Supplemental Data: Zebrafish Lab Manual, Equipment Needed, Worksheets, Course Evaluation Form, and Solution Recipes

Contents:

(1) Preface 1
(2) Overview of Lab Schedule 1
(3) Zebrafish Husbandry 1
(4) List of Fish Stocks, Equipment, and Solutions 1
(5) Lab Manual for Lab Managers and Instructors (Includes Worksheets) 2
(6) Course Evaluation Sheet 18
(7) Recipes for Solutions for the Lab Course 18
(8) References 19

Preface

In this supplemental data, we provide detailed information necessary to set up this laboratory course. We list the equipment and materials necessary for the different sessions as well as provide our detailed Lab Manual for Lab Mangers and Instructors. We provide our worksheets, including answers, and recipes for all the solutions needed. This manual was made possible by the many instructors and TAs who have taught the course as well as the hard work of Scotty Davis and Jason Crawley our Lab Managers.

Overview of zebrafish lab schedule

Week	*Lab Topic and Assignments*
1	**Introduction to Zebrafish** Basics of development—lecture by instructor Lecture by zebrafish researcher, Dr. Iain Shepherd
2	**Lab 1: Zebrafish Embryonic Development** Worksheet 1—in class and homework Reading Assignment: (1) "Headwaters of the zebrafish—emergence of a new model vertebrate" by Grunwald DJ and Eisen JS, 2002. (2) "The art and design of genetic screens: zebrafish" by Patton EE and Zon LI, 2001. PDF files of papers are posted.
3	**Lab 2: Mutation and Development I** Worksheet 1 and lab report due Worksheet 2—in class and homework
4	**Lab 3: Mutation and Development II** Worksheet 2 and lab report due Worksheet 3—homework
5	**Lab 4: Environmental Effects on Development*** Worksheet 3 and lab report due Prepare a group presentation for next week
6	**Group Presentations on Experiments of Week 7***

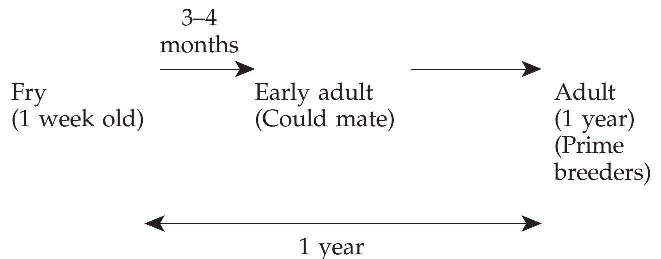
*The class will be divided into four groups of six students.

Zebrafish husbandry

All relevant information can be obtained from the Zebrafish Information Network website (www.zfin.org). This site has an online copy of the Zebrafish Handbook that describes in detail zebrafish animal husbandry and breeding. Embryos, fry, and adults can be purchased from Zebrafish International Resource Center (ZIRC) (www.zebrafish.org). Thoren Aquatic Systems, Inc., PA, is our recommended vendor for breeding tanks and complete fish facilities (www.thorenaquatics.com). An alternative vendor for establishing a fish facility is Aquatic Eco-Systems, Inc., FL, which sells a bench-top system or Aquatic Habitats, Inc., FL, which sells various sizes of fish-handling systems.

Male fish are thinner and have a yellow belly, while females are rounder and silver. To breed, set up four breeding tanks with two pairs of male and female fish in each. To obtain around 5000 embryos, you will need to set up 10 such tanks. The tanks are set up the night before, with males separated from females by a divider. Tanks are kept in a room with a fixed light–dark cycle. At 9.00 a.m. when the lights turn on, change the water and clean the tank. Then lift the divider for the fish to mate. You can stagger the time when the divider is lifted in each tank, to obtain embryos at different stages.

Timeline



Breeding slows with adults that are 1.5–2 years.

List of fish stocks, equipment, and solutions for each session

LAB 1: Zebrafish Embryonic Development

- Fish facility with controlled light–dark cycle
- Breeding tanks with dividers
- Adult wild-type zebrafish for breeding
- Petri dishes
- Embryo media
- 28.5°C incubator
- Pokers (need one per microscope plus spares). These are glass capillary tubes (WPI 1B200-6) that have fishing line inserted through them so that a small

amount extends from the end of the tube. The fishing line is stuck to the glass by applying superglue.
- Transparent photocopies on acetate of graph paper to permit measurements
- Stereomicroscopes (Olympus SZ30, Olympus USA) one per pair of students
- Apple iSight camera attached to the Instructor's dissecting scope

LAB 2: Mutation and Development I

- *tbx24* morpholino
- Injection apparatus to inject morpholino
- Adult wild-type zebrafish for breeding

As an alternative to these, first three items have a stock of adult breeding *tbx24* mutant carrier fish.

- 4% Paraformaldehyde
- *myoD* antisense riboprobe
- 65°C Waterbath for processing *in situs*
- Hybridization buffer
- 1× PBS
- 10× SSC
- 20% Tween 20
- PBTw (1× PBS and 0.2% Tween 20)
- Methanol
- Goat serum
- BSA
- Anti-DIG antiserum
- Rocker to shake tubes; one per lab
- Eppendorf microtubes
- P200 and P1000 pipetteman and tips
- Petri dishes
- *In situ* color reaction buffer
- Stereomicroscopes (Olympus SZ30) one per pair of students
- Apple iSight camera attached to the Instructor's dissecting scope

LAB 3: Mutation and Development II

- Breeding stock of *lessen* mutant carrier fish
- Acid–alcohol (100 mL 70% ethanol and 1 mL concentrated HCl [±37%])
- Alcian green (0.1 g Alcian green 2GX [Sigma-Aldrich, St. Louis, MO] and 100 mL acid–alcohol)
- Glycerol–KOH (50 mL 0.5% KOH and 50 mL glycerol)
- Eppendorf microtubes
- P200 and P1000 pipetteman and tips
- Petri dishes
- White card to place under Petri dish
- Stereomicroscopes (Olympus SZ30) one per pair of students
- Apple iSight camera attached to the Instructor's dissecting scope
- Compound scope
- Glass depression slides for compound microscope

LAB 4: Environmental Effects on Development

- Incubator at 28°C
- 70°C water bath (for cyclopamine)
- 35-mm Petri dishes
- Embryo media
- 0.3 M Lithium chloride (Sigma-Aldrich)
- 100 μM Cyclopamine (Toronto Research Chemicals, Toronto, Ontario, Canada)
- 0.006% Phenyl-thiourea (Toronto Research Chemicals)
- 1 μM Retinoic acid (Toronto Research Chemicals)
- Pipette pumps and glass pasture pipettes
- Stereomicroscopes (Olympus SZ30) one per pair of students
- Apple iSight camera attached to the Instructor's dissecting scope
- Pokers
- Transparent photocopy on acetate of graph paper

Lab Manual for Lab Manager and Instructors

Lab 1: Zebrafish: Early Embryogenesis and Gastrulation

Students will be observing live fish embryos at the following embryological stages:

(a) 0–1 hour postfertilization (hpf), (b) 6–8 hpf, (c) 24–26 hpf, and (d) 48–50 hpf.

Directions for prep managers before class

(1) Fish setup and breakdown

Set up at least one complete stock tank for each setup day ~10–12 breeding tanks at around 5 p.m. the previous day. Each breeding tank should have four females plus three males. The day of lab, change water 2 h before each embryo collection.

For the 0–1 and 6–8 hpf embryos, lift the dividers 2 and 5 h, respectively, before lab begins.

For the 24 and 48 hpf embryos, change over water and remove cage dividers, 24 and 48 h before lab.

Once embryos are collected, place them in Petri dishes in standard embryo media. Label the dishes appropriately as (A) 0–2 hpf embryos, (B) 6–8 hpf embryos, (C) 24 hpf embryos, and (D) 48 hpf embryos. Put into 28.5°C incubator until shortly before class.

AT THE END OF CLASS EACH DAY COLLECT ALL THE EMBRYOS UP. THESE EMBRYOS WILL BE REUSED FOR THE FOLLOWING DAY'S CLASS! CLEAN THE DISHES UP BEFORE PUTTING BACK INTO 28.5°C INCUBATOR (i.e., remove dead/unfertilized embryos).

Additional items that need to be made

1. Pokers need to be made before each lab; you need one per microscope plus spares. People will lose them!
2. Transparent photocopies on acetate of graph paper to permit measurements.

(2) Fish setup and breakdown for lab 2

If labs are held in consecutive weeks, then fish need to be set up for next week's lab (see under lab 2).

Directions for Instructors

You should be familiar with the fundamental developmental processes being looked at. You should have an introductory lecture prepared to help the students. Refer to Zebrafish Information Network (ZFIN), http://zfin.org/, and Kimmel *et al*. (1995) to staging development.

Key concepts

(1) Multicellular embryos arise from the cell division of a single-cell zygote.

(2) Cleavage divides up the one-cell zygote cytoplasm with little overall increase in embryo size.

(3) Initial planes of cell division occur in precise planes of orientation.

(4) Cell cycle length initially is (fairly) synchronous and uniform length of time. However, at midblastula stages cell cycle length changes and becomes asynchronous and nonuniform.

(5) The evolution of the process of gastrulation was a key event in animal evolution. The evolution of this developmental process enabled the formation of triploblastic organisms, that is, those that have three germ layers, ectoderm, endoderm, and mesoderm.

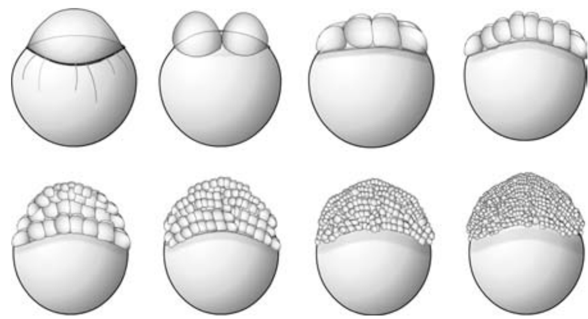
(6) Gastrulation requires dramatic cell movements.

(7) Gastrulation reflects the first morphological indication of the establishment of the major body axes.

