and add 100 µL PBT and 10 µL NGS to re-block and place on orbital shaker for 35 minutes.
3. Add 1 mL of **secondary antibody** solution (HRP-conjugated α–Goat) and incubate for 2 hours at room temperature.
4. Place the embryos on a rotator for two hours with periodic (a total of five) PBT washes.
5. Remove the PBT as much as possible, leaving ~100 µL solution.

Methods: Visualizing the reporter

1. **Fume hood:** Add 100 µL of DAB (PBT containing 0.33 mg/mL diaminobenzidine) and 200 µL PBT to the embryos.
2. DAB is a carcinogen, handle with care and use gloves. Neutralize any unused DAB with bleach.
3. Start the reaction by adding 3 mL of H2O2 (3%).
4. Monitor the reaction through the dissecting microscope. Once the desired signal and depth of colour is attained, stop the reaction by diluting the embryos with PBT.
5. Wash the embryos thrice with PBT.
6. Remove as much PBT as possible and dehydrate embryos with EtOH in gradation: 50%, 70%, 90%, 95%, and 3× 100%.
7. Remove as much EtOH as possible and preserve embryos in methyl salicylate

Week Two | Activity 3: Imaging Drosophila samples

For the final week of this laboratory exercise, you will photograph the completed **immunohistochemistry** staining of the *Drosophila* embryo developmental stages. From these photographs, you will submit a scientific figure appropriate for scientific publication. This figure should be well labelled and be generated as if you were reporting the expression pattern for the first time.

Deliverables

At the end of the two-week lab period, you will prepare a mini-lab report that will be separated into two parts.

FRUIT FLY LAB REPORT (/20)

Part 1: Research Quality Figure (/10) (1 page)

With your photographs, you will make a figure with at least 3 panels that shows the expression pattern of tup-GFP at different stages of development (refer to **Appendix B** for staging) as seen by sagittal and frontal views. The figure will require a detailed legend indicating the scale, orientation, and significant features of each panel. Figure must be followed by a descriptive caption (figure legend) detailing each panel. Do **NOT** include protocols in your figure legend. If there is morphology, identify the orientation and magnification of the sample. Keep the orientation and the views of the embryos consistent. You can use any software available (such as ImageJ, PowerPoint, and Word) to generate these figures. Please check **Appendix C** for tips and clues on how to assemble a "publication worthy figure".

Part 2: Worksheet Questions (/10) (1– 2 pages)

Alongside your figure, submit answers to the following questions.

1. What regulated the spatial pattern of reporter expression? (/1)
2. Can reporter constructs stably inserted in an organism genome be deleterious to this organism? Explain. (/3)
3. With information gleaned from FlyBase, Flymove and Interactive Fly websites (see posted reference files in Avenue sourced from those), summarize the nature of the *tup* gene product. (What kind of protein and signal transduction pathway?) (/3)
4. Under what circumstances can the pattern of expression of a hypothetical gene detected using immuno-labelling differ from that detected using a reporter construct? (/3)

Laboratory 4 Demo Video

One or more interactive elements has been excluded from this version of the text. You can view them online here: https://ecampusontario.pressbooks.pub/molbiol3m03/?p=293

PART V

LABORATORY #5 DANIO RERIO : HOW MORPHOGEN GRADIENTS SHAPE THE SIZE OF PATTERNING

Overview

Danio rerio is model organism for the study of vertebrate developmental biology. The embryos of the *D. rerio*, the zebrafish, develop rapidly and exist independently of their mother. Most importantly, however, these embryos are transparent and can be observed under light microscopy. For this laboratory, you will be familiarizing yourself with how cell signaling dynamics instruct the embryonic patterning and how it can be impacted by pharmacological perturbations.

Lab Components and Objectives

This lab will have 3 activities spread over the course of two weeks.

Activity 1. Drug suppression of FGF signaling in live embryos

1. Observe metameric backbone planning common in vertebrates
2. Through microscopy, observe various developmental stages and learn about basic aspects of the zebrafish embryogenesis
3. Observe the effects of SU5402 on embryogenesis of zebrafish

Activity 2. Immunohistochemistry of phosphorylated ERK in zebrafish tail

1. Learn how to perform immunohistochemistry against a phospho-protein in zebrafish embryos

Activity 3. Imaging p-ERK immunostaining

Measure and quantify the effects of SU5402 treatment on the gradient of phosphorylated ERK in the tail of zebrafish

Introduction

All vertebrate embryos pattern their longitudinal (head-to-tail) embryonic axis with **somites**, metameric structures that segment out of the **presomitic mesoderm** (PSM). A sequential process known as somitogenesis connects gastrulation to organogenesis during embryo development (see **Appendix D**). In this process, the **PSM** segments into bilateral pairs of **somites**, from the right and left side of the embryonic midline. While the size and total counts of **somites** vary between species, the regulation of this process is well conserved. **Somites** are functionally important during embryogenesis as they mark positional information for other cells to migrate and pattern organs during organogenesis. Ultimately, **somites** themselves give rise to back muscle, skin, vertebrae, connective and adipose tissues.

In all vertebrates, somitogenesis is guided by a molecular clock. The Hairy-enhancer-split-related gene family (Her/Hes) transcriptional regulators dimerize and drive clock-like molecular oscillations during embryonic development. The oscillation frequencies observed are species dependent – the rate of Her/Hes mediated oscillations in zebrafish are ten-times faster than in humans, with two oscillations taking place every hour. The quick embryogenesis of zebrafish enables us to observe embryonic development rapidly, making them a powerful model system for understanding vertebrate embryogenesis.

You will be investigating the FGF signalling cascade pathway in zebrafish embryogenesis. FGF is a **morphogen** that triggers the phosphorylation of extracellular receptor kinase (ERK) (Figure 1). You will conduct immunostaining on fixed D. *rerio* embryos treated with SU5402, a pan-FGF receptor inhibitor, against the phosphorylated ERK (p-ERK) as a read out of **morphogen** concentration. You will additionally assess the morphological consequences of SU5402 treatment during embryogenesis by treating live embryos with the drug. As the p-ERK **morphogen** gradient in **tail bud** shrinks, one would expect cells commit into making **somites** precociously. This would result in formation of bigger **somites**. Altogether, in these lab activities you will learn the functional role of the FGF signalling cascade during embryogenesis and observe the morphological consequences of its perturbation.

