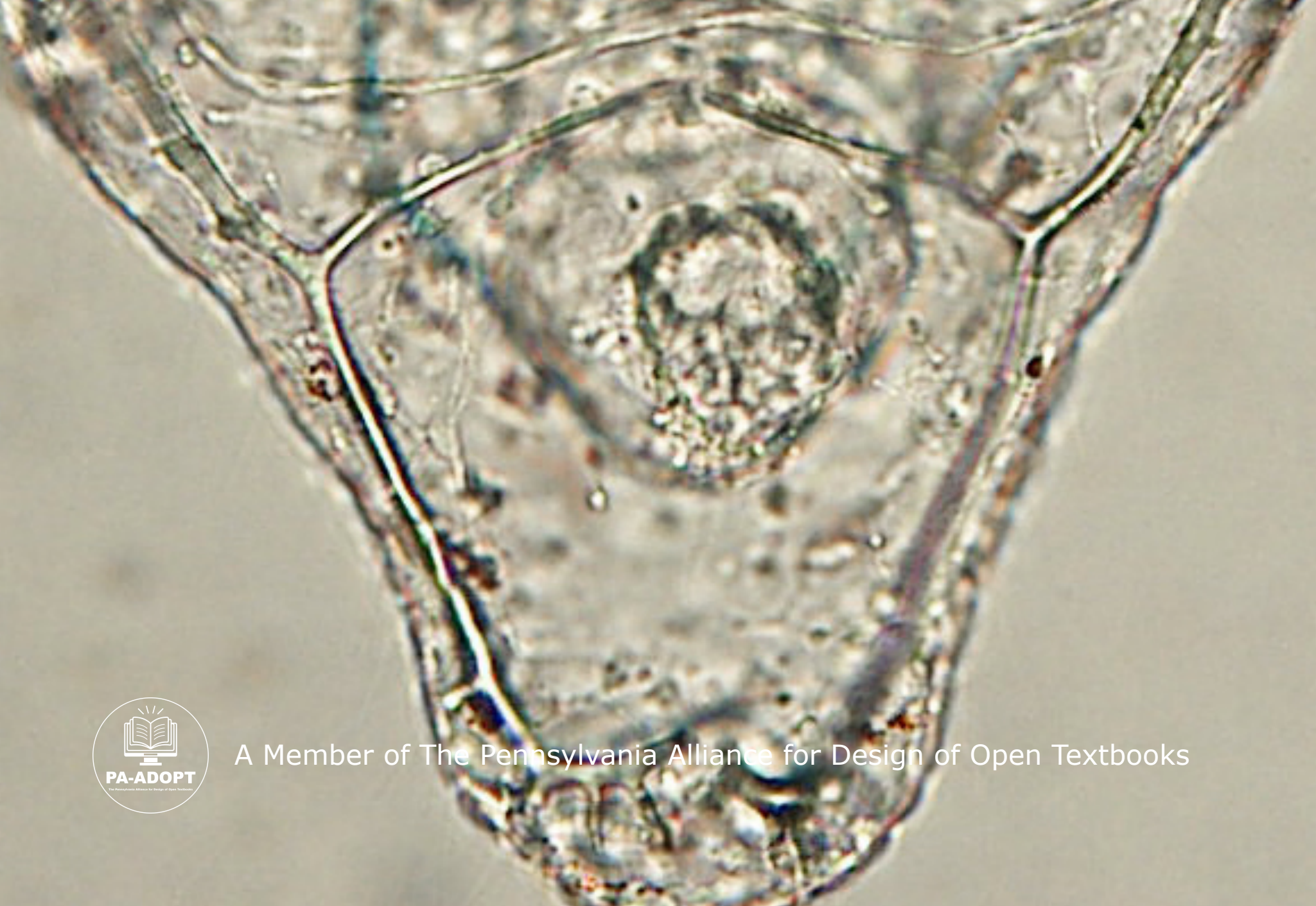
Developmental Biology Laboratory

Experiments in Classical and Experimental Embryology

Judith A. Cebra-Thomas
Matthew E. Smith



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Cover Image: “Sea Urchin Pluteus Larva” by J. Cebra-Thomas “

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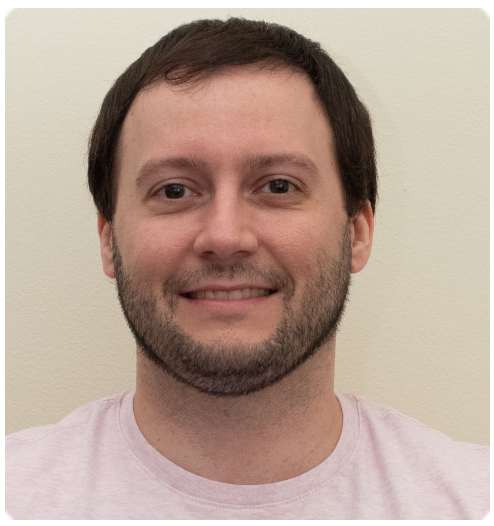
Judith Cebra-Thomas is an Associate Professor of Biology at Millersville University of Pennsylvania. Dr. Cebra-Thomas is an evolutionary developmental biologist who researches the formation of the bony shell in turtle embryos. She teaches Foundations of Biology, Cell and Developmental Biology, Immunology, Cell & Molecular Techniques, and, of course, Developmental Biology.



Mr. Smith

Matthew Smith is a graduate student working on his PhD in Biological Sciences and his MS in Bioinformatics & Computational Biology at the University of Delaware. He has taught and/or was a teaching assistant for courses in introductory biology, developmental biology, genetics, cell biology, molecular biology, and anatomy. His current research utilizes *Xenopus* as a model to uncover how the transcription factor Six1 and the Notch signaling interact during embryonic development. The overarching goal of his research is to identify novel causative genes to the unresolved cases of Six1-related birth defects, such as branchio-oto-renal disorder. He also has prior research experience with chicken, zebrafish, and turtle embryos.

About the Illustrator

Ms. Bleacher

Helena Bleacher is an undergraduate student majoring in molecular biology at Millersville University of Pennsylvania. She was previously accepted to Parsons School of Design for illustration before shifting her focus to an education in biology. She intends to pursue graduate school and advance her career in molecular biology or genetics as a research scientist.

Photographs by the author (JCT).

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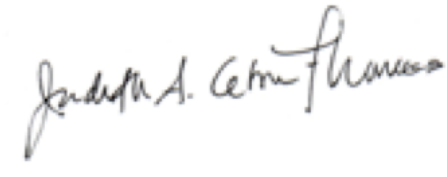

About the Lab Manual

Development is the way that living things become different. This course focuses on embryology, the process by which a fertilized egg becomes an entire organism. The creation of a new organism from a single cell requires coordination of growth, differentiation and morphogenesis, the generation of form. We will explore several model animal systems and a variety of scientific approaches that have been used to study them. The experiments described in the lab manual are designed for 2- to 3-hour laboratory periods (ones that involve isolating sea urchin gametes to begin will require the full 3 hours). The introductory protocols in each chapter can be adapted to Introductory Biology or Cell Biology courses, while the later protocols are more suited to an advanced course in Developmental Biology. The one essential ingredient is time; for most experiments, you will have to budget class time for follow up or have students come in outside of class to observe the results.

Chicken embryo

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Chapter 1 Sea Urchin Development

Sea Urchin Fertilization and Development

Fertilization is the union of two gametes, the sperm and the egg, to create a new organism. Although some unicellular animals reproduce asexually, sexual reproduction is the preferred method of propagation in most multicellular animal species. The resulting zygote contains genetic information from both parents. Sea urchins and other echinoderms have long been favorite subjects for the study of fertilization and early development. They produce large numbers of gametes, which can be combined to create embryos that rapidly develop in real or artificial sea water. The embryos are transparent, thus allowing the direct observation of internal and external structures.

The union of sperm and egg presents several challenges for sea urchins, primarily because fertilization is external. They live and spawn in tide pools and reefs in the ocean, where there is a tremendous amount of water rushing about. To prevent the sperm and eggs from being washed away and diluted, sea urchins have evolved mechanisms to bring the gametes together, including synchronized spawning and the **chemotaxis** of the sperm towards the egg. At the same time, many animals (including many echinoderms) coexist in the same habitats, so there is a need for safeguards to ensure **species-specificity** and prevent association between gametes of different species. Both the sperm and the egg have specific receptors for the other that must bind and transduce a signal sequentially for fertilization to occur. In addition, all those attractive forces can work too well and bring many sperm to each egg. Two is good, but more is not better; fusion with multiple sperm will bring in multiple genomes and multiple centrioles and result in the death of the embryo. Therefore, mechanisms to prevent **polyspermy** (fertilization with more than one sperm) are also needed.

The sperm is basically a stripped-down vehicle for transporting genes. It contains a **haploid set of chromosomes** that have been ultracompacted by the addition of a novel set of DNA-binding proteins: a **flagella** to supply motility and **mitochondria** to provide energy. The **basal body** of the flagella in sea urchin sperm, but not mammalian sperm, forms one of the asters for cell division. Finally, the sperm contains a very large exocytic vesicle known as the **acrosome** at the very front, just under the plasma membrane.

The sea urchin egg is 10,000 times bigger than the sperm. It is covered with the jelly coat and the vitelline envelope (a protein “shell”) just outside the plasma

membrane. The vitelline envelope contains proteins that bind to sperm of the same species. Because the egg must supply all the needs of the embryo until it has developed a mouth and digestive system, it is jammed full of yolk granules, mitochondria, metabolic precursors, endoplasmic reticulum, and protein synthetic machinery. In addition to the nucleus, the sea urchin egg also contains 25,000–50,000 maternal messenger RNA (mRNA) species that control early development. Finally, the egg contains a set of secretory vesicles, the cortical granules, lined up just inside the plasma membrane.

When a sperm cell encounters an egg of the same species, components of the jelly coat bind to specific "egg receptors" in the plasma membrane. This encounter triggers a signal transduction cascade resulting in a series of events that facilitate fertilization. First, an influx of calcium ions (Ca^{2+}) induces the fusion of the plasma membrane with the membrane surrounding the acrosome, releasing a set of hydrolytic enzymes that digest a channel in the jelly coat. This also results in the replacement of the plasma membrane over the front of the sperm by what had previously been the inside of the acrosome vesicle membrane. This exposes a new "egg receptor" to the environment. Actin filaments polymerize at the tip of the sperm head, extending the acrosomal process—a long proboscis that penetrates through the jelly to contact the vitelline membrane of the egg. The tip of the acrosomal process binds to the egg plasma membrane.

The egg also has specific "**sperm receptors**" that extend through both the plasma membrane of the egg and the vitelline envelope. These bind to newly exposed surface proteins that had previously been inside the acrosome. This selects sperm that have undergone the acrosome reaction. Therefore, **the two binding events have to occur sequentially** and help to **ensure species-specificity.**

The binding of sperm to the receptor triggers a second signal transduction cascade resulting in (1) the opening of sodium **Na^+ channels**, causing a transient depolarization; (2) a wave of "free" Ca^{2+}; and (3) a rise in intracellular pH. The membrane depolarization acts as the **fast block** to polyspermy; it is thought to interfere with electrostatic interactions between surface proteins of the sperm and egg. Polyspermy is bad because, in addition to the extra set of chromosomes, a sea urchin sperm donates a centriole. The presence of additional centrioles during the first cell division will result in additional cleavage furrows and incorrect partitioning of the chromosomes.

Ca^{2+} ions are released from the egg endoplasmic reticulum in a wave beginning at the point of sperm binding. The wave of Ca^{2+} induces the fusion of a set of exocytic vesicles (known as the **cortical granules**) with the plasma membrane. The contents of the cortical granules, which are released into the space between the vitelline envelope and the plasma membrane, include a trypsin-like protease that clips protein "posts" anchoring the vitelline envelope and removes the sperm-binding sites. The cortical granules also release mucopolysaccharides (carbohydrates) that absorb water and cause the vitelline envelope to expand and become the **fertilization envelope**; this is the **slow block** to polyspermy. The **sperm and egg plasma membranes also fuse,** and the sperm nucleus enters and fuses with the egg nucleus. The wave of Ca^{2+} and the rise in intracellular pH **activate the metabolism** of the fertilized egg.

The union of sperm and egg creates a single cell, the **zygote,** which gives rise to a new organism through the process of embryogenesis. The formation of a new organism requires both **morphogenesis** (the creation of form) and **cell differentiation**. In most animals, embryogenesis can be divided into three major stages:

1. **Cleavage**, in which rapid cell divisions divide the mass of the zygote into many cells and establish the basic embryonic axis;
2. **Gastrulation**, in which the gut is formed and the embryo becomes multilayered; and
3. **Organogenesis**, in which the major organ systems are formed.

The embryo cannot feed until it develops a digestive system, so all of the nutrition that it will need must be packed in the egg. This is true in the sea urchin, but the food material (yolk) does not disrupt the partitioning of cytoplasm (cytokinesis) after each cell division. This pattern of cell division (complete and equal) is known as **holoblastic cleavage**. In the sea urchin, early cell divisions are rapid; the cell cycle alternates between S phase, where new DNA is synthesized, and mitosis. As a consequence, the embryonic DNA is not transcriptionally active; it is not actively "read" to produce new mRNAs. The proteins that are synthesized during cleavage utilize mRNAs in the cytoplasm provided by the mother.

