

WHY LAB NOTEBOOKS?

Laboratory notebooks are diaries for scientists. We keep records of everything we do in lab. We record ideas, calculations, experimental plans, and, most importantly, we record data. One of the primary reasons we use lab notebooks in this class is to establish good practices for your independent projects and your thesis (as well as your future scientific endeavors).

YOUR LAB NOTEBOOK SHOULD:

1. Have your name and the course title on the front.
2. An accurate table of contents
3. Contain all information related to what you did in each lab.
4. Include relevant parts of the lab handout stapled or taped in appropriate places.

EACH ENTRY IN YOUR LAB NOTEBOOK SHOULD:

1. Begin with a title and or question, the date, and the name(s) of your lab partner(s).
2. Describe what you're planning to do in lab with an outline/flow chart.
3. Contain a **complete** record of what you did and what you observed. You are more than welcome to cut up pieces of the lab handout and tape/paste/staple them into your notebook. You should be taking notes and recording data as you perform your experiments.
4. Answer all questions posed in the lab handout. As long as you analyze your data, make conclusions based on your data, there is no "right" answer. This is because biology is based on observations of data from experiments that you tested. As a scientist, you perform experiments and then gain insights into particular processes based on your results.
5. Use your lab notebook for ALL of the labs, including your independent projects. There will be regular lab notebook checks throughout the semester and they are meant to help you optimize the use your notebook.

INTRODUCTION AND BACKGROUND: Fertilization is often considered the logical starting point for a course in developmental biology. Fertilization occurs when haploid gametes – the sperm and the egg – fuse to create a new diploid cell. This cell is the beginning of a new individual, formed with genetic instructions derived from both parents. Fertilization typically consists of four major events: (1) contact and recognition between egg and sperm, (2) polyspermy block, (3) fusion of sperm and egg genetic material (4) activation of egg metabolism to initiate development. All of these events occur prior to the first cell division.

Today you will conduct experiments designed to probe the mechanisms underlying fertilization and polyspermy block. Polyspermy is fertilization by more than one sperm, a dangerous proposition that can result in cleavage stage embryos with too many chromosomes and the appearance of four (or more) cells at the two-cell stage. The polyspermy block is essential for normal development. Sea urchin eggs employ two mechanisms for blocking polyspermy – one fast and transient, one slow and permanent. You will interrogate both the fast and slow polyspermy blocks today by:

(1) testing how various concentrations of Na^+ influence the fast block and **(2)** determining whether internal or external sources of Ca^{2+} trigger the slow block.

Table 1. Timing of early events during sea urchin embryogenesis at 20°C.

Approximate time	Event
0	Contact between egg and sperm
1 sec	Fusion of sperm and egg membrane
20-60 sec	Cortical granule exocytosis
30-50 sec	Vitelline membrane begins to raise
60-90 sec	Vitelline membrane completely raised
2 min	Hyaline layer begins to form
5 min	Fertilization membrane begins to harden
120-135 min	First cell division (Cleavage)
1 st Cleavage + 30 min	Second cell division (2 nd Cleavage)

In the first series of exercises, you will test how $[\text{Na}^+]$ impacts the fast block to polyspermy. The **fast block to polyspermy** has been shown to occur when the electric potential of the egg cell membrane changes when the first sperm binds to receptors on the egg surface. Normally the $[\text{Na}^+]$ inside the egg is much lower than outside (sea water has a much higher concentration of sodium ions relative to the egg cytoplasm). The resting membrane potential of sea urchin eggs is approximately -70 mV. Less than 3 seconds after the first sperm binds and fuses with the egg surface, the cytoplasmic $[\text{Na}^+]$ increases to an extent that raises the egg's membrane potential to approximately +20 mV. The positively charged membrane is now impervious to all other sperm. How might one prevent this fast block to polyspermy?

In the second series of exercises, you will test the contribution of Ca^{2+} to the slow block to polyspermy. Unlike the fast block, the **slow block to polyspermy** is long-lasting. It mechanically removes any sperm remaining on or near the surface of the egg with the cortical granule reaction. Cortical granules, small vesicles filled with proteases and a variety of other components required for maturation of the fertilization envelope, are located directly beneath the egg cell membrane. As soon as one sperm fertilizes the egg, cytosolic $[\text{Ca}^{2+}]$ increases, triggering membrane fusion between the cortical granules and plasma membrane. Waves of Ca^{2+} then propagate across the egg, beginning at the site of

sperm entry. This flux of Ca^{2+} activates a series of events such as egg cell cycle re-entry, NADP⁺ production, stimulation of protein synthesis, DNA replication, and movement of morphogenetic materials (eg., proteins, RNAs, and organelles).

In our experiments, we will use eggs and sperm from one of two marine invertebrates: *Lytechinus pictus*, a small greenish sea urchin that lives in lower intertidal and nearshore subtidal zones from British Columbia to Panama and *Dendraster excentricus*, a purplish sand dollar found in deeper waters and range from Alaska to Baja. *L. pictus* spawns during the spring and fall when water temperatures hover around 20°C and sand dollars are typically gravid throughout the year. We will culture the urchin larvae at 15°C and the sand dollar embryos will be kept in the cold room. The urchins and dollars we are using today come from the coast of Southern California (near Long Beach). We will use real and artificial seawater for our experiments.

Table 2. Primary salts in real and artificial seawaters

Solution	[NaCl]	[KCl]	[CaCl ₂]	[MgCl ₂]
Real sea water	485 mM	10 mM	8 mM	56 mM
Artificial sea water (ASW)	485 mM	10 mM	8 mM	56 mM
Na-free ASW	0	10 mM	8 mM	56 mM
Ca-free ASW	453 mM	9 mM	0	48 mM

METHODS: We will first observe the eggs and sperm, familiarizing ourselves with various types of microscopy. Next, using various dilutions of the solutions listed in Table 2, you will (A) determine the relationship between $[\text{Na}^+]$ and the fast block and (B) test whether eggs rely exclusively on internal or external stores of Ca^{2+} for the slow block.

Before coming to lab, make an outline and/or flowchart of what you'll be doing in your lab notebook. To get an idea of the types of things we'll be doing, please run through the interactive video exercise at <http://web.stanford.edu/group/inquiry2insight/cgi-bin/vu-r1a/vu.php?view=fertlab> (be sure to click on parts 2 and 3 (upper right corner) for the entire exercise). Although we'll be using microfuge tubes for our fertilization reactions instead of beakers and although the interactivity may seem a bit clunky, this is a great teaching tool (if you're interested the menu on the left-side has links to other interesting tutorials). Please draw Table 3 in your lab notebook, being sure to leave a few extra rows in case you need to do a few practice runs to get your technique down). Choose various $[\text{Na}^+]$ between 50 mM and 485 mM to test (at least 5 separate concentrations, including 50 and 485 mM) and then calculate the volumes of each solution you will need to mix to achieve the appropriate final concentration in a final volume of 400 ul (round up to the nearest integer for ease of pipetting). These are simply $C_1V_1 = C_2V_2$ type calculations that you've learned and practiced in other courses.

Table 3. Concentrations and volumes of solutions for testing $[\text{Na}^+]$ dependence of the fast block.

Desired $[\text{Na}^+]$	ASW	Na-free ASW	Eggs	Na-free Eggs	Sperm in RSW	Total Rxn Volume
Example: 100 mM Na^+	52 ul	218 ul	0 ul	100 ul	30 ul	400 ul
Example: 150 mM Na^+	94 ul	176 ul	0 ul	100 ul	30 ul	400 ul
Practice: 485 mM Na^+	270 ul	0 ul	100 ul	0 ul	30 ul	400 ul

Sea urchins can be coaxed into shedding their gametes by injecting their coelomic cavity with 0.5 M KCl (insert the needle through the peristomial membrane around the mouth). The KCl causes the muscles around the gonads to contract, forcing the gametes out the gonopores (located on the aboral side of the urchin). Because the animals do not exhibit any visible sex differences, male and female cannot be distinguished until they begin shedding tiny orange eggs or white semen. For our experiments today, eggs were spawned into beakers filled with artificial seawater (ASW), ASW minus Na⁺, and ASW minus Ca⁺. Semen was collected in petri dishes. Depending on the urchins available, you may be able to see KCl-induced spawning as a demonstration during the lab.

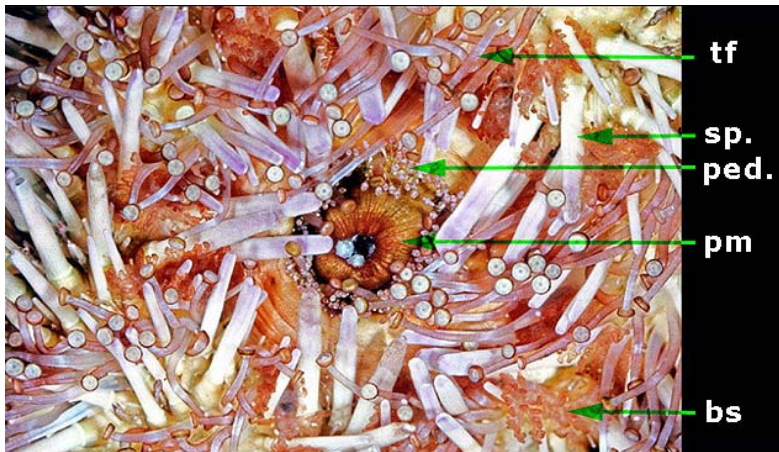


Photo of Sea Urchin Oral Surface. Green arrows indicate tube feet (tf), spines (sp), pedicellariae (ped), peristomial membrane (pm), and buccal sacs (bs). Image courtesy of <http://www.nhm.ac.uk/research-curation/research/projects/echinoid-directory/intro/softext1.html>

LAB WORK:

FAST BLOCK EXPERIMENT:

(1) Make all your test solutions WITHOUT eggs or sperm. Tubes of ASW and Na-free ASW are in the 15°C incubator. Make the solutions in microfuge tubes by mixing the calculated amounts of ASW and Na-free ASW in each tube. Remember to label each tube with the appropriate concentration.

(2) Obtain eggs and sperm.

Eggs: Once you've aliquoted all of your solutions into microfuge tubes, obtain ~0.5 ml of eggs in ASW into a clean microfuge tube by swirling the beaker of eggs and pipetting from the middle with a long Pasteur pipette. Keep the stock egg tube on its side (for better surface:volume ratio for better gas exchange).

Sperm: Next obtain 1.0 ml of **real sea water (RSW)**. For sperm to fertilize eggs they must be activated with RSW. To activate the sperm, dilute 1 ul of undiluted semen (available on ice at the front of the room) into 1.0 ml of RSW. Mix by vortexing for 1-2 sec. As soon as the sperm hit the water, they'll start swimming. Pipette 30ul of diluted sperm into a depression slide and check to make sure they're swimming. Keep sperm on ice.

(3) Perform the 485 mM [Na⁺] practice first. Verify that the eggs generate fertilization membranes.

-For successful in vitro fertilizations:

- (a) Gently mix eggs immediately before pipetting (ensures you actually pipet eggs).
- (b) Record the time at which sperm is added.
- (c) Immediately check for motile sperm and fertilization after mixing each sample. Mix the sample once by inversion and then immediately pipette 25 ul onto a

depression slide and observe. Close the iris diaphragm to clearly see the membranes.

(d) If you can see fertilization membranes on $\geq 75\%$ of the eggs, put your tube in the 15°C incubator. If 50% or fewer eggs display fertilization membranes, add another 1 μl of undiluted semen to your RSW tube. Vortex and add another 30 μl of diluted sperm to your sample (this second addition of sperm won't change the $[\text{Na}^+]$ concentration of the practice 485 mM $[\text{Na}^+]$ sample, but it would for all other concentrations).

(e) Lay your tube on its side in the 15°C incubator. Incubate until embryos are well into the first cleavage (see Table 1 to estimate the time).

(4) After performing in vitro fertilizations in 485 mM $[\text{Na}^+]$, work down from the next highest concentration ending with 50 mM $[\text{Na}^+]$. Complete steps a-e (above) for each $[\text{Na}^+]$ individually. Do one tube at a time (e.g., add eggs, add sperm, check for fertilization...then repeat for the next concentration).

-Collect ~0.5 ml of Na-free ASW eggs immediately before you need them.

- If percentage of fertilized eggs starts to decline, make a fresh tube of diluted sperm.

(5) When all tubes are fertilized, start the slow block experiments (next page). You'll come back to score the success of the fast block ~2h after fertilization.