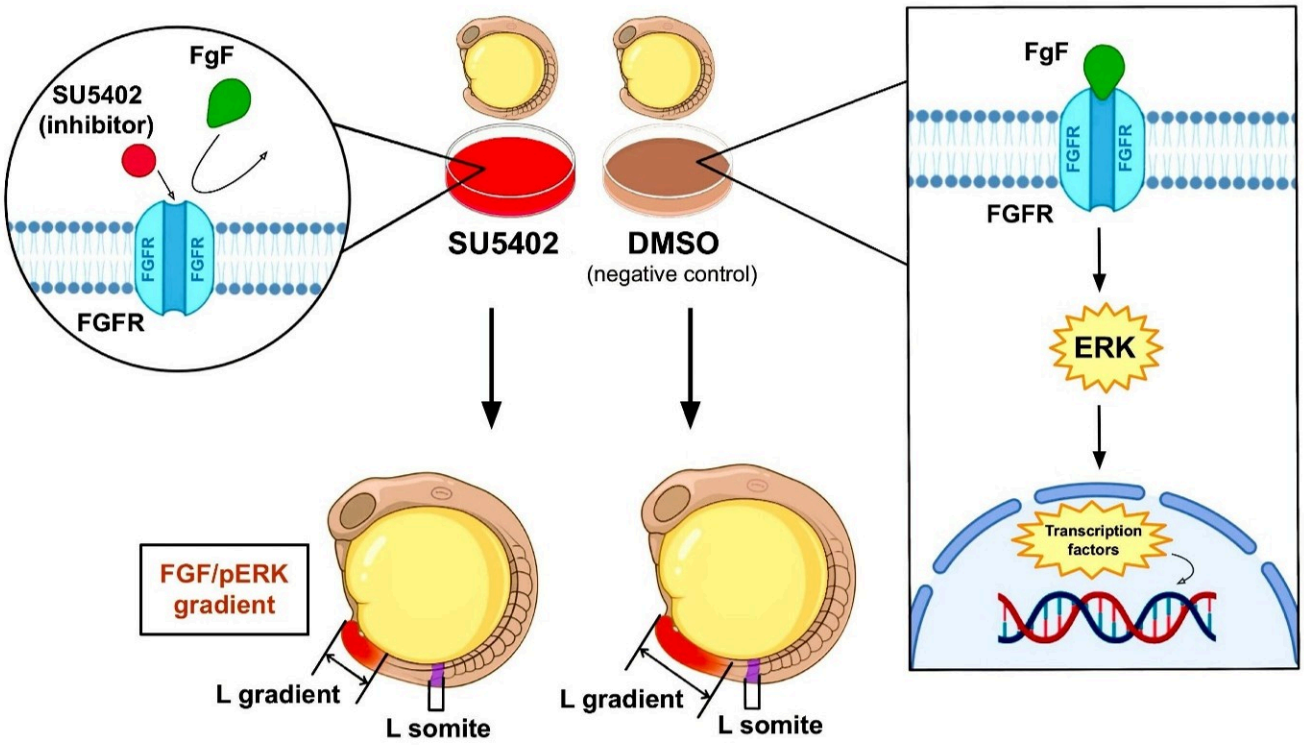


Figure 1 | Pharmaceutical investigation of FGF/ERK signaling gradient's role on segmental patterning.

Key Terms and Concepts

Morphogen: is a chemical signal that governs development of cell-types and tissues based on its concentration gradient during morphogenesis.

Embryonic mesoderm: is the middle embryonic germ layer that gives rise to the development of organ and tissue structures during embryogenesis.

Presomitic mesoderm: is the tail (posterior) portion of the paraxial mesoderm flanking embryo axis, formed with the neural tube in the neurulating embryo.

Somites: are a set of bilaterally paired blocks of presomitic mesoderm formed on the head-to-tail axis during embryogenesis.

Tail bud cells: are the cells that end up in the elongating tail mesoderm with gastrulation movements. The chemical signalling gradients sourced from these cells inform cells to commit in making somites.

Week One | Activity 1: Drug treatment of live zebrafish embryos

This week of experiments will be divided into two procedures, one requiring treatment of living embryos with SU5402 and the other immunostaining fixed embryos. **Designate a pipette for each given activity, such that you use the same pipette throughout the treatment of live embryos and a different pipette for the treatment of fixed embryos.**

Drug treatment of live zebrafish embryos

For this exercise, you will be treating live zebrafish embryos with SU5402 drug and visualizing the morphological consequences of disrupting the FGF-signaling cascade. Using the compound microscope, you will record observed developmental differences between the SU5402 and control DMSO treated samples.

Handle all drugs and solutions with proper care! You MUST wear gloves for this procedure.

Methods: Suppression of the FGF signaling pathway

1. Prepare 25 micromolar SU5402 solution in treatment dish by diluting the 10 mM stock solution in 4 mL fish system water. Prepare an equal dilution of dimethyl sulfoxide (DMSO, the drug-carrier-solvent) in equal volume of system water as a separate negative control dish.

SU5402 is a light-sensitive drug. To minimize exposure, wrap the stock solution in aluminum foil and put the treatment dish in a opaque box.

2. Inspect dechorionated zebrafish embryos under the stereoscope and group some 30 embryos staged as close as possible to 14 **somites** stage (12-16 **somite** stage) (use **Appendix D** as a guide for proper staging) with the help of a syringe needle. Transfer those embryos into a separate petri dish of fish system water

using glass Pasteur pipettes.

Care should be taken for not damaging live embryos during transfer processes.

3. With glass Pasteur pipette, Move 15 embryos into the SU5402 treatment dish. Keep embryos at the tip of glass pipette and only transfer the embryos. This measure is essential to minimize additional liquid transfer which may dilute the drug concentration. Start timer for 30 minutes drug treatment.
4. Transfer the remaining 15 embryos into the negative control (DMSO) dish.
5. Wait 30 minutes for the treatment.

At this stage, start working on Activity 2 Day One at step 1. Be sure to change the glass pipette tip, as any detergents or salt solutions mixing into live embryos would kill the embryos and abort the successful experiment. ***Change tips!***

6. After 30 minutes, move the embryos out of the drug/DMSO solutions into a fresh fish system water petri dish.
7. Wash the embryos by swirling the dish.
8. Inspect the embryos under a stereoscope and remove any of the damaged/yolk leaking/unhealthy looking embryos to a separate dish for proper disposal.
9. Dispose of the drug solutions in a properly labeled drug waste falcon tube (keep the tube in dark).
10. Immediately transfer embryos into the 32 °C incubator and incubate 75 minutes for further development of **somites**.

During this 75-minutes period, return to working on fixed embryos to complete Activity 2 Day One: (continued) *(from step 1). Be sure to change the glass Pasteur pipette tip!*

11. Remove the samples from the incubator and prepare to observe the embryos under the compound microscope.

1. Your TAs will provide a 3% methyl cellulose in fish system water as a live-embryo friendly viscous medium. Use this to orient the embryos laterally with the help of a syringe.

Avoid bubbles! Instead of pipetting, decanting might be preferred.

2. Using a compound light microscope, take images of the antero-posterior lengths of last formed 3-4 **somites** for each embryo. **These will be used in your lab report.**

Week One | Activity 2: Immunohistochemistry in zebrafish embryos

For this exercise, you will be immunostaining previously fixed zebra fish embryo treated with SU5402 against phosphorylated extracellular receptor kinase (ERK), which is the downstream signaling receptor of the FGF signaling cascade. This procedure will take place over the course of two days, and the procedure of Day 1 will be carried out by you and your lab mates. Day 2 will be carried out by the course lab assistants. In the following week, you will visualize the stained fixed embryos. **Clearly label your tubes so you may track them the following week.**

Methods: Preparing the fixed embryos

Day One:

1. Rehydrate the zebrafish embryos in 2 mL vials which were dehydrated in 100% MeOH in following 3 steps on orbital shaker:
    1. Replace 250 µL of 1 mL MeOH with PBSTx (0.1% Triton-X-100 detergent in phosphate buffer saline) and incubate for 5 mins.
    2. Replace 500 µL of 1 mL MeOH-PBSTx with PBSTx and incubate for 5 minutes.
    3. Replace all solution with 1 mL of PBSTx and incubate for 5 minutes.
2. Replace the PBSTx wash buffer with 1 mL of permeabilization buffer (PBS-Tx2%, with higher detergent concentration) and incubate for 30 minutes on orbital shaker.

At this stage, you can return to Activity 1 at step 6. ***Change pipette tips!***

Methods: Immunohistostaining phosphorylated ERK (p-ERK) proteins in zebrafish **tail**

Day One: (continued)

1. Prepare 2 mL serum block solution by diluting 2% fetal bovine serum and 1% goat serum in MABDT (Maleic acid buffer, 1%DMSO, 0.1% Triton-X 100).