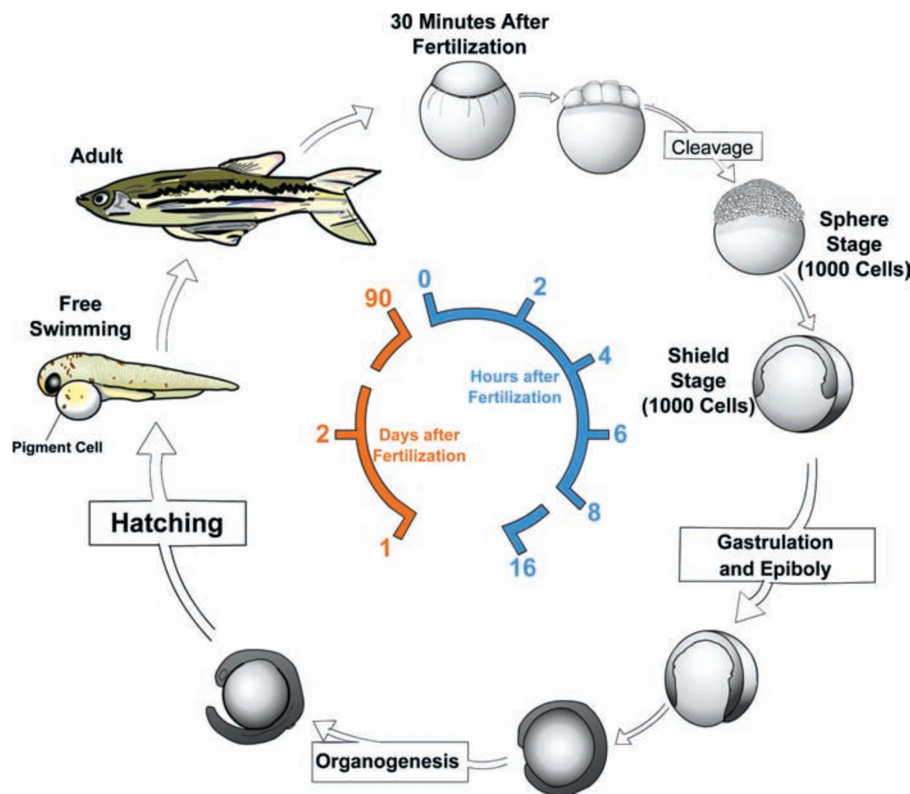
(8) Subsequent to gastrulation, organogenesis can occur, and the different major organs of the body can arise.

Discussion/Lecture

At the beginning of the class, you should introduce the key concepts being investigated. Below is a rough overview of



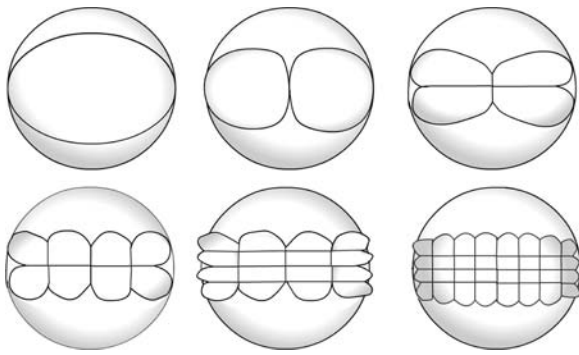
zebrafish embryogenesis. Sphere stage is the blastula stage of embryogenesis, while shield stage is the first stage of gastrulation.



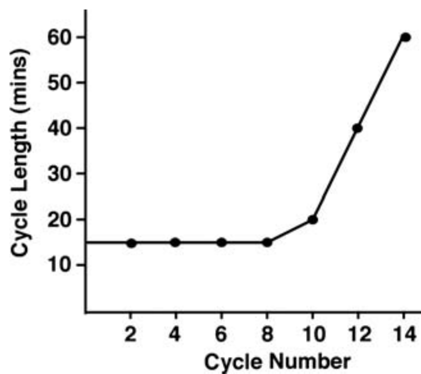
(1) Multicellular embryos arise from the cell division of a single-cell zygote. Remind students that zebrafish are a eukaryotic organism that develops via sexual reproduction. During fertilization gametes fuse to generate a diploid zygote. Fertilization needs to take place for normal development to occur.

(2) Cleavage divides up the one-cell zygote cytoplasm with little overall increase in embryo size. In the series of drawing below that are to scale the overall size of the embryo does not change that much. We are just generating more cells so that the subsequent stages of development and the final embryo form can be sculpted this "cellular raw material." Can use analogy that the development process like a sculptor who needs to beginning with an unformed block of stone/marble from which he/she carves out the final form.

(3) Initial planes of cell division occur in precise planes of orientation. Students are going to be asked to record the



initial planes of division. In the series of images below the planes of cell division for the first five cell divisions are illustrated. The view is as if we were looking down from



above from the animal pole of the embryo. Note how they are at 90° to the previous plane of cell division.

(4) Cell cycle length initially is (fairly) synchronous and takes a uniform length of time. However, at midblastula stages cell cycle length changes and becomes nonuniform and asynchronous. The students are going to be asked to record the length of time for each initial cell division for the 0–1.75 hpf embryos. The synchronicity and time length uniformity result from the fact that the initial cell divisions are driven by uniformly deposited maternal transcripts and proteins. The asynchronicity and nonuniformity of length of time of cell division starts to occur when the cells begin zygotic gene transcription so that there is nonuniform genes transcription starting to occur in the cells of the embryo. The graph below reflects this initial situation and the change over time.

(5) The evolution of the process of gastrulation was a key event in animal evolution. The evolution of this developmental process enabled the formation of triploblastic organisms, that is, those that have three germ layers, ectoderm, endoderm, and mesoderm. The students are going to be looking at embryos around 6–8 hpf. This is the stage when embryos first start to gastrulate. To form the three germ layers, mesodermal cells must involute so as to give rise to theses three layers. Point out the difference in diploid organism, things like sponges where there is only an ectoderm and an endoderm ~no mesoderm. Point out what each of these germ layers gives rise to and summaries how the process takes place using the frog gastrulation. Depicted below is a schematic of the example that they will be discussing in class of *Xenopus* gastrulation.

(6) Gastrulation requires dramatic cell movements. The situation in zebrafish is similar to that seen in frogs. Unlike frogs that are opaque, the zebrafish embryos are transparent; therefore, you can see the involuting mesoderm. The images on the following link show development during the gastrula period of zebrafish embryo genesis:

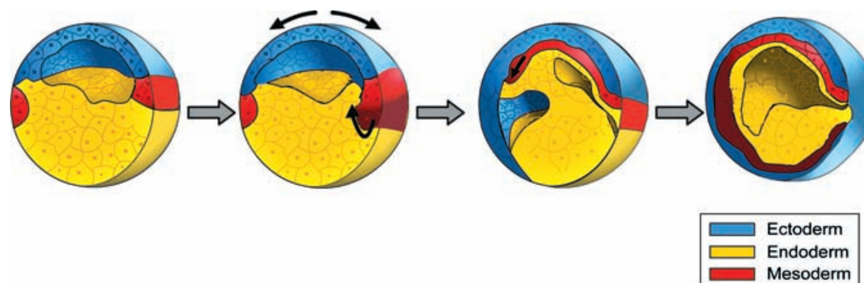
http://zfin.org/zf_info/zfbook/stages/figs/fig11.html.

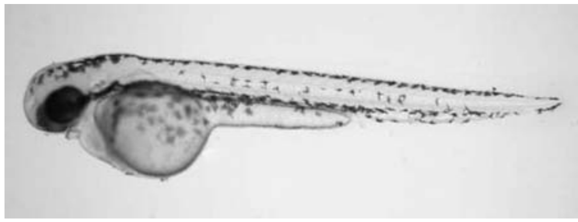
Panels D and E in the above link show the involuting mesoderm from lateral and dorsal and lateral perspective at what is know as the shield stage approximately 6 hpf.

Students will be asked to draw the lateral view of the embryos, identify the leading edge of the involuting mesoderm know as the shield in zebrafish, and then measure/note that cell movements drive the involution forward.

(7) Gastrulation reflects the first morphological indication of the establishment of the major body axes. The location where the shield forms is the first morphological indicator that indicates the establishment of the anterior–posterior axis. It also lays down the medial and lateral axis and dorsal–ventral axes. The point where the shield is located will eventually end up being the posterior ends of the embryo at the midline. The first involuting tissue mesoderm will give rise to the anterior part of the embryo, so the embryo develops and is specified in an anterior to posterior manner. Obviously, involuting tissue is ventral to the dorsal overlying ectoderm.

(8) Subsequent to gastrulation, organogenesis can occur, and the different major organs of the body can arise. The three germ layers give rise to specific organs and structures in specific locations. These organs are completely dependent on the gastrulation process and the establishment of the axes. The 24- and 48-h-old embryos can be used to illustrate the different organs that have specific axial locations. The students will be expected to identify the eyes, ears, heart, intestine, somites, notochord, anterior–posterior axes, and pigment cells, both melanocytes and iridophores. Photograph below shows a 48-h-old embryo and illustrates these structures.





Directions for students

Each pair should collect two to five embryos for each age from the large Petri dishes at the beginning of the class. Use the transfer pipette to collect the embryos and put into the small dishes. **BE CAREFUL! THESE DISHES ARE EASY TO SPILL AND THESE ARE THE ONLY EMBRYOS YOU HAVE FOR THE CLASS. YOU MUST RETURN THEM AT THE END OF THE CLASS.**

(1) Using the dissecting microscope, examine the four different dishes of embryos in front of you. Adjust the optics and the light so that the embryos appear transparent. (Using stereoscopes can sometimes be difficult. If this is the first time you have used, one make sure you have adjusted the width of the eye pieces so that it fits your eyes comfortably. If you do not adjust them correctly, you will not be able to see in stereo.)

You will undertake the following series of experiments simultaneously over the next 2 h. Make sure you keep an accurate note on the timing of the experiments, as this is critical. Read through the entire protocol and then start.

(2) Examine the dish labeled A. These embryos are between 0 and 2 hpf. Select one fertilized embryo. Watch this embryo over the next 2 h. Every 15 min draw the embryo both from a side view and from a view from the animal pole using a poker roll the embryos, so you can see it from above. At each of the 15-min time point make notes on the following.

(a) How many cells does the embryo have?
(b) If the embryo has undergone cell division, where did the division take place, that is, the plane in relation to the previous cell boundaries?
(c) Are the cell divisions synchronous?
(d) What is the time interval between each cell division?
(e) When looking at the dorsal side view of the embryo, is there any obvious difference?
(f) When looking at the lateral side view of the embryo, is there any obvious difference?
(g) How big is the embryo? Use the acetate grid to get a measurement.
(h) After each division, is the embryo getting bigger?

(3) While you are waiting during the first 15-min interval of the above experiment, examine the embryos in the B dish. These embryos are 6–8 hpf. Select one embryo. You are going to watch this embryo over the next 2 h. Every 30 min draw the embryo from a side view and from a dorsal (animal pole) view. Use a poker roll the embryos so you can see it from above. At each of the 30-min time point, make notes on the following.

(a) Where is the embryo in relation to the animal and vegetal pole of the embryo?
(b) When looking at the dorsal side view of the embryo, is there any obvious difference?
(c) When looking at the lateral side view of the embryo, is there any obvious difference?
(d) Record on your drawings where these differences are and what happens to these differences over the next 2 h.

(4) In the remaining time between your observations and drawings of the 0–2 hpf and 6–8 hpf embryos, look at the C and D dish embryos. These are 24 and 48 hpf, respectively. Draw these embryos from a side view. Ask your TA to help dechorinate the embryos for you if they have not been dechorinated already. Label the axes of the embryo and then identify the following structures and label them on your drawings.