(6) Score the success of the fast block. Gently invert the tube and pipet 30 μl of the reaction onto a depression slide. Look at the contents of the tubes one at a time (otherwise slides will dry out). Count the number of cells in which the first cleavage generated two cells and the number of eggs in which the first cleavage generated more than two cells. Unfertilized eggs generally do not cleave and should not be counted. Record your results as number of 2-celled embryos versus number of polyspermic eggs. Repeat for all concentrations.

*****IMPORTANT****

If the embryos go past their first cleavage (2-cell stage), it will be impossible to tell whether an egg is a normal 4-cell embryo or a polyspermic egg. Therefore, if the controls get to the 4-cell stage, immediately refrigerate ALL experimental tubes and score them one at a time.

(7) Do NOT discard your tubes of fertilized eggs. We will keep the embryos that continue to develop, hopefully culturing the sea urchin embryos to the pluteus larval stage (come back throughout the week to check on their development and observe the beautiful pluteus).

ANALYSIS AND CONCLUSIONS PART I: Analyze and interpret your data by calculating the percent polyspermy for each of the solutions tested. You can use JMP or Excel to perform regression analysis (see Bio Binder pages H13-14, Bivariate Regression for JMP instructions (accessible on the courses server). Remember that simple bivariate regression is only appropriate for linear relationships. What can you conclude about the relationship between $[\text{Na}^+]$ and the fast block? Frog eggs use a similar mechanism of changing the membrane potential from negative to positive to block polyspermy. Given that frogs live in fresh water (with low concentrations of Na^+), how might this be possible? Include your answer to this question along with the graph of your data and an interpretation of your results in your lab notebook.

SLOW BLOCK EXPERIMENT: This experiment addresses whether egg activation during fertilization relies on internal or external Ca^{2+} . Sperm-egg fusion initiates an increase in cytosolic Ca^{2+} , thus stimulating cortical granule exocytosis and fertilization membrane formation. Eggs can be artificially activated by adding a calcimycin (also known as A23187). Calcimycin functions as a mobile cation carrier, integrating into membranes and allowing Ca^{2+} to flow down its electrochemical gradient. Remember that Ca^{2+} concentrations outside the cell are higher than the inside (RSW and ASW ~ 8 mM; cytoplasm $\sim 10^{-4}$ mM; and endoplasmic reticulum stores ~ 0.1 mM).

**** IMPORTANT ****

A23187 is TOXIC. It is dissolved in DMSO, which facilitates its solubility and enhances its toxicity. WEAR GLOVES. Do not put in your mouth or near your eyes.

In this experiment, you will attempt to induce egg activation (scored by formation of the fertilization membrane) in the presence and absence of external Ca^{2+} . You will need to do four treatments: two experimental treatments, which are listed below and two control treatments, which you should define.

Treatments:

(A) 150 ul ASW eggs + 6 ul calcimycin (2 m M)

(B) ASW control = _____

(C) 150 ul Ca-free ASW eggs + 6 ul calcimycin (2 mM)

(D) Ca-free ASW control = _____

-Calculate the final concentration of calcimycin in all of the treatments and record along with your other calculation and observations in your lab notebook.

NB: Perform each of the treatments individually and complete the quantitation before moving onto the next treatment.

- (1) Add 150 ul egg suspension to a microfuge tube.
- (2) Near the upper lip of the tube, pipet 6 ul of calcimycin onto the side of the tube. Do NOT put the calcimycin directly into the egg suspension (Kara will demonstrate this technique at the beginning of the lab). Cap the tube and immediately vortex the tube for 1 sec.
- (3) Check for activation immediately. Pour the contents of the tube onto a depression slide and count the number of eggs that are activated (with a fertilization membrane) and those without a fertilization membrane. Remember to close the iris diaphragm to be able to see the fertilization membrane. Count at least 100 eggs per field of view and count several fields of view. Do not count eggs that have exploded.
- (4) Record your data.
- (5) If your control tubes show egg activation, modify your vortexing procedure so that you mix for 1 sec or less.
- (6) Repeat each treatment at least 2X (to increase your sample size and confirm repeatability).

(7) When finished, rinse your depression slides with distilled water and dry them.

(8) You should have time to analyze your data for the slow block experiment before returning to the fast block experiment.

ANALYSIS AND CONCLUSIONS PART II: What is the relationship between the presence of external calcium and the formation of the fertilization membrane? Are these variables independent or is the fertilization membrane formation dependent on external calcium? Are internal stores of calcium enough to elicit fertilization membrane formation?

Chi-squares contingency table analysis will allow you to test dependence/independence of the two variables (external calcium and fertilization membrane formation). For a refresher on how to do this calculation using JMP, see your Bio Binder H-29. You can also perform the Chi-squares analysis with Excel or R if you know how. Remember to use the actual number of events not percentage fertilization when performing this type of analysis. Record your observations and answers in your lab notebook.

(9) Do NOT leave the lab before transferring all of your remaining embryos to the RSW beaker at the front. If you would like to help with culturing the urchin embryos, please let Kara know.

Procedure we'll use for culturing sea urchin embryos:

1. On the first night, remove sperm water using the nylon-mesh contraption and replace with RSW.
2. At each time point, remove ~530 ul of embryos from the beaker. Observe ~30 ul with dark field and bright field microscopy. Allow the embryos to settle to the bottom of the tube and remove the seawater. Replace with ~500 ul of 100% ice cold MeOH (stored in the -20°C freezer).
3. IFF the embryos have gastrulated and are swimming larvae, obtain a clean beaker, decant the "swimmers" into the clean beaker and discard the remaining debris (urchin waste container near the sink). You can always ask Kara for help if you're unsure of the stage of the embryos.
4. Place labeled tube in rack in -20°C freezer (in a few weeks when we investigate gastrulation and induction, we'll stain these with DAPI and observe).

SYNTHESIS AND CONCLUSIONS: To cement what you've learned from this lab, write a paragraph that summarizes your results and then write one or more paragraphs that synthesize your understanding of fertilization. Include drawings and reference the tables and graphs. Think about what might happen if eggs only had one mechanism to block polyspermy. Which would be best and why? What might be the evolutionary consequences of an organism that did not have a way to block polyspermy?

INTRODUCTION AND BACKGROUND: To probe the mechanisms of organismal development, scientists rely on basic genetic and molecular biology manipulations. The common fruit fly, *Drosophila melanogaster*, is one model organism that facilitates investigations of the genes that control development. Firmly cemented in the seminal work by Thomas Hunt Morgan from the early 1900s, thousands of research labs use fruit fly genetics to tackle diverse problems including body plan organization, cell-cell signaling, and learning. Three “modern” *Drosophila* researchers – Ed Lewis, Christiane Nüsslein-Volhard and Eric Weischaus – won the Nobel Prize in 1995 for their “discoveries concerning the genetic control of early embryonic development”.

The **purpose of today’s lab** is three-fold: (1) become familiar with *Drosophila* as a model organism by examining wild-type and mutant flies of various stages, (2) investigate how the products of multiple genes work together to regulate fly development by examining gene expression patterns in wild-type and mutant fly embryos, and (3) discuss how genetic engineering and molecular biology enable *in vivo* study of gene regulation.

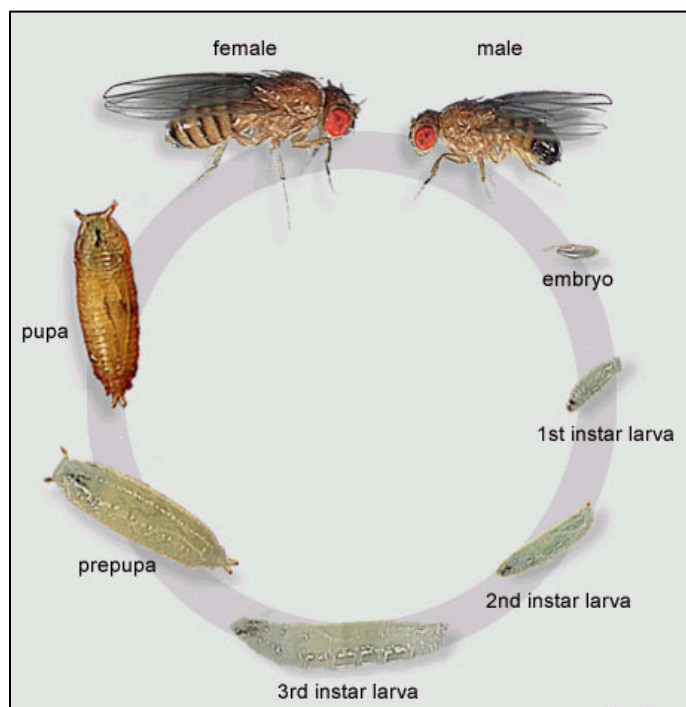


Figure 1. Fly life cycle from <http://flymove.uni-muenster.de/>. At 25°C (room temperature) the embryo, 1st and 2nd instar larvae each take one day. 3rd instar larvae require ~3 days before pupating. Five days later, adult flies eclose and unfurl their wings. Notice the difference in size and coloration between the male and female. For more details about phenotypic differences between the sexes, see Figure 2.

Drosophila melanogaster is an incredibly powerful model system. The flies have a rapid generation time, passing through embryonic, larval, pupal, and adult stages over the course of 10-20 days. Males, females, and virgin females are easy to identify and cross. Females lay many eggs and the flies are hardy and easy to manipulate. Most importantly mutant fly stocks can be maintained with the help of balancer chromosomes. Balancer chromosomes have three key features that distinguish them: (1) they express at least one dominant marker (such as curly wings or white eyes) that can easily be observed; (2) they are typically lethal when homozygous; and (3) they carry many inverted repeats, which suppress recombination between homologues, preventing loss of a mutation from crossing over or homologous recombination. Balancer chromosomes are so named because they make it easy to “balance” fly

stocks, ensuring that lethal mutations are maintained heterozygously. Many balanced lines are stored in central repositories such as the Bloomington Stock Center (<http://flystocks.bio.indiana.edu/>). In our experiments, we’ll use stocks carrying balancers for chromosomes 2 (CyO) and 3 (TM3, Sb). The CyO balancer carries a dominant mutation that gives adults curly wings. The TM3, Sb balancer carries a dominant mutation that gives flies stubby bristles on their backs.

METHODS AND LAB WORK:

Developmental biologists studying flies and many other organisms often rely on microscopes, forceps, and homemade tools to manipulate embryos, larvae, and adults. Before we begin any experiments, Kara will demonstrate how to use your forceps, probes, and make (and use) eyelash needles and tungsten microscalpels. Please note that you will need these tools for future labs and during your independent projects. Preparing and keeping quality tools (including sharp forceps) will make your life in lab much easier.

Eyebrow/Eyelash knives: Use as Microscalpels. Embed an eyebrow hair or eyelash into a 5 ml glass transfer pipette and seal with wax.

Tungsten microscalpels

Cut a ~2.5 cm piece of tungsten wire using the wire cutters. Insert the wire into your pin holders. Sharpen the wire electrolytically with a solution of saturated KOH and a 9V power supply. Plug electrodes with alligator clips into the power supply. Then attach the black electrode alligator clip to the paper clip, immerse into paper clip into the KOH, and then attach the red electrode alligator clip to your pin holder. Turn on the power supply. Dip the tip of the tungsten needle into and out of the NaOH solution at an angle (bubbles should appear) until tapered. Rinse with PBS or dH₂O before use.

In PART 1, you will observe the various developmental stages of fruit flies – adults, larvae, and embryos. Next, in PART 2, you will investigate gene expression patterns in both wild-type and mutant embryos. Finally, the lab will conclude with a shocking look at some mutant flies and a discussion of the molecular biology and genetics that biologists use to understand the mechanisms of development.

PART 1: GETTING TO KNOW YOUR FLIES.