The first three cell divisions bisect the embryo equally (Figure 1-1). The first two cleavage planes run from the "top" (known as the **animal pole**) to the "bottom" (known as the **vegetal pole**), while the third runs across the equator and separates the embryo into "animal" and "vegetal" halves. The fourth cleavage is

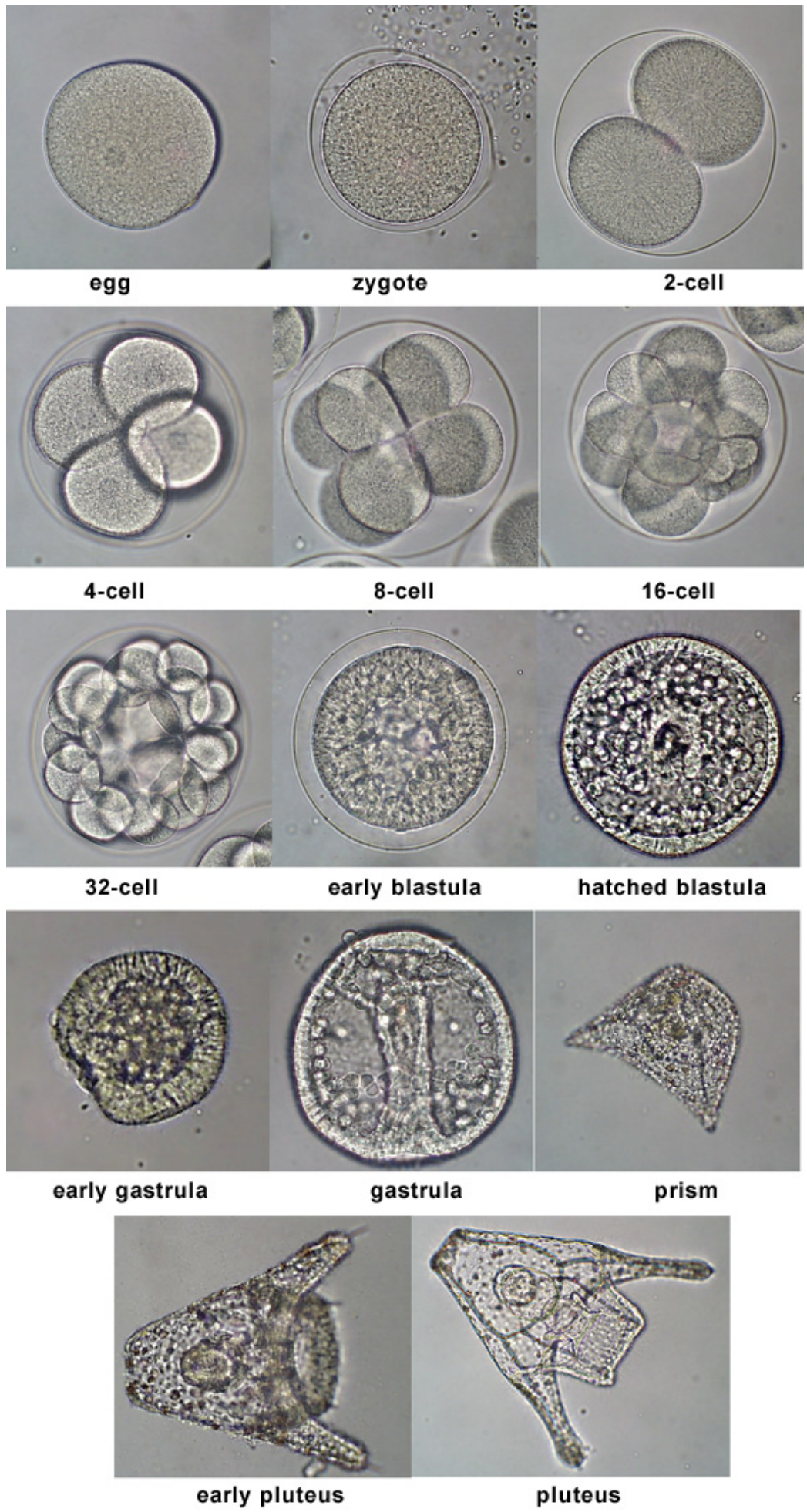

Figure 1-1. Cleavage and gastrulation in Lytechinus sea urchin embryos. Invertebrate embryos develop at different rates depending on the temperature of incubation. Descriptive stages based on the number of cells and their arrangement in the embryo are used to enable researchers to compare results between experiments. Cleavage-stage sea urchin embryos are described based on the number of cells, followed by the blastula (central cavity surrounded by an epithelial layer), gastrula (formation of the archenteron) and larval stages. Feeding larvae are defined morphologically as first prism, and then pluteus larva.

more unusual (Figure 1-1). The cells in the top half divide equally, but those in the bottom half divide unequally, creating large cells (**macromeres**) and small cells (**micromeres**). This is accomplished by one of the centrioles positioning itself in the middle of the cell, so that the spindle is displaced to one side. The cells continue to divide until they form a hollow ball known as the **blastula** with a central cavity (the **blastocoel**). Each of the cells produces a cilia. At this point, the genome is activated and starts to express new genes. One of these genes codes for a protease that digests a hole in the fertilization envelope; the embryo "hatches" and begins to swim.

Shortly after hatching, the descendants of the micromeres at the vegetal end detach from the epithelial sheet and move into the blastocoel (through **ingression**). These are known as **primary mesenchyme cells,** and they form the calcium carbonate **spicules** of the larval skeleton. The decendants of the macromeres thicken to form the **vegetal plate**, which invaginates to form the **archenteron** or gut. This process is known as **gastrulation** and, in addition to forming the gut, it results in a multilayered body plan. The archenteron extends by cell rearrangement and by connections between the cells at the archenteron tip (secondary mesenchyme cells) and the extracellular matrix lining the blastocoel (Figure 1-2). Once the archenteron reaches the other side, the mouth is formed. As the skeleton is laid down, the embryo's shape changes to form the prism and then **pluteus larvae** (Figure 1-1).

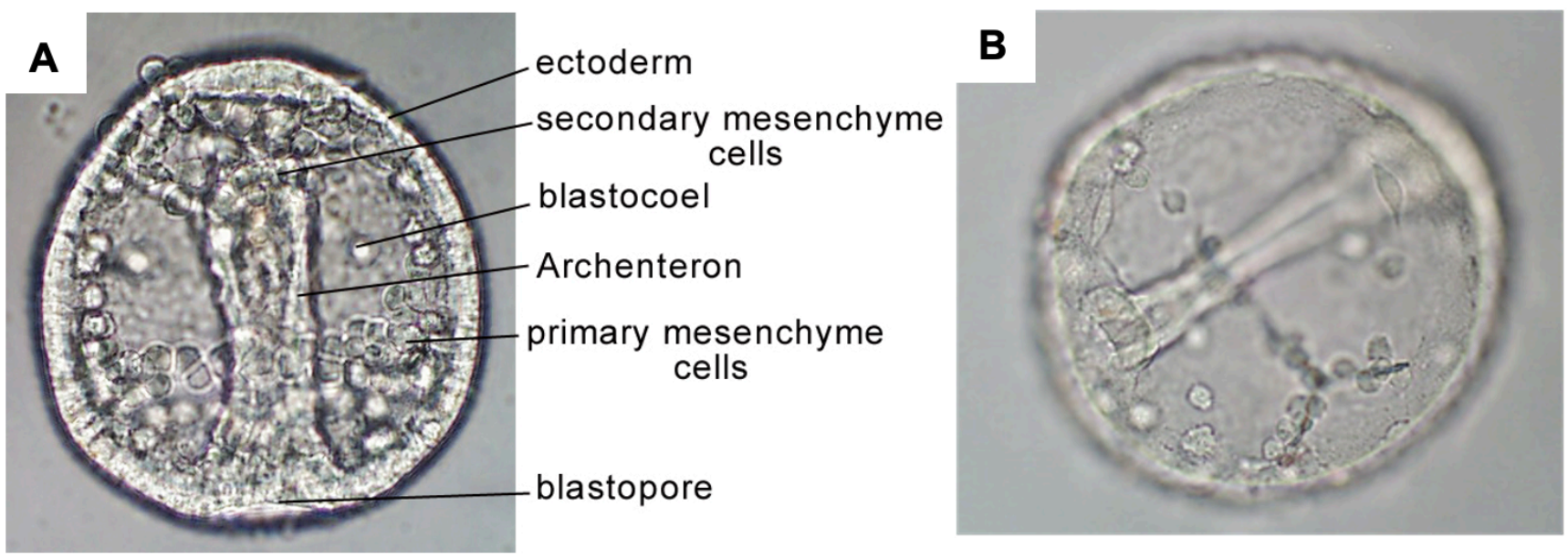

Figure 1-2. Sea urchin gastrulation. (A) By late gastrulation, the three germ layers have become established. Note the secondary mesenchyme cells at the top of the archenteron. They send out filopodia, which attach to the extracellular matrix lining the blastocoel wall and pull the archenteron up towards the site of the future mouth to form the gut cavity. (B) The primary mesenchyme cells form the spicules on the inside surface of the ectoderm.

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Experiment 1-1. Fertilization and Early Development

Objective:

To observe sea urchin gametes, fertilization, and early development

Background:

Sea urchins and sand dollars provide an excellent introduction to embryology. Male and female gametes can easily be isolated, allowing for the observation of fertilization and early development during a class period or over the next few days. One of the advantages of echinoderms is that fertile animals are available virtually year-round. *Lytechinus variegatus* embryos will develop at room temperature; *L. pictus* and *Strongylocentrotus purpuratus* gametes and embryos need to be kept at lower temperatures.

Gamete Collection:

Inject 0.5 M KCl into the adults at several sites in the leathery tissue surrounding the mouth on the ventral side to induce spawning (Figure 1-3) and set the urchin down on paper towels. The sex of the adults cannot be determined until the gametes start to be extruded from openings (the gonadopores) on the dorsal surface; eggs are tan to orange (depending on the species), while sperm are milky and white. Eggs are collected by allowing an inverted female to release them into a beaker of artificial sea water (ASW). The eggs are washed by allowing them to settle, decanting the ASW, and replacing it three times. Sperm are collected by inverting a male over a dry petri dish.

Figure 1-3. Preparation of sea urchin sperm and eggs

The sperm can be transferred to a microfuge tube and stored at 4 °C for up to two weeks. Eggs should normally be used on the same day they are collected. Consult the Sea Urchin Embryology website for a protocol for collecting the eggs with antibiotics, which allows them to be stored for several weeks before use.

Experimental Procedure:

1. Transfer 5 mL of a diluted egg suspension to a test tube and allow the eggs to settle. Remove all but approximately 2 mL of ASW. Resuspend and transfer 1 drop of fresh eggs into the well of a depression slide and cover with a coverslip. Examine under lowest power first. Rotate the nosepiece to change to a higher magnification. Be careful; sea water will corrode your microscope and damage the objectives. Before proceeding, check to make sure that the population does not contain a large percentage of immature eggs (Figure 1-4); they will not fertilize.

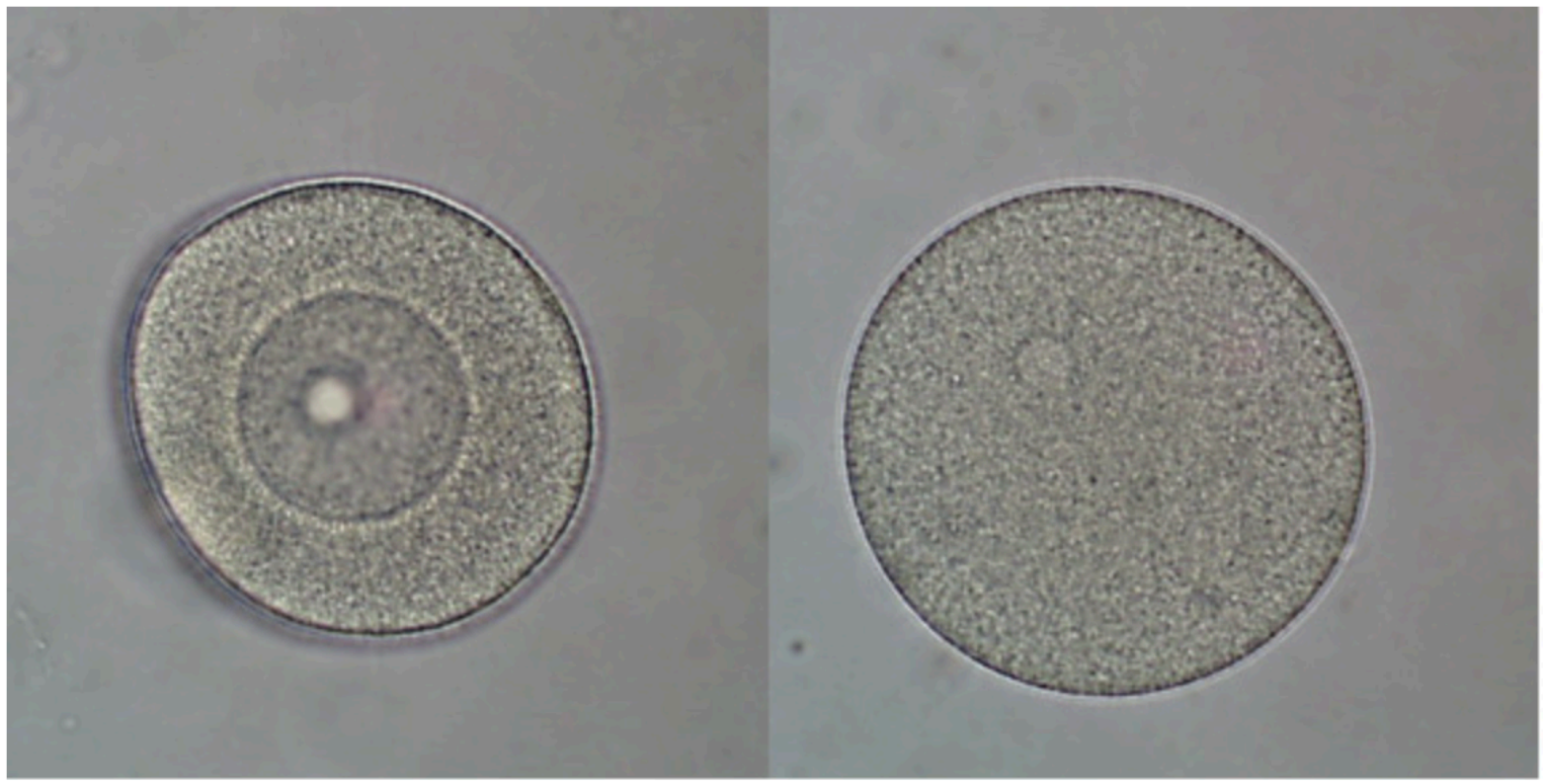

Figure 1-4. Sea urchin eggs. Immature (left) and mature (right) sea urchin eggs. Note the compacted acentric nucleus in the mature egg compared with a large central nucleus with visible nucleolus in the immature egg.