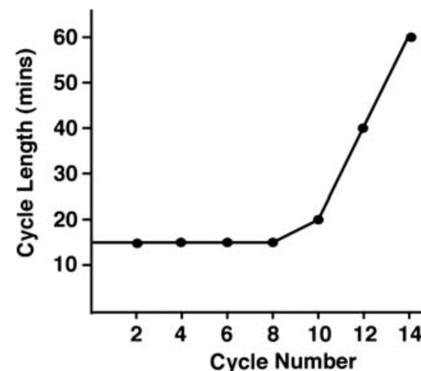
(a) Eye
(b) Otic vesicle (ear)
(c) Heart
(d) Intestine
(e) Notochord
(f) Somites
(g) Melanocytes
(h) Iridophores
(i) Pharyngeal arches (only on the 48 hpf embryo drawing)

Laboratory Worksheet 1 — Name ______________
Zebrafish Embryo Development — Lab Section & Day____

Work through these questions on your own. Do not copy the answers from anyone else since this will be considered plagiarism. Answers are shown in italics and underlined.

(1) In lab you were asked to label the following structures on a 48 hpf zebrafish embryo. Which germ layer are each of these structures derived from?

Structure	Germ layer
Eye	*Ectoderm*
Otic vesicle (ear)	*Ectoderm*
Heart	*Mesoderm*
Intestine	*Endoderm*
Notochord	*Mesoderm*
Somites	*Mesoderm*
Melanocytes	*Ectoderm*
Iridophores	*Ectoderm*
Pharyngeal arches	*Endoderm and ectoderm (jaw cartilage present in the arch is neural crest derived)*



(2) A student is observing a zebrafish embryo through a microscope over many hours. Assume that the student is not keeping track of time as the embryo develops. By what observations could the student measure development and know that the embryo is going through?

(a) Cleavage—*Count the number of blastomeres being formed with each cell division. Observe the divisions occurring perpendicular to each other. Note that cell division begins synchronously but then becomes asynchronous*.
(b) Gastrulation—*Cell movement, measuring involution*.
(c) Segmentation—*Developing somites*.

(3) Use the graph to answer the following questions:
(a) Describe the cell divisions during cell cycles # 6–9. Give a reason for your answer. (1 pt)

Cells are dividing at exactly the same time (synchronously), and the period of time between each division is constant. This is because they contain all the proteins required for cell division from maternal transcripts and proteins that are uniformly deposited in these cells.

(b) How many blastomeres would an embryo have **after** cell cycle #6? (0.5 pt)

64

(c) At what point (cell cycle #) does asynchronous and nonuniform cell division begin? Why? (1 pt)

Cell cycle #10.
Asynchronous and nonuniform length of time of cell division starts to occur when the cells begin zygotic gene transcription so that there is nonuniform gene transcription starting to occur in the cells of the embryo.

Lab 2: Mutations and Development I

Students will be completing a whole-mount *in situ* hybridization experiment by carrying out the washes and color reaction part of the process. The purpose of this experiment is to

(a) Demonstrate how we can identify tissue-specific gene expression by *in situ* hybridization.

Students will be determining the expression pattern of the *myoD* gene.

(b) Introduce them to the concept of screening for mutants.
(c) Introduce them to the concept of transcriptional regulation as a key process in development.

Students will be processing a clutch of embryos a fraction of which are mutated for the gene *tbx24* that regulates *myoD* expression. They will have to identify the mutants and write a detailed lab report in which they describe the effect of the mutation on the morphology of the embryos and link this to mutation's effect on the *myoD* gene expression.

Reading:

(1) Freeman S. Biological Science, 2nd edition. Upper Saddle, NJ: Pearson Education Inc., chapter 22.
(2) Grunwald DJ, Eisen JS. Headwaters of the zebrafish—emergence of a new model vertebrate. Nat Rev Genet 2002;3:717–724.
(3) Patton EE, Zon LI. The art and design of genetics screens: zebrafish. Nat Rev Genet 2001;2:956–966.

Directions for prep managers before class

(1) To generate *tbx24* mutants for *in situ*

Mutants are generated by injecting morphilinos purchased from Gene Tools, LLC (Philomath, OR), using previously published sequence for *tbx24* (Nikaido *et al.*, 2002). Inject 2.5 μg/μL concentration of tbx24 MO.

(2) Fish setup and breakdown for lab 2 (done the previous week)

A dish containing live wild-type (3/4) and mutant (1/4) embryos is needed for each lab room.

Set up fish 2 days before lab day, and inject one tray (approximately 120 embryos) a day before lab. Leave injected embryos and wild-type control embryos out on the bench at room temperature.

(3) Fish setup and breakdown for lab 3

See lab 3 instructions.

(4) *In situ* processing schedule

Start the *in situ* procedure (Thisse *et al.*, 1995) 2 days before the lab day, and stop after adding antibody; the steps after that are done by the students. These embryos can be stored in antibody for up to 2 days (therefore can be divided in half and used for 2 consecutive lab days). Sort the wild-type and mutant embryos so that each group of three to four students will receive a 1.5 mL Eppendorf tube with 5 mutant and 15 wild-type embryos.

(1) Rehydrate embryos in methanol

1×5 min in 66% methanol
1×5 min in 33% methanol
4×5 min PBTw (1× PBS, 0.2% Tween 20*)*use a 20% stock of Tween 20

(2) Prehybridization

1 to 5 h at 65°C in hybridization mix (HM)

(3) Hybridization

Overnight at 65°C with 1 μL of probe in 100 μL of HM (Heat probe at 65°C for 5 min before adding it to the embryos)
Save Probe after hybridization

(4) Washes

(Prewarm solutions to 65°C)
300 μL of solution in water bath
65°C 1×10 min with 75% HM/25% 2× SSC
1×10 min with 50% HM/50% 2× SSC
1×10 min with 25% HM/75% 2× SSC
1×10 min with 2× SSC
1×20 min with 0.2×SSC + 0.1% Tween
2×20 min with 0.1×SSC + 0.1% Tween

500 μL of solution on rocker
Room temp. 1×5 min with 66% 0.2×SSC/33% PBTw
1×5 min with 33% 0.2×SSC/66% PBTw
1×5 min with PBTw

(5) Preincubation of hybridized embryos

Incubate embryos in PBTw with 5% goat serum and 2 mg/mL BSA for several hours on the rocker at room temperature.

(6) Incubation with preadsorbed anti-DIG antiserum

Incubate embryos overnight—two nights at 4°C in a-DIG antiserum diluted 1:5000 in PBT w/2% goat serum and 2 mg/mL BSA.

(7) Sort embryos into 1.5 mL Eppendorf tubes with 5 mutants and 15 wild type sufficient for one tube per 2/3 people. Sort in PBTw.

Additional materials that need to be prepared

(1) Each microscope experimental group needs a p200 and p1000 pipetteman and tips.
(2) Each group needs a razor blade to cut the P1000 pipette tip.
(3) Each group will need a piece of white card to under the Petri dish on the scope to provide contrast.
(4) Each group will need a bottle/tube of PBTw.
(5) Each group will need a small Petri dish.
(6) Each group will need a waste container/Beaker.
(7) Each group will need a bottle/tube of buffer C (color reaction buffer with out NBT/BCIP solution added).
(8) Each lab will need a white Coors plate in a foil-wrapped large Petri dish. Each well should be numbered with a Sharpie.
(9) Each lab will need a tube of NBT/BCIP color reaction buffer.
(10) Each lab needs a rocker!
(11) Prepare a dish per lab of 25% mutant embryos (*tbx24* MO injected) and 75% wild-type embryos.

Directions for instructors

This week the students will be finishing a whole-mount *in situ* hybridization of 8–10 somite embryos that have been hybridized with an *in situ* probe to the transcription factor *MyoD*. The purpose of this experiment apart from showing the students this experimental technique is to show how gene expression patterns can reveal a mutant phenotype before morphological differences can be detected. You will introduce the concept of how you would screen for a mutant phenotype if you were undertaking a homozygous recessive screen. You will also talk about how transcription factors such as *Tbx24* (the gene affected in a quarter of these embryos) control the expression pattern of other genes such as MyoD.

Key concepts

(1) Antisense RNA probes can be synthesized to complement a specific gene's sense transcript. These probes can be used to reveal a gene's tissue distribution. The probes are synthesized with a tagged U base. The tag allows the message to be immunologically detected, that is, the theory behind *in situ* hybridization.

(2) Amplification of a tag using immunological methods can make very rare messages become visible.

(3) Differential gene expression can be used as a method to screen for mutants before morphological differences.

(4) Recap Mendelian genetics. In this study we have a homozygous recessive mutation that exhibits a phenotype. Mention that haploinsufficiencies can also have a phenotype; that is, the heterozygous state can have a phenotype though it may not be as severe as homozygous phenotype.

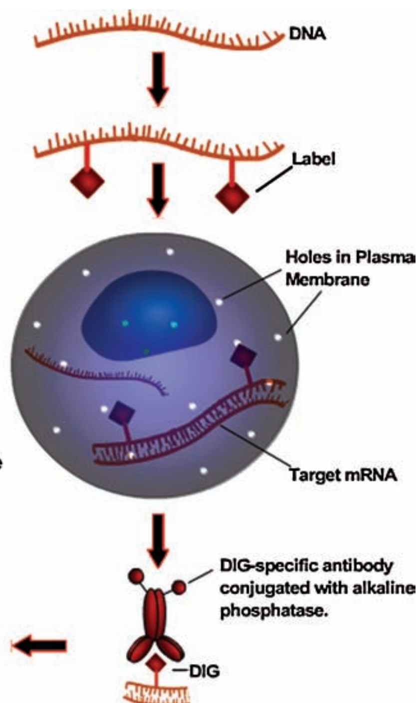
(5) Transcription factors act as regulators of gene expression. If a master regulator transcription factor fails to be expressed at critical stages of development, it will lead to obvious phenotypic consequences.

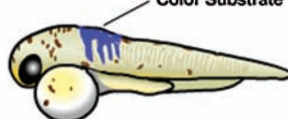
Discussion/Lecture

At the beginning of the class you should introduce the key concepts being investigated.

(1) Antisense RNA probes can be synthesized to complement a specific gene's sense transcript. These probes can be used to reveal a gene's tissue distribution. The probes are synthesized with a tagged U base. The tag allows the message to be immunologically detected, that is, the theory behind *in situ* hybridization.

You need to talk about how you can alter specificity of a probes binding by washing at different stringencies and hybridizing at different temperatures. You need to point out that



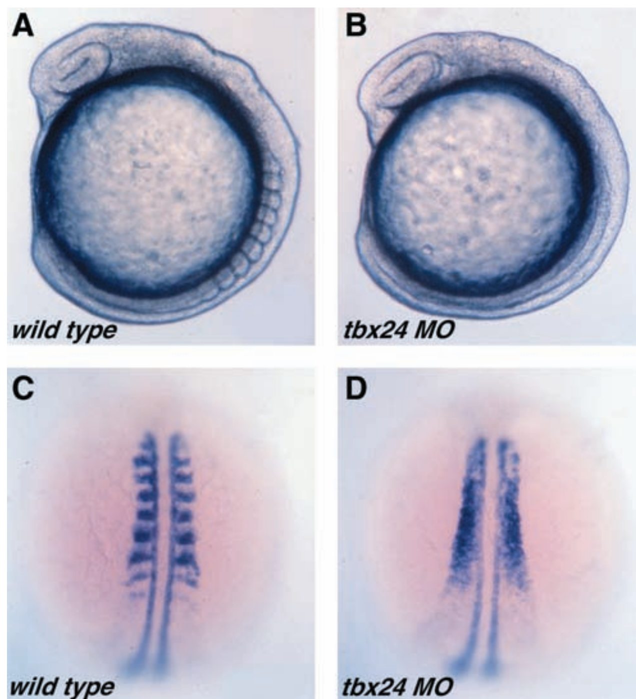


one of the bases is tagged and that it is incorporated in the probe when it is being synthesized.