1. ADULTS. Obtain a small vial of fruit flies from the front table. Note the genotype in your lab notebook and be sure to keep the flies of different genotypes separate (other groups

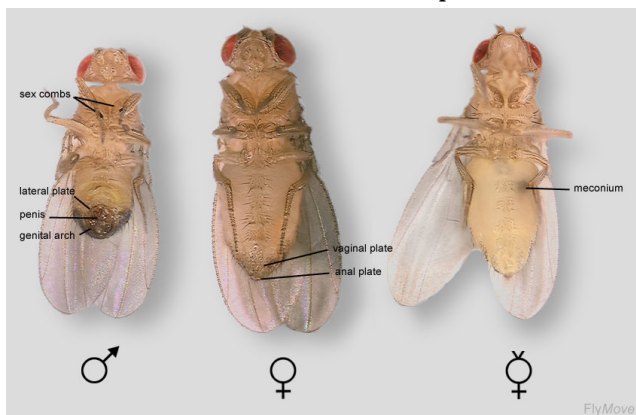


Figure 2. Ventral views of anesthetized flies from <http://flymove.uni-muenster.de/>.

will have flies of different genotypes). At one of the adult fly stations set up in the back corner, anesthetize the flies using the needle-end of the hose connected to the CO₂ tank. Transfer some of the adult flies onto a “flypad” with a gentle tap. Look at the flies with the dissecting scope and with a small paintbrush, sort the flies into groups of male, female, and virgin female. As you look at the different flies, think about the following questions and then answer them in your lab notebook.

- A) Why is it important for scientists using fruit flies in their research to collect virgins?
- B) Take a minute to notice the phenotypic effects of the CyO or TM3, Sb balancers. Do

the balancer phenotypes appear in Mendelian ratios? Why or why not? Record your thoughts and observations in your lab notebook.

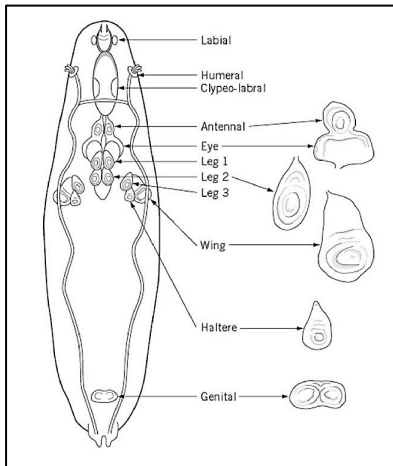


Figure 3. Organization of 3rd instar larva. <http://what-when-how.com/molecular-biology/imaginal-disc-molecular-biology/>

2. Place a drop of water on a depression slide and bring it to the front table. Brush a few 3rd instar larvae from the vial at the front table into the water droplet. Flies (like many insects) undergo complete metamorphosis with a small proportion of the larval tissues giving rise to the adult. 21 clusters of cells called imaginal disc, so called because they give rise to the imago (final winged stage) not because they are imaginary. These remarkable tissues undergo tremendous growth during development and form nearly all the parts of the adult fly.

We will use the “Christmas cracker” or “Party popper” method for revealing imaginal discs (Kara will demonstrate).

Once you pop the larva apart, carefully clean away the brain and guts with forceps, probe, and eyelash needles to fully expose the imaginal discs. Add small drops of

water as needed. Kara will demonstrate when you’re ready. Draw an example of your imaginal disc dissection in your lab notebook, noting the similarities and differences between the eye, leg, and wing discs. Choose one of the imaginal discs, carefully dissect it away from the connective tissue with forceps and probe. Place it in a 25ul drop of water on a slide, add 5ul of the dilute DAPI solution (at the front). Gently move the disc around and position it so that when you place a coverslip over it, you will be able to view the entire disc. Observe your imaginal disc prep using your compound microscope and the fluorescence compound microscope (at the front). Note of what you see.

3. EMBRYOS. Obtain one apple juice-agar plates from the back table. Note the strain number (5337 or 5344) on the plate. The number refers to the parental line that laid eggs this morning. Examine the plates under the stereomicroscope and see if you can find the antenna-like respiratory appendages of the egg. These allow gas exchange when the embryos are buried in rotting and fermenting fruit. We will use these embryos for PART 2. Draw an example of a *Drosophila* embryo in your lab notebook. Remember that you’re looking at the egg shell or chorion and that the recently cellularized embryo is inside

PART 2: DEVELOPMENTAL GENETICS AND EARLY EMBRYONIC PATTERNING

Drosophila set up the anterior-posterior and dorsal-ventral body axes very early in development with maternal determinants regulating expression of gap and pair-rule genes. The stereotypical, segmental expression of the gap and pair-rule genes determines how segment polarity and homeotic genes are expressed, ensuring appropriate identity and number of larval body segments. **In this section of the lab we will examine how the expression pattern of *fushi tarazu (ftz)*, a pair-rule gene with homeotic character,**

changes in flies lacking expression of other patterning genes. We will collect data as a class and then based on the staining patterns of *ftz* in two different strains, you'll make hypotheses about the identity of the mutation carried by each strain. By the end of the lab, you'll be motivated (hopefully) to learn more about how developmental biologists have used the mutants originally isolated by Ed Lewis, Christiane Nusslein-Volhard and Eric Weischaus to tease apart the genetic cascades that govern embryonic development. The results of this lab will feed directly into our lectures about embryonic patterning and fly development.

How are we detecting the expression of *ftz*? Although in situ hybridization is often used to detect gene expression patterns, we will use another method. Our flies have been genetically modified to carry a transgenic reporter that is expressed in the same pattern as *ftz*. Reporter transgenes often encode fluorescent proteins or an enzyme with an activity that can be easily detected, and they are typically inserted into the genome downstream of specific cis-regulatory elements (those that control *ftz* expression in our case). The reporter transgene we will use today is *LacZ*, an *E. coli* gene that codes for the β -galactosidase (β -gal for short) enzyme. In *E. coli* and in vitro, β -gal cleaves lactose to generate glucose and galactose. When β -gal is incubated with the lactose analog X-gal, a colorimetric reaction occurs such that a colorless solution turns blue. Today, the mutant flies we are examining carry the *LacZ* transgene under control of the cis regulatory elements for *ftz* and therefore the cells that express *ftz* will turn blue.

THE EXPERIMENT:

1. Preparation. Collect the following equipment and reagents from the side bench:

- squirt bottle filled with PBST (PBS+0.3% TX-100)
- egg collection basket and weigh boat
- 50 ml beaker
- small squirt bottle of 50% bleach

2. Collect Eggs. Use a squirt bottle to flood the apple juice-agar plate with PBST. Dislodge any remaining eggs stuck to the surface by rubbing the surface of the plate with a small paintbrush. Tip the plate over an egg collection basket standing in a weigh boat. Squirt the plate with more PBST to wash the eggs into the basket.

3. Dechorionate Eggs. Set the egg collection basket (containing the eggs) in a 50ml beaker. Squirt enough bleach into the egg collection basket to completely cover the embryos. After 3 minutes, check the embryos under the dissecting scope. As soon as the respiratory appendages disappear, dechoriation is complete. To stop the reaction, immediately transfer the egg collection basket (with the dechorionated embryos) into the weigh boat and wash with PBST.

4. Collect Embryos. Add 500ul of PBST to a microfuge tube. Carefully remove the mesh from the egg collection basket and gently brush the dechorionated embryos into the PBST.

5. Repeat steps 1-4 for each of the dishes of embryos you have. Keep track of their stock numbers by closing and labeling the microfuge tube as soon as you transfer in the embryos.

6. Fixation. Attach a small yellow tip to the end of disposable glass pipette and remove the PBST from each tube. Don gloves. Add 1ml of formaldehyde-saturated heptane from the bottle on the back bench (be sure to take the top phase). Cap tube and shake by hand (NOT vortex) for 5 minutes.

7. Let the embryos settle to the bottom of the tube (~30 sec) and then remove the formaldehyde-saturated heptane with a small yellow tip attached to the end of disposable glass pipette.

8. Rinse embryos with PBST at least 3 times. Add ~1ml of PBST and shake for 2 min. Remove PBST. Repeat rinse at least 2 more times.

9. Coloration reaction. Remove PBST. Add 0.5ml of X-gal staining solution. Cover your tubes with foil and/or place in a dark place. Blue color should start to appear in ~30 minutes at room temperature. While you wait, do PART 3.

10. Observing and Imaging. After the staining reaction is complete, discard the X-gal staining solution and rinse embryos 2 times with PBST. Remove the PBST and add 100 ul of 70% glycerol. Gently flick to mix the glycerol and embryos. Then, using a glass Pasteur pipette, transfer embryos to a microscope slide. Make very small clay feet for your coverslip (Kara will demonstrate) and observe your embryos using brightfield illumination (compound microscope). Draw and make notes in your lab notebook. You may want to count the number of distinct staining patterns you see in each tube. Record your data and based on what we've discussed in class about embryonic patterning, make some hypotheses about which genes have been mutated.

PART 3. WHILE YOU WAIT FOR THE STAINING...

A major goal of this lab is to illustrate how the power of molecular biology and genetics can be applied to developmental biology questions. We can easily generate and study mutant flies, learning how specific genes regulate embryonic patterning, growth, morphogenesis, and differentiation. We can also easily construct transgenic flies that serve as read-outs for both gene function and the sequences that ensure genes are expressed in particular places at particular times. The *ftz-LacZ* strains are one example. While we wait for the X-gal to change colors, we will look at some photos of strange fly and zebrafish embryos, some of which resulted from chemical mutagenesis and others that were specifically designed. We'll discuss the utility of such genetic "monsters" and also discuss genetic screens in flies and fish in more detail, addressing questions you have about the two primary papers that were assigned for this week.

CONCLUSIONS: Answer the following questions in your lab notebook.

-Based on the class dataset, speculate about which genes are mutated in the three different strains that we examined. Remember that *ftz* is not mutated in any of the strains.

-Draw a potential genetic relationship between *ftz* and the two genes we examined that would ensure *eve* and *ftz* are found in alternating patterns of 7 stripes each. For this question, do not let yourself be influenced by what is in the literature...just make a reasonable hypothesis. (During week 4, we'll have time in lab to further discuss the relationships among axial patterning genes in flies and vertebrates).

-Today we examined mutations in 2 genes that control embryonic patterning and segmentation, but there are other genes involved in these complicated processes. How would you design a genetic screen to identify these additional genes?

Optional Readings:

Rivera-Pomar, R. and Jäckle, H. From gradients to stripes in *Drosophila* embryogenesis: filling in the gaps. ***Trends in Genetics***. 1996 Nov; 12(11):478-83.

Frasch M and Levine M. Complementary patterns of *even-skipped* and *fushi tarazu* expression involve their differential regulation by a common set of segmentation genes in *Drosophila*. ***Genes and Development***. 1987 Nov;1(9):981-95.

Halder, G., Callaerts, P., and Gehring W.J. Induction of Ectopic Eyes by Targeted Expression of the *eyeless* Gene in *Drosophila*. ***Science***. 1995 Mar 24;267(5205):1788-92.

INTRODUCTION AND BACKGROUND: One of the first organism-level events required for animal development is establishment of the anterior-posterior and dorsal-ventral axes. Hans Spemann and Hilde Mangold were the first to investigate the basis for formation of heads, tails, bellies, and backsides in vertebrates. In the early 1900's, they performed precise grafting experiments that identified a small region in early gastrula-stage salamander embryos capable of inducing and patterning the body axes. This small group of cells was named, "the organizer". The molecules enabling the organizer to establish axial identity were enigmatic until the 1990s when brute force screens identified a number of gene products (both secreted proteins and transcription factors) that were expressed by the organizer and influenced anterior-posterior (A-P) and dorsal-ventral (D-V) character. Today, armed with multiple molecular and cellular biological tools, scientists continue to probe how axial patterning occurs.

To identify which gene products are both necessary and sufficient for body axis establishment, scientists can perform genetic, biochemical, and embryological manipulations. Some of the most common experiments include showing that a gene is expressed in an appropriate region to pattern the body axes and assessing gain-of-function and loss-of-function phenotypes. Microinjection of mRNAs, DNA, antisense morpholinos (mo), or a mixture of gRNA and Cas9 protein can modulate levels of gene expression/activity.

In today's lab, we will assay how two components of the bone morphogenetic protein (BMP) signaling pathway influence D-V patterning. Although initially identified as inducers of bone formation, we now appreciate the diverse functions of BMPs, including their key role in dorsal-ventral axial patterning. When BMPs bind their cell-surface receptors, a cascade of phosphorylation and protein interactions ensues, ultimately regulating gene expression and cell shape changes (Figure 1).

Some of the first genes implicated in dorsal-ventral patterning are components of the BMP pathway, and we will assay the effects of overexpression and inhibition of two BMP pathway components, *smad5* and *chordino*. For overexpression experiments, we will microinject mRNAs and for knock-down experiments, we will inject *smad5* and *chordino* mo. The **purpose of today's lab** is two-fold. You will (1) practice microinjections into zebrafish embryos and (2) examine zebrafish embryos to learn how the BMP pathway influences dorsal-ventral axis specification.