2. Replace the permeabilization buffer in vials with 1 mL serum block solution (MABDT-F-G) and incubate for 1 hours.
3. During serum block incubation, prepare antibody stock solution (0.3 mL per vial, i.e., 0.6 mL in total) by diluting 0.6 µL of p-ERK antibody 1:1000 in MABDT-F-G.
4. Replace the serum block fully with the 300 microliters of antibody solution, **make sure the vials are properly labeled with drug vs. DMSO condition and your lab group number**, leave vials for overnight incubation at 4 °C while rocking.

Now you can return to working on live embryos to complete Activity 1 (step 11). Be sure to change the glass Pasteur pipette tip!

Day Two: (steps to be completed by Course Lab Assistant)

1. Wash three times in MABDT for 5 min at room temperature.
2. Incubate the embryos in MABDT solution with an HRP-conjugated anti-mouse secondary antibody for 2 h at room temperature or overnight at 4 °C while rocking.
3. Wash three times in PBSTx for 5 min at room temperature.
4. During the washes prepare the DAB solution: Mix 50 µL of 1% DAB (3,3'-diaminobenzidine) dissolved in RO-grade water and 50 µL of 0.3% hydrogen peroxide and bring to 1 mL with PBS.
5. Fully submerge the embryos with the DAB solution prepared and monitor vials under the stereoscope for color development (typically ~5 minutes).

DAB is hazardous. Make sure to wear gloves and dispose of DAB contaminated liquids in properly labelled waste tubes.

6. After reaching the desired levels of color development, stop the coloring reaction by rinsing the embryos for 3-5 minutes in PBS.
7. Re-fix the embryos by transferring into 4% PFA and keep in the fridge until Week 2 of experiments.

Week Two | Activity 3: Imaging p-Erk staining after IHC

You will be visualizing the p-ERK gradient in the zebrafish **tail** following drug treatment with SU5402 and compare to the DMSO-treated control. You will measure the length (A-P span) of p-ERK gradient in SU5402 treated and DMSO control embryos and these measurements will be important for your lab report.

Methods: Visualizing p-ERK

1. Wash the embryos out of IHC immersed in 4% PFA three times in PBSTx for 5 min each on shaker.
2. Inspect embryos from SU5402 treated vial and DMSO control vial by transferring them one-by-one or in groups of couple embryos at once with glass Pasteur pipettes into a petri dish with 85% glycerol (minimize additional liquid transfer and avoid making bubbles).
3. With the help of syringe needle and viscous glycerol solution, orient the embryos laterally for proper imaging under the microscope.
4. For each embryo in both treatment groups, take a picture of the DAB-stained p-ERK gradient in the **PSM** of a laterally oriented embryo. **These pictures will be used for the measurements in your lab report, ensure that they are clear.**

Deliverables

ZEBRAFISH LAB REPORT (/20)

Your lab report should include 5 sections as detailed below. At least 11pt font-size, with 1.5 line-spacing for the text. Total length of the report should be ~ 4 pages (±1) including your references.

1. **Introduction** (1/2 page, 2-3 paragraphs) (/3)
2. **Materials and Methods** (1/2 – 1 page max., not a replicate of the procedure above, check methods section of any published developmental biology paper if unsure about how this works) (/2)
3. **Results** (provide your measurements for each sample as described in the protocol in tables followed by proper statistics, mean and standard deviation) (/4)
4. **Discussion** (answer 10 questions posited below) (/10)
5. **References** (APA style, with IN-TEXT reference) (/1)

For the lab report, you will use the images you took from Activity 1 and Activity 2. With them, you will measure and compare the mean **somite** size of your live zebrafish embryos between the SU5402 treated vs. DMSO and measure the length (A-P span) of p-ERK gradient in SU5402 treated and DMSO control embryos. **Each image must be included in your sampling.** You can measure the distances with software like FiJi or ImageJ. **Apply the proper statistical tests to compare the effects of SU5402 treatment vs. DMSO treatment on embryogenesis.** Use these images to answer the following questions:

1. Determine which **somite** size is impacted from the drug treatment performed at 14 **somite** stage.
2. Why do you see the delay between drug treatment stage and impacted **somite** stage?
3. What is the average length increase due to 30 min SU5402 treatment at 25 micromolar?
4. What would you expect to see if you
    1. kept the embryos in the drug longer?
    2. had increased the concentration of drug twice?
5. Do you see any visual differences in between two gradients in the strength of staining, span of the gradient etc.?
6. Report how the drug treatment impact FGF/p-ERK signalling in the **PSM** tissue.
7. How long is the average span of control embryo gradient?
8. How long is the average span of the p-Erk gradient for drug-treatment group?
9. Are they significantly (do the statistics!) different from each other?
10. Does the change in the length of the signaling gradient come close to the **somite** size changes you observed in Week 1 of the live experiments? Comment on why or why not.

Laboratory 5 Demo Video

One or more interactive elements has been excluded from this version of the text. You can view them online here: https://ecampusontario.pressbooks.pub/molbiol3m03/?p=330

PART VI

LABORATORY #6 CAENORHABDITIS ELEGANS : CURING A MUTANT PHENOTYPE BY RNA INTERFERENCE

Overview

Caenorhabditis (see-no-rab-dye-tis) *elegans* is a non-parasitic, non-pathogenic roundworm model organism used to understand physiology, neurology, and developmental biology. They are cheaply propagated, can self-fertilize as a hermaphrodite, and are easily observed under a microscope throughout their lifecycle. Importantly, a *C. elegans* comprises a precise number of somatic cells (959 in adult hermaphrodite), making it a powerful organism for cell determination and specification. While many genetic tools have been developed for this system, one of the most powerful is *C. elegans* susceptibility to RNA interference (RNAi). This gene silencing technique can be carried out in a variety of ways (**feeding**, microinjection, soaking) and allows for researchers to assess a variety of phenotypes without directly mutagenizing the organism. For this laboratory, you will conduct a RNAi experiment through **feeding** and explore the effects of gene silencing on a *C. elegans* phenotype.

Lab Components and Objectives

This laboratory will have three activities which will take place over the course of two weeks.

Activity 1. Scoring *C. elegans* behavioral phenotypes

1. Observe *C. elegans* ***rol-6*** **mutant** and compare growth to wild-type roundworm
2. Generate a scoring sheet to assess range of aberrant movement in *C. elegans* for Activity 1

Activity 2. Phenotype suppression through RNAi

1. Conduct an RNA interference procedure on **rol-6** ***C. elegans***
2. Become familiar with growing, handling, and manipulating *C. elegans* eggs and adults

Activity 3. Observe developmental phenotypes in adult worms

1. Score and assess effect of RNAi on adult ***rol-6*** **mutant** worms based on assessment criteria developed in Activity 1

Introduction

The model organism *C. elegans* has been widely used to investigate many health-related phenomena, including aging, neurodegeneration, and physiology. You will be working with a *C. elegans* strain with abnormal **cuticle** production. Many proteins mediate the formation of a healthy **cuticle** and body, and today you will be observing the effects of the ***rol-6* (su1006) II mutant** allele on *C. elegans* morphology. Wild-type ***rol-6*** encodes for the **cuticle** collagen rol-6 protein, but the **su1006 allele** results in the production of an aberrant collagen that still integrates into the **cuticle** matrix. Its incorporation results in a helically twisted **cuticle** and body, which alters the locomotion pattern. While wild-type *C. elegans* move in a sinusoidal wavelike pattern, the muscle contractions of ***rol-6* (su1006)** are abnormal and instead cause the nematode to roll over about its longitudinal axis and move in counterclockwise circles (right-handed rollers – RRol). Notably, when *C. elegans* lacks *rol-6* expression, their movement patterns are indistinguishable from that of wild type. This suggests *rol-6* is non-essential in *C. elegans* and the collagen produced by ***rol-6* (su1006)** compromises the whole **cuticle** structure.