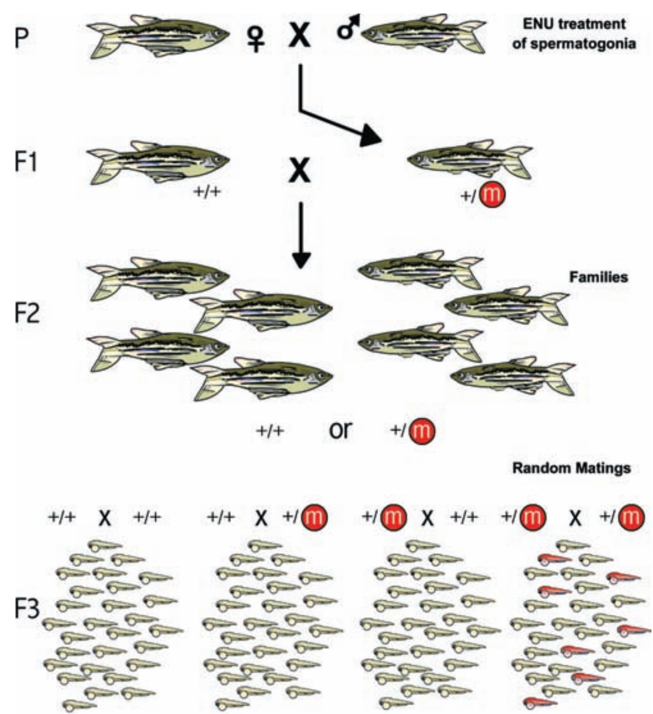
(2) Amplification of a tag using immunological methods can make very rare messages become visible. You need to point out that mRNAs are rare, so to view their tissue distribution you need to amplify the signal using antibodies that recognize the RNA probe's tag (digoxigenin) and amplify the immunological signal by utilizing the enzyme that is conjugated to the antibody to precipitate a color reaction product.

The key experimental steps are summarized in the schematic below:

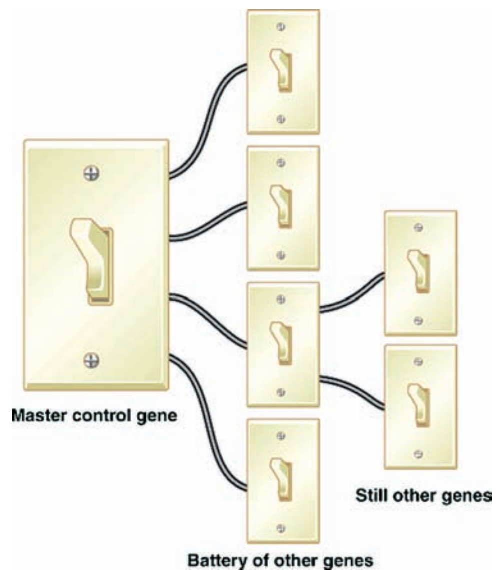
(3) Differential gene expression can be used as a method to screen for mutants before morphological differences. The purpose of doing this experiment is twofold: one to show the technique and second to show how it can be used to screen for mutants. Obviously, if you look at a group of embryos, you can determine if there has been a change in the expression pattern of the gene expression. This change in expression pattern may subsequently lead to an overt phenotypic difference in these embryos. The picture below shows the morphological phenotype that is observed in *tbx24* morphants (**B**) as compared to wild-type control (**A**) at 14 hpf ~ morphants fail to form somites. This morphological phenotype is reflected at earlier stages of embryogenesis around 12 hpf by completely unsegmented expression of *myoD* gene expression in the paraxial mesoderm (**D**) as compared to wild-type siblings (**C**).



(4) Recap Mendelian genetics. In this study we have a homozygous recessive mutation that exhibits a phenotype. Mention that haploinsufficiencies can also have a phenotype; that is, the heterozygous state can have a phenotype though it may not be as severe as homozygous phenotype. You should introduce the class to the idea of forward genetics and that if you screen for a phenotype you can subsequently identify the affected genes using classical genetic mapping techniques. No need to go into positional cloning methods. Use the following scheme to introduce the idea of how mutations are generated, and how you can screen for mutations in the F3 clutches. Talk about different mutagens that can be used and how they work. You should end up talking about how in human disease many conditions arise from haploinsufficiencies.



(5) Transcription factors act as regulators of gene expression. If a master regulator transcription factor fails to be expressed at critical stages of development, it will lead to obvious phenotypic consequences. The mutant (morphant)



phenotype that is being looked at today is the result of loss of function of the tbx24 transcription factor. Students need to be familiar with the idea that transcriptional regulation is a key regulatory mechanism in development. Tbx24 acts as a master regulator controlling somite segmentation from the paraxial mesoderm. In addition MyoD, the *in situ* marker being used, is a

master control gene responsible for muscle cell differentiation. You should introduce this key concept to the students as well.

Directions for students

Each group will be given a tube containing 20 embryos. Follow the detailed directions below. **BE CAREFUL!** The embryos are very small and delicate. Your mission is to complete the color reaction of a whole-mount *in situ* hybridization that has been undertaken using an antisense probe to *myoD*. The embryos are between 12 and 14 hpf.

(1) Carefully remove the supernatant from the tube and discard in waste container. Use the P200 pipetteman to remove the supernatant. Add 1 mL of PBTw to the tube immediately so that the embryos do not dry out.
(2) Remove the 1 mL PBTw after 10 seconds, discard in the waste container this fluid, and replace with another fresh 1 mL of PBTw and leave on rocker for 5 min.
(3) Repeat this wash five times with a fresh 1 mL of PBTw each time. Each wash should be 5-min long.
(4) After the sixth PBTw wash remove the PBTw and replace with 1 mL of buffer C and put back on rocker for 5 min.
(5) Repeat wash with buffer C two more times. Each wash should be 5 min.
(6) At the end of the third wash **carefully** remove the embryos with the P1000 pipetteman that has a razor cut tip. This razor cut tip should be large enough to permit the embryos to go through the tip mouth unimpeded. Place the embryos in one well of the white Coors plate in the foil-wrapped large Petri dish on the Instructor's bench.
(7) Remove all the buffer C and replace with 0.5 mL of the color reaction buffer.
(8) Check the embryos every 20 min to see if there is any change in the embryos.
(9) While waiting for the color reaction to develop, complete the worksheet regarding *in situ* hybridization and look at the dish of mixed live mutant and wild-type embryos on the Instructor's bench. What is the mutant phenotype? Record what you believe is the phenotype. When writing you lab report, compare the mutant phenotype you observe in the live embryos with your results from the *in situ* hybridization experiment.
(10) After 1.5 h remove the embryos from the white plate and transfer to a small Petri dish that contains 3 mL of PBTw using the P1000 pipetteman.
(11) Return to your bench and examine the embryos using your dissecting microscope. Use the microscopes direct light source to illuminate the embryos and place the white card below the Petri dish to aid in seeing the *in situ* expression pattern.
(12) Draw the pattern of the *myoD* expression in both mutant and wild-type embryos. You will be able to determine which embryos are mutant and which ones are wild-type based on the pattern of this gene's expression.
(13) Count the number of wild-type embryos versus mutant embryos in your group of 20 embryos and record the data.
(14) Complete a lab report incorporating your results and explaining how *tbx24* (the gene that is mutated in your mutant embryos) might control the expression pattern of *myoD* and what the result of the mutation will be morphologically.

Laboratory Worksheet II **Name________________**
Mutation and Development I **Lab Section & Day_____**

Work through these questions on your own. Do not copy the answers from anyone else since this will be considered plagiarism. Answers are shown in italics and underlined.

(1) In lab you developed the color reaction of an *in situ* hybridization on a clutch of zebrafish embryos and determined *myoD* expression.

(a) Total no. of embryos observed = ________
(b) No. of embryos in which *myoD* expression looked normal = _________
(c) No. of embryos in which *myoD* expression looked abnormal = ______
(d) % of embryos that are mutant for *tbx24* = *25%*
(e) By Mendelian genetics, the *tbx24* mutation would be classified as *homozygous recessive*
Explain your answer.

(2) In this exercise you are given the genomic sequence of the gene *tbx24*. *The sequence is in a Word file and can be uploaded from the conference.* You will use Internet-based software to determine the gene structure, that is, intron–exon boundaries and predict the amino acid sequence of the Tbx24 protein.

Instructions:

Obtain the *tbx24* genomic DNA sequence your Instructor has posted for you

- Select the entire DNA sequence and copy.
- Go to the GenScan website. http://genes.mit.edu/GENSCAN.html
  This is a gene prediction program that uses Neural Net or Hidden Markov Model (HMM) algorithms to predict the existence of introns, exons, and coding sequences. The programs work by recognizing gene features such as open reading frames and consensus splice sites at intron–exon junctions.
- Choose **organism** "vertebrate."
- For **print options**, choose "Predicted peptides only."
- Paste DNA sequence into the "sequence text" box.
- Click "run Genscan"
- Read the output. To see the intron–exon structure graphically, click to see PDF image of predicted gene.
  (a) How many exons does your gene have?
  9
  (b) How many introns does your gene have?
  8
  (c) How many amino acids in your predicted protein?
  752 aa
  (d) What is the minimum number of nucleotides in your predicted RNA?
  752 × 3 = 2256 bp

The next step is to look for **Domains** in the protein sequence. Domains are small stretches of amino acids that are highly conserved in many proteins and that perform a specific function. For example, a certain domain may be important in catalyzing a chemical reaction in the case of a protein that is an enzyme.

Do a domain prediction to see if your protein has any domains of known function that might indicate how your protein works.

Instructions:

- From the GenScan output page, highlight and copy the entire predicted amino acid sequence of your protein.
- Open a new window in your browser, so you can go back to GenScan later.
- Go to http://www.ncbi.nlm.nih.gov/. Click BLAST (at top of screen). Under **Protein** click the first one "Protein–protein BLAST (Blastp)." This compares your amino acid sequence to all other proteins.
- Paste your amino acid sequence into the "search" box.
- Choose which database you want to search, in this case choose "nr" in the "database" pull-down menu. This is a nonredundant database of all protein sequences.
- Check the "Do CD-Search" box. This will find "conserved domains."
- Click "BLAST." This opens up a separate window "NCBI CD-Search." This window shows any conserved domains in your protein. Note that the "Query" is your sequence.
  - (e) What domain(s) do you have in your protein? *Tbox*
  - (f) At what amino acid (number) does this domain begin in your protein? *62 aa*
  - (g) How many amino acids long is the domain? *247 − 62 = 185 aa*
  - (h) What are the features of a Tbox domain? *Copy from the page*
  - (i) What kind of protein is Tbx24? *A transcription factor*

(3) *In situ* hybridization is a technique that enables researchers to study the pattern of gene expression. In lab, we looked at *myoD* expression in 12–14 hpf zebrafish embryos. Since this technique takes 3 days to complete our prep mangers carried out most of the technique and you developed the color reaction. To better understand the technique, let us go through what was done behind the scenes before lab.