2. Sperm will only remain viable for a short time (less than 1 hour) in water. Five minutes before use, dilute 30 µL of sperm in 5 mL ASW (to produce diluted sperm). Put a drop of diluted sperm on a clean microscope slide and cover with a coverslip. Observe with your compound microscope. Focus on sperm using a 40X objective. Check for evidence of motility. With permission from your instructor, observe under 1,000X magnification. Rotate the objectives to a point between the 40X and 100X objectives. Add a drop of immersion oil to the center of the field of view, then carefully rotate the 100X objective into

position and use the fine adjust focus. BE CAREFUL and do not get immersion oil on the other objectives. When you are done, CAREFULLY blot all the oil from the objective using lens paper. Use several regions of the sheet. Do not wipe back and forth as this may scratch the lens.

3. To “capture” early events after fertilization, transfer a drop of fresh eggs on the depression slide. Add a drop of activated sperm. Cover with a coverslip and observe immediately. Watch for the appearance of a fertilization envelope (Figure 1-5).

4. Use 3 drops of diluted sperm to fertilize the tube of eggs. After 5 minutes, add additional ASW until the tube is approximately 90% full. Transfer a drop of eggs to the depression slide. What percentage of the eggs are fertilized, based on the appearance of a fertilization envelope?

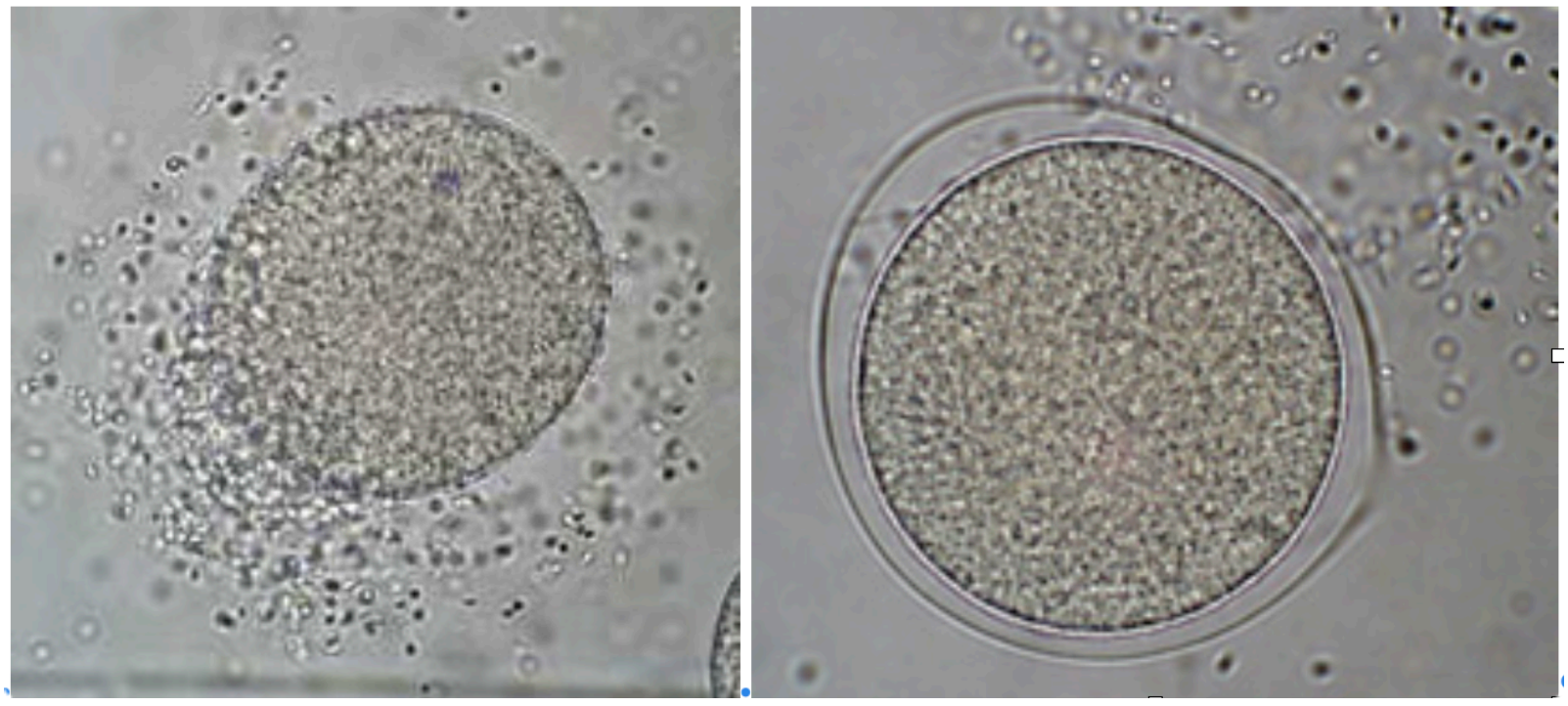

Figure 1-5. Sea urchin fertilization. (Left) Sea urchin egg surrounded by sperm burrowing through the jelly coat. (Right) Zygote with a fertilization envelope.

5. Allow the fertilized eggs to settle in the tube and carefully remove the ASW. Add 5 mL ASW and continue to incubate for 70–90 minutes. Remove a sample periodically and examine microscopically. Check for the presence (or absence) of cell nuclei, the appearance of the asters (cytoplasmic disturbances caused by the formation of the microtubule arrays during mitosis), and the formation of a cleavage furrow. Construct a table in your lab notebook and record your observations. How long after fertilization did it take for the first signs of cell division in the control? What percentage of the fertilized eggs divided?

6. Transfer the embryos to a sterile petri dish and dilute with additional ASW for overnight incubation. Over the following days, check for swimming embryos

that have “hatched” out of the fertilization envelope. What stage of development have they reached (Figure 1-1)?

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Cebra-Thomas, J. 2006. Sea urchin gamete collection. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Urchin/Urchin_gamete.html

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Prep Checklist for 1-1:

For preparation of gametes:

- ☐ 25-gauge needles
- ☐ 3 mL syringes
- ☐ Needle disposal container
- ☐ Aluminum foil
- ☐ Controlled-drop Pasteur pipets or transfer pipets
- ☐ Pipet bulbs
- ☐ Clean 250 mL beakers (never washed with soap), sterile
- ☐ Artificial sea water (ASW)
- ☐ 0.5 M KCl

For each table:

- ☐ Test-tube rack
- ☐ Markers
- ☐ Labelling tape
- ☐ Paper towels (C-fold)
- ☐ Kimwipes
- ☐ 100 mm tubes (Fisher 14-961-27, never washed with soap)
- ☐ Controlled-drop Pasteur pipets with bulbs or transfer pipets
- ☐ Pipet Bulbs
- ☐ Depression slides (never washed with soap)
- ☐ Microscope slides
- ☐ Coverslips
- ☐ Glass 60 mm petri dishes (never washed with soap), autoclaved

For the room:

- ☐ Gloves: small, medium, and large (1 box each)
- ☐ ASW

Equipment:

- ☐ Compound microscopes

Experiment 1-2. Inhibition of the Fast and Slow Blocks to Polyspermy

Objective:

To test the effects of inhibiting the fast and slow blocks to polyspermy on sea urchin development.

Experimental Procedure:

1. Obtain sperm and eggs as described in Experiment 1-1.

2. To inhibit the fast block to polyspermy, resuspend eggs in artificial sea water (ASW) or in nicotine at 0.25 mM, 0.5 mM, and 1 mM (dilute 1.25 µL, 2.5 µL, and 5 µL of a 40% solution of nicotine free base in 10 mL ASW and use immediately). Fertilize eggs with diluted sperm as described in Experiment 1. Be sure to allow sperm to activate for at least 5 minutes before adding to eggs.

3. To inhibit the slow block to polyspermy, resuspend eggs in ASW or in soybean trypsin inhibitor (SBTI) at 0.4 mg/mL in ASW (1/10 volume of 4 mg/mL SBTI stock). Fertilize eggs with diluted sperm as described in Experiment 1. Be sure to allow sperm to activate for at least 5 minutes before adding to eggs.

4. Examine eggs after 10 minutes for the presence (or absence) of the fertilization envelope and periodically over the next 90 minutes to observe the events associated with the first cleavage division. Document the percentage fertilized and the percentage undergoing normal vs. abnormal cleavage divisions in your notebook (see Figure 1-6). Photograph or sketch.

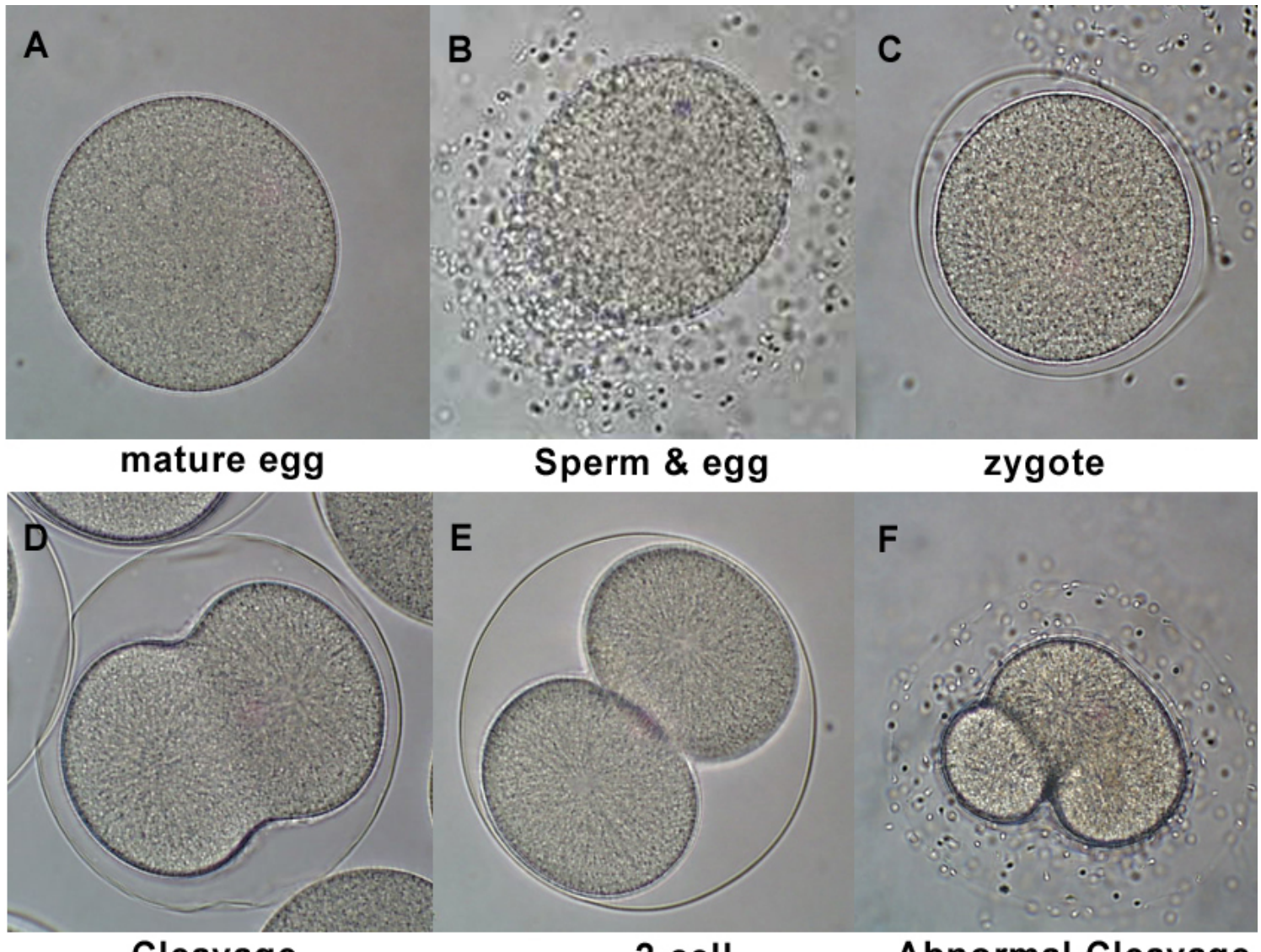

Figure 1-6. Fertilization and early development in *L. variegatus*. (A) Mature egg; (B) mature egg with sperm embedded in egg jelly; (C) zygote with a fertilization envelope; (D) beginning of the first cell cleavage division; (E) 2-cell stage; (F) abnormal cell division resulting from interference with the blocks to polyspermy.

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Cebra-Thomas, J. 2006. Inhibition of the fast and slow blocks to polyspermy. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Urchin/Polyspermy.html

Prep Checklist for 1-2:

For preparation of gametes:

- ☐ 25 g needles
- ☐ 3 mL syringes
- ☐ Needle disposal container
- ☐ Aluminum foil
- ☐ Controlled-drop Pasteur pipets
- ☐ Pipet bulbs
- ☐ Clean 250 mL beakers (never washed with soap), sterile
- ☐ Artificial sea water (ASW)
- ☐ 0.5 M KCl
- ☐ Gloves: small, medium, and large (1 box each)

For each table:

- ☐ Test-tube rack
- ☐ Markers
- ☐ Labelling tape
- ☐ Paper towels (C-fold)
- ☐ Kimwipes
- ☐ 100 mm tubes (Fisher 14-961-27, never washed with soap)
- ☐ Controlled-drop Pasteur pipets and bulbs
- ☐ Depression slides (never washed with soap)
- ☐ Microscope slides
- ☐ Coverslips
- ☐ Glass 60 mm petri dishes (never washed with soap), autoclaved

Solutions:

- ☐ ASW
- ☐ Nicotine solution, 40% free base
- ☐ Soybean trypsin inhibitor stock (4 mg/mL in ASW), aliquots stored frozen

Equipment:

- ☐ Compound microscopes

Experiment 1-3. Gastrulation and Cell Differentiation in Sea Urchin Embryos

Objective:

To examine sea urchin development through gastrulation and the establishment of distinct cell lineages.