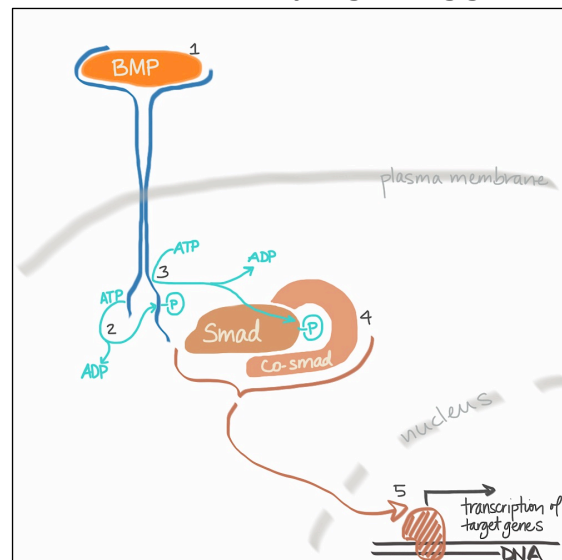


Figure 1. General overview of BMP signaling transduction. Secreted BMP binds a Type II receptor and recruits a Type I receptor to the complex (1). The Type II receptor then phosphorylates the Type I receptor (2), which activates the kinase activity of the Type I receptor (3) to enable its phosphorylation of Smads (there are multiple Smads, which are typically divided into two classes – smads 1,5,8 and smads 2,3). Phosphorylated Smads recruit a co-smad (typically Smad 4). The phospho-smad-co-smad complex translocates to the nucleus where it regulates target gene expression.

METHODS AND LAB WORK: Successful microinjections require calibrated glass needles, appropriately aligned 1-2 cell stage embryos, familiarity with the micromanipulator and dissecting scope, and patience.

Zebrafish typically spawn in the morning. Since our lab is scheduled for the afternoon, we have used dividers in the breeding boxes to separate the males and females. Hopefully, when we remove the dividers, the fish will be sufficiently interested in spawning to provide us with enough embryos for our experiments.

Before today's lab, Kara tested the mRNAs and morpholinos (generously donated by GeneTools) and prepared the needles. The mRNAs and morpholinos have been diluted into phenol red (for tracing our injections) and aliquoted. The tubes on the front bench; the mRNA is on ice and the morpholinos are at room temperature.

INJECTIONS:

(1) Preparing to inject:

- Switch on the microinjector. Turn the house air valve to ensure that airflows into the machine (the pressure should be between 10-30 psi).
- Check that the foot pedal is positioned so you can reach it while looking through the dissecting microscope.

(2) Filling your needle:

- Use a p20 and the long "gel loading" tip to deposit 2-3 μ l of either mRNA or morpholino into your needle.
- Insert your needle into the needle holder attached to the micromanipulator. Be sure to position it past the orange gasket and then tighten the tip.

(3) Breaking your needle:

- Using the highest magnification on your dissecting scope to view your needle, gently scrape the tip of needle with clean forceps to break off a tiny amount of glass.
- Press the injection pedal to be sure that tip has broken and nucleic acid solution can flow out of the needle.

(4) Calibrating your needle (to inject 1 - 2 nl of nucleic acid solution):

- Obtain a calibration micrometer slide.
- Place a drop of mineral oil over the micrometer.
- Focus the microscope on the slide so that you can see the scale that divides 1mm into 100 units.
- Carefully lower the needle tip into the oil.
- Depress the foot pedal and observe the size of the drop in the oil. The drop should hang in the oil to form a perfect sphere. Use **Table 1** to estimate the volume of your droplet. You can change the volume of the drop by adjusting the pressure and/or number of pulses on the foot pedal. As a last resort, you may want to break off a little bit more of your needle if your drop is very small. Before you do this, please consult with Kara.
- If your needle should break, you will need to begin the process again, so be careful.
- If you need to wait for embryos, submerge your needle in a drop of embryo medium to prevent it from drying out.

(5) Organizing your embryos

- Move your needle up and out of the injecting space.
- Arrange your embryos in a column along the side of glass microscope slide in the lid of a petri dish by holding the dish and your plastic pipette at an angle (Kara will demonstrate). Use your eye lash needle or the micropipette “embryo pokers”.
- Remove nearly all embryo medium for injection. This provides surface tension that holds the embryo/chorion in place and prevents it from sticking to the needle.

(6) Injecting into the embryo’s yolk

- Carefully position your needle so that it is aligned with the embryos.
- By turning the micromanipulator, extend the needle through the chorion and into the yolk. You must use both eyes to look through the oculars to monitor what you are doing. Using both eyes provide the necessary depth perception for positioning the needle. When the needle is in the yolk, depress the injection pedal.
- Confirm that your nucleic acid mixture is inside the embryo. Because the mRNAs and morpholinos have been diluted in phenol red, you should see a faint red ball inside the yolk. If the phenol red diffuses immediately, your needle was inside the chorion, but not inside the embryo. Keep trying.
- Once you have finished injecting the column of embryos, flush them into a clean petri dish with fresh embryo medium.
- Label the dish with your names, date, and identity of injection solution (e.g., *smad5* or *chordin*; mRNA or morpholino)
- Obtain more embryos (if available) and repeat. Both you and your partner should practice all aspects of microinjection.

Drop diameter (mm)	Drop volume (nL)
0.1	0.41
0.11	0.56
0.12	0.72
0.13	0.92
0.14	1.15
0.15	1.41
0.16	1.71
0.17	2.1
0.18	2.4

EXAMINING YOUR EMBRYOS:

- (1) Place your embryos in the 28°C incubator at the end of the lab period. You will need to return tomorrow (noon or before) to observe and score your embryos for D-V patterning defects.
- (2) Before capturing photos of your embryos, you will need to remove the chorion (Before you leave, make sure that Kara demonstrates how to use your forceps to dechorionate ~1 dpf zebrafish embryos)
- (3) Use the photos and information in Figure 2 to classify embryos as dorsalized (lacking ventral character) or ventralized (lacking dorsal character). Count and record the number of different classes of dorsalized and ventralized embryos in each of your dishes of injected embryos.
- (4) After classifying and counting your embryos, fill in the google spreadsheet ([DV patterning data](#)) so that all members of the class will have access to all the data. Upload any photos to the google drive folder ([DV patterning zfish photos](#)).

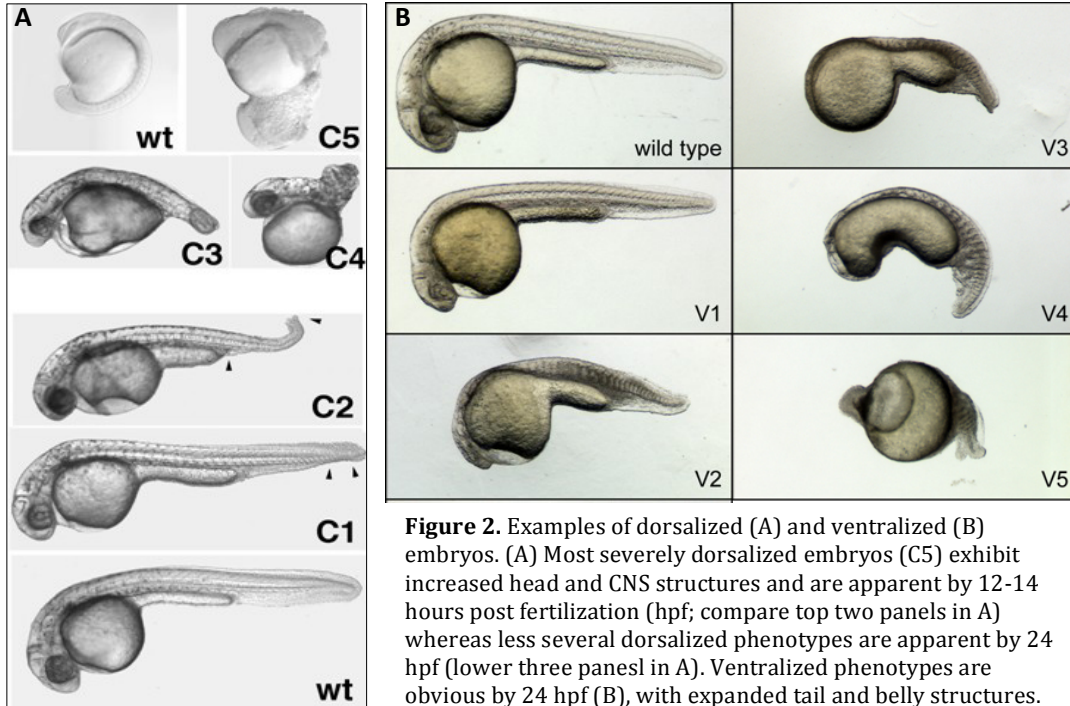


Figure 2. Examples of dorsalized (A) and ventralized (B) embryos. (A) Most severely dorsalized embryos (C5) exhibit increased head and CNS structures and are apparent by 12-14 hours post fertilization (hpf; compare top two panels in A) whereas less severe dorsalized phenotypes are apparent by 24 hpf (lower three panels in A). Ventralized phenotypes are obvious by 24 hpf (B), with expanded tail and belly structures.

ANALYSES AND CONCLUSIONS:

Download the final class data set from google drive. Use the data to graphically depict how changes in BMP influence D-V patterning and write a paragraph that explains your graph(s) and address the questions listed below. **Please print out the analysis (and accompanying paragraph) and turn in at the beginning of your next lab period.**

- What percentage of embryos injected with *chordin* mRNA are dorsalized?
- What percentage of embryos injected with *chordin* morpholino are ventralized?
- What percentage of embryos injected with *smad5* morpholino are ventralized?
- What percentage of embryos injected with *smad5* mRNA are dorsalized?
- Are the effects of manipulating *chordin* stronger than manipulating *smad5*? Why?
- What additional experiments could/should we have performed to verify that BMP pathway activation and/or inhibition directly regulates D-V axis formation?

Finally, over the next week, read the paper by Hashiguchi and Mullins (*Development*, 2013) that proposes how D-V and A-P patterning are linked during vertebrate embryo development. Provide evidence of your understanding of the paper by reflecting on *how gastrulation-mediated movements enable FGF inhibition of BMP pathway activity, promoting coordination of D-V and A-P axis patterning* in your lab notebook. You will want to revisit your understanding of this paper after weeks 4 and 5 of the course.

INTRODUCTION AND BACKGROUND: After fertilization, a series of cell divisions (cleavages) generates a blastula (ball of cells) that has to create an embryo with endoderm on the inside, ectoderm on the outside, and mesoderm in between. To understand how these processes occur, we will (1) build clay models of urchin and frog gastrula, (2) examine fixed specimens of sea urchin or sand dollar blastula and gastrula, (3) examine transverse sections of frog gastrulae, and (4) cut a series of sagittal and transverse sections of your model blastula and gastrula stage embryos.

Building models of gastrula will help solidify your understanding of how a ball of cells generates the three germ layers – ectoderm, endoderm, and mesoderm – to form a more complex organism with a gut on the inside. You will construct multicolored clay models of gastrula-stage sea urchin and frog embryos and then cut sagittal and transverse sections of your models, imitating the paraffin sections that researchers cut to see inside embryos.

Table 1: Traditional colors for specifying tissue fates in embryology

Tissue Layers and Spaces	Color
Ectoderm	Blue
Mesoderm	Red
Endoderm	Yellow
Nervous tissue (from ectoderm)	Green
Blastocoel	Grey
Archenteron	White

During the second part of today's lab we will examine some sea urchin embryos, using a fluorescent stain to highlight nuclei in the fixed embryos. We will incubate embryos with dilute 4',6-diamidino-2-phenylindole (DAPI), a chemical with a conjugated ring structure that binds/intercalates into the minor groove of A-T rich DNA. In addition, we will observe slides of blastula and gastrula-stage frog embryos and compare them to the sections we cut of our clay models.

METHODS AND LAB WORK: Everyone will build their own clay models (they make great study aids and dorm room decorations). The next pages list step-by-step instructions for building the gastrulation models. While you are rolling out balls and sheets of clay, I would encourage you to also look carefully at the pictures in your text and in this handout. Try to translate what you see in two dimensions into three dimensions in your mind before you build the model. To confirm your ideas, examine the wax and plastic models of developing frog embryos on the rear bench. Once your model is built, you can assess whether the understanding you acquired of gastrulae spatial organization from 2-D images was accurate. As with all experiments, it is important to record what you do and observe in your lab notebook, and be sure to answer the questions in the Synthesis and Conclusion section in your lab notebook.