Fill in the blanks.

(a) *In situ* hybridization was carried out on____________ __________________ (live/*preserved*) embryos made permeable to probes by treatment with detergent.

(b) A labeled 500-bp-long *myoD* antisense RNA probe was synthesized to have a sequence that is_________ (identical/*complementary*) to the transcript.

(c) Probe was then added to the embryos and hybridized overnight at 65°C. A successful *in situ* hybridization depends on specificity of the probe, which depends on temperature of hybridization. Low temperatures can lead to _______ (*nonspecific*/no) hybridization and very high temperature can lead to_________ (nonspecific/*no*) hybridization.

(d) Once hybridization is complete, the embryos are washed several times with a solution containing salt and detergent, each wash being of higher stringency than the previous wash. High stringency washes contain _______ (high/*low*) concentrations of salt and add to increasing the specificity of the probe.

(e) Since most mRNAs are rare, the probe signal must be amplified by ___________________________ (PCR/*immunological methods*) that you carried out in lab.

Lab 3: Mutation and Development II

Experimentally, in this class the students will be carrying out an Alcian green stain to reveal the cartilage elements in the jaw of a clutch of *lessen (lsn)* mutant and wild-type embryos. The purpose of this experiment is to first of all demonstrate how we can use vital dyes to reveal the complex jaw morphology of the zebrafish. This in turn can then be used to screen for mutants. The students will have to identify the *lsn* mutants and describe what the effect of the mutation is on the jaw morphology. They will also have to determine the number of mutants in the clutch of embryos they are processing. In addition to the Alcian green staining, the students will examine a clutch of live *lsn* embryos that will be 4 or 5 days old depending on which day the class meets. Students will be expected to identify the mutants in this clutch of embryos.

Directions for prep managers

(1) Fish setup and breakdown (done the previous week)

You will be setting up four pairs of *lsn* fish, that is, four tanks each with one male and one *lsn* female on the two fish setup days. You will need to clean the embryos up (i.e., remove the chorions from which the embryos have hatched) when the embryos reach 2 days of age. Do this toward the end of the day. Incubate the embryos through out their development in normal embryo media.

Set up fish 5 days before lab day. Three days before lab, change water and breakdown. Two days before lab, remove chorions. The embryos can be used for 2 consecutive lab days.

(2) Solutions to be made

Recipes are for 100 mL. This is sufficient for two groups. Scale up as necessary.

(1) Acid–Alcohol
100 mL 70% ethanol
1 mL concentrated HCl (±37%)

(2) Alcian green
0.1 g Alcian green 2GX (Sigma Chemical)
100 mL acid–alcohol

(3) Glycerol–KOH
50 mL 0.5% KOH
50 mL glycerol

(3) Setup per group

(1) Each microscope experimental group needs a p200 and p1000 pipetteman and tips.
(2) Each group needs 50 mL acid–alcohol and a 250 waste beaker labeled "Acid–Alcohol waste."
(3) 50 mL Alcian green and a 250 waste beaker labeled "Alcian green waste."
(4) 50 mL Glycerol–KOH.
(5) Pokers to manipulate embryos.
(6) Small 35-mm Petri dishes.
(7) White card to place under Petri dish.
(8) Glass depression slides to permit compound scope examination of preps.

(4) Fish setup and breakdown for lab 4

See lab 4 instructions.

Directions for instructors

This week the students will be undertaking an Alcian green staining of a prefixed clutch of 5-day-old *lsn* embryos. The purpose of this experiment apart from showing the students this experimental technique is to show how morphological changes can be accessed in mutant embryos using vital stains and not just by looking at gene expression patterns. You will reintroduce the concept of how you would screen for a mutant phenotype if you were undertaking a homozygous recessive screen. You will also talk about how neural crest cells are an embryonic stem cell population that gives rise to not only the jaw cartilage but also the whole of the peripheral nervous system and pigment cells. Bring home the point that a mutation in a gene may have multiple phenotypes depending which cells it is expressed in.

Key concepts

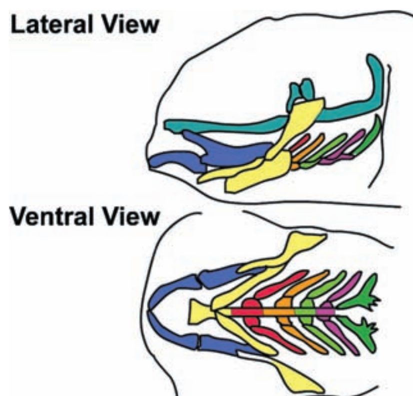
1. **Alcian green stain reveals the pattern of cartilages in the zebrafish jaw.**
2. **Differential staining can be used as a method to screen for mutants.**
3. **Recap again Mendelian genetics. In this study we have a homozygous recessive mutation that exhibits a morphological phenotype.**
4. **There are stem cells in a developing embryo (such as the neural crest) that are pluripotent/multipotent; that is, they can give rise to multiple cell types. Contrast this to totipotent stem cells that can give rise to all cell types.**
5. **Mutations can have multiple cellular phenotypes depending on where the gene is expressed. Further, the mutant phenotype potentially could be an indirect effect of the mutation.**

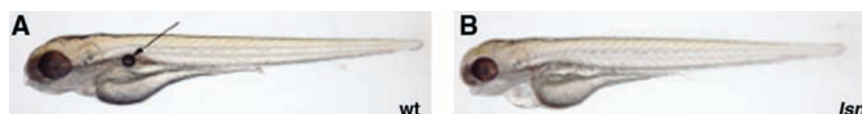
Discussion/Lecture

At the beginning of the class you should introduce the key concepts being investigated.

(1) Alcian green stain reveals the pattern of cartilages in the zebrafish jaw.

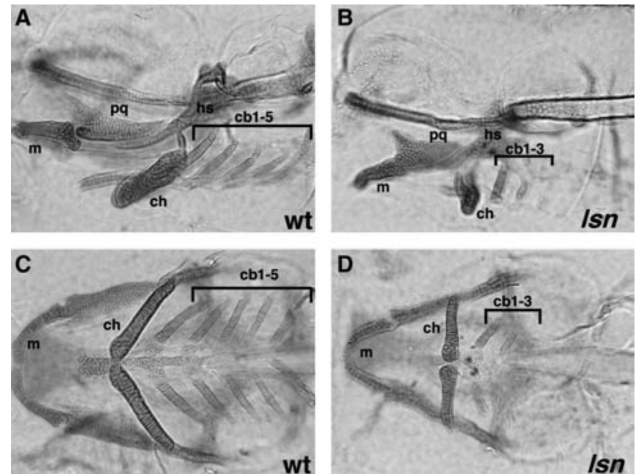
Zebrafish have a very defined number of jaw cartilages. The figure below illustrates the normal arrangement of jaw cartilages:



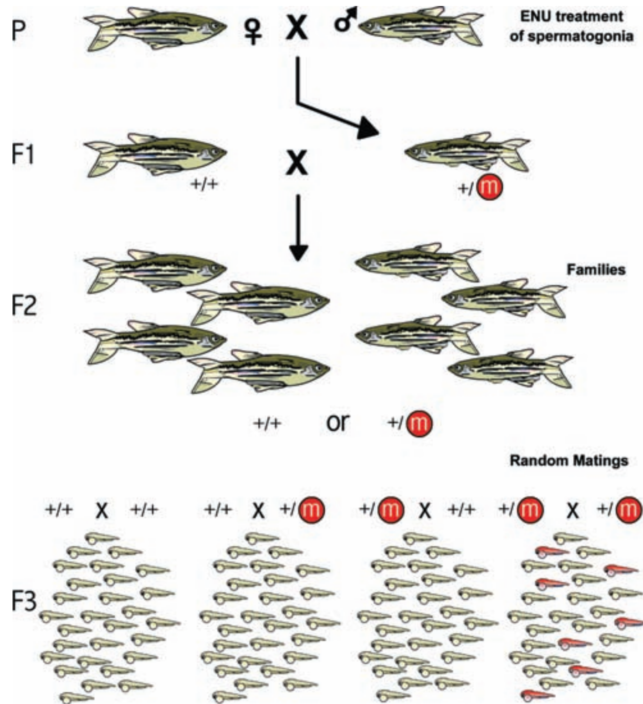


The jaw cartilages shown in this figure are the lateral and ventral views. The stain used in this lab is Alcian green, a vital stain that binds to mucins, which are heavily glycosylated proteins, for example, cartilaginous tissues in the embryo.

(2) Differential staining can be used as a method to screen for mutants.



Just like last week the purpose of doing this experiment is twofold: one to show the technique and second to show how it can be used to screen for mutants. Obviously, if you look at a group of embryos, you can determine whether there has been



a change in the expression pattern of the jaw staining. In the case of *lsn* mutant embryos the jaw is clearly affected as in the live fish; the mutants can be seen to have an "open jaw" phenotype.

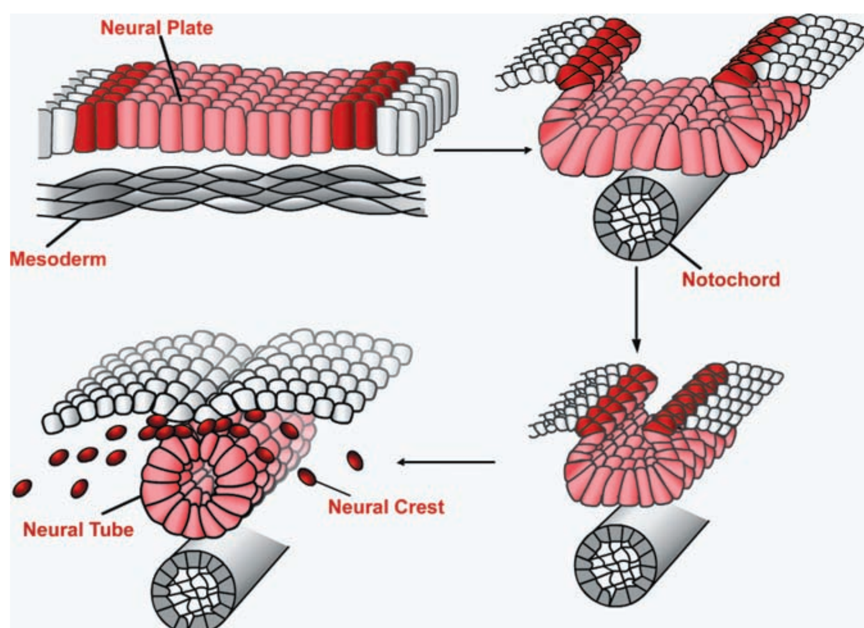
When you stain 5-day-old embryos with Alcian green, you should clearly see that mutants have lost the posterior cartilage elements. This can be difficult to see but should look like

This loss of cartilage elements leads to the open jaw phenotype seen in the live fish

they can give rise to multiple cell types. Contrast this to totipotent stem cells that can give rise to all cell types. The jaw cartilage is a neural crest–derived structure. Introduce the class to what neural crest cells are.