Experimental Procedure:

A. Fertilization and Preparation of Embryos

1. Obtain sperm and eggs as described in Experiment 1-1.
2. Wash eggs several times in artificial sea water (ASW) in a small beaker and suspend approximately 5 mL of packed eggs in 50 mL ASW.
3. Dilute 30 µL of sperm in 5 mL ASW to activate. Check for motility as described in Experiment 1.
4. Add 3–5 mL of diluted sperm to egg suspension and swirl to mix.
5. Check for fertilization after 5 minutes. Add additional activated sperm if necessary. Allow eggs to settle and pour off excess sperm. Replace with fresh 50 mL of ASW. Check for cell division after 70–90 minutes.
6. Transfer the embryos to finger bowls or large beakers and dilute 10-fold; relatively shallow cell suspensions with a large surface area are better for oxygen transfer and embryonic development.
7. If developing at room temperature, the embryos should reach the hatched blastula stage by Day 2, gastrula by Day 3 (Figure 1-7), and prism and pluteus larva by Days 4–5. Living embryos will be swimming in the water, not lying on the bottom. Observe with a dissecting microscope and try to capture swimming embryos with a transfer or Pasteur pipet. Mount them on labeled depression slides for observation with a compound microscope. Add Protoslo® or a few strands of cotton wool teased from the end of an applicator stick to slow down the swimming embryos. If available, observe under polarizing optics to examine spicule development.

B. Preparation of Fixed Embryos for AP Staining

1. Transfer 50 mL of embryo cultures at different stages to centrifuge tubes. Spin at 1,500 rpm for 5 minutes. Check that you can see a pellet of embryos at the bottom.

2. Quickly pour off the ASW. Try to remove as much as possible, but don’t worry about a little ASW left in the tube.

3. Gently swirl the tube to resuspend the embryos. Add 40 mL of ice-cold methanol and allow the embryos to fix on ice for no more than 20 minutes. By this time, the embryos should have settled to the bottom of the tube.

4. Decant off the methanol and resuspend the embryos in approximately 25 mL of ice-cold ASW.

5. ON ICE, let the embryos settle to the bottom of the tube by gravity.

6. Decant off the ASW and resuspend the embryos in fresh ice-cold ASW. (At this point, embryos can be stored in refrigerator).

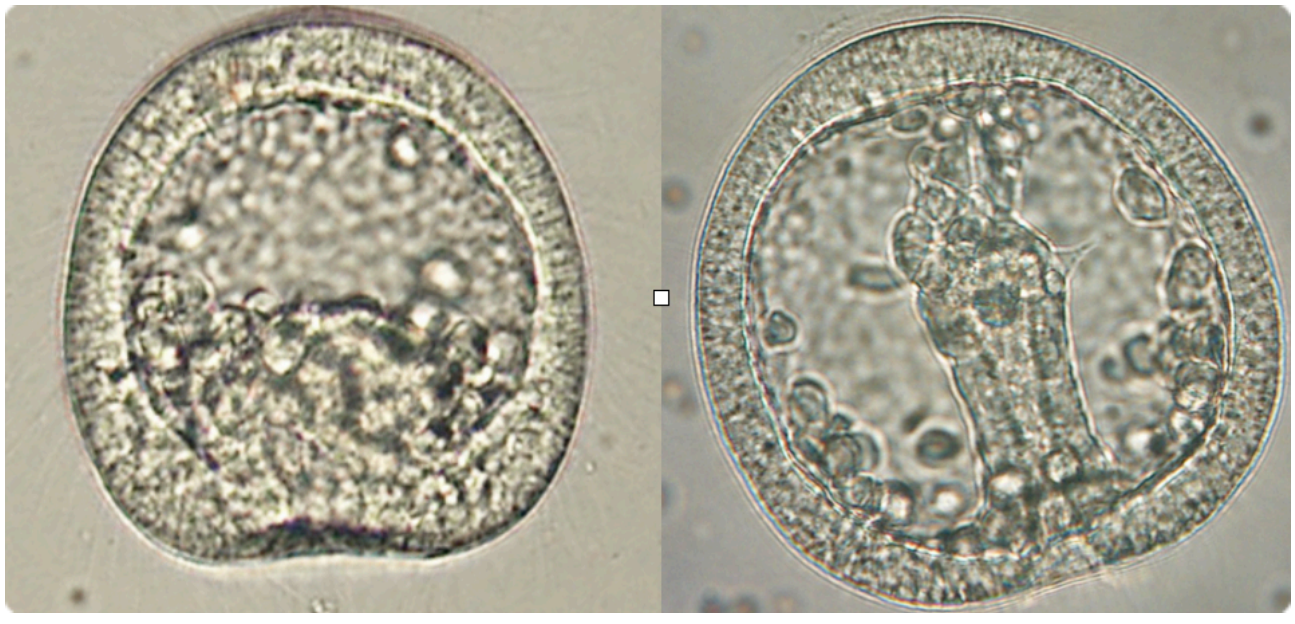


Figure 1-7. Sea urchin gastrulation. (Left) In an early gastrula the vegetal plate starts to buckle inwards and the primary mesenchyme cells ingress into the blastocoel. (Right) The archenteron extends towards the animal pole, and the primary mesenchyme cells migrate upwards and cluster.

C. Histochemical Staining of Sea Urchin Embryos for Alkaline Phosphatase (AP) Enzyme Activity

1. Decant ASW from the 50 mL tube of fixed embryos, then add ASW to about 10 mL. Transfer 1 mL aliquots to microfuge tubes.

2. Obtain embryo samples, a tube of AP substrate buffer, and a tube of phosphate-buffered saline (PBS) for each group. Allow embryos to settle. Carefully remove excess liquid with a transfer pipet. It is not necessary to remove all of the liquid; don't get so close to the pellet that you suck up the embryos.

3. Resuspend in 0.5 mL of AP substrate buffer. Allow embryos to settle for 10 minutes. Remove excess buffer.

4. Add 100 mL of AP substrate to tubes. Check for staining after 5 minutes by transferring a small sample to a depression slide and observing at low power. Be careful not to get AP substrate on your hands (wear gloves) or on your microscope. Do not leave the light turned on between observations. To stop the reaction, return embryos to the tube and add 0.5 mL of PBS.

5. Allow embryos to settle for 10 minutes. Remove buffer to about 100 mL, return to depression slides, and observe. Look for evidence of morphogenesis (archenteron invagination) and tissue differentiation (gut alkaline phosphatase activity and spicule formation).

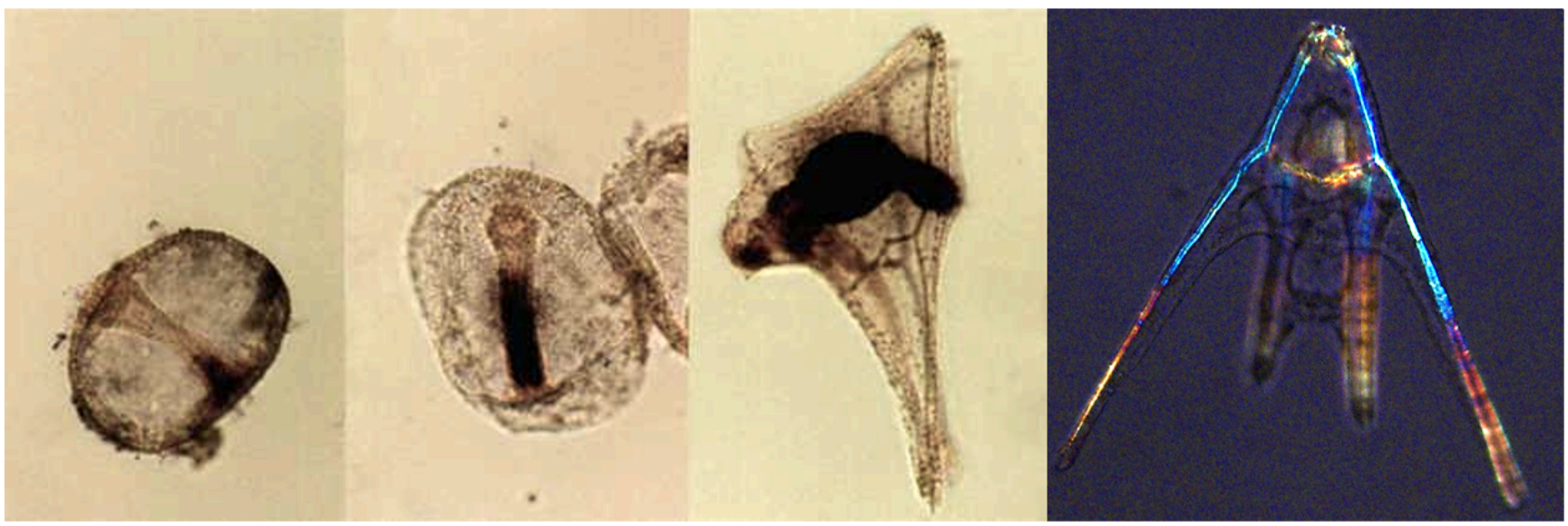


Figure 1-8. Cell differentiation during sea urchin gastrulation and larval development. (Left) Histochemical staining for alkaline phosphatase activity in sea urchin gastrula and pluteus larva. (Right) Pluteus larva under polarized light.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Cebra-Thomas, J. 2006. Histochemical staining of sea urchin embryos for alkaline phosphatase (AP) enzyme activity. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Urchin/Urchin_AP.html

Drawbridge, J. 2003. The color purple: analyzing alkaline phosphatase expression in experimentally manipulated sea urchin embryos in an undergraduate developmental biology course. The International Journal of Developmental Biology, 47, 161–164.

Prep Checklist for 1-3 Week 1:

For preparation of gametes:

- ☐ 25 g needles
- ☐ 3 mL syringes
- ☐ Needle disposal container
- ☐ Aluminum foil
- ☐ Controlled-drop Pasteur pipets
- ☐ Pipet bulbs
- ☐ Clean 250 mL beakers (never washed with soap), sterile
- ☐ Artificial sea water (ASW)
- ☐ 0.5 M KCl

For each table:

- ☐ Test-tube rack
- ☐ Markers
- ☐ Labelling tape
- ☐ Paper towels (C-fold)
- ☐ Kimwipes
- ☐ 100 mm tubes (Fisher 14-961-27, never washed with soap)
- ☐ Controlled-drop Pasteur pipets and bulbs
- ☐ Depression slides (never washed with soap)
- ☐ Microscope slides
- ☐ Coverslips
- ☐ Finger bowl or 600 mL beakers
- ☐ 50 mL conical screw-top centrifuge tubes

For the room:

- ☐ Gloves: small, medium, and large (1 box each)

Solutions:

- ☐ ASW
- ☐ Cold ASW
- ☐ Cold 100% Methanol

Equipment:

- ☐ Compound microscope
- ☐ Dissecting microscope
- ☐ Refrigerated centrifuge

Prep Checklist for 1-3 Week 2:

For each table:

- ☐ 1.5 mL microfuge tubes and rack
- ☐ Marker
- ☐ Labeling tape
- ☐ Paper towels (C-fold)
- ☐ Wide-bore transfer pipets
- ☐ P200 Pipetman and wide-orifice tips (“Cell-saver”)
- ☐ Depression slides
- ☐ Coverslips
- ☐ Gloves: small, medium, and large (1 box each)

Solutions:

- ☐ Cold ASW
- ☐ Cold methanol
- ☐ Alkaline phosphatase substrate buffer (100 mL/section)
- ☐ AP substrate (5 mL/section)
- ☐ 1X PBS

Equipment:

- ☐ Compound microscopes

Experiment 1-4. Perturbation of Gastrulation in Sea Urchin Embryos

Objective:

Exposure to LiCl and deprivation of sulfate both affect sea urchin gastrulation. The effects of LiCl on sea urchin gastrulation will be examined by treating embryos from the two-cell stage to the mesenchyme blastula stage. The effect of sulfate deprivation on archenteron formation will be tested by fertilizing eggs and rearing embryos in sulfate-free seawater. Alkaline phosphatase enzyme activity will be used to monitor the developing digestive tract (Drawbridge, 2003) in the control and treated embryos. In addition, the number and distribution of pigment-containing cells will be analyzed.

Experimental Procedure:

A. Sulfate-Deprivation (Based on Karp & Solursh, 1974)

1. Induce sea urchin gamete release by injection of KCl and obtain eggs and sperm as described in Experiment 1.
2. Wash eggs 3 times in sulfate-free water.
3. Fertilize eggs as described in Experiment 3.
4. Check for fertilization under the microscope.
5. Fill (and label) a beaker with artificial sea water (ASW) and a beaker with sulfate-free water; transfer fertilized eggs into each beaker with a pipet.
6. Cover and incubate overnight.
7. Allow embryos to develop until the control group in ASW has gastrulated.
8. Extract swimming embryos and place them on labeled depression slides for observation.
9. Fix samples of each group of embryos as described below for staining next week.

B. Lithium Treatment (Based on Cameron & Davidson, 1997)

1. Use a 60 mM solution of LiCl in ASW to prepare 30 mM and 15 mM solutions in ASW.

2. Fertilize eggs as described in Experiment 3.

3. At the two-cell stage, transfer embryos from ASW to seawater with 15 mM and 30 mM LiCl. A third of the embryos should remain in ASW as a control group.

4. At the mesenchyme blastula stage, transfer the treated embryos back to ASW.

5. Observe samples of embryos from a control (ASW with no LiCl added) culture and each of the experimental cultures the next day.

6. Use 2–3 drops of eggs/embryos on a depression slide to compare developmental rate and morphology of the groups; focus on the vegetal area morphology of embryos.