Within the following laboratory exercises, you will use RNA interference (RNAi) to suppress gene expression. RNAi largely works by the introduction of double-stranded RNA (dsRNA) to block translation. In *C. elegans*, RNAi can be carried out through **feeding**, where the nematodes are fed bacteria producing the dsRNA and ingestion facilitates the uptake of the targeting dsRNA. For this exercise, you will feed *Escherichia coli* harboring either an empty vector or a plasmid-borne inducible dsRNA (anti-rol-6) to wild-type and ***rol-6* (su1006) II mutant** allele *C. elegans*.

Key Terms and Concepts

Genetic Terms

Feeding: is the experimental protocol where roundworms are fed transgenic bacteria that express target proteins or nucleic acids.

Reverse genetics: is the "gene-forward" study of a phenotype. The gene is directly targeted, and its role is then assessed.

Cuticle: is the barrier between *C. elegans* environment and body. It is an extracellular matrix built by crosslinked small collagen-like proteins that maintain the shape of the nematode.

C. elegans Strain Nomenclature

rol-6* (su1006) II *mutant:

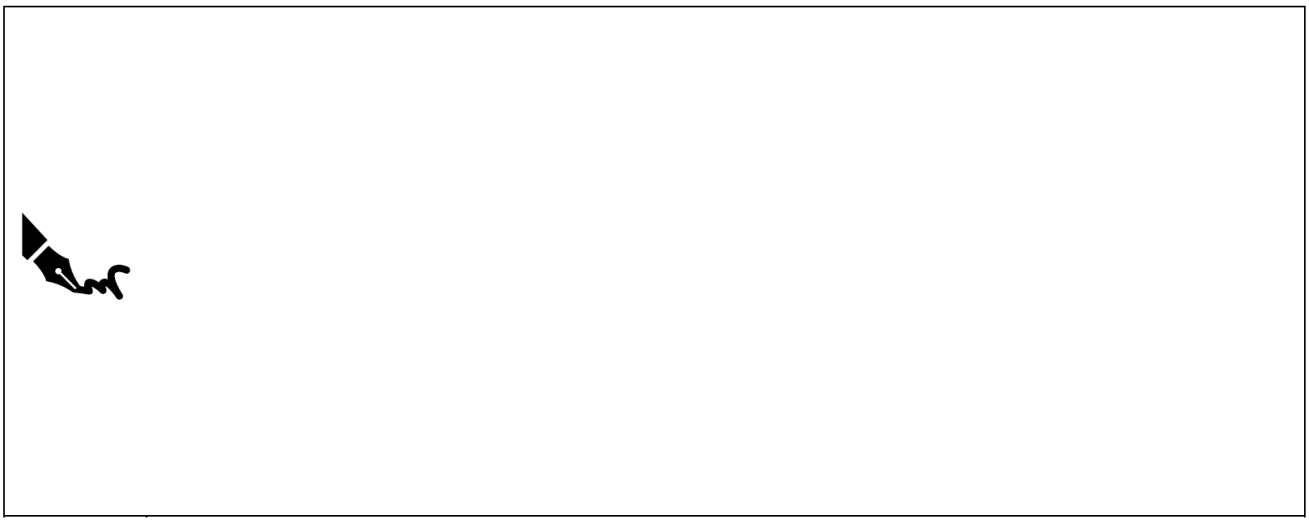
rol refers to the phenotypic abnormality (rollers) **-6** refers to the specific gene that causes the phenotype **(su1006)** the specific allele causing the mutation **II** indicates the linkage group.

Week One | Activity 1: C. elegans behavioural phenotype

In this week, you will begin by observing a handful of *C. elegans* mutants and generating a scoring mechanism to score locomotion. **This scoring sheet will be used to assess the effects of RNAi treatments during Week 2.** Following this, you will prepare your RNAi experiments. **Begin Activity 2 as soon as you can, since it takes quite a bit of time.**

C. elegans behavioral phenotype

Use your dissecting microscope to view the various *C. elegans* mutants that have been provided. Your TA will provide you with a list of the mutants and describe their phenotype. **You will decide how you will score movement patterns for the lab next week based on these observations and further independent research.**



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Week One | Activity 2: RNAi suppression of the mutant phenotype

The course lab assistants will have prepared the necessary plates for this activity. These plates contain isopropyl β-D-1-thiogalactopyranoside (IPTG) – a non-hydrolysable lactose analog that induces expression of the T7 DNA polymerase in our *E. coli* strain. In turn, this will result in the transcription the plasmid-encoded dsRNA, which is under the control of a T7-specific promoter. Altogether, this system requires IPTG to induce expression of the T7 polymerase, which then transcribes the dsRNA that interferes with **rol-6** expression in *C. elegans*. You will be given two sets of these plates, one of which will have a lawn of the empty-vector (EV) *E. coli* control and the anti-rol-6 encoded dsRNA encoding (anti-rol-6) *E. coli*.

The purpose of this week will be to set up the RNAi experiment. You will first isolate eggs from the two *C. elegans* strains: wild-type and ***rol-6* (su1006) II mutant**. You will seed these eggs on the plates prepared for you. For this experiment, our negative control will be ***rol-6* (su1006) II mutants** fed with EV *E. coli* that does not transcribe the dsRNA. The positive control will be wild type *C. elegans* fed with anti-rol-6 *E. coli*. For our experimental group, ***rol-6* (su1006) II mutants** will be fed anti-rol-6 *E. coli*. These experiments will be done in duplicate.

Read through all the steps below before beginning.