I. Sea urchin gastrula model: The series of drawings below illustrate the dynamic changes taking place during gastrulation. Although you can choose to make any stage represented below, I would suggest constructing an early gastrula stage, such as a or b.

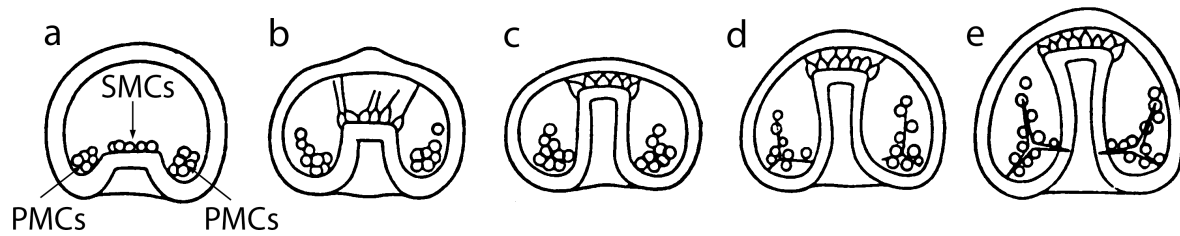


Figure 1. Gastrulation of a sea urchin containing a minimal amount of yolk. Primary and secondary mesenchyme cells (PMCs and SMCs) are inside the blastocoel. In panel b, SMCs have extended filopodia to pull the archenteron across the blastocoel. In panels d and e, PMCs are secreting the skeletal rods known as spicules.

(1) Make the blastocoel first. . Following the color conventions outlined in Table 1, use grey clay to form a small sphere approximately 2.5 cm in diameter (about the diameter of a quarter).

(2) To represent the vegetal pole, slightly flatten the grey sphere at one end.

(3) Now make space for the archenteron. Press the closed end of a small test tube into the base about mid-way into the blastocoel.

(4) Using a razor blade, bisect the punched-in sphere along the animal-vegetal axis. Lay the two halves with the grooved side up.

(5) Now make the archenteron lumen. Make a small cylinder of white clay that is slightly smaller in diameter and a few mm longer than the groove you made in step 3.

(6) Now make the endoderm that forms the archenteron wall. Roll out a sheet of yellow clay into a ~3x3 cm square. This piece should be thin ~2 mm thick.

(7) Place the white cylinder (from step 5) onto the yellow square so that one end of the archenteron is offset from the edge of the square by a few mm. Roll the yellow endoderm around the cylinder and pinch the overhanging endoderm closed over the top of the cylinder.

(8) Insert the endodermal covered archenteron into the groove in one-half of the blastocoel. Make sure that the end covered in yellow goes in first. Also make sure that the archenteron extends a few mm below (outside) the vegetal pole.

(9) Now make the mesenchymal cells. Following the color conventions outlined in Table 1, use the clay to make ~16 small red beads ~1 mm in diameter. Divide them into three piles, with 2 piles of 6 beads representing the primary mesenchyme and 1 pile of 4 representing the secondary mesenchyme.

(10) Make small indentations in the lobes of blastocoel on either side of the archenteron. Press the PMCs into these depressions. These PMCs will form the spicules (skeletal elements) in the sea urchin larva.

(11) Press the SMCs into the blastocoel at the tip of the archenteron. SMCs contribute to multiple tissues including the coelomic sacs, pigment cells, and muscles.

(12) Align the other grey blastocoel half and press the two halves together. Seal the seam by pinching the two lips together. **Keep track of the seam during the next step.**

(13) Now make the ectoderm. Roll out a sheet of blue clay ~3 mm thick that will cover the entire surface of the grey blastocoel ball leaving a space for the blastopore. Carefully wrap the blue ectoderm around the embryo, avoid wrinkles if at all possible. You may need to trim the blue ectoderm in places, especially at the blastopore. You want it to cover the yellow endoderm but not the white archenteron. Remember, at the blastopore the endoderm and ectoderm are one continuous sheet of cells.

(14) Mark your blastocoel seam on the surface of the blue layer with your fingernail (this will guide your sectioning (described in step 16)).

(15) Chill your model in the refrigerator while you make your frog gastrula models and perform the DAPI and AP staining of the fixed sea urchin embryos. When you are finished with those parts of the lab, retrieve your model from the refrigerator and complete step 16.

(16) Section your model with a razor blade. At this step, it is best to work in pairs. For one model, I recommend making a series of sagittal (meridional) slices (in the plane of the animal-vegetal axis) that are parallel to the seam you made. For the other model, I recommend making a series of transverse (equatorial) slices (cut perpendicular to the animal-vegetal axis).

(17) Draw representations of these sections in your lab notebook. Now forget about the specific steps listed above and think about how these tissues are generated in the embryo. Talk about the following questions with your lab partner.

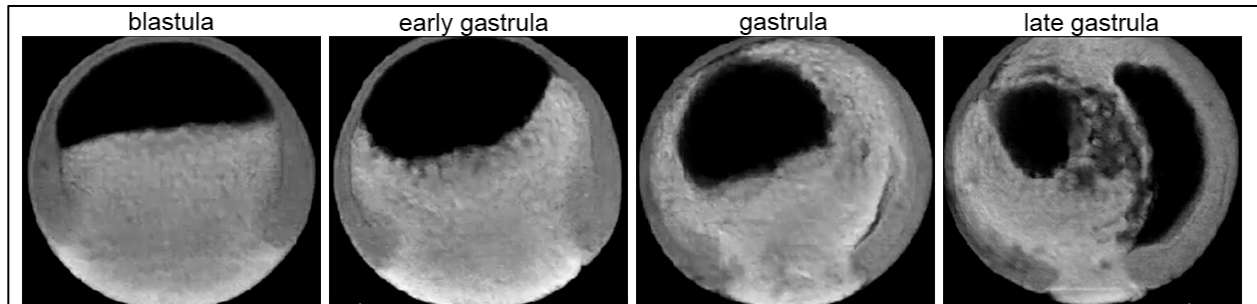
How do you think the blastocoel forms?

Where do the PMCs and SMCs come from?

How are endoderm and ectoderm part of a continuous sheet of cells?

What makes the cells on the inside versus the outside different from each other?

II. Amphibian (frog) gastrulation model: Now that you've had some practice building clay models of gastrula, try your hand at translating two of the diagrams below into 3-D clay models. Build one for blastula stage and then choose ONE of the later stages. You can also find diagrams of transverse sections of these same stages on **page 309** of your text (*Wolpert's Principles of Development, 4th Edition*). The large models in the lab, which are accurate for *Xenopus laevis*, may also help you figure out how to represent frog gastrulae with different colors of clay. In addition to the features listed on the diagrams, include the neural ectoderm (in green) in the appropriate location(s).



Confocal optical sections through a developing *Xenopus* embryo. Courtesy of M. Danilchik.

Hints for frog gastrula construction:

- Use the color conventions in Table 1.
- One way to construction of a stage 8 blastula, would be to place a sphere of blastocoel on top of a sphere of yolky vegetal cells. Then add ring of mesoderm, more endoderm and then ectoderm.
- Try constructing a spherical model with or without joining mirror-symmetric hemispheres
- *In our models we must use solid clay to represent empty spaces (blastocoel and archenteron).

If you're interested in learning more about frog development you might want to explore Xenbase: <http://www.xenbase.org/anatomy/alldev.do>

III. Staining embryos with DAPI to highlight nuclei:

- (1) Retrieve aliquots of the various stages of fixed embryos Allow each tube to equilibrate to room temperature by removing the supernatant from above the settled embryos and resuspending the embryos in 500 µl of PBS + 0.1% Triton X-100 (PBST).
- (2) Let embryos settle to bottom of tube. Remove supernatant and add 300 µl of PBST + 1.5 µl DAPI (5 ug/ml). Incubate at room temperature for 3 min.
- (3) Remove DAPI solution and rinse 3X with 300-500 µl of PBS. Cover these tubes with foil and keep them on your bench until ready to use the fluorescence scope.
- (4) When ready to observe and count the fluorescent nuclei in the blastula and gastrula staged embryos, place 15 µl of stained embryos into a depression slide. We'll most likely do this step as a group and project the images on the screen.
- (5) Draw an example of each embryo stage. Estimate the number of nuclei in the embryos you observe.

IV. Observing fixed transverse sections of frog embryos:

- (1) Obtain a slide from Kara and observe using the dissecting scopes and compound scopes near the front of the room.
- (2) Can you identify the different germ layers? Can you see individual nuclei?
- (3) How do your models compare to these samples? What, if anything, would you need to do to make your models more realistic?

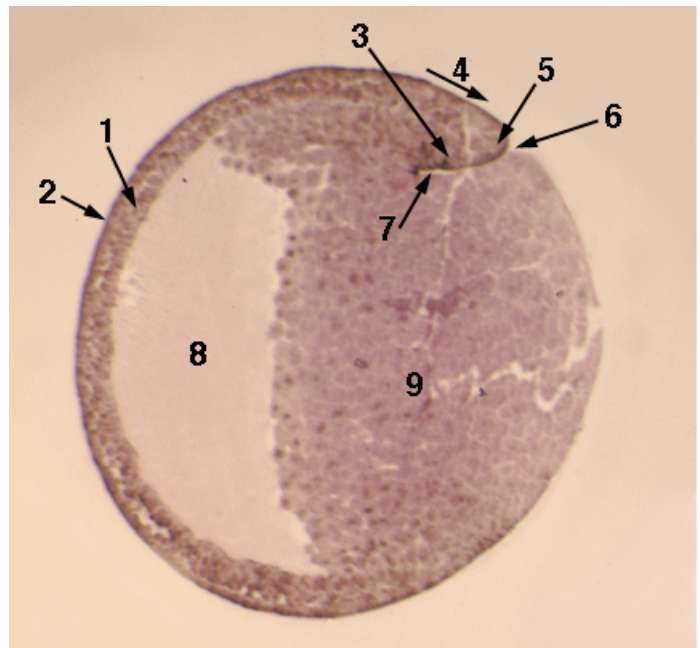
SYNTHESIS AND CONCLUSIONS: For the conclusion of this lab, write a paragraph in your lab notebook (you can type, print it out and paste it in) that synthesizes your understanding of gastrulation. Then answer the following questions:

- What might happen if the bottle cells didn't form properly. What would you predict would happen? Would all three germ layers still be present?
- Compare and contrast the sea urchin and frog gastrulae. Are these organisms protostomes or deuterostomes? How are the three germ layers organized with respect to each other in each organism?
- In both of our models we have to use solid clay to represent empty spaces (blastocoel and archenteron). How do these empty spaces normally develop?
- Think about how one might remove small pieces of the gastrula and graft them onto another embryo. What might you predict would happen if the dorsal blastopore lip were removed and grafted onto another organism in a different location?

Finally, test your understanding of the germ layers and gastrulation with a little "quiz". Cut and paste the photo and your answers into your lab notebook.

For each numbered arrow, identify the germ layer/tissue type/space for this sagittal section of a *Xenopus* embryo.

- 1.
- 2.
- 3.
4. Direction of epiboly
- 5.
6. Blastopore
- 7.
- 8.
- 9.



ADDENDUM: Embryology and Fiddly Techniques

Today we will have time in lab to watch two videos about the people and embryological techniques used since the early 1900s to probe vertebrate embryogenesis (including patterning and gastrulation). Kara will also demonstrate cell transplantations, showing you how to remove a small group of fluorescently labeled cells from a donor embryo (generally a cell-cycle mutant) and squirt them into an unlabeled host embryo. These embryos will be observed over the next several days, and depending on the particular cells transplanted, proliferation, migration, and differentiation of the transplanted cells will be analyzed. The ability to make **genetic chimeras** is a very powerful technique, especially useful for fate mapping and analyses of single cell behaviors. If you have steady hands, you may opt to perform transplant experiments for your independent project (please confer with Kara if this is something you're interested in and would like to participate in on-going research in her lab).

INTRODUCTION AND BACKGROUND: Cell-cell signaling and interplay among signaling pathways coordinates a number of developmental events including tissue specification and organogenesis. Proteins and small molecules manufactured within specific regions of the embryo can act in a graded manner to control specific developmental events, including tissue specification and cellular differentiation. Scientists can manipulate signaling pathway activity genetically and pharmacologically. In today's lab, we will perform pharmacological manipulations to investigate how two well-characterized signaling pathways – **Wnt/ β -catenin** and **FGF** – impact zebrafish embryo development. In addition to regulating key developmental events, over-activation of these pathways is implicated in oncogenesis, the initiation of cancerous tumors.