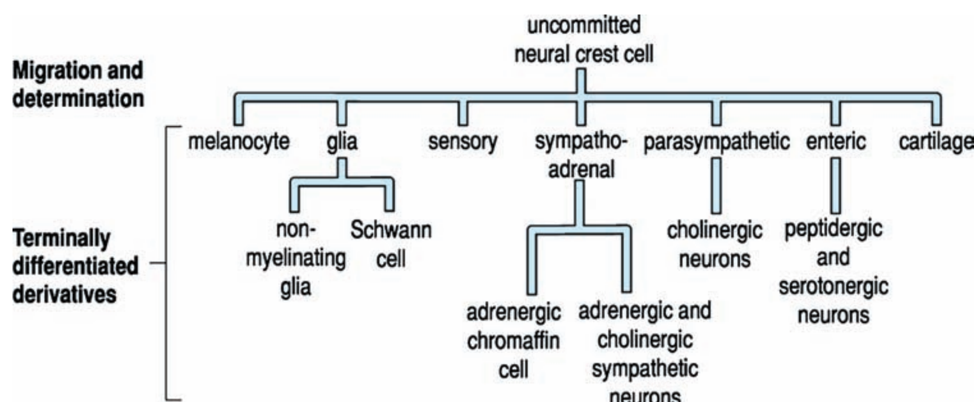
You should then introduce the class to the concept that the neural crest cells can contribute to many different types of tissue.

You should introduce the idea that the environment that a neural crest cell encounters while it migrates will determine its eventual cell fate. You should also mention that



(3) Recap again Mendelian genetics. In this study we have a homozygous recessive mutation that exhibits a clear morphological phenotype. Just like last week, re-introduce the

depending on where the neural crest cells are coming from with respect to the anterior–posterior axis would determine which cell types the neural crest will give rise to. In the

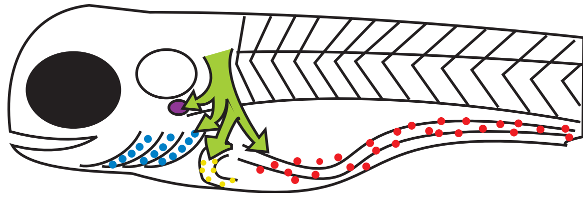


class to the idea of forward genetics and that if you screen for a phenotype, you can subsequently identify the affected genes using classical genetic mapping techniques. Again use the following scheme to introduce the idea of how mutations are generated and how you can screen for mutations in the F3 clutches.

(4) There are stem cells in a developing embryo (such as the neural crest) that are pluripotent/multipotent; that is,

case of the *lsn* mutation it appears that the mutation principally affects postotic-derived neural crest cells. This population of axial neural crest cells gives rise to nervous system of the intestine or enteric nervous system that is also affected in the mutants, hence the name *lessen* ~ less enteric neurons.

If the students want to know what the lessen mutation is, you can tell them that it affects a protein that is required to



enhance gene transcription for genes whose expression is controlled by nuclear hormones, that is, things like thyroid hormone and vitamin D.

(5) Mutations can have multiple cellular phenotypes depending on where the gene is expressed. Further, a mutant phenotype potentially could be an indirect effect of the mutation. If you screen for a mutation in a specific tissue type, it is necessary to remember that a mutated gene could be expressed in multiple tissues. As a result, there could be other phenotypes that result from the mutation not just the phenotype you are screening for. In addition, how a mutated gene causes a phenotype may be indirect; that is, a mutated gene might not actually directly affect the tissue you are looking at. Instead the mutant phenotype could be a result of some defect in another tissue that consequently affects the tissue you are interested in.

Directions for students

Each group will be given a tube containing 20 embryos. Follow the detailed directions below. **BE CAREFUL!** The embryos are delicate and can be lost/stick to the pipetteman tips easily. Your mission is to complete the Alcian green staining of this group of wild-type and mutant embryos. You need to determine what the effect of the mutation is on the jaw cartilage. The embryos are 5 days old.

(1) Carefully remove the supernatant from the tube and discard in waste container. Use the P200 pipetteman to remove the supernatant. Add 1 mL of acid–alcohol and wash the embryos for 3 min.
(2) Remove the 1 mL acid–alcohol after 3 min, discard in the waste container, and replace with 1 mL of 0.1% Alcian green to stain the tissue. Leave on rocker for 1 h.
(3) While the tissue is staining look at the live clutch of 4–5-day-old *lsn* embryos on the Instructor's bench. Identify the mutant, draw embryos as compared to wild-type embryos recording in detail as part of your lab, and report the mutant phenotype.
(4) After an hour, remove the Alcian green staining+ solution from your tube of embryos and dispose of it in the waste container. "Differentiate" the tissue by washing the embryos with 1 mL of fresh acid–alcohol for 5 min.
(5) Repeat this wash two more times with a fresh 1 mL of acid–alcohol each time. Each wash should be 5-min long.
(6) After the final acid–alcohol wash, transfer the embryos into a small Petri dish that you have filled with 3–4 mL of glycerol–KOH. Watch the embryos under the dissecting microscope as the tissue clears. You should see the embryo becomes transparent.
(7) Identify the mutant embryos as compared to the wild-type embryos. Draw the pattern of cartilages in wild-type and mutant embryos from a lateral and a ventral perspective. (If necessary transfer the embryos you are drawing to a glass depression slide, which contains some glycerol–KOH so that you can look at the cartilages in more detail.)
(8) Count the number of wild-type embryos versus mutant embryos in your group of 20 embryos, and record the data.
(9) Complete a lab report incorporating your results and hypothesize how *lsn* mutation might cause the jaw phenotype you have observed.

Laboratory Worksheet III **Name**______________
Mutation and Development II **Lab Section & Day**___
Environmental effects of drugs

Work through these questions on your own. Do not copy the answers from anyone else since this will be considered plagiarism. Answers are shown in italics and underlined.

(1) A clutch of zebrafish embryos can be screened for mutants by whole-mount *in situ* hybridization as well as using vital stains. In what way do these two methods differ in what they can detect?

In situ *can reveal changes in patterns of gene expression linked to the mutation. Vital stains detect changes in tissue structure or morphology caused by the mutation.*

(2) What are unique properties of stem cells?

They are capable of dividing and renewing themselves for long periods, they are unspecialized, and they can give rise to specialized cell types.

(3) What is the difference between totipotent and multipotent stem cells? What kind are neural crest cells and from which germ layer do they arise?

Totipotent cells give rise to all cell types.
Multipotent cells give rise to multiple cell types—neural crest. Ectoderm.

(4) Describe the origin of neural crest and explain how their final fate is determined to give rise to many different kinds of tissues.

Notochord is formed from mesoderm and releases chemicals that induce formation of the neural plate and closure of the neural tube. Neural crest tissue pinches off at the top of the neural tube.
Cells migrate from the neural crest to become various tissues such as neurons of the peripheral nervous system and jaw cartilage.
The final fate of neural crest is determined by the environment they encounter during migration and well as the position where they originated along the A–P axis.

(5) The next time you come to lab (week 7) you will study the effects of four drugs on zebrafish embryonic development. Due to time constraints, the embryos will already be treated with the drugs before you come to lab. In order for you to know what was done behind the scenes and how each drug treatment was given, complete the following calculations.

(a) Embryos were treated with **1 μM retinoic acid (RA)** for 1 h. How much of a 10^{-4} M stock must be added to 3 mL embryo media, to give a working concentration of 1 μM RA? Molecular weight of 13-cis RA = 300.42.

$X \times 100\ \mu M = 3000\ \mu L \times 1\ \mu M$

$X = 30\ \mu L$

(b) How much of 100 mM **cyclopamine** must be added to 3 mL of embryo media to give a working concentration of 100 μM? Your calculations should also show how the 100 mM cyclopamine stock solution was made. Molecular weight of cyclopamine = 411.63

100 mM = 0.1 M = 41.1 mg/mL

X × 100 mM = 3000 × 0.1 mM

X = 3 µL

(c) Embryos were treated with **0.006% phenylthiourea** (PTU). Beginning with 1 mL of a 10% stock solution of PTU, how would you make a 0.006% PTU solution? Your calculations should also show how you made the 10% PTU stock solution. Molecular weight of PTU = 152.22.

10% stock = 100 mg/mL

X × 10% = 0.006% × 15 mL

X = 9 µL

(d) Each Petri dish of embryos was treated with 3 mL of **0.3 M lithium chloride (LiCl)** for 10 min. How much LiCl you would weigh out to make a solution to treat five Petri dishes? Molecular weight of LiCl = 42.39.

5 Petri × 3 mL 0.3 M = 15 mL of 0.3 M needed.

0.3 M stock = 12.71 mg/mL

For 15 mL of 0.3 M, you will need to weigh out 190.65 mg

Lab 4: Environmental Effects of Drugs

In this laboratory, students will carry out a detailed description and analysis of 48 hpf embryos that have been treated with different chemicals/pharmacological reagents as compared to control embryos. The students will be expected to determine which reagent has resulted in which embryological phenotype. Students will be required to identify and draw the various anatomical structures that are perturbed by the treatments. They will be expected to start a literature search using PubMed to determine the effect of the different pharmacological treatments and prepare 15–20-min presentations for the following week that will be graded by the class as well as the TAs.

Directions for lab prep managers

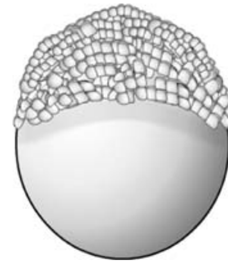
(1) Fish setup and breakdown

Set up fish 3 days before lab day. Set up at least one complete tank for each ~10–12 breeding cages at around 5 p.m. Each breeding tank should have four females plus three males. Two days before lab, at ~9 a.m., change over water breakdown (remove cage dividers).

(2) Drug treatments

For each day divide embryos up so that there are **twenty-five** 35-mm dishes with equal numbers in each (aim for 20 embryos per dish). Incubate at 28°C.

(a) 0.3 M LiCl Treatment (Stachel *et al*., 1995): When embryos reach **4 hpf** ~ sphere stage remove embryo media from five dishes and replace with 3 mL of **0.3 M LiCl stock embryo media per dish** for **10 min**. LiCl was purchased from Sigma-Aldrich. After 10 min discard the LiCl embryo media and



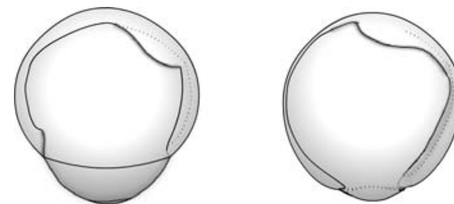
Drawing of 4 hpf sphere stage embryo.

wash/replace three times with fresh normal embryo media. After final wash **label dishes A** and return to the 28°C incubator.