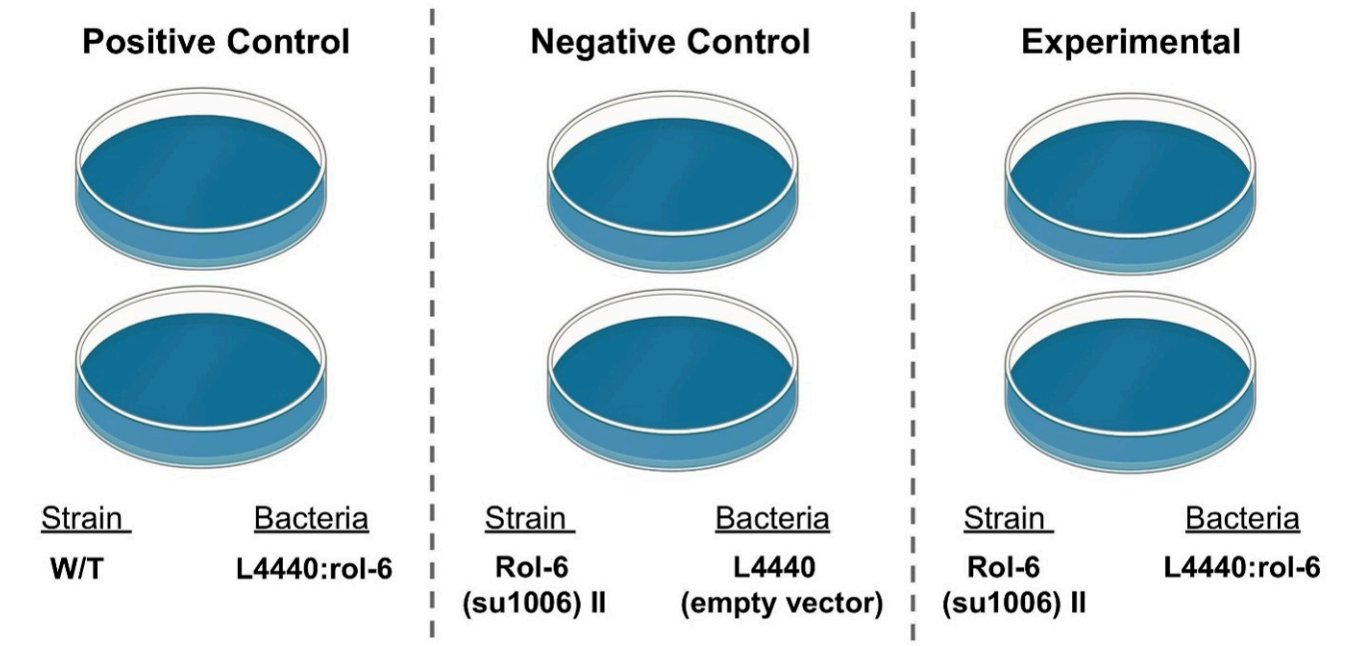


Figure 1 | Overview of the experimental setup with positive and negative controls and experimental plate, two duplicates for each.

Methods: Isolating the wild-type *C. elegans* eggs

You will be working with 10M NaOH, so you must wear a lab coat and googles.

1. From your TAs, obtain: two plates of wild-type (W/T) adult *C. elegans*, a 15 mL falcon tube. Label the 15 mL tube with your strain (W/T) and initials.
2. After removing the lid, add 3 mL of M9 liquid media into a single plate.
3. Dislodge the worms from the agar surface by laying the plate flat on the bench and gently moving it in a figure-eight pattern.
4. Transfer this same solution to the second plate of worms and swirl again.
5. Carefully, transfer the solution into your labeled falcon tube.
6. Repeat step 2 – 5 two more times to thoroughly wash the agar plates and extract all worms. You should have ~ 9 – 15 mL in your falcon tube.
7. Place your initialed falcon tube in the clinical microcentrifuge and balance it with another tube containing an equal amount of water.
8. To isolate and pellet the worms, close the lid and set the dial on the centrifuge to #2. Run the spin for three minutes.
9. When removing the falcon tube from the centrifuge, keep it vertical. Hold the tube up to the light to see if the worms have pelleted to the bottom. If not, centrifuge for an additional 2 minutes.
10. Careful not to dislodge the pellet remove the supernatant until you have ~1 mL of worm solution left.
11. Pipetting carefully, take up 775 µL of the worm solution and put it in a 1.5 mL tube. Label this tube with your initials and worm strain (W/T).

*These next steps involve bleaching the eggs and are **very time sensitive**. Make sure you have your pipettes (P1000, P200), tips, and solutions (bleach, NaOH and M9) ready. If you expose the eggs to bleach for more than five minutes they will be destroyed.*

12. To dissolve the worms, add 25 µL of 10N NaOH and 200 µL of bleach into the tube. Close the lid and start a timer for **five minutes**, inverting gently.
13. Every minute, observe the tube underneath the dissecting microscope. **Once the worms are dissolved, or after 4 minutes**, place the tube in the microcentrifuge and balance the centrifuge with another tube containing 1 mL of $^{dd}H_2O$. Centrifuge at 3500 RPM for 1 min. Make sure the microcentrifuge is balanced.
14. Carefully remove your tube from the microcentrifuge so as not to disturb the pellet. You should be able to see a small, white pellet at the bottom of the tube –these are the eggs.

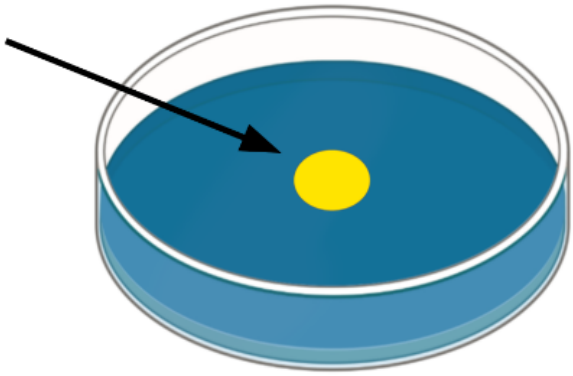
15. To wash the cells, remove 850 µL of supernatant from the tube, being careful not to disturb the pellet. Add 850 µL of M9, close the lid and gently invert and mix the eggs.
16. Centrifuge again at 3500 RPM for 1 minute.
17. Repeat steps 14-16 to wash the eggs a total of three times.
18. Remove 850 µL and add 200 µL of M9.
19. Gently mix the eggs until the solution is homogenous.

Methods: Quantifying the wild-type *C. elegans* eggs

1. Remove a 10 µL aliquot from the egg solution and place it on a coverslip. Observe under the dissecting microscope at 40×.
2. You will want to spot 10 µL on your bacterial plates to add approximately 50 – 100 eggs. **Determine the volume of mixture** you will need to add to your RNAi plates.
    1. If your cells are too concentrated, dilute the mixture and re-quantify. If they are too dilute, add more volume to the RNAi plates (i.e., Add 30 µL if you have 15 – 35 eggs in a 10 µL droplet).

Methods: Seeding the wild-type *C. elegans*

1. Light the Bunsen burner to create a sterile environment. Ensure that you are operating with proper aseptic technique at the following steps.
2. For seeding W/T *C. elegans* eggs, obtain two plates with *E. coli* anti-rol-6 lawns. Label the bottom of these plates with your initials, the bacterial strain, the *C. elegans* strain and the date.
3. In the sterile environment carefully remove the lid from each plate and add the calculated volume (from step 2 of **Methods**: Quantifying the wild-type *C. elegans* eggs) to the center.
4. Place the lid back on, turn off the Bunsen burner, and move the plates to the side of your desk and allow



the egg solution to dry.

Methods: Isolating the mutant *C. elegans* eggs

1. From your TAs, obtain: two plates of ***rol-6* (su1006) II** adult *C. elegans*, a 15 mL falcon tube. Label the 15 mL tube with your strain (***rol-6* mutant**) and initials.
2. Repeat steps 2 – 19 from **Methods**: Isolating the wild-type *C. elegans* eggs for the ***rol-6* mutant** *C. elegans*.

Methods: Quantifying the mutant *C. elegans* eggs

1. Remove a 10 µL aliquot from the egg solution and place it on a coverslip. Observe under the dissecting microscope at 40×.
2. You will want to spot 10 µL on your bacterial plates to add approximately 50 – 100 eggs. **Determine the volume of mixture** you will need to add to your RNAi plates.
    1. If your cells are too concentrated, dilute the mixture and re-quantify. If they are too dilute, add more volume to the RNAi plates (i.e., Add 30 µL if you have 15 – 35 eggs in a 10 µL droplet).

Methods: Seeding the mutant *C. elegans*

1. Light the Bunsen burner to create a sterile environment. Ensure that you are operating with proper aseptic technique at the following steps.
2. For seeding ***rol-6* mutant** *C. elegans* eggs, obtain four plates: two plates with lawns of EV *E. coli* and two plates with lawns of *E. coli* anti-rol-6. Label the bottom of these plates with your initials, the bacterial strain, the *C. elegans* strain and the date.
3. In the sterile environment carefully remove the lid from each plate and add the calculated volume (from from step 2 of **Methods**: Quantifying the mutant *C. elegans* eggs) to the center.
4. Place the lid back on, turn off the Bunsen burner, and move the plates to the side of your desk and allow the egg solution to dry.
5. In the container for your lab section, place your plates with the bottom side facing up. They will be incubated at 17 °C for a week to allow the *C. elegans* to reach adulthood.