Wnt/ β -catenin signaling governs axial patterning and, as a result, promotes distinct cell fates within the developing nervous system. When the Wnt ligand (a secreted protein) binds its cell-surface receptor (Frizzled, Fz) and co-receptor (LRP5/6) on the cell surface, a signal is transduced that encourages β -catenin protein accumulation in the cytosol. As the cytosolic levels of β -catenin increase, the protein diffuses into the nucleus where it activates target gene expression. In the absence of Wnt-Fz binding, β -catenin is phosphorylated by a kinase called GSK3 β . The additional phosphate groups on β -catenin trigger its degradation by the proteasome. In an embryo's most dorsal and anterior region, down-regulated Wnt/ β -catenin pathway activity is linked to the formation of a number of neural structures, including the eyes.

Fibroblast growth factor (FGF) pathway activity also contributes to axial patterning and influences the specification of posterior structures in the nervous system. When an FGF ligand (a secreted protein) binds its cell surface receptor (FGFR), a signaling is transduced that ultimately stimulates phosphorylation of Mitogen-activated protein (MAP) kinase and activation of target genes. Because MAP kinase is a serine-threonine kinase, it can influence the phosphorylation status of numerous proteins, including those that regulate other signaling pathways (including effectors of BMP (Hashiguchi and Mullins, 2013) and Wnt pathways (Squarzone et al., 2011) and influence cell shape changes (by targeting non-muscle myosin (e.g., Klemke et al., 1997) and other components that regulate the cytoskeleton). Throughout vertebrate development, FGF is secreted by small groups of cells that exert organizing effects in discrete regions of the embryo. In today's lab we are most interested in how FGF impacts development of the central nervous system (CNS).

Treating embryos with pharmacological agents that activate or inhibit cell-cell signaling events is a simple way to investigate the effects of a particular pathway and provides a non-genetic way to probe potential cross-talk among signaling pathways. In today's lab, we will (1) activate the Wnt pathway in early embryos with LiCl, an inhibitor of the protein kinase, GSK3 β and (2) inhibit the FGF pathway with SU5402 or FIIN1-HCl, compounds that block activity of the FGF receptor. You will then have the opportunity to design and perform treatments with these compounds, either singly or in combination to further investigate how these pathways impact vertebrate development.

METHODS AND LAB WORK:

Many pharmacological agents used to manipulate cell-cell signaling pathways exhibit narrow concentration ranges of solubility and activity. Scientists must therefore precisely dilute the compounds before using them. Before coming to lab, answer the questions below by performing the necessary calculations.

How many g of **LiCl**¹ did I use to make up 50 ml of 1M LiCl?

How much 1M LiCl will you need to make 15 ml of 300 mM LiCl?

How many mg of **SU5402**² did I use to make up 5 ml of 0.5 mM stock solution in DMSO?

How much 0.5 mM **SU5402** will you need to make 20 ml of 5 μ M **FGF inhibitor** in embryo medium?

Since the 0.5 mM **FGF inhibitor** stocks are made in DMSO. How much DMSO and how much embryo medium will you need to make a control treatment solution?

¹ Molecular Mass of LiCl is 42.39 g/mol and it is soluble in water (and embryo medium).

² Molecular Mass of SU5402 is 296.32 g/mol and it is soluble in DMSO to 100 mM.

I. DEFINED PHARMACOLOGICAL TREATMENTS:

1. Before beginning the treatments to manipulate signaling pathways in vivo, you will (re)familiarize yourself with zebrafish embryos and see the results of treating early gastrula-stage embryos with a pulse of LiCl. Record your observations and answers to the questions below in your lab notebook.
2. Obtain some 1 day post fertilization (dpf) embryos from Kara. These embryos were treated with either 300 mM LiCl or 300 mM NaCl or left untreated for 15 min at 50% epiboly and then washed and allowed to develop in embryo medium.
3. Compare the embryos. What do you notice about the LiCl treated embryos?
4. Why do you think we used NaCl treatment as a control?
5. Next perform the FGF pathway inhibition experiments by diluting the SU5402 into E3 embryo medium as outlined above (be sure to check your calculations with Kara before beginning). Obtain a small brown tube of 0.5 mM SU5402 (in the freezer). Be sure to wrap your diluted drug in foil.
6. Make your control treatment solution (DMSO is available on the front bench).
7. Obtain a 6-well plate and 30-40 late gastrula-stage embryos from Kara.
8. Transfer 15-20 embryos into two different wells of your dish. Use a fine-tipped Pasteur pipette to remove as much embryo medium from the wells as possible. It can help to pool the embryos by tilting the dish at a 45° angle.
9. Add your FGF inhibitor solution to one well and then your control solution to the other well. Label the wells with the treatment they received and label your dish with your names. After you complete the next part of the lab, you will incubate your embryos overnight (~12-14 h) at 28°C by placing them in the appropriate incubator.

II. YOUR OWN PHARMACOLOGICAL TREATMENTS: Design and perform an experiment to examine how time, concentration, or perdurance of activity of the Wnt and/or FGF pathway impacts embryonic development. Alternatively you may want to examine the interdependence of FGF and Wnt pathways, examining the extent to which GSK3 β , which is essential for regulating Wnt/ β -cat pathway activity and plays an auxiliary role in modulating and/or integrating FGF and Wnt pathway activities.

- (1) Decide which aspect of signal transduction during development you'd like to investigate. Discuss the pros and cons of pharmacologically manipulating zebrafish embryos for these types of experiments. Keep in mind that our amounts of SU5402 are limited and that the impact of both LiCl and SU5402 can be modulated by changing the concentration, timing, and/or duration of incubation. Depending on the number of early and late gastrula stage embryos available for this experiment, we may need to decide as a class on one experiment. Therefore, after everyone has had time to perform the literature search described below, we'll have a class discussion about which experiment(s) we'll perform.

(2) Perform a search using Web of Science or PubMed to find three papers related to pharmacological manipulation of Wnt/ β -cat and FGF signaling pathways. Read the abstract of each paper and take notes on any information relevant to the type of experiment you'd like to do. Below are some example papers that might help stimulate your creative juices.

- Picker and Brand, 2005. Fgf signals from a novel signaling center determine axial patterning of the prospective neural retina. *Development* 132: 4952-62.

- Kim et al., 2002. Specification of an anterior neuroectoderm patterning by Frizzled8a-mediated Wnt8b signalling during late gastrulation in zebrafish. *Development* 129: 4443-4455.

- Gunhaga et al., 2003. Specification of dorsal telencephalic character by sequential Wnt and FGF signaling. *Nature Neuroscience* 7:701-7.

(3) Use your experience from the first part of the lab to guide experimental design. You will want to perform simple measurements of the embryos as they develop, including the presence or absence of particular features such as eyes, ears, or melanocytes (pigment). Think about how many time points to sample and which treatments are appropriate negative controls. To have a meaningful dataset with the appropriate controls, we'll most likely need to pool our data as a class. For these experiments to be a success, you'll need to organize your time to return at specified intervals throughout the week to check on your experiment and record your observations/capture images etc. All raw data will need to be available (uploaded to google drive) by the beginning of lab next week.

CONCLUSIONS AND WRITE UP: Scientists communicate their research in two primary forms – multimedia presentations (talks and posters) and papers. By this point in the laboratory section of the course, you will have had first-hand experience with concepts such as the genetic basis for development, embryonic patterning, induction, gastrulation, and tissue specification. You will also have a sense of some of the ways in which these processes can go awry. It is your task to place your experiments in the appropriate context and explain your results to your scientific colleagues by **following the format of a paper published in *Development* (<http://dev.biologists.org/>)**. For your write up you will need to: (1) provide an **Introduction** with relevant background information, context, and the hypothesis/question your experiments address; (2) explain your experimental procedures in the **Materials and Methods**; (3) report your data, using text, photos, graphs (in which you've analyzed your data), and diagrams in the **Results**; (4) explain what you think your results mean and propose future directions in the **Discussion**; (5) cite any **References** used in preparing the lab report; and (6) list **Contributions** of all people who helped with the lab and lab write-up.

-Remember that you are writing in the style of a scientific paper (not just a lab report). Therefore, you will want to write in prose throughout the document, being sure to describe your data with words and use figure legends and/or table captions as appropriate in the **results section**. In the **discussion section**, please propose a model that explains your findings. You may also want to explain what your findings mean for basic biomedical research and think about any applications they might have for treating/diagnosing

diseases. You should draw on what you know about other morphogenetic processes and signaling pathways.

-Seek out original references where possible and try not to **cite** textbooks or the lab handout if at all possible. The best way is to search for key terms on the Web of Science (via Thomson Reuters Web of Knowledge) and/or PubMed (via the NCBI).

-Finally, no paper is complete without a **Title** and an **Abstract**. Although these two pieces of writing are the first things your reader sees, it is best to write them last.

-Please save your file as a pdf, naming it with your lab day (W or Th) followed by your and your partner's last names. Then upload the pdf of your report to the google drive folder ([Dev Bio Lab Reports](#)) no later than **6pm on Oct 14, 2016**.

REFERENCES

Hashiguchi, M. and Mullins, M. C. (2013). Anteroposterior and dorsoventral patterning are coordinated by an identical patterning clock. *Development* **140**, 1970-1980.

Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P. and Cheresh, D. A. (1997). Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* **137**, 481-492.

Squarzoni, P., Parveen, F., Zanetti, L., Ristoratore, F. and Spagnuolo, A. (2011). FGF/MAPK/Ets signaling renders pigment cell precursors competent to respond to Wnt signal by directly controlling Ci-Tcf transcription. *Development* **138**, 1421-1432.

INTRODUCTION AND BACKGROUND: This week we will examine chick development, focusing primarily on the formation of the cardiovascular system, the first organ-system to form. Chicks are widely used in developmental biology studies because the egg/embryo can be surgically and chemically manipulated with relative ease and because eggs are often available. Moreover, early chick development is thought to be similar to human development. To gain a perspective on the utility of chick as a model system, watch the following movie of chick development: <http://www.cellimagelibrary.org/images/12317>.

The cardiovascular system facilitates the transport of oxygen, nutrients, and signaling molecules throughout the young embryo. Loosely connected mesodermal cells undergo specific morphogenetic changes to become more epithelial, forming tubes that grow, branch, twist, and segment to ultimately give rise to the heart and an intricate network of arteries, veins, and capillaries. This complex vascular network is formed by hemangioblasts. These cells arise from mesodermal cells in response to vascular endothelial growth factor (VEGF), which is released initially by axial structures (primarily the somites). Depending on where in the embryo the hemangioblasts form, they can give rise to blood cells, endothelial cells, or cardiac precursor cells. Once the foundation of the circulatory system is laid down, smooth muscle cells and pericytes ensheath the endothelial cells, reinforcing the vascular tubes and regulating blood flow.

The vascular network continues to grow in response to hypoxia, which also induces expression of vascular endothelial growth factor (VEGF). Hypoxia (low [O₂]) is a necessary signal for patterning the vascular system during embryogenesis. Hypoxia-induced blood vessel growth can, unfortunately, be exploited by many of the anoxic/hypoxic tumors, some of which also secrete VEGF. Morphogenesis of blood vessels is a direct result of localized proliferation (facilitated by VEGF, Notch, and Wnt pathways), communication between adjacent cells (Notch-Delta pathway), and adhesive interactions between endothelial cells and the extracellular matrix (integrins and guidance molecules).

The heart arises when two pools of lateral plate mesoderm, which migrated through the anterior portion of the primitive streak, coalesce across the midline. The medial cells contribute to the anterior heart tube whereas the more lateral cells contribute to the posterior heart tube. The posterior heart tube forms the inflow tract (left atrium and ventricle); the anterior heart tube forms the outflow tract (right atrium and ventricle). In addition, neural crest cells contribute to the outflow tracts and the septum that separates the aorta from the pulmonary trunk.

Although the tissue origins of the heart are relatively well understood, the mechanisms that guide heart morphogenesis are less clear, and thus the pathways that are perturbed in congenital cardiac defects are an active area of investigation. Fetal alcohol syndrome and reduced levels of oxygen often result in a wide variety of cardiac defects, including lack of septa or bifurcated hearts (cardia bifida). A number of developmental defects, including heart malformations, have been found in infants born to mothers with gestational diabetes.