7. Allow embryos to develop until the control group has gastrulated.

8. Fix samples of each group of embryos as described below for staining next week.

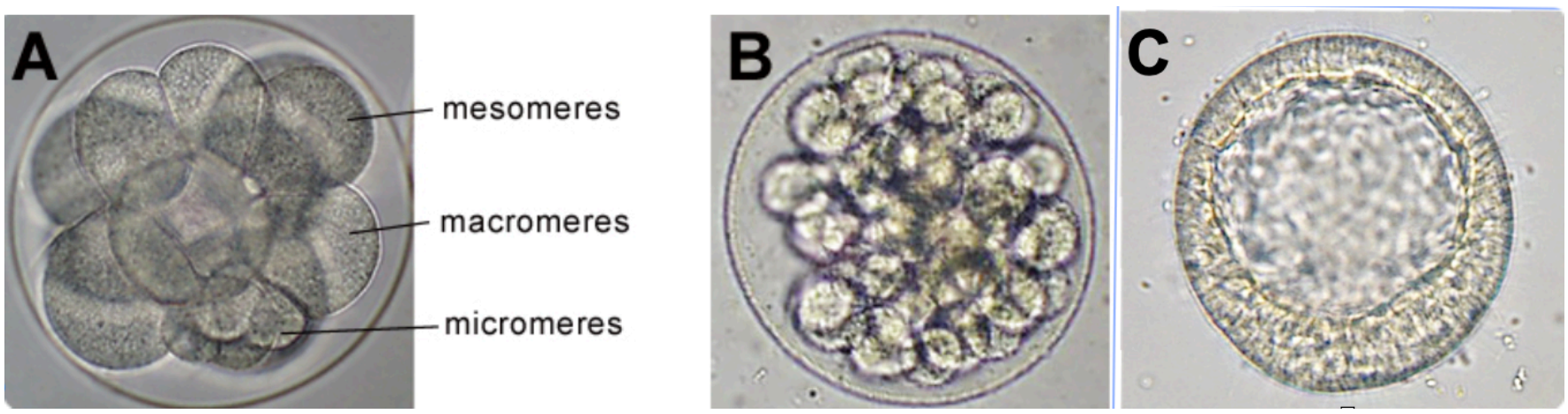


Figure 1-9. Cleavage and blastula stage sea urchin embryos. (A) 16-cell stage *L. variegatus* embryo showing the three types of blastomeres. (B) A 64-cell stage *L. variegatus* embryo consists of a ball of loosely connected cells; the descendants of the three types of blastomeres remain in the same relative position in the embryo. (C) Hatched blastula stage embryo showing thickening of the epithelium in the vegetal half of the embryo.

C. Preparation of Fixed Embryos for AP Staining

1. Transfer 50 mL of embryo cultures at different stages to centrifuge tubes. Spin at 1,500 rpm for 5 minutes. Check that you can see a pellet of embryos at the bottom.

2. Quickly pour off the ASW. Try to remove as much as possible, but don’t worry about a little ASW left in the tube.

3. Gently swirl the tube to resuspend the embryos. Add 40 mL of ice-cold methanol and allow them to fix on ice for no more than 20 minutes. By this time, the embryos should have settled to the bottom of the tube.

4. Decant off the methanol and resuspend the embryos in approximately 25 mL of ice-cold ASW.

5. ON ICE, let the embryos settle to the bottom of the tube by gravity.

6. Decant off the ASW and resuspend the embryos in 25–50 mL of fresh ice-cold ASW. (At this point embryos can be stored in refrigerator)

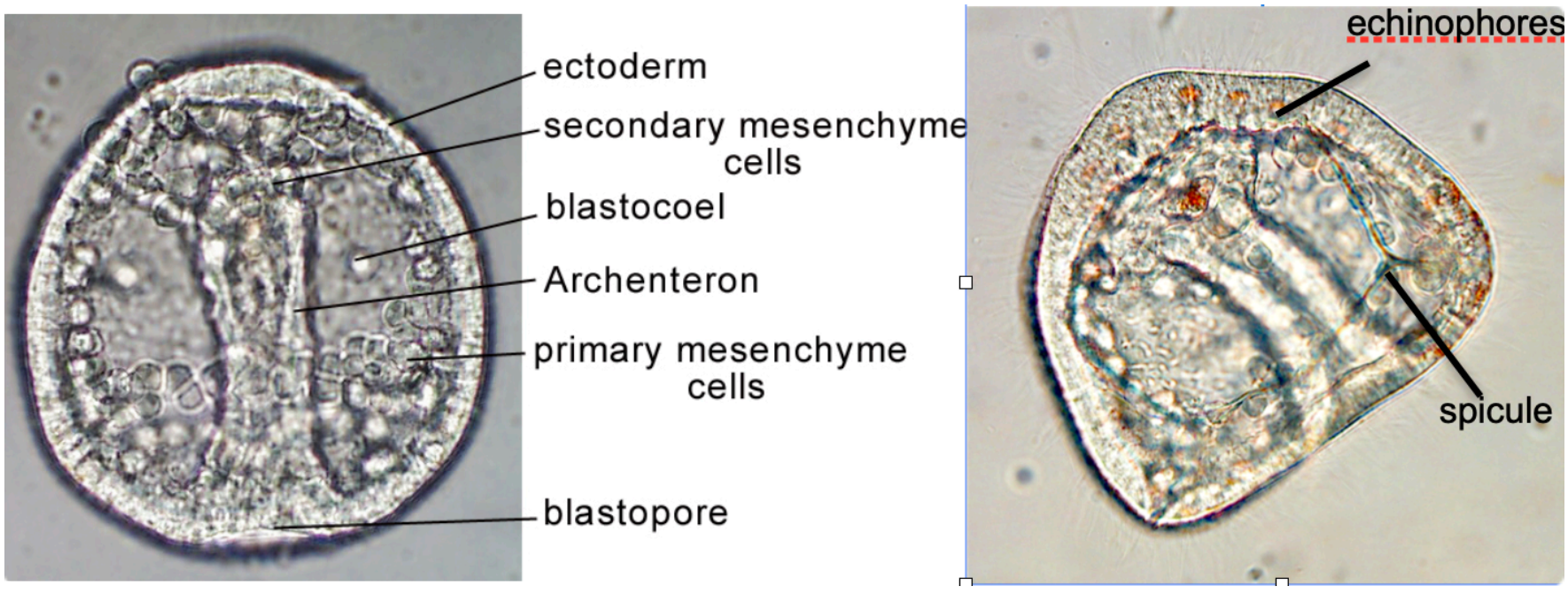


Figure 1-10. Sea urchin gastrulation and echinophore differentiation. (Left) The archenteron extends to the site of the future mouth on the roof of the blastocoel. Note the secondary mesenchyme cells at the top of the archenteron. They send out filopodia, that attach to the extracellular matrix lining the blastocoel wall and pull the archenteron up towards the site of the future mouth to form the gut cavity. (Right) By the early prism larval stage, echinophores have migrated throughout the ectoderm and differentiated to produce pigment.

D. Histochemical Staining of Sea Urchin Embryos for Alkaline Phosphatase (AP) Enzyme Activity (See Experiment 1-3)

1. Decant ASW from 50 mL tube of fixed embryos, then add ASW to about 10 mL. Transfer 1 mL aliquots to microfuge tubes.

2. Obtain embryo samples, a tube of AP substrate buffer, and a tube of phosphate-buffered saline (PBS) for each group. Allow embryos to settle. Carefully remove excess liquid with transfer pipet. It is not necessary to remove all of the liquid; don't get so close to the pellet that you suck up the embryos.

3. Resuspend in 0.5 mL of AP substrate buffer. Allow embryos to settle for 10 minutes. Remove excess buffer.

4. Add 100 µL of AP substrate to tubes. Check for staining after 5 minutes by transferring a small sample to a depression slide and observing at low power. Be careful not to get AP substrate on your hands (wear gloves) or on your microscope. Do not leave the light turned on between observations. To stop the reaction, return embryos to the tube and add 0.5 mL of PBS.

5. Allow embryos to settle for 10 minutes. Remove buffer to about 100 µl, return to depression slides, and observe. Look for evidence of morphogenesis (archenteron invagination) and tissue differentiation (gut alkaline phosphatase activity and spicule formation, see Figure 1-8).

References

Barresi, M. and S. Gilbert. 2023. Developmental Biology, 13th edition, Oxford University Press.

Cebra-Thomas, J. 2006. The effect of lithium chloride on sea urchin embryos. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Urchin/Ur_lithium.html

Cebra-Thomas, J. 2012. Look Ma, No Archenteron! Sulfate's role in sea urchin early development. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Urchin/Matthews/sulfate.html

Drawbridge, J. 2003. The color purple: analyzing alkaline phosphatase expression in experimentally manipulated sea urchin embryos in an undergraduate developmental biology course. The International Journal of Developmental Biology, 47, 161–164.

Prep Checklist for 1-4 Week 1:

For preparation of gametes:

- ☐ 25 g needles
- ☐ 3 mL syringes
- ☐ Needle disposal container
- ☐ Aluminum foil
- ☐ Controlled-drop Pasteur pipets
- ☐ Pipet bulbs
- ☐ Clean 250 mL beakers (never washed with soap), sterile
- ☐ Artificial sea water (ASW)
- ☐ 0.5 M KCl

For each table:

- ☐ Test-tube rack
- ☐ Markers
- ☐ Labelling tape
- ☐ Paper towels (C-fold)
- ☐ Kimwipes
- ☐ 100 mm tubes (Fisher 14-961-27, never washed with soap)
- ☐ Controlled-drop Pasteur pipets and bulbs
- ☐ Depression slides (never washed with soap)
- ☐ Microscope slides
- ☐ Coverslips
- ☐ Finger bowls or 600 mL beakers
- ☐ 50 mL conical screw-top centrifuge tubes

For the room:

- ☐ Gloves; small, medium, and large (1 box each)

Solutions:

- ☐ ASW
- ☐ Sulfate-free ASW
- ☐ 60 mM LiCl

- ☐ Cold ASW

Cold 100% Methanol

Equipment:

- ☐ Compound microscope
- ☐ Dissecting microscope
- ☐ Refrigerated centrifuge

Prep Checklist for 1-4 Week 2:

For each table:

- ☐ 1.5 mL microfuge tubes and rack
- ☐ Marker
- ☐ Labeling tape
- ☐ Paper towels (C-fold)
- ☐ Wide-bore transfer pipets
- ☐ P200 Pipetman and wide-orifice tips (“Cell-saver”)
- ☐ Depression slides
- ☐ Coverslips
- ☐ Gloves: small, medium, and large (1 box each)

Solutions:

- ☐ Cold ASW
- ☐ Cold methanol
- ☐ Alkaline phosphatase substrate buffer (100 mL/section)
- ☐ AP substrate (5 mL/section)
- ☐ 1X PBS

Equipment:

- ☐ Compound microscopes

Chapter 2 Chicken Development

Chick Development

Birds, such as chickens, are **amniotes**, like mammals and reptiles. Birds and reptiles have eggs that are packaged to prevent desiccation and allow the embryo to develop for several weeks without external food. The yolk is the actual egg cell, and it is fertilized internally as it is released from the ovary into the oviduct. The eggs are **telolecithal**, with the majority of the volume given over to the energy-rich yolk. The non-yolky cytoplasm that will produce the embryo is initially confined to a small region on one side. If you examine an unfertilized chicken egg yolk, you will notice a small ring made of hardened yolk that lies underneath the embryonic cytoplasm. The yolk is surrounded by a protein coat known as the **vitelline membrane** that helps prevent the yolk from rupturing.

As the yolk continues down the oviduct, first the egg white and then the shell is deposited around it. The yolk is kept suspended in the center by ropes of albumin known as chalaza. If the yolk cell has been fertilized, the embryonic cytoplasm begins to be subdivided by cell division. The cell division is initially **meroblastic** (incomplete cleavage with the bottom of the cells remaining open to the yolk) and **discoidal** (occurring in a single plane), producing a **blastodisc**, a circular structure defining the animal pole of the embryo. Eventually, the cells fully separate from the yolk and form a polarized epithelium. They transfer ions and fluid from the apical side near the egg white to the basal side below, forming a space (the **subgerminal space**) between the embryonic cells and the yolk. At the end of the cleavage phase, the embryo consists of a disc of cells known as the **epiblast**, which is equivalent to the blastula, and the subgerminal space is analogous to the blastocoel. The future ventral side of the embryo is defined as the side adjacent to the subgerminal space.

As the egg continues down the oviduct, it rotates, creating a force that causes cells to build up at one end, known as the **posterior marginal zone (PMZ)**. This helps to establish the anterior-posterior axis. A second, lower layer, known as the **hypoblast**, is created by cells moving anteriorly from the PMZ and dropping down from the epiblast. At the initiation of gastrulation, the epiblast, which will form the embryo proper, is underlain by the lower hypoblast layer, with a thickened arc at the posterior end known as **Koller’s sickle**. Gastrulation begins as the cells of the epiblast push towards the midline at the posterior, forming a mound known as the **primitive streak**. As the cells push together from each side, the cells at the midline fold inwards, forming the primitive

groove. The cells in the primitive streak ingress through the groove and drop down into the blastocoel. The cells that will form the embryonic endoderm drop all the way down to the hypoblast and push the existing cells to either side. The cells that will form the mesoderm spread out on the underside of the epiblast.