Week Two | Activity 3

This week we will score the young adult or adult stage worms to determine the effect of our RNA interference experiment based on the criteria you devised in the previous week. Recall that you have 6 plates (3 groups in duplicate):

	***C. elegans* Genotype**	***E. coli***
Experimental	***rol-6* (su1006) II**	Anti-rol 6
Positive Control	Wild type	Anti-rol 6
Negative Control	***rol-6* (su1006) II**	Empty vector

For this activity, you will be observing the *C. elegans* you had plated in the previous week. You will want to transfer the worms onto fresh plates and observe their movements under the microscope. Before beginning this, consider what kind of locomotory phenotype do you expect to see for each of the three groups after ingestion of the bacteria on the plate?

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Methods: Transferring the worms

Read through all the steps below before beginning.

1. From your TAs, retrieve your six plates and six 1.5 mL Eppendorf tubes.
2. To each of the plates, add 1.5 mL of M9 buffer gently swirl to dislodge the worms.
3. Pipette 1 mL of the worm solution from the plate into a 1.5 mL labeled with which experimental replicate and group you are transferring.
4. Gently mix the tube by inverting several times. Using a P20, remove a 10 µL aliquot, place it on a fresh NGM plate.
5. Using your dissecting microscope, make a rough count of the number of worms in the 10 µL droplet. To observe their locomotion, you will want ~ 10-20 worms per 10 µL droplet.

Methods: Observing the worms

1. For both replicates, observe the movements of the different experimental groups through your dissecting microscope. Fill out the information either in the table below or in the form (if you choose to fill out the information in the table, please append it to the Lab 6 Notes Export file):

Condition	Sinusoidal Movement		Right-hand Rollers		No Movement	
	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
Positive Control						
Negative Control						
Experimental						

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Deliverables

C. ELEGANS **LAB REPORT (/20)**

Your lab report should include five sections as detailed below. At least 11pt font-size, with 1.5 line-spacing for the text. Total length of the report should be ~ 5-6 pages (±1) including your references.

1. **Introduction** (1/2 page, 2-3 paragraphs) (/3)
    1. Include known theory and the relevant background material for the experiments being reported. You will need to do some extra research for this section.
    2. State the purpose of the study in this section.
2. **Materials and Methods** (1/2 – 1 page max., not a replicate of the procedure above, check methods section of any published developmental biology paper if unsure about how this works) (/2)
3. **Results** (1-2 pages) (/5)
    1. Write this section in paragraph form. Include figures, images, tables in an appendix.
4. **Discussion** (1-2 pages, answer the 8 questions posted below) (/8)
5. **References** (APA style, with in-text references) (/2)

Questions for discussion:

1. Explain how dsRNA is produced by the *E.coli* used in our experiment and why you used IPTG.
2. Why do we need to use a positive and a negative control?
3. What kind of locomotory phenotype do you expect to see for each of the three groups after ingestion of the bacteria on the plate?
4. How does the dsRNA enter the *C. elegans* in this experiment?
5. Should RNAi targeting the *rol-6* mRNA affect the phenotype of wild type strains?
6. Should RNAi targeting the *rol-6* mRNA suppress the phenotype of the ***rol-6*** **(su1006)** worms?
7. What kind of mutation is ***rol-6*** **(su1006)** regarding the pattern of inheritance?
8. What do you think the results of your experiments would be if the collagen encoded by the *rol-6* gene were an extremely stable protein (i.e., long half-life)?

Laboratory 6 Demo Video

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PART VII

LABORATORY MANUAL APPENDICES

APPENDIX A

Stages of chick development according to Hamburger and Hamilton (1951). Refer to the resource paper shared on Avenue for images.

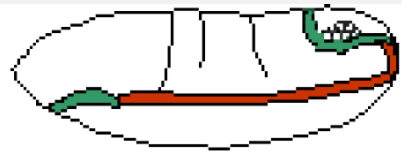
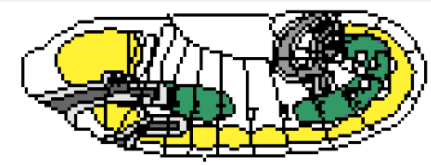
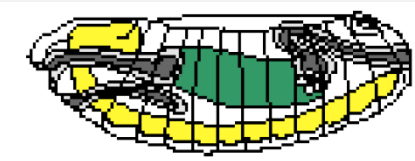
Hamburger – Hamilton Stages	Age	Identification of Stages
Before Laying		
Early cleavage	3.5-4.5 h	Shell membrane of egg formed in isthmus of oviduct
During cleavage		Germ wall formed from marginal periblast
Late cleavage	4.5-24.0 h	Shell of egg formed in uterus
After Laying		
1		Pre-primitive streak (embryonic shield)
2	6-7 h	Initial primitive streak, 0.3-0.5 mm long
3	12-13 h	Intermediate primitive streak
4	18-19 h	Definitive primitive streak, ±1.88 mm long
5	19-22 h	Head process (notochord)
6	23-25 h	Head fold
7	23-26 h	1 somite; neural folds
7 to 8-	ca. 23-26 h	1-3 somites; coelom
8	26-29 h	4 somites; blood islands
9	29-33 h	7 somites; primary optic vesicles
9+ to 10-	ca. 33 h	8-9 somites; anterior amniotic fold
10	33-38 h	10 somites; 3 primary brain vesicles
11	40-45 h	13 somites; 5 neuromeres of hindbrain
12	45-49 h	16 somites; telencephalon
13	48-52 h	19 somites; atrioventricular canal
13+ to 14-	ca. 50-52 h	20-21 somites; tail bud
14	50-53 h	22 somites; trunk flexure; visceral arches I and II, clefts 1 and 2
14+ to 15-	ca. 50-54 h	23 somites; premandibular head cavities
15	50-55 h	24-27 somites; visceral arch III, cleft 3
16	51-56 h	26-28 somites; wing bud; posterior amniotic fold
17	52-64 h	29-32 somites; leg bud; epiphysis
18	3 d	30-36 somites extending beyond level of leg bud; allantois
19	3.0-3.5 d	37-40 somites extending into tail; maxillary process
20	3.0-3.5 d	40-43 somites; rotation completed; eye pigment
21	3.5 d	43-44 somites; visceral arch IV, cleft 4