(b) 100 µM Cyclopamine Treatment (Chen *et al*., 2001, Incardona *et al*., 1998): When embryos reach 4 hpf ~ sphere stage, remove embryo media from 5 dishes and replace with 3 mL of embryo media that has had added to it 30 µL of **100 mM cyclopamine stock** solution per dish. Cyclopamine was purchased from Toronto Research Chemicals.

Note: To prepare the cyclopamine containing embryo media, 3 mL embryo media should put in a 15 mL conical tube swirled in a 70°C water bath for 30 seconds before adding cyclopamine. This drug media should be added immediately to the embryos. **Label dishes B** and return to the 28°C incubator. A white precipitate may well appear in these dishes. This is not a problem.

(c) 0.006% PTU Treatement (Karlsson *et al*., 2001): When



Drawings of 8–9 hpf 80–90% epiboly stage embryo.

embryos reach **4 hpf** ~ sphere stage, remove embryo media from five dishes and replace with 3 mL of **0.006% PTU embryo media**. PTU can be purchased from Toronto Research Chemicals. **Label the dishes C** and return to the 28°C incubator.

(d) 1 µM RA Treatment (Hyatt *et al*., 1992, Marsh-Armstrong *et al*., 1994, Akimenko and Ekker 1995, Neumann *et al*., 1999): RA was purchased from Toronto Research Chemicals.

When embryos reach **8.5 hpf** ~80–90% epiboly, remove the embryo media and replace the media of 5 dishes with 3 mL of embryo media that has **30 µL of 10^{-4} M Stock RA added**. Leave for **1 h**. Discard RA media and wash/replace 3 with fresh normal embryo media. After final wash, **label dishes D** and return to the 28°C incubator.

(e) Control: The remaining five dishes will act as control embryos. Leave embryos in normal embryo media, **label dishes control**, and leave in 28°C incubator.

(3) Check embryos after 24 hpf.

Remove any dead embryos after 24 hpf.

(4) Setup for each group:

Each lab will be divided into four groups of six students. For each group of six 1 SV40 Olympus group per two students.

Each bench should have a stock bottle of embryo media and a waste bucket.

Each bench should have one green pipette pump and a box of pasture pipettes.

Each bench should have an additional sleeve of 35-mm Petri dishes.

Each bench/microscope will need pokers.

Each microscope will need a transparent photocopy on acetate of graph paper to permit measurements.

Directions for instructors

This week the students will be asked to determine the effect of different pharmacological reagents on early developing embryos and subsequently identify which reagents cause which phenotypes. These experiments will compliment material being covered in the 142 lectures, but you should be familiar with the fundamental developmental pathways and the developmental cell types that are being perturbed in these experiments. As a result, please read the attached photocopies of chapters detailing the pathways being examined/perturbed and read carefully and get familiar with the following information.

Key concepts

(1) Different signaling mechanisms are required at specific points in development to determine specific cell fates/developmental out comes. Three of the regents affect different critical signaling pathways: LiCl affects the Wingless/Wnt signaling pathway by perturbing β-catenin signaling (Stachel *et al*., 1993); RA affects *Hox* gene expression (Hyatt *et al*., 1992, Marsh-Armstrong *et al*., 1994, Akimenko and Ekker, 1995, Neumann *et al*., 1999); Cyclopamine perturbs the hedgehog signaling pathway (Chen *et al*., 2001, Incardona *et al*., 1998). The fourth reagent, PTU, affects melanin synthesis (Karlsson *et al*., 2001) and as such is not affecting a specific signaling pathway that is involved in a fundamental developmental process. **DO NOT TELL THE STUDENTS HOW EACH OF THE CHEMICAL REAGENTS ACT, AS THEY ARE TO FIND THIS OUT AS PART OF THEIR LAB REPORTS!**

(2) Even short periods of perturbation of these signaling pathways can lead to dramatic effects. (Point out that the LiCl treatment was only for 10 min at 4 hpf, and RA treatment was for an hour at 8.5 hpf.)

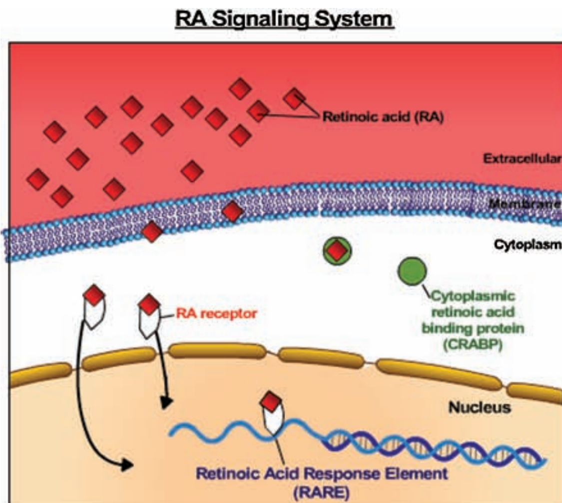
(3) Secreted molecules such as Wnts and Hedgehog act as morphogens. Morphogens are morphogenetic molecules that "tell" tissues to switch on certain genes that will eventually cause that tissue to give rise to a specific structure. Morphogens function in a concentration-dependent manner, that is, a tissue exposed to a low concentration of the morphogen will switch on one set of genes that will lead to a certain developmental outcome, while the same tissue exposed to a higher concentration of a morphogen will switch on a different set of genes that in turn will give rise to a different developmental outcome.

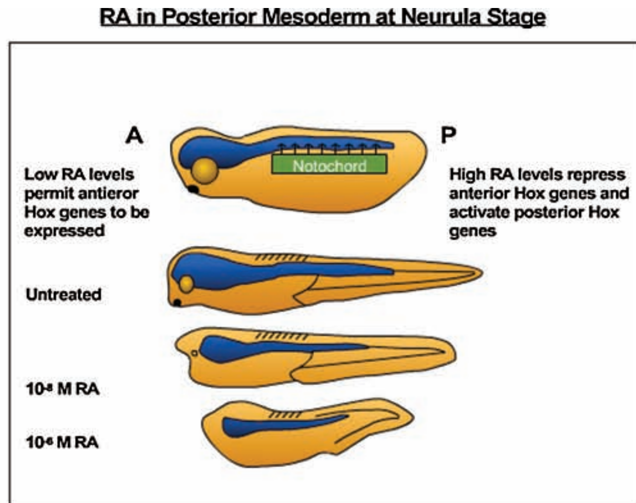
(4) Highlight the link that environmental factors can have serious consequences on embryogenesis and link to human birth defects. You should have specific examples to give to the class.

Wild-type embryo at 48 hpf. Note straight anterior–posterior axis. There is a clear fore and midbrain with clear distinctly formed eyes that are symmetrical either side of the midline. Further, there is a clear ear structure anterior to the first somite. Neural crest–derived melanocyte pigment cells are apparent in distinct stripes along the length of the embryo as are "silver" reflective pigment cells the iridophores.

Discussion / Lecture

At the beginning of the class you should introduce the key concepts. Next you should also talk about the importance of setting up the initial embryological axes, that is, anterior–posterior and dorsal–ventral. If the axes are not specified correctly, signaling centers that are subsequently need for



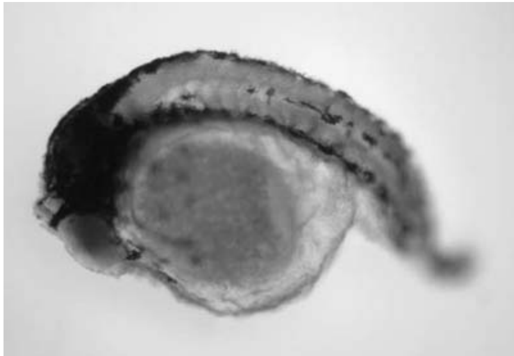


development to proceed appropriately will not be positioned correctly. These signaling centers are required to give rise to specific structures so that by 48 hpf normal wild-type embryos appear like below:

Some of the experimental conditions being looked at today affect the setting up and maintenance of these embryonic axes:

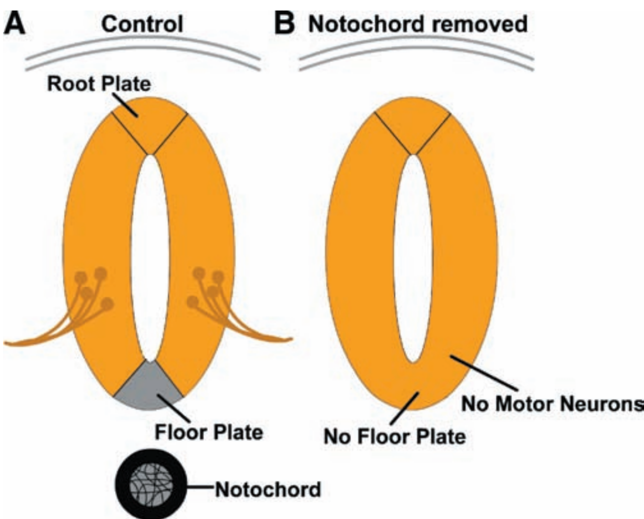
Example 1: RA ∼ derived from vitamin A acts as a transcription factor when it binds to its receptor and translocates to the nucleus. One key action of RA is that it is required to maintain *Hox* gene expression. It is also acts in a number of other developmental pathways to control gene expression. This is illustrated below.

In today's experiment key point is that RA treatment at 90% epiboly for just an hour perturbs gene expression required to



RA-treated embryo at 48 hpf. Note the lack of anterior head structures, that is, no eyes fore or midbrain, or no ear. Shortened body length. Posterior body is patterned relatively normally; that is, there are normal somites; however, tail growth is perturbed affected (later requirement of RA). We have "posteriorized" the embryo. Note also that dorso-ventral patterning in the embryo is pretty much normal and neural crest–derived melanocytes are normally formed.

set up and maintain the normal anterior–posterior axis. This is due to *Hox* genes being inappropriately expressed in anterior axial segments. *Hox* genes are required to specify specific axial segments identities. General rule of thumb is that *Hox* genes that specify more posterior axial segments require higher



Cyclopamine-treated embryo at 48 hpf. Note comparative normal anterior–posterior axis, anterior head structures, eyes, forebrain, midbrain, and ear. However, the body axis is curved due to lack of ventral midline structures such as notochord and spinal cord floor plate. In addition, though not dramatically apparent in this image, the eyes are abnormal, as the eye fields have fused at the midline, so the embryo has cyclopia. Note that the neural crest–derived melanocytes are normally formed though the exact pigment patterning is perturbed.

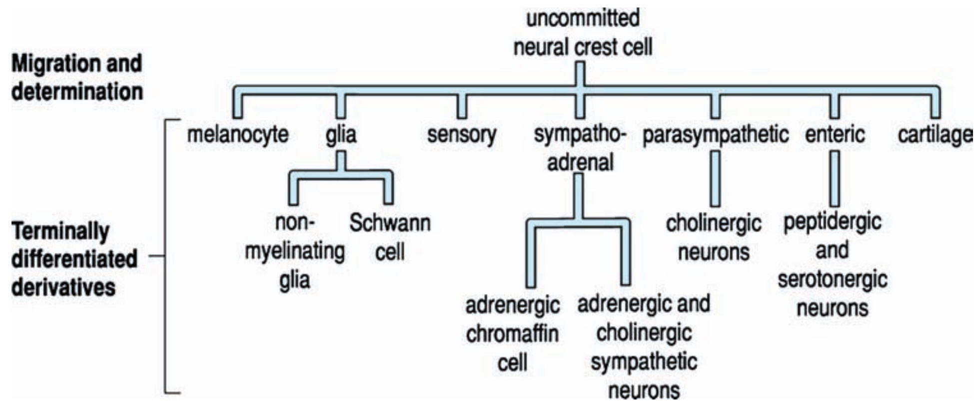
concentrations of RA. As a result, we get an embryo that looks like this at 48 hpf.