As gastrulation progresses, the primitive streak elongates towards the future head, eventually extending along approximately 80% of the midline of the embryo (see Figure 2-1, stages 3 & 4). As the epiblast cells converge towards the primitive streak, the embryo becomes longer and narrower. The cells at the anterior end of the primitive streak are derived from Koller's sickle and form a structure known as **Hensen's node**. It is the source of signaling molecules, including **sonic hedgehog** (SHH), and serves as an organizing structure for the embryo. Like the amphibian dorsal blastopore lip, it is the site where gastrulation initiates and transplantation can result in the formation of a second embryonic axis. As the mesoderm and endoderm cells ingress through the primitive streak, they are influenced by the signals from Hensen's node, and their fate is determined by their relative proximity. Cells that ingress directly through the node region become pharyngeal endoderm, head mesoderm and **notochord** (the most 'dorsal' fates), while cells that pass though adjacent to the node form the two stripes of **paraxial mesoderm** that are laid down on either side of the notochord. Cells that ingress through the middle and posterior end of the primitive streak assume progressively more ventral fates: intermediate mesoderm, lateral plate mesoderm and extraembryonic mesoderm. After attaining its maximal length, the primitive streak begins to regress towards the posterior end of the embryo, leaving endoderm and mesoderm, including a strip of notochord along the midline, in its wake (see Figure 2-1, stages 5 & 6). The remnants of the primitive streak continue to proliferate at the posterior of the embryo and end up in the tail bud.

At this point, the embryo has transformed itself through gastrulation from a single pancake (the epiblast) into a stack of three pancakes (the ectoderm, mesoderm and endoderm). It then becomes a more recognizable vertebrate embryo through convergence towards the midline from the sides. The central region of anterior ectoderm gives rise to the **neural plate**. As the plate begins to round up into a tube, the lateral edges elevate at the anterior end of the embryo and form the headfolds (Figure 2-1, stage 8) and then the chambers of the brain (Figure 2-1, stage 9) as the head becomes recognizable. The **neural tube** continues to round up in a progressive fashion, forming the spinal cord. Also beginning at stage 8, the two longitudinal stripes of paraxial mesoderm condense into darker paired **somites** that will form the vertebrae of the neck and body of

the embryo. As the embryo grows, its embryonic axis extends posteriorly and new pairs of somites are produced at regular intervals. As the neural tube and the somites form, the lateral edges of the embryo push inward and the entire embryo elevates and rounds up (Figure 2-1, stages 12-14), producing a **pharyngula**-stage embryo. The head curls forward as the embryo continues to grow.

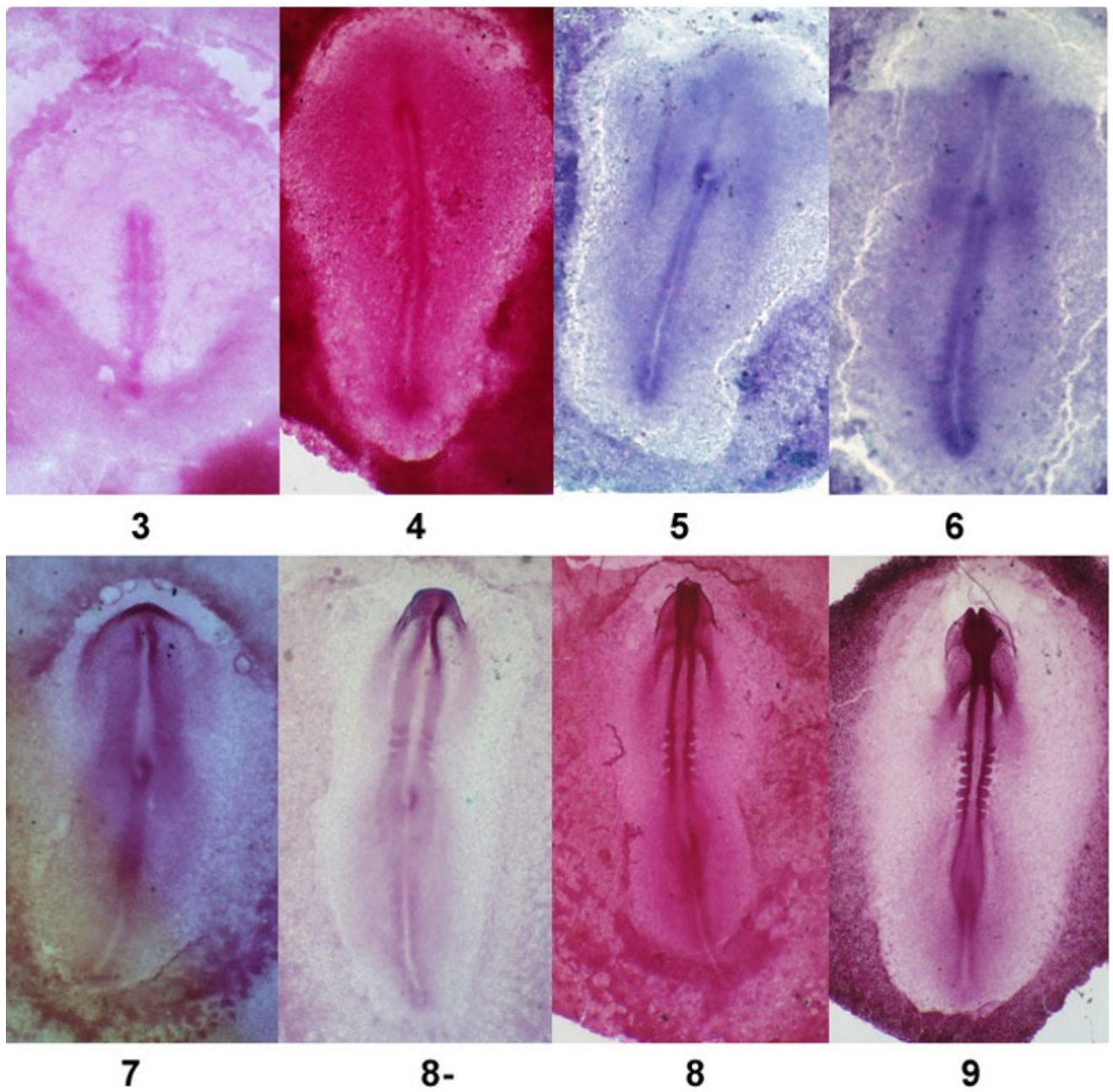


Figure 2-1. Stages of early chicken development based on the Hamburger and Hamilton staging series from gastrulation through the pharyngula stage and early organogenesis.

The earliest recognizable organs to form are the eyes and the heart. The **optic lobes** extend laterally from the forebrain (Figure 2-1, stage 10). The eyes form through reciprocal induction between the optic lobes (producing the retina) and the surface ectoderm of the head (producing the lens and cornea). The two wings of mesoderm below the head converge and form the heart tube (Figure 2-1, stage 10), which bulges out and begins to loop (Figure 2-1, stage 11). Later, the lateral plate mesoderm gives rise to the blood vessels (Figure 2-1, stage 14), then thickens and produces the paired wing and leg buds (Figure 2-1, stage 18).

The intermediate mesoderm produces the kidneys and gonads, while the extraembryonic mesoderm produces the **allantois** adjacent to the hindgut (Figure 2-1, stage 20) and the other extraembryonic membranes.

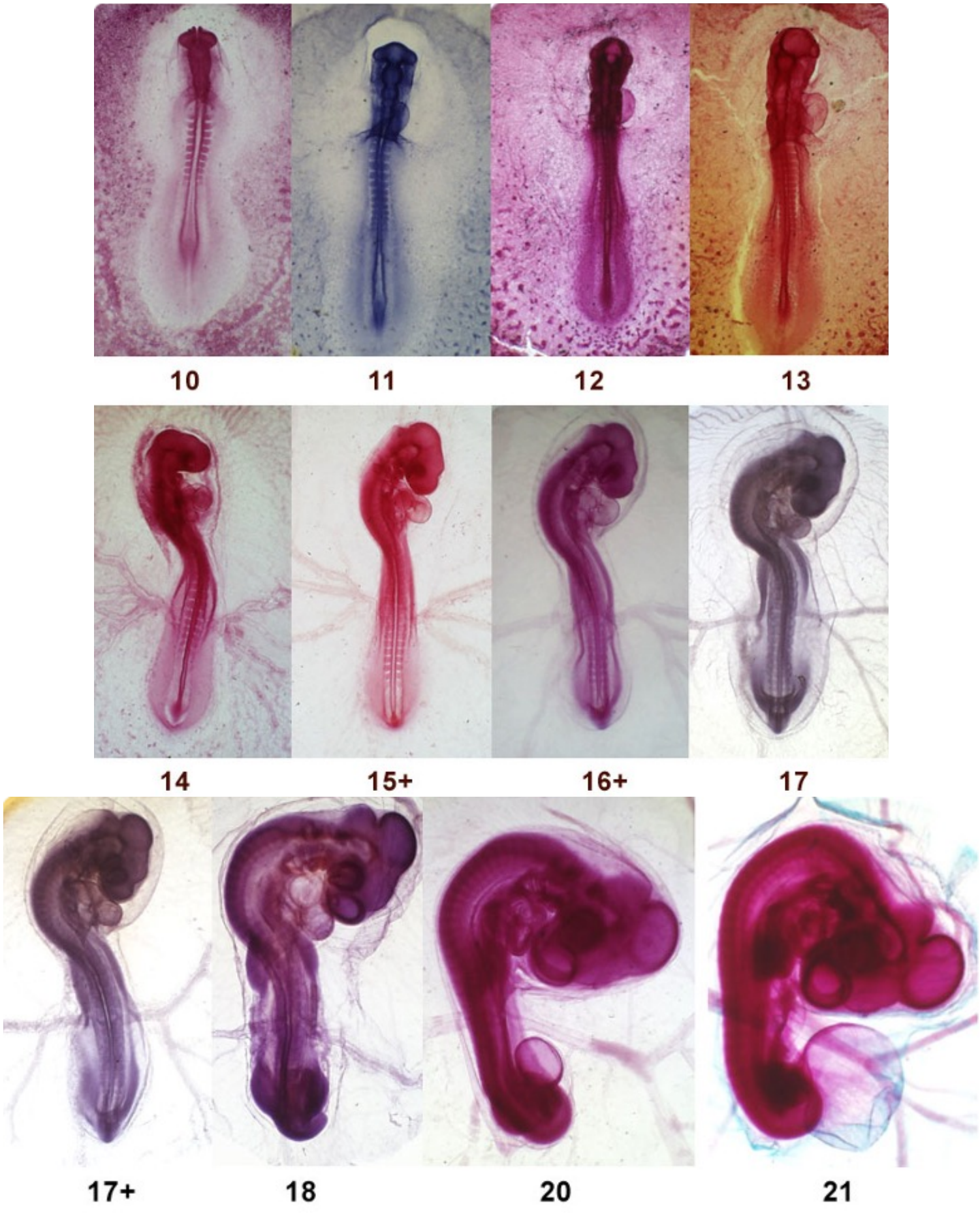


Figure 2-1 (continued). Stages of early chicken development based on the Hamburger and Hamilton staging series from gastrulation through the pharyngula stage and early organogenesis.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Hamburger, V. and H. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *Journal of Morpholo*gy 88, 49-92.

Normal Stages of Chick Embryo Development. 2006. *Developmental Dynamics*. www.anatomy.org. [accessed 2025 Jun 15]. https://anatomypubs.onlinelibrary.wiley.com/pb-assets/assets/10970177/pdf/Chick_stage_poster-1629379840910.png

Experiment 2-1. Observation of Living and Plastic-Embedded Chick Embryos

Objective:

To observe early development of chicken embryos as the basic vertebrate body plan is being established.

Background:

The development of chick embryos has been studied since Aristotle. It is one of the most intensely studied organisms. One reason for this extensive study is the great similarities between avian and mammalian embryology. Another advantage is that the embryo is relatively accessible and can be operated on or treated with teratogens (substances that perturb development) while still *in ovo* (in the egg). Finally, fertilized chicken eggs are available year-round and can be "held" at 14°C for several weeks before being set, producing embryos upon demand.

Observation of Plastic Embedded Specimens:

Examine plastic embedded specimens of "13- or 16-hour," "24-hour," "33-hour," "48-hour," and "72-hour" chick embryos. These terms refer to classic studies of chick development. In reality, chick embryos will develop at different rates for a number of reasons, including incubation temperature. In modern times, chicks always take longer to reach these stages. Two more accurate ways of describing the relative developmental age of a chick embryo are (1) the number of somites and (2) comparison to a staging series, such as the one devised by Hamburger and Hamilton (H&H). The somites are the blocks of tissue on either side of the neural tube. Try to determine the number of somites and the H&H stage number of your embryos, and then identify the structures indicated in the diagrams. Pay particular attention to the developing circulatory system. Try to determine which germ layer forms each structure. Highlights include the following:

13- to 16-hour. Identify the area opaca and area pellucida. Locate the primitive streak. Hensen's node is located at the anterior end of the streak.

24-hour. Locate the neural folds and Hensen's node. Identify the anterior end of the embryo.

33-hour. The embryo is lying along the center of the blastodisc, dorsal side up. The heart is to the right, along the side of the hindbrain. It is a

simple, looped tube, connected to the vitelline vein and the ventral aorta. What is the function of the vitelline vein? Identify the forebrain, midbrain, and hindbrain. The optic vesicles are outpockets on either side of the forebrain. The neural plate runs along the dorsal midline and is not yet closed. You may also be able to see the notochord running along the midline.

48-hour. The head has bent forward, and the body has begun to twist. The optic cup (eye) and the otic vesicle (ear) have formed. The neural tube has closed, except in the region of the hindbrain. A series of aortic arches run between the pouches in the pharynx and converge into the dorsal aorta. The heart has begun to differentiate into an atrium and a ventricle.

72-hour. The flexure of the embryo is now pronounced, and the embryo is lying on its left side. The brain has further subdivided, and the olfactory pit (nose) is present over the tip of the forebrain. The lens of the eye should be visible. Lateral swellings representing the limb buds and a curved tail are also present.

Observation of Living Embryos (3-Day and Older):

1. Obtain an egg and clean scissors, forceps, and plastic spoon. First prepare a small petri dish with Howard Ringer's (HR) solution. Gently rotate egg, clean with 70% ethanol, and set down for a few minutes to allow the embryo to float to the top. Open up the blunt end of the shell and carefully peel back the shell membranes (Figure 2-2). Observe the embryo under the dissecting scope. Measure the heart rate and observe any other movement. Grab the outer ring of the blastodisc near embryo with fine forceps in your non-cutting hand. Try not to pinch any blood vessels. Quickly cut around embryo.