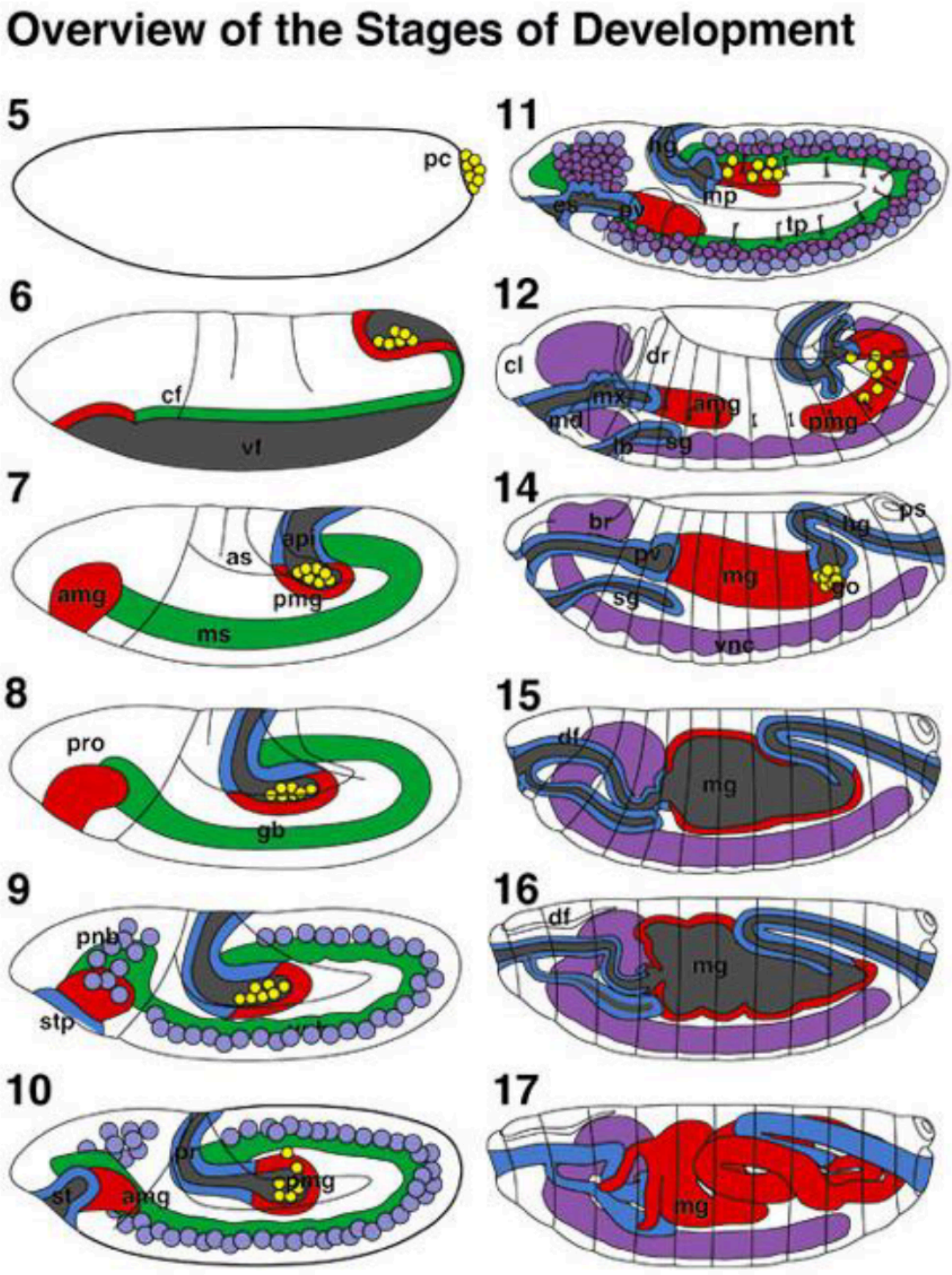
Hamburger – Hamilton Stages	Age	Identification of Stages
22	3.5-4.0 d	Somites extend to tip of tail
23	4 d	Dorsal contour from hindbrain to tail is a curved line
24	4.5 d	Toe plate
25	4.5-5.0 d	Elbow and knee joints
26	5 d	1st 3 toes
27	5.0-5.5 d	Beak
28	5.5-6.0 d	3 digits, 4 toes
29	6.0-6.5 d	Rudiment of 5th toe
30	6.5-7.0 d	Feather germs; scleral papillae; egg tooth
31	7.0-7.5 d	Web between 1st and 2nd digits
32	7.5 d	Anterior tip of mandible has reached beak
33	7.5-8.0 d	Web on radial margin of wing and 1st digit
34	8 d	Nictitating membrane
35	8.5-9.0 d	Phalanges in toes
36	10 d	Length of 3rd toe from tip to middle of metatarsal joint = 5.4 ±0.3 mm; length of beak from anterior angle of nostril to tip of bill = 2.5mm; primordium of comb; labial groove; uropygial gland
37	11 d	Length of 3rd toe = 7.4 ±0.3mm; length of beak = 3.0 mm
38	12 d	Length of 3rd toe = 8.4 ± 0.3 mm; length of beak = 3.1 mm
39	13 d	Length of 3rd toe = 9.8 ± 0.3 mm; length of beak = 3.5 mm
40	14 d	Length of beak = 4.0 mm; length of 3rd toe = 12.7 ± 0.5 mm
41	15 d	Length of beak from anterior angle of nostril to tip of upper bill = 4.5 mm; length of 3rd toe = 14.9 ± 0.8 mm
42	16 d	Length of beak = 4.8 mm; length of 3rd toe = 16.7 ± 0.8 mm
43	17 d	Length of beak = 5.0 mm; length of 3rd toe = 18.6 ± 0.8 mm
44	18 d	Length of beak = 5.7 mm; length of 3rd toe = 20.4 ± 0.8 mm
45	19-20 d	Yolk sac half enclosed in body cavity; chorio-allantoic membrane contains less blood and is “sticky” in living embryo
46	20-21 d	Newly-hatched chick

APPENDIX B

Timetable of *Drosophila melanogaster* development (Reference 1) and detailed stages (Reference 2).

Timetable of embryogenesis

	Stage	Time	Developmental events
	1 – 4	0:00 – 2:10 h	Cleavage
	5	2:10 – 2:50 h	Blastoderm
	6 – 7	2:50 – 3:10 h	Gastrulation
	8 – 11	3:10 – 7:20 h	Germ band elongation
	12 – 13	7:20 – 10:20 h	Germ band retraction
	14 – 15	10:20 – 13:00 h	Head involution and dorsal closure
	16 – 17	13:00 – 22:00 h	Differentiation



Atlas of Drosophila Development by Volker Hartenstein | *Stages of Embryonic Development. All embryos are in lateral*

view (anterior to the left). Endoderm, midgut (red); mesoderm (green); central nervous system (purple); foregut, hindgut (blue); pole cells (yellow). (amg) (Anterior midgut rudiment; (br) brain; (cf) cephalic furrow; (cl) clypeolabrum; (df) dorsal fold; (dr) dorsal ridge; (es) esophagus; (gb) germ band; (go) gonads; (hg) hindgut; (lb) labial bud; (md) mandibular bud; (mg) midgut; (mg) Malpighian tubules; (mx) maxillary bud; (pc) pole cells; (pmg) posterior midgut rudiment; (pnb) procephalic neuroblasts; (pro) procephalon; (ps) posterior spiracle; (po) proventriculus; (sg) salivary gland; (stp) stomodeal plate; (st) stomodeum; (tp) tracheal pits; (vf) ventral furrow; (vnb) ventral neuroblasts; (vnc) ventral nerve.