METHODS: In this lab, you'll have the opportunity to study the developing circulatory system (and overall development) in the chicken (*Gallus gallus domesticus*). You will observe the developing cardiovascular system at a variety of developmental stages and then design and perform experiments testing one of the following: **1**) how varying

concentrations and/or durations of ethanol exposure can impact cardiovascular development; 2) how VEGF or FGF pathway activity can influence the formation and morphogenesis of blood vessels; or 3) how elevated levels of glucose impact heart and vessel formation.

The chicken embryos are from a commercial hatchery in Iowa. We hope that the embryos are a bit older than 2 days, but we won't know until we open the eggs. Depending on the age of the chicken embryo, you may be able to see blood vessels in the chorioallantoic membrane (directly beneath the shell), the conus arteriosus, the developing ventricle, and other cardiovascular structures.

Before coming to lab, familiarize yourself with the initial stages of heart development. Depending on the stages of embryos that are available, you are likely to see some of those depicted below, as well as earlier and later stages.

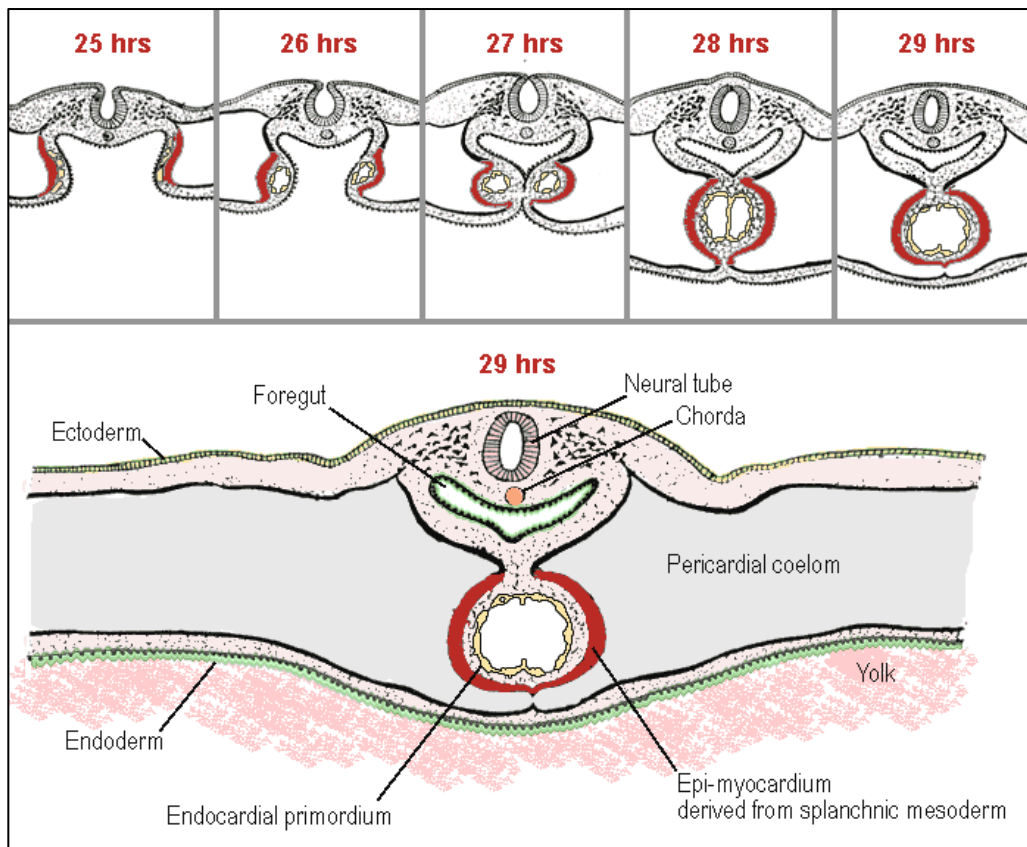


Figure 1. Transverse sections of chick heart development from 25-29 hpf according to Patten, B.M. (1920). *The Early Embryology of the Chick*. Philadelphia: P. Blakiston's Son and Company Publishers.

LAB WORK: Before you begin, obtain a staging series printout so that you'll be able to easily refer to them throughout the lab. Today's lab is divided into two parts, the first consists primarily of familiarizing yourself with the chick embryo and observing their hearts and associated vessels. The second part requires you to design and then start an

experiment. You will need to come back to the lab at various intervals throughout the week/weekend to collect data.

I. OBSERVATIONS OF CHICK EMBRYOS:

- (1) Obtain an egg, small glass petri dish, and 2 glass finger bowls. Fill the petri dish with Howard's Ringers solution.
- (2) Gently clean the egg by applying a small squirt of 70% ethanol and gently wiping with a kimwipe.
- (3) Rest the egg blunt side up for ~10 minutes to allow the embryo to float to the upper surface. While you are waiting, obtain a slide of a fixed and stained 2 dpf chicken embryo. How is it different from the images in Figure 1? What structures do you see? After 10 minutes have passed, use the scissors to cut open the blunt side of the egg. Carefully peel back the shell membranes until the blastodisc/embryo is visible. Observe the embryo under the dissecting scope.
- (4) To culture the embryo in vitro, cut a small ring of filter paper that will fit around the blastodisc. Pick it up with your forceps and then drop it onto the egg so that the embryo is encircled by a filter paper ring.
- (5) Once the disc of filter paper adheres to the egg, hold onto it with your fine forceps and cut around the outside of the filter paper with the sharp scissors.
- (6) Use the plastic spoon to scoop the filter paper and embryo into the glass petri dish filled with Ringer's solution. Have your partner stand by with the scissors in case the embryo is still attached to the egg.
- (7) If any yolk has remained, gently wash it off with Ringer's. To make observing the embryo easier, you may need to obtain another glass petri dish filled with Ringer's and transfer the embryo away from yolk etc.
- (8) Examine the embryo. Using the staging series provided, estimate the Hamburger-Hamilton (HH) stage. Make sketches in your notebook, identifying the developing heart, neural tube, somites, Hensen's node, and/or the primitive streak (if still visible), and any other structures of interest. Structures such as the eyes and limb buds may not yet be visible.
- (9) Obtain an albumin-agar small petri dish. Use a syringe to remove ~1 ml of fresh albumin from the egg and add it to the dish.
- (10) Gently lift the embryo by the filter paper ring and transfer to the albumin-agar dish filter paper-side down (vegetal/ventral surface of the embryo is facing up).
- (11) Observe the structures in the developing embryo. If it is aged between 5-11 somites (HH 8 -10), you may ask what happens if the two halves of the developing heart tube are physically separated. You can make a small incision along the midline A-P axis with your eyelash knife.
- (12) Cover and transfer your embryo in a dish to the 37°C incubator. At the end of the lab period, you will want to retrieve this embryo, flood the dish with Ringer's solution

and flip the embryo so that it's dorsal surface faces up (unless the embryo is very young, and then you will need to come back the following morning).

- (13) Chick embryos can be cultured like this for a couple of days and you can more easily observe the morphogenetic movements of organism development. Additional information about this culture method, can be found in Chapman et al., 2001 *Developmental Dynamics*.

II. DESIGN AND CONDUCT AN EXPERIMENT TO INVESTIGATE HEART AND/OR BLOOD VESSEL DEVELOPMENT IN *Gallus gallus*.

- (1) Decide whether you'd like to conduct an experiment that examines blood vessels or one that examines the heart. Discuss the pros and cons of using chick embryos for each type of experiment. Keep in mind that our amounts of VEGF¹ are limited and that filter paper soaked in VEGF (or other substances) can be inserted into chicken eggs or near a chicken embryo developing in vitro. Other substances such as glucose² or ethanol³ can be added to Ringer's Solution of cultured embryos. Due to the limited number of eggs, we'll need to decide as a class on one experiment. Similar to last week, lab groups will perform the literature search described below and then we'll have a class discussion about which question we'll address with a class-wide experiment.
- (2) Perform a search using Web of Science or PubMed to find three papers related to the effects of various substances on cardiovascular development. Read the abstract of each paper and take notes on any information relevant to the type of experiment you'd like to do. I've listed some example papers below. These might help stimulate your creative juices and choose experimental conditions.

-Memon S and Pratten MK (2013). Teratogenic effects of diabetic conditions in chick heart in ovo and in micromass culture may be prevented by addition of vitamin C and folic acid. *Reprod Toxicol* 35:117-24.

-Giles S, Boehm P, Brogan C, Bannigan J (2008). The effects of ethanol on CNS development in the chick embryo. *Reprod Toxicol* 25(2):224-30.

-Parsons-Wingenter P, Elliott KE, Clark JI, Farr AG (2000) Fibroblast growth factor-2 selectively stimulates angiogenesis of small vessels in arterial tree. *Arterioscler Thromb Vasc Biol* 20(5); 1250-6.

- (3) You will want to perform simple measurements of the embryos as they develop, including length, number of somites, developmental stage (can you think of others?). We'll need to pool our data as a class, so before we begin the experiments, we'll have a discussion about who will do which treatments. Remember to include the appropriate negative controls. Can you think of possible positive controls? How many time points will you include? You'll need to organize your time to return at specified

¹ If you choose with work with VEGF, a good range to assay is 0.25 – 0.75 ug/ml (the stock is 1 ug/ml in DMEM).

² If you choose to work with glucose, a good range to assay is 400-800 mM glucose made in Ringers and used to culture embryos.

³ If you choose to work with ethanol, a good range to assay is 20-200ul of 10-40% EtOH (diluted made in Ringers Solution).

intervals throughout the week to check on your experiment and record your observations/capture images etc. At the beginning of next week's lab period, we will exchange data raw data.

CONCLUSIONS AND SUMMARY: Scientists communicate their research in two primary forms – multimedia presentations (talks and posters) and papers. By this point in the laboratory section of the course, you will have had first-hand experience with concepts such as the genetic basis for development, embryonic patterning, induction, gastrulation, and organ formation. You will also have a sense of some of the ways in which these processes can go awry. It is your task to place your experiments in the appropriate context and explain your results to your scientific colleagues by preparing a short presentation that will use **4 key slides** (see template uploaded in the dev bio google drive folder). You will need to: (1) provide an introduction with relevant background information, context, and the hypothesis/question your experiments address; (2) explain how you addressed your question with specific experimental procedures; (3) report your results, with pictures, graphs, and diagrams; and (4) explain what you think your results mean.

You will have plenty of time to make your slides and practice your presentation that you and your partner will give to Kara. Before leaving lab today, please sign-up for a presentation time slot. Although groups will be presenting to Kara at different times, please save your slides as a pdf and then upload the file to the google drive folder ([Dev Bio Lab Reports](#)) no later than **6pm on Oct 25, 2016**.

INTRODUCTION AND BACKGROUND: The nervous system in vertebrates is comprised of millions of neurons and glia. The connections among these specialized cells govern sensory perception and promote learning. To understand how the nervous system works, we need to understand how it forms. During gastrulation, ectodermal cells are induced to become neural, based on their proximity to the forming mesoderm. These neural cells will proliferate, and at distinct stages of development, differentiate into neurons – cells that will extend processes (axons) and form connections (synapses) with each other.

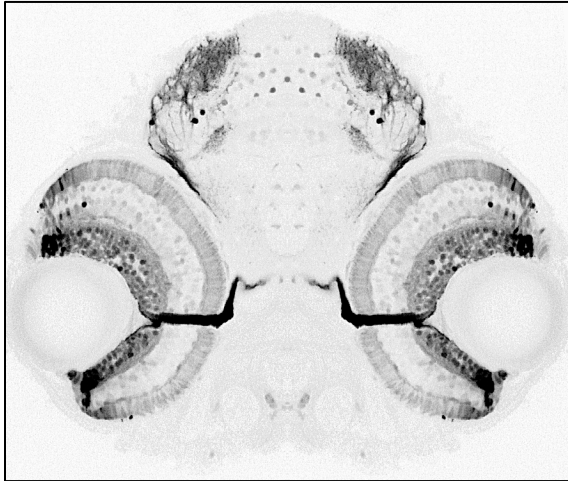


Figure 1. One of the most accessible parts of the vertebrate brain is the retina, the neural tissue of the eye. Transverse thin section of the head of a 5 day old zebrafish embryo expressing the *atoh7:GFP* transgene. Notice the optic nerve that fasciculates in the eye and then projects to the tectum where it defasciculates, connecting each retinal ganglion cell to its retinotopically matched partner.

Developmental neurobiologists employ multiple techniques to trace lineages and understand how specific parts of the nervous system form. We can capture snapshots or movies of the developing nervous system within an embryo by performing various types of microscopy on embryos in which gene expression or protein and/or cell localizations can be visualized¹. Although zebrafish are one of the most recently-developed model organisms, they have become highly favored for studying nervous system development and function. This is primarily because of the near optical transparency of the embryos coupled with their rapid development enables unprecedented microscopy imaging possibilities.