Example 2: Hedgehog secreted by the notochord and subsequently by the floor plate of the spinal cord is required to specify ventral structures in the spinal cord/CNS. If this signal is missing, the spinal cord becomes "dorsalized" and midline structures fail to form normally.

Cyclopamine affects the posttranslational processing of the hedgehog protein, so it is nonfunctional. As a consequence, cyclopamine treatment results in an embryonic phenotype that look like this at 48 hpf.

After talking about setting up the embryonic axes, you should talk about the importance of establishing signaling centers in specific locations in the embryo. These signaling centers can then specify the tissue surrounding them so that they acquire specific cell fates, that is, develop a specific structure. In today's lab we will examine the effect of perturbing one signaling pathway that is required to establish the eye filed in the early developing embryo. The eye obviously develops in a very specific location in the embryos. Its specification occurs at an early stage of embryogenesis before there being any obvious embryonic patterning. By treating embryos with a high concentration of LiCl for only 10 min at this early stage of embryogenesis stage, we perturb the Wnt signaling pathway required to specify the eye field inducing signaling center as a result 48 hpf embryos look like this:

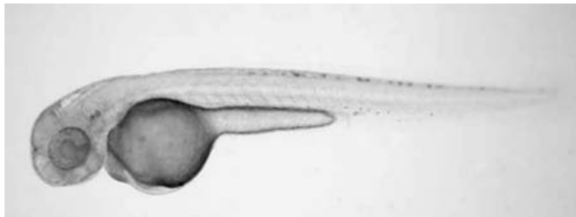
LiCl-treated embryo at 48 hpf. Note normal anterior–posterior axis, anterior head structures such as fore midbrain and ear, but a complete lack of eyes. Dorso-ventral patterning is also completely



normal, and the pattern and number of the neural crest–derived melanocytes is normal.

You should finally talk about the development of terminal cell fates, that is, the final type of cell type a precursor population generates. Use the example of neural crest cells already talked about in week-4 experiments. Neural crest cells are a pluripotent embryonic stem cell population that generates a wide variety of different cell types. See below:

Tell the students that signals in the environment determine what cell types they give rise to. In today's class the treatment with PTU prevents melanocytes (black pigment cells from forming). Point out that environment even at later stages of embryogenesis can affect cell fate decisions. A 48 hpf embryo treated with PTU looks like:



PTU-treated embryo at 48 hpf. Note completely normal looking embryo apart from the lack of melanocytes. Not that nonmelanin containing iridophores are still present and normally distributed.

Having introduced the concepts you need to remind the students about the importance of chemical concentrations that can determine the phenotypic effect. The lab work sheet will require them to determine how the stock and working concentrations of each chemical were determined.

Remind the students the importance of accurately recording the complete phenotype of the embryos.

Finally, need to remind students about how to effectively use the PubMed and other NCBI online resources to be able to obtain information necessary to generate their PowerPoint presentations the following week.

Directions for students

(1) Examine the dish of control embryos. Identify, draw, label, and make notes on the following structures:

(a) Anterior–posterior axis ~ record the length of the axis and record the body shape.

(b) Eyes size and location in relation to the midline of the embryo.

(c) Ear location and size.

(d) Pigment cells melanocytes and iridophores.

(2) Examine each of the four dishes of experimental embryos labeled A–D. For each condition identify, draw, label, and make notes on the following structures:

(a) Anterior–posterior axis ~ record the length of the axis and record the body shape.

(b) Eyes size and location in relation to the midline of the embryo.

(c) Ear location and size.

(d) Pigment cells melanocytes and iridophores.

Each group will have **15–20 min** to draw and record this information for the embryos for each condition.

(3) The four chemical reagents that were used to cause the embryological phenotypes were PTU, cyclopamine, LiCl, and RA. Complete the lab worksheet calculations to determine the amounts and volumes that were required to make the stock solutions for these reagents, and then how much of these stock solutions were used to give rise the final work concentrations of these regents that were used to elicit the observed embryological phenotypes.

(4) Undertake a literature search to determine which chemical reagent caused which phenotype. Write a lab report describing in detail how each of these pharmacological agents causes their respective phenotypes.

(5) In addition to the lab report, your group will be randomly assigned one of the four mutants. Working with your group, you will be expected to prepare a class presentation that will be given to class next week. Each presentation should last approximately 15–20 min with 5 min of questions and will be based around PowerPoint presentation. You are expected to work with your group to prepare a presentation and your classmates as well as the TA will be asked to critique your presentation and assign a grade.

Lab 5: Group Presentations

This is the final class of the zebrafish laboratory module. There will be no experiments this week, but the students will make presentations. Each of the classes' four groups will be randomly assigned one of the four mutants. Working within these groups, the students will have been expected to prepare a class presentation. Each of these presentations should last approximately 15–20 min with 5 min of questions. The presentations should be based around a PowerPoint

presentation. The students are expected to work as a group to prepare a presentation. The novel aspect of this presentation is that the grade for the presentation will be assigned based not only on your Instructor's assessment but also on the classmate's assessment. This is to introduce the class to the concept of peer review.

Dr. Iain Shepherd — Spring 2007
BIO 142 Lab *Foundations in Modern Biology II*

Course Evaluation—Zebrafish Module

Rate the following as 5 = Almost always, 4 = Very often, 3 = Occasionally, 2 = Seldom, and 1 = Never. Circle the number.

(1) Do you think the labs were

(a)	Interesting?	1	2	3	4	5
(b)	Well-organized?	1	2	3	4	5
(c)	Co-related with lecture?	1	2	3	4	5
(d)	Added to what was taught in lecture?	1	2	3	4	5
(e)	Hands-on and interactive?	1	2	3	4	5

(2) Did you like

(a)	Working with live material?	1	2	3	4	5
(b)	Viewing real-time embryonic development?	1	2	3	4	5
(c)	Working with mutants and observing changesin patterns of gene expression?	1	2	3	4	5
(d)	Studying the effects of mutagens/ pharmacologicalagents on development?	1	2	3	4	5

(3) Did the lab make you want to take the 323 Developmental Biology course and why?
(4) What suggestions do you have for improving this module?
(5) Additional comments:

Recipes for Solutions for the Lab Course

All chemicals are purchased from Sigma apart from where noted.

20× E2 Medium Stock

For **1** L (can scale up):
(1) Add about 700 mL of dH_2O to a clean 1-L flask. Add a stir bar and start mixing on stir plate.
(2) Weigh out the following chemicals as precisely as possible. Add in this order to the flask

$MgSO_4$*7H_2O	4.9 g
KH_2PO_4	0.41 g
Na_2HPO_4 (anhydrous)	0.12 g
$CaCl_2$*2H_2O	2.9 g
KCl	0.75 g
NaCl	17.5 g

If have to use Na_2HPO_4•2H_2O instead of Na_2HPO_4 (anhydrous), use 0.15 g.

(3) Dilute to 1 L. Can use heat for a short time if it does not go into solution. Store in a labeled plastic bottle in the refrigerator.

Embryo medium

100 mL 20× E2 medium stock
4 mL 500× $NaHCO_3$
dH_2O to 2 L

(1) Add 1 L of dH_2O to 2-L graduated cylinder.
(2) Add 4 mL of the 500×$NaHCO_3$ (3 g/100 mL water = 500×) to the graduated cylinder.
Store stock solutions in the refrigerator.
(3) Add 100 mL of 20× E2 medium stock (in fridge) to the cylinder, and then add enough dH_2O to make 2 L.
(4) Pour out old medium. Fill glass containers on the microscope table. Label and date container.

Mesab

0.2 g Ethyl-m-aminobenzoate methanesulfonate
0.4 g Na_2HPO_4
dH_2O to 50 mL

1. Weigh out 0.2 g of ethyl-m-aminobenzoate methanesulfonate (3-aminobenzoic acid ethyl ester) and add to 45 mL of dH_2O in a 150 mL flask. Mix on stir plate.
2. Weigh out 0.4 g of Na_2HPO_4 (or 0.5 g of Na_2HPO_4 *2H_2O) and add to flask.
3. Add dH_2O to make 50 mL (total volume). Mix well. Label and date. Store in the refrigerator.

PBS (5×)
Per 1 L: 40.0 g NaCl
1.0 g KCl
7.2 g Na_2HPO_4
1.2 g KH_2PO_4
dH_2O to 1000 mL

Usually make **4** or more liters at a time.

1. Add some water to a flask with a stir bar.
2. Weigh out the reagents, adding them to the flask as you weigh them out.
3. Let dissolve well. Then bring up the volume to the appropriate level.
4. Pour into the container labeled 5× PBS.

SSC (20×)
Per 1 L of 20× SSC: 175.3 g NaCl
88.2 g sodium citrate
dH_2O to a final volume 1 L
pH should be 7.0, adjust with NaOH or HCl

Instructions for **4** L:

1. Usually make 4 L at a time. Add 3.5 L of dH_2O and a stir bar to a 4 L flask.
2. Weigh out a total of 701.2 g of NaCl and add to flask.
3. Weigh out a total of 352.8 g of sodium citrate. Add to flask and mix.
4. Check the pH. If the pH is higher than 7.0, use NaOH to adjust, or if below, use HCl. Then add dH_2O to make the total volume 4 L. Check the pH again.
5. Once all the reagents have dissolved, transfer solution to the container labeled 20× SSC.

***In Situ* Protocol Recipes**

PBST: 1× PBS, 0.5% Triton X

Hyb. Mix:	**Final concentration:**		
	Formamide	50%	for 10 mL use: 5 mL of Formamide
	SSC	5×	2.5 mL of 20× SSC
	Heparin	50 μg/mL	10 μL of Heparin 50 mg/mL
	TRNA	500 μg/mL	100 μL of TRNA 50 mg/mL
	Tween 20	0.1%	50 μL of Tween 20
			92 μL Citric acid to pH 6
			Add sterile water to reach 10 mL
Block:	PBST, 2% goat serum, 2 mg/mL BSA		
Coloration buffer:	5 mL 1 M Tris-HCl pH 9.5 2.5 mL 1 M $MgCl_2$ 1 mL 5 M NaCl 0.25 mL 20% Tween 20 **to 50 mL** sterile water		
Coloration reagent:	10 mL coloration buffer 45 μL NBT stock (50 mg of nitro blue tetrazolium [Roche] in 0.7 mL of dimethylformamide and 0.3 mL of water) 35 μL BCIP (50 mg of 5-bromo 4-chloro 3-indolyl phosphate [Roche] in 1 mL of dimethylformamide)		

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