APPENDIX C

Creating a Publication-worthy Figure

Things to include:

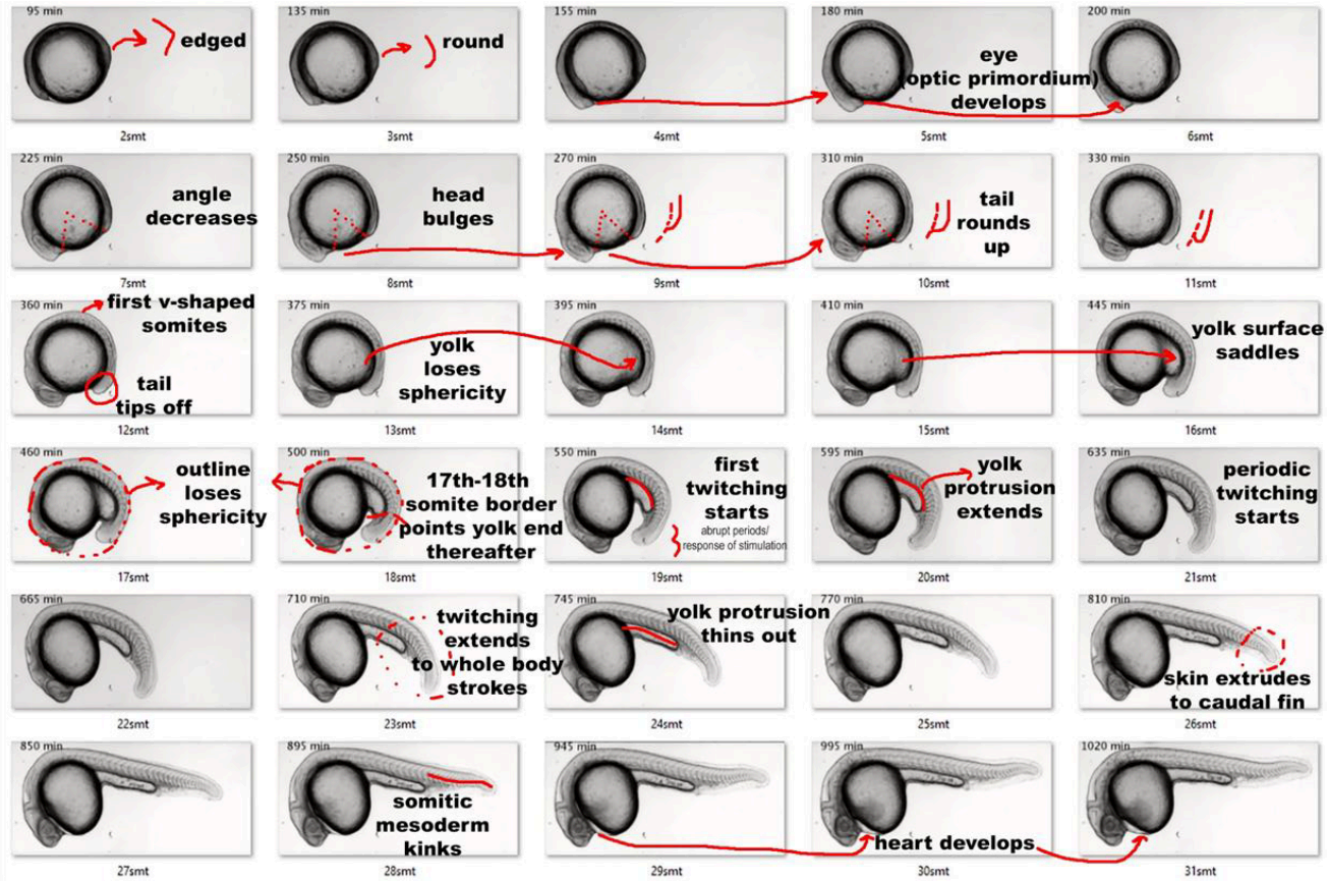
- A descriptive title that states exactly what the figure is showing.
    - For graphs the title goes below.
    - For tables the title goes above.
- The title of the figure should be bolded.
- Graphs need axes labels and units where relevant.
    - Images with a scale bar should state the units.
- Each figure panel should have a label that is easily legible.
    - The figure caption should refer to these labels when discussing the figure.
- A descriptive figure caption
    - Should have enough information for the figure to "stand alone" (it can be interpreted without any other information).
- Images used in figure panels should have the same orientation, be facing the same direction, be cropped to show the part that is of interest.
- Arrows or other markers can be used to denote a feature of interest but there should not be text explanations on the figure panels themselves.
    - Any marker needs to be described in the figure caption.

Things to avoid:

- Don't include unnecessary information – things like methods do not belong in the figure caption.
- You don't need to interpret the data in the figure caption and should not highlight every single result.

APPENDIX D

Somitogenesis stages of a zebrafish embryo with descriptive annotations (pictures modified from Principles of Development, 5th Edition, OUP, movie courtesy: Andy Oates).



Glossary

Aboral side

The furthest side from the oral cavity of the organism.

Aperture

The hole that controls the amount of light reaching the specimen.

Blastodisc

The blastula found at the top of the yolk in chicken eggs.

Compound microscope

A high magnification microscope that uses two lenses to multiply (compound) the magnification.

Cuticle

The barrier between C. elegans environment and body. It is an extracellular matrix built by crosslinked small collagen-like proteins that maintain the shape of the nematode.

Embryonic mesoderm

The middle embryonic germ layer that gives rise to the development of organ and tissue structures during embryogenesis.

Enhancer trap

A type of P element which results in the transposition of a reporter gene downstream of known regulatory elements to determine their spatial and temporal expression patterns.

Epiblast

Top blastodisc layer, later differentiates into the primary germ layers.

Feeding

The experimental protocol where roundworms are fed transgenic bacteria that express target proteins or nucleic acids.

Field of view (FOV)

The diameter of the visible area of the specimen you are viewing through a microscope.

Gamete

A haploid cell (either male or female) that can contribute to the formation of a zygote with a gamete of the opposite sex.

Gonophore

A reproductive structure found in the Hydrozoa class.

Hensen's node

The organizer node at the tip of streak; cells of this region release biochemical signals that mediate neural differentiation and drive gastrulation.

Hypoblast

Bottom blastodisc layer which gives rise to the yolk sac.

Immunohistochemistry

An *in situ* protein staining method which relies on the recognition of specific proteins in the target sample by primary antibodies. This allows for the downstream visualization of detection of these targets with a secondary antibody.

Morphogen

A chemical signal that governs development of cell-types and tissues based on its concentration gradient during morphogenesis.

Ocular micrometer

A glass disk with a marked scale that is inserted into a microscope's eye piece.

P element

A transposon originally identified in Drosophila that has now been used as a tool in fruit fly molecular biology.

Presomitic mesoderm

The tail (posterior) portion of the paraxial mesoderm flanking embryo axis, formed with the neural tube in the neurulating embryo.

Primary antibody

An antibody sensitive to a specific epitope target.

Primitive streak

The linear furrow trailing the node which marks the gastrulation site and initiates germ layer formation.

Reverse genetics

The "gene-forward" study of a phenotype. The gene is directly targeted, and its role is then assessed.

rol-6 (su1006) II mutant

rol refers to the phenotypic abnormality (rollers) -6 refers to the specific gene that causes the phenotype (su1006) the specific allele causing the mutation II indicates the linkage group.

Secondary antibody

An antibody that will recognize the primary antibody and harbors a means of visualization.

Section

A slice of sample is cut and mounted on a slide.

Smear

A suspension of cells or samples is spread on the slide as a film.

Somites

A set of bilaterally paired blocks of presomitic mesoderm formed on the head-to-tail axis during embryogenesis.

Stage micrometer

A microscope slide with a scale marked on the surface for measurements.

Stereoscope

A low magnification microscope enabling 3-D depth view through its eyepiece for proper sample investigation and dissection.

Tail bud cells

Cells end up in the elongating tail mesoderm with gastrulation movements. The chemical signalling gradients sourced from these cells inform cells to commit in making somites.

Transgenic

An organism whose genetic make-up has been altered by the introduction of non-endogenous genetic elements.

Whole mount

The entire sample is mounted or embedded in clear mounting medium on the slide.