The big picture question that guides research in the Cerveny lab is: How does the neural retina grow and develop? **In this lab**, you will practice some of the embryological techniques we use to probe mechanisms of growth control in the visual

system. The eye develops from both neural and non-neural ectoderm within the first day of zebrafish development. The neural part of the eye, the retina, is a trilaminar tissue, comprised of light sensing photoreceptors (rods and cones) in the outer nuclear layer. These cells are connected to projection neurons called retinal ganglion cells (RGCs) via a number of different types of interneurons located in the inner nuclear layer. Anatomical constraints necessitate continuous remodeling of the connections between the RGCs that their targets in the optic tectum. An important property of zebrafish that facilitates my research is their continuous growth². To support life-long growth, the fish nervous system contains a number of stem cell niches that exquisitely balance proliferation and differentiation.

METHODS AND LAB WORK: Over the next two weeks (punctuated by Fall Break), you will perform two distinct sets of experiments that may contribute to on-going research in the

¹ These features are visualized with techniques such as in situ hybridization (to detect mRNA expression), immunohistochemistry (to detect protein localizations with antibodies), or cell labeling (injection or fluorescent dyes or proteins into specific cells/groups of cells).

² Also known as indeterminate growth. This property is also a feature of many amphibians and plants.

Cervený lab, with the goal of exposing you to additional, standard developmental biology techniques. The experiments we'll be performing are directly linked to understanding the mechanisms governing the transition from proliferation toward differentiation in the zebrafish visual system.

In this week's lab, you will examine several sets of embryos – some expressing a fluorescent transgene, some that have been hybridized with antisense RNAs (for in situ hybridization), and some that have been subjected to immunohistochemistry for a particular protein. For the in situ hybridized and immunostained embryos, you will perform the final developing and imaging steps of these protocols³. You will observe and document differences within each set of embryos, distinguishing between mutants and their wild-type siblings.

During this week's lab you will also have time in lab to plan your independent projects⁴, and you will have a rough draft of your project proposal. By the end of week 8 (the week after fall break), you will have submitted your final proposal and discussed it with Kara.

In situ hybridization and immunohistochemistry.

Gene expression dynamics influence myriad developmental processes. Two of the most common techniques to observe how genes are expressed in the embryo are: in situ hybridization (within tissue hybridization) and immunohistochemistry (also known as antibody staining). In situ hybridization relies on base pairing between the mRNA of interest and a probe, which is comprised of complementary *in vitro* synthesized RNA molecules labeled with a small moiety (often digoxigenin) to detect where a gene is expressed (in both time and space). Detection of labeled RNA-RNA hybrids with specific enzyme-coupled antibodies. Immunohistochemistry uses antibodies to reveal the presence and location of specific proteins. Detailed protocols for these techniques are available in the literature⁵ and from Kara.

In today's lab, we will be performing the final “developing” step for in situ hybridization and immunohistochemistry reactions that were started in the Cervený lab. You and your partner will visualize expression patterns of genes (*ccnD1*⁶ or *sox2*⁷) and proteins (*Atoh7*), all of which are required for development of a functional visual system in zebrafish.

LAB WORK:

1. Obtain 2 tubes of embryos (one marked “in situ” and one marked “immuno”). The embryos in these tubes were fixed with paraformaldehyde, subjected to various staining techniques, and are currently in PBST⁸.
2. For the tube marked “in situ”, you will be able to visualize the expression pattern of a highly expressed mRNA with an alkaline phosphatase catalyzed color change reaction.

³ The protocols for in situ and immunostaining require an extensive time commitment spread over 2-3 days. Therefore, we've performed all the steps except for the final (most variable) step so that you can see how gene expression and protein patterns can be gradually revealed and understand first-hand why these techniques are not quantitative.

⁴ Please see the independent project guidelines, distributed separately.

⁵ <http://zfin.org/ZFIN/Methods/ThisseProtocol.html>

⁶ *ccnD1* codes for a G1-phase cyclin important for regulating cell cycle progression

⁷ *sox2* encodes an SRY box transcription factor and is expressed in many neural progenitors

⁸ PBST in this instance = Phosphate Buffered Saline supplemented with 0.5% TX-100.

3. Record the age of the embryos (in days post fertilization, dpf) and any other information written on the tube.
4. Wash the embryos 3x5 minutes in AP buffer (made fresh before lab)⁹
5. Don gloves and prepare developing solution by adding 1ul NBT + 3.5ul BCIP to 1ml of AP buffer
6. Remove the last wash of AP buffer and replace with developing solution.
7. Cover your tube with tin foil or place in a drawer. Check to see if the embryos are changing color after 10-15 minutes by pipetting them into a glass well plate and checking them with the dissecting scope. If no color is visible, check again in another 15 minutes. We chose antisense probes that recognize highly expressed genes with obvious expression patterns that should become visible within 30-60 minutes.
8. *While you are waiting, start developing the embryos that have been incubated with antibodies (see step 13).*
9. Once color and pattern are apparent¹⁰, stop the reaction and wash out developing solution with several rinses of PBST.
10. To fix color, incubate embryos in 4% PFA¹¹ at room temperature for 1 h (or 4°C overnight).
11. Remove PFA and place in waste container in hood. Wash 3x5' in PBS.
12. Serially dehydrate embryos into 25, 50, 75, 95% glycerol (~5 min for each wash).
13. For the tube marked "immuno", you will be able to visualize the expression pattern of a neuronal protein by a horseradish-peroxidase catalyzed precipitation reaction.
14. Don gloves and lay out a paper towel on which to work (to catch any spills). Make up 1mL DAB¹² solution by carefully pipetting 16.6 µl of DAB stock into 1ml of PBS in a microfuge tube.
15. Transfer embryos to 3 well glass dish.
16. Remove PBST and add 500 µl of DAB solution. Incubate at room temperature for 20 minutes.
17. Add 1.5 µl 30% H₂O₂ to the remaining 500 µl DAB solution in the microfuge tube.
[H₂O₂] final=0.0009%.
18. Carefully remove the DAB solution from the embryos (into the waste container in the hood) and replace with 500 µl DAB+ H₂O₂

⁹ 50ml of AP buffer contains:

-1M Tris-HCL, pH=9.5	5 ml
-1M MgCl ₂	2.5 ml
-4M NaCl	1.25 ml
-20% TX-100	250 ul
-H ₂ O	41 ml

¹⁰ If you have questions about whether your embryos are developed enough, please see the examples Kara has under the teaching scope and ask her to view your samples.

¹¹ 4% Paraformaldehyde is toxic. Wear gloves and dispose of any waste in appropriately labeled containers in the hood.

¹² DAB is 3,3'-Diaminobenzidine. An organic compound that gives a dark brown color when oxidized by H₂O₂ that is toxic. Wear gloves and dispose of all waste in the containers provided in the hood. The stock DAB solution is 50 mg/ml.

19. Monitor reaction closely until brown color (in a specific pattern) becomes visible under the dissecting scope.
20. When the reaction has progressed far enough, rinse the embryos with PBST. Transfer back to microfuge tube.
21. Fix color by incubating in 4% PFA at room temperature for 45 min (or 4°C overnight).
22. Remove PFA and place in waste container in hood. Wash 3x5 minutes in PBS.
23. Serially dehydrate into 25, 50, 75, 95% glycerol (~5 min for each wash).

CONCLUSIONS AND FUTURE DIRECTIONS:

After completing the developing steps, you will observe your embryos using the dissecting microscopes and capture some photos, either with your smart phone or with the veroptics cameras connected to designated dissecting and compound scopes. Save your photos so that you'll be able to access them later to make a figure (which will be printed and taped/stapled into your lab notebook). See the attached appendix for help with image capture and presentation.

1. To observe the embryos with your dissecting scope, use a glass pipette to transfer the embryos from the tube to the well of a depression slide or multi-well glass dish.
2. Observe the staining patterns of the embryos that were subjected to in situ hybridization. Use your eyelash needle or poker to move the embryos around, observing the dorsal, ventral, and lateral surfaces of the embryos. Decide which features of the staining pattern are most important to you. Sort the embryos into categories based on these features. Describe the different phenotypes in your lab notebook and note the numbers of embryos in each group. Think about the ratios (do you see $\frac{1}{4}$ or $\frac{3}{4}$ or $\frac{1}{2}$ or all of the of the embryos with the same patterns?)
3. In class we have discussed other techniques to study gene expression, namely qRT-PCR and northern blotting. Compare and contrast these two techniques with in situ hybridization. Speculate why scientists would choose one method over the other.
4. Observe the embryos subjected to immunohistochemistry and compare them with the transgenic embryos carrying *Tg[atox7:GFP]* or *Tg[atox7:RFP-caax]* available at the front of the room. View your immunohistochemistry samples with white light and the transgenic samples with blue or green light. What do you notice when comparing the fluorescent living embryos with your immunostained embryos. Speculate why scientists would choose one method over the other.

OPTIONAL RESOURCES AND READINGS:

Kemp et al., 2009. Generating Chimeric Zebrafish by Transplantation. *Journal of Visualized Experiments*. Available at <http://europepmc.org/articles/PMC2770904?pdf=render>

Complete In situ hybridization protocol and flowchart

<https://wiki.zfin.org/display/prot/Thisse+Lab++In+Situ+Hybridization+Protocol++2010+update>

Silbereis et al., 2016. The Cellular and Molecular Landscapes of the Developing Human Central Nervous System. *Neuron* 89(2):248-68.

Valdivia et al., 2016. Antagonism between Gdf6a and retinoic acid pathways controls timing of retinal neurogenesis and growth of the eye in zebrafish. *Development* 143(7):1087-98.

Cervený et al., 2012. Continued growth and circuit building in the amniote visual system. *Developmental Neurobiology* 72(3):328-45.

APPENDIX: IMAGE PROCESSING AND PRESENTATION:

-If you captured photos from your phone, you can either import your image into iPhoto on your own laptop OR share it with your Google Drive account on bench top computers.

-For iPhoto, you'll need to Export the image
(File -> Export).

-Then save as a TIF (tagged image file, which preserves the most information) with a name that is informative (e.g., zfish_compound_klc_lab1.TIF)

-For Google Drive, you'll need to Download the image, Open in Preview, and then Export as TIFF. First, click on the download arrow at the top of the window. Then, click on the downloaded file icon at the bottom left and it should automatically open in Preview. Then Export the image (File -> Export)

-Then in the Export As box, type a name that is informative (e.g., zfish_klc_lab1)

-Open the Downloads folder (should be visible in the dock) and find your image.

-To process and analyze the image:

-Open the file in ImageJ:

Open ImageJ

Drag and drop the file onto the ImageJ toolbar.

-Once the image is open, you can adjust the image to view the features you want to highlight with the following manipulations:

1. (Image -> Adjust -> Brightness/Contrast) Watch the histogram and see how the image changes and the dynamic range stretches across all of the grey (or color) space.

2. (Process -> Enhance Contrast) Perform non-linear contrast enhancement by unchecking the "equalize histogram" box. Then perform linear contrast enhancement by checking the "equalize histogram" box. Do you notice any difference? (Non-linear contrast enhancement is similar to adjusting the gamma to make digital images appear more like what we see with our eyes).

3. (Process -> Math -> Gamma¹³). Check the box that allows you to preview the image. Slowly change the slider and observe what happens as you increase and decrease gamma. Note how changing the gamma is very similar to changing the contrast.

-Save your modified image. If you have time and would like, you can experiment with the other Processing algorithms in ImageJ such as "find edges" or other operations in the math submenu. Think about why some of these might be useful for scientists.

-Before our discussion of image processing, I'll demonstrate some of the most common, acceptable image modifications in Photoshop. You will be able to use ImageJ and/or Photoshop for other images you capture in this course. ImageJ is freely available for downloading to your personal computer. In addition, ImageJ and Photoshop are available for use on the ETC computers. For more information on how to use ImageJ and the science behind what the various operations that it can perform, navigate to the following link:

<http://imagej.nih.gov/ij/docs/menus/>.

¹³ Gamma is a non-linear factor that describes the relationship between pixel luminance (amount of light we perceive) and the numerical value assigned by the digital detector. Gamma is required to adjust the bright and dark tones of a digitally captured image to make them appear more like what we perceive (because our eyes capture light non-linearly whereas digital detectors capture linear information).