Note: an alternate method of embryo isolation is to break the egg open into a finger bowl of HR. This is similar to cracking an egg for culinary purposes, but the yolks of warm eggs are more fragile than those of refrigerated ones. First, place the egg in a horizontal position to allow the embryo to float to one side. Next, keeping the same orientation, crack the egg gently against the side of a finger bowl filled with HR. Submerge the egg and gently pull the ends apart to crack the egg open. Isolate the embryos as above.

2. Keeping hold of the embryo with the forceps, pick up a spoon with your cutting hand and slide it under the embryo. Continue to hold onto embryo, lift with the spoon and transfer to the petri dish filled with HR and examine with

the dissecting microscope. Have your lab partner stand by with scissors in case the embryo wasn't completely detached. If the dish is cloudy with yolk, transfer embryo to a fresh dish of HR. Carefully trim away membranes that surround the embryo.

3. Examine embryos. Pay particular attention to the heart and circulation, and to the developing neural tube. Measure the heart rate. Which side was towards the yolk?

4. For each embryo, determine the H&H stage (see figures 2-3 to 2-5 to get started). How do these embryos compare to the stained specimens? Photograph the best representative of each stage.

5. Clean your instruments well with warm water, distilled water, and 70% ethanol. Dry before returning to case. Discard the shells in the trash, and the yolk/albumin remains in the sink.

Figure 2-2. Isolation of chick embryo. (Left) embryo in opened shell; (right) egg cracked into finger bowl.

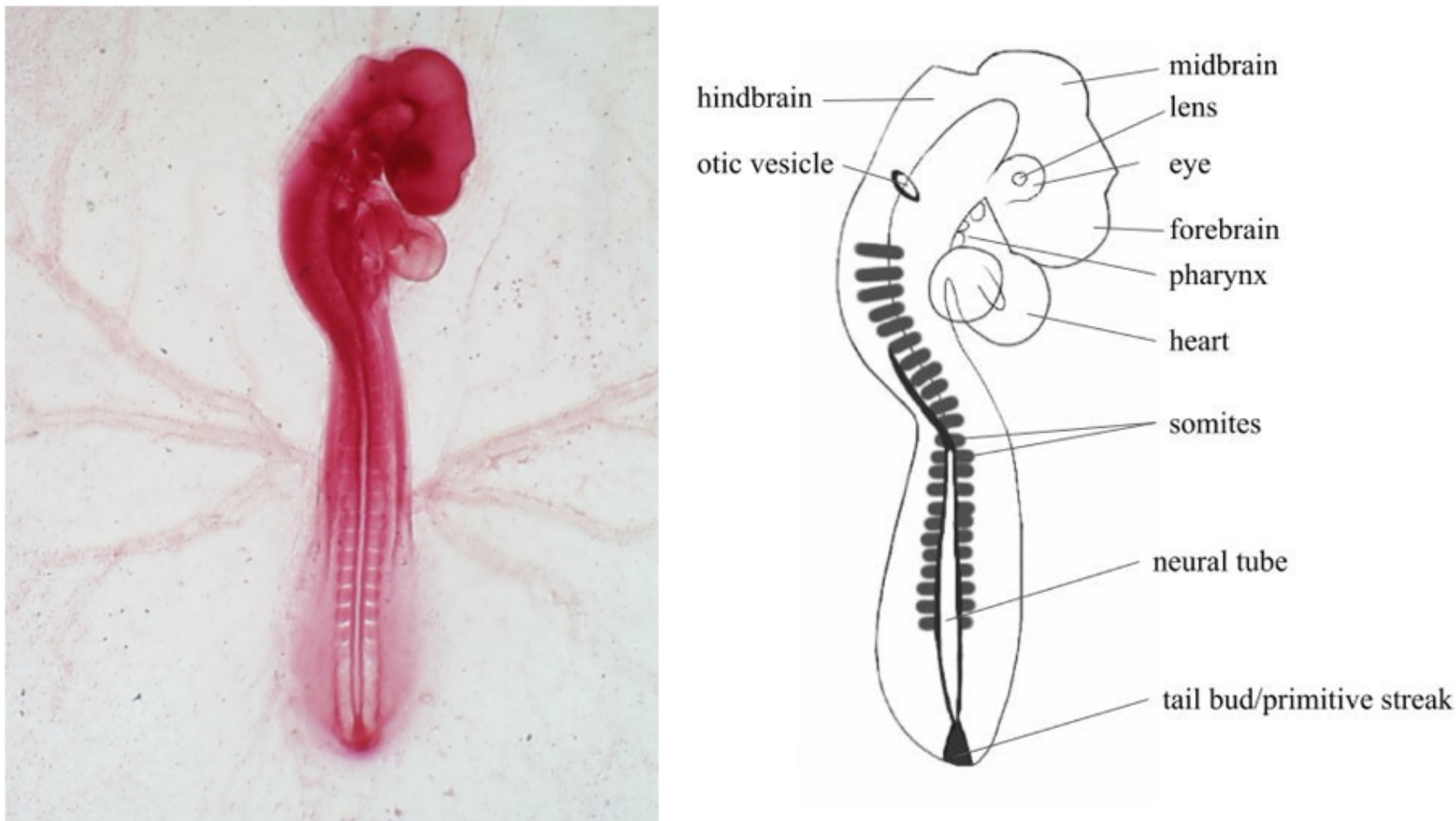


Figure 2-3. Normal chick embryo development (H&H stage 15). Chicken embryo after 3 days of incubation at 37-38°C; this is classically referred to as a "48-hour chick embryo."

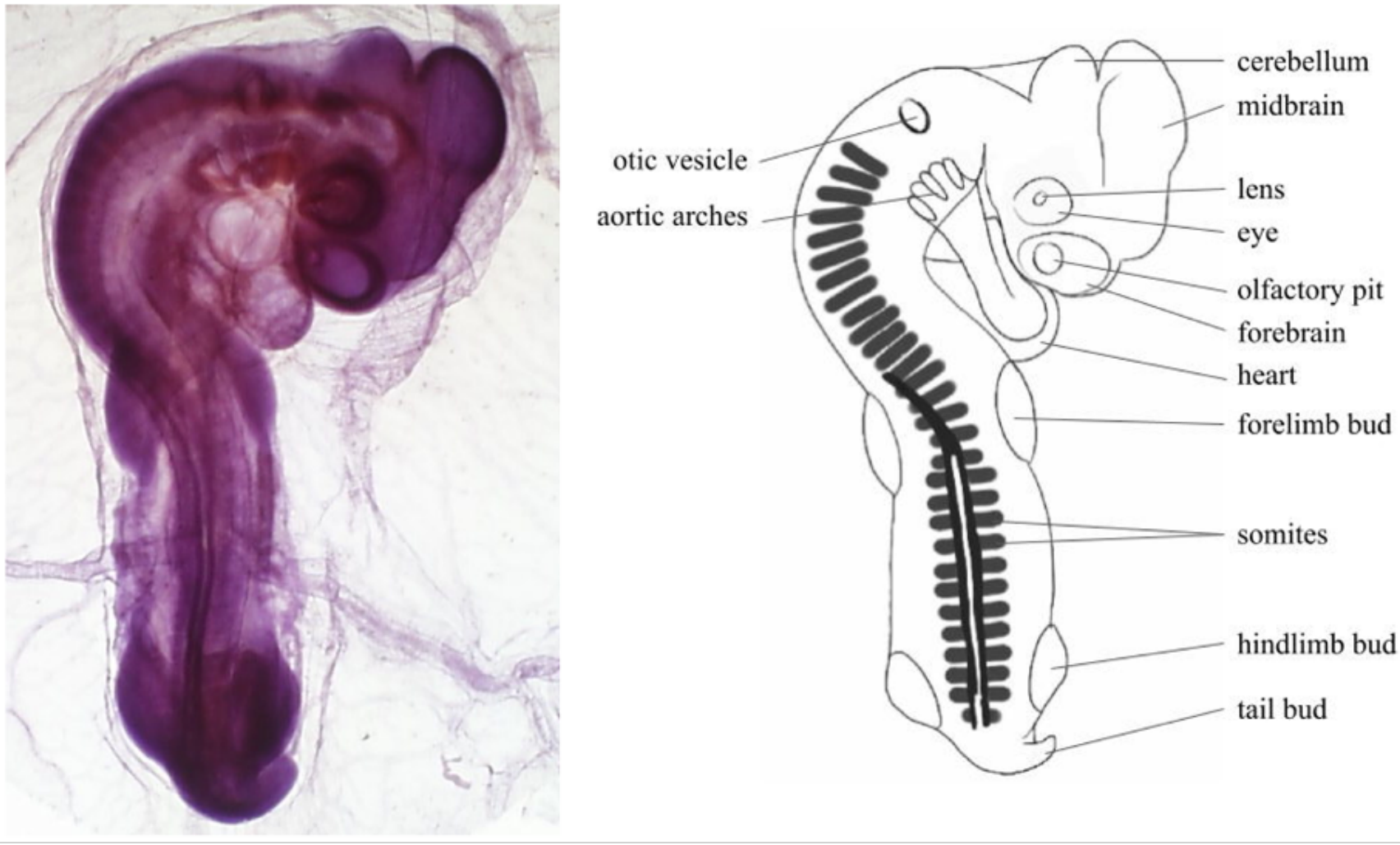
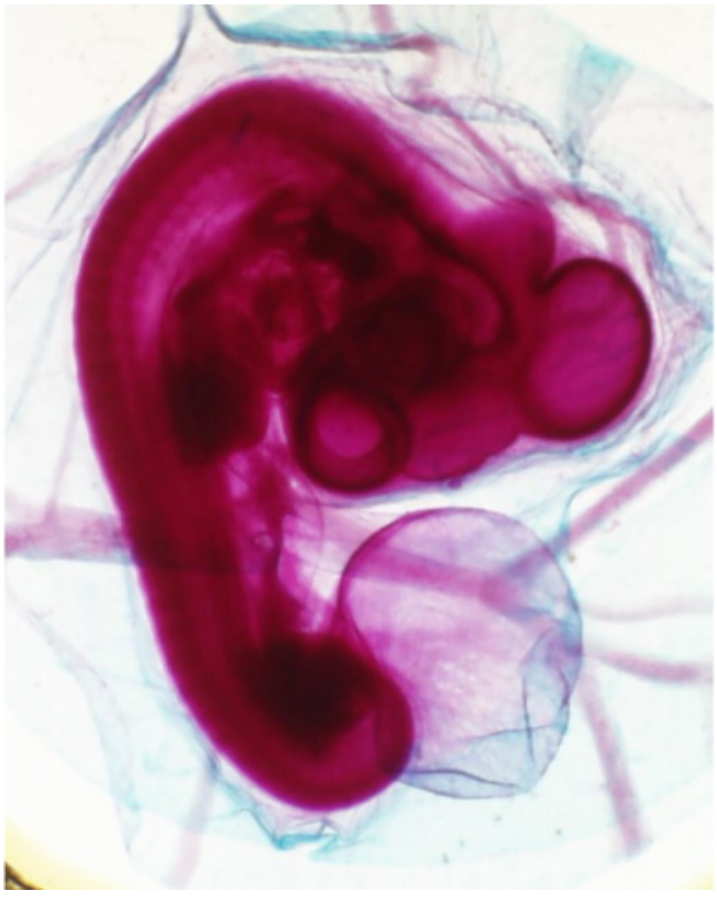


Figure 2-4. Normal chick embryo development (H&H stage 18). Chicken embryo after 3-4 days of incubation at 37-38°C.



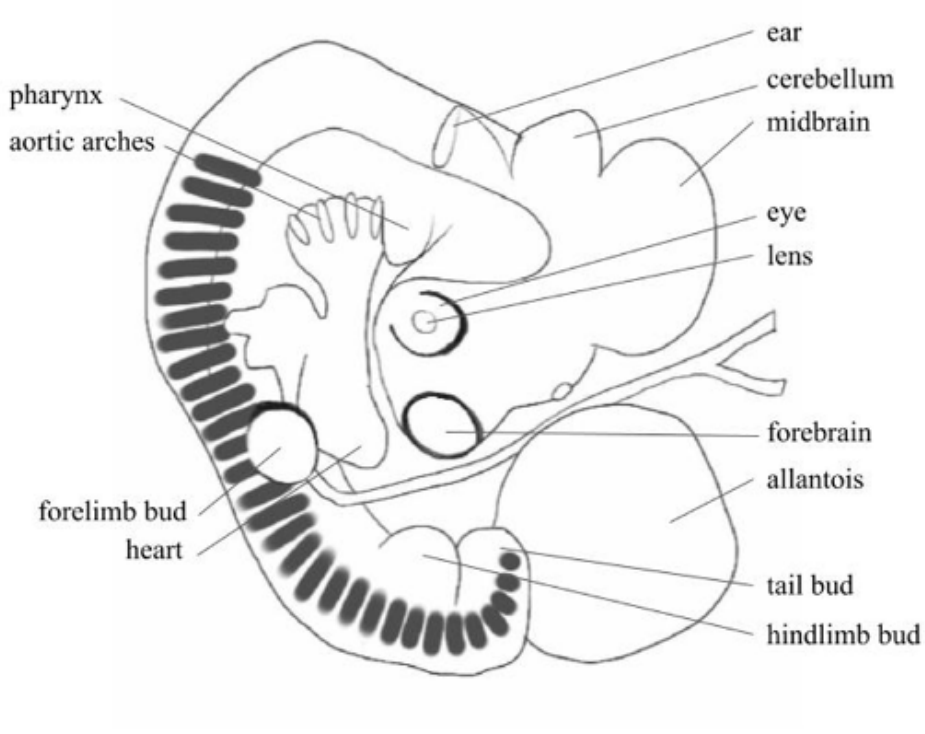


Figure 2-5. Normal chick embryo development (H&H stage 21). Chicken embryo after 4 days of incubation at 37-38°C; this is classically referred to as a "72-hour chick embryo."

References

Cebra-Thomas, J. 2000. Observation of living and plastic-embedded chick embryos. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Chick/Chick_Observe.html

Hamburger, V. and H. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *Journal of Morpholo*gy 88, 49-92.

Prep Checklist for 2-1:

For each group or pair of students:

- ☐ Dissecting microscope
- ☐ 60 mm and 100 mm plastic petri dishes
- ☐ Transfer pipets
- ☐ Kimwipes
- ☐ Bench paper sections
- ☐ 70% ethanol in squirt bottle

Dissecting tools:

- ☐ Plastic spoon or Moria spoon
- ☐ 2 Dumont #5 fine forceps
- ☐ 1 Dumont #6 bent forceps
- ☐ 1 pair iris scissors (curved best)

For class:

- ☐ Howard Ringer’s solution
- ☐ 3-day, 4-day, and 5-day eggs incubated at 37°C
- ☐ Stained, mounted chick embryos "18-96 hours"
- ☐ Copies of Hamburger & Hamilton staging series

Experiment 2-2. Shell-Less Cultures of Chick Embryos

Objective:

To observe chicken embryonic development through organogenesis.

Experimental Procedure:

1. Squirt section of PVC pipe with 70% ethanol and allow to dry in laminar flow hood. Label with your name and the date.

2. Create a cradle with generic plastic wrap; be careful not to touch the middle of what will be the inside of the cradle. Secure with a rubber band. Cover with the lid of a sterile petri plate.

3. Wipe 4-day egg with 70% ethanol. Allow it to sit on its side in the 37°C incubator for 5-10 minutes to enable the embryo to rotate to the top side.

4. Remove lid from cradle and leave in hood. **Firmly** crack lower side on edge of finger bowl. **Resolutely** open and transfer contents into cradle (Figure 2-6). The embryo will float to the surface. Obviously, the embryo will develop best if the yolk is not broken. Warm, incubated eggs break more easily than refrigerated ones, but embryos will usually survive for a while if there are only small tears in the yolk.

5. Add 1 drop of penicillin-streptomycin to prevent infection. Cover with lid and return to 37°C incubator.

6. Observe periodically to watch for organ development and extraembryonic membrane formation. Do not keep outside the incubator for more than 5 minutes. Remove dead embryos promptly to freezer. We will examine the survivors next week and compare them to control embryos incubated in the shell.

7. If the yolk is too damaged to permit survival, isolate the embryo. Locate the peripheral ring of blood vessels. Hold onto the membrane near the blood ring and cut around it with sharp scissors. Position the spoon near the embryo with your cutting hand and lead the embryo onto it. Do not let go with forceps. Quickly transfer to small petri dish filled with HR. Position under dissecting scope. Pipet out yolk and straighten embryo gently with forceps. Add additional solution if necessary or transfer to fresh dish.

Figure 2-6. Three-day embryo in shell-less culture.

References

Cebra-Thomas, J. 2004. Shell-less cultures of chick embryos. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Chick/Shell-less.html

Tuan, R.S., and M.H. Lynch. 1983. Effect of experimentally induced calcium deficiency on the developmental expression of collagen types in chick embryonic skeleton. *Developmental Biology* 100, 374-386.

Prep Checklist for 2-2:

For each group or pair of students:

- ☐ Dissecting microscope
- ☐ 60 mm and 100 mm plastic petri dishes
- ☐ Transfer pipets
- ☐ Kimwipes
- ☐ Bench paper sections
- ☐ 70% ethanol in squirt bottle
- ☐ PVC pipe sections
- ☐ 100 mm glass petri dishes (sterile)
- ☐ Large rubber bands
- ☐ Plastic wrap (generic, gas permeable)

Dissecting tools:

- ☐ Plastic spoon or Moria spoon
- ☐ 2 Dumont #5 fine forceps
- ☐ 1 Dumont #6 bent forceps
- ☐ 1 pair iris scissors (curved best)

For class:

- ☐ Howard Ringer’s solution
- ☐ 4-day eggs incubated at 37°C
- ☐ Copies of Hamburger & Hamilton staging series

Experiment 2-3. Observation and Culture of Early Chick Embryos (2-Days and Younger)

Objective:

To isolate and examine younger chick embryos. Embryos cultured on albumen-agar dishes can be used to study the development of the head and heart, examine the effect of added growth factors, and construct a fate map by application of dye marks.

Experimental Procedure:

1. Use sterile technique. Prewarm agar/albumin culture dish to 37°C. Set up several small petri dishes with Howard Ringer's (HR). Clean your dissecting equipment and wipe with 70% ethanol.

2. Clean a 2-day egg and allow the embryo to float to one side. Crack egg into large sterile petri dish. Use cut-off sterile disposable pipet to remove some of the albumin so that top half of yolk is uncovered. Save some sterile albumen for culture dishes.

3. The embryo may not be visible to the naked eye. The blastodisc is located above a small ring of white yolk and generally looks like a cloudy circle on top of the yellow yolk. Drop a filter paper disc around your embryo. Hold on to the filter paper with fine forceps and cut around the ring with your sharp scissors. Transfer the filter paper ring with embryo to a small petri dish with HR (see Figure 2-7). Determine the stage of the embryo. Gently wash away yolk.

4. Examine the embryos.Determine the stage (see Figures 2-8 and 2-9 to get started). Identify the heart and the developing neural tube. Do you see evidence of blood circulation? The reddish spots on the 2-day blastodisc are the blood islands, the sites of hematopoiesis. The embryo is covered with a clear protein layer known as the vitelline membrane. This may start to peel away from the embryo.

5. Transfer to a new dish with fresh HR if necessary. Photograph embryo.

6. Transfer a small quantity of albumen to agar/albumin dish. Gently lift the embryo by the filter paper ring and transfer to dish, filter-paper side down (ventral side of embryo is up).

7. Place bottom of 35 mm dish (with albumen-agar and embryo) into 60 mm dish. Add a few drops of HR to outside dish. Incubate in 37°C incubator overnight. Check for evidence of development the next day and thereafter.

8. Clean your instruments well with warm water, distilled water and 70% ethanol. Dry before returning to case. Discard the shells in the trash and the yolk/ albumin remains down the drain with cold water.

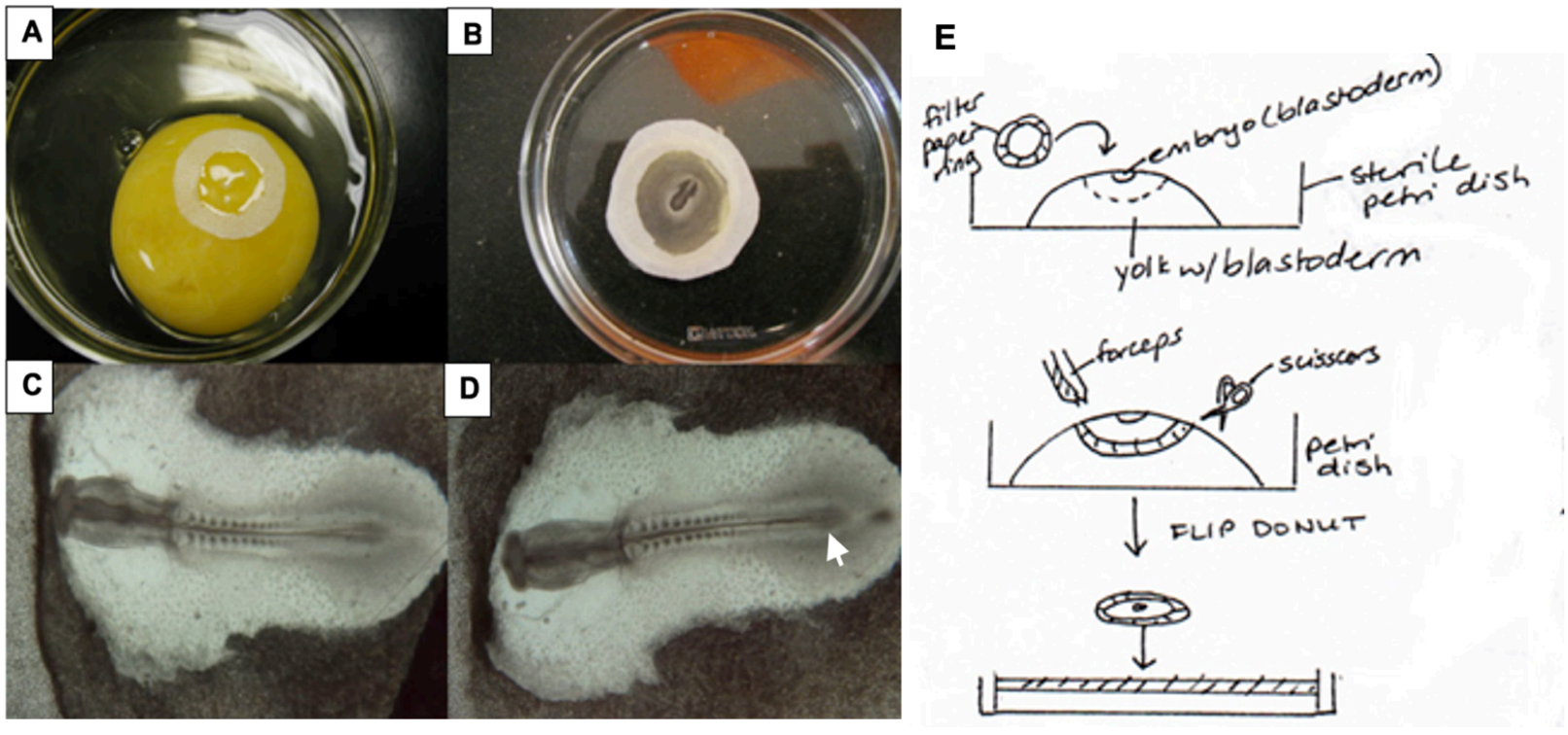


Figure 2-7. Isolation of 2-day chick embryo. (A) Fertilized chick egg cracked into a petri dish with filter paper ring around the embryo blastodisc; (B) isolated embryo transferred to petri dish with Howard Ringer's; (C) dorsal and (D) ventral view of isolated embryo. (E) Diagram of procedure. Arrow = regressing primitive streak**.**

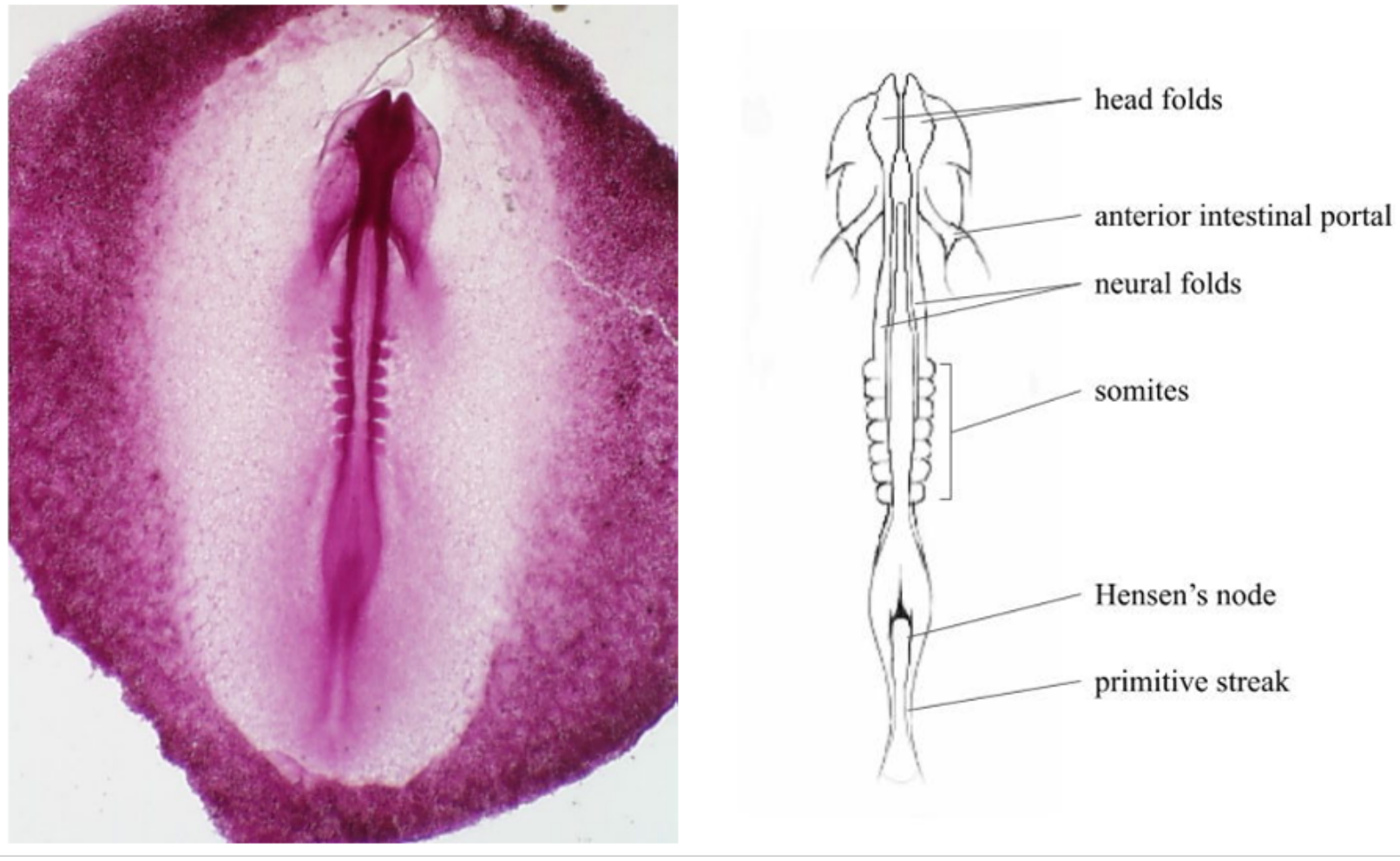


Figure 2-8. Normal Chick embryo development (H&H stage 9). Chicken embryo after 1-2 days of incubation at 37-38°C; this is classically referred to as a "24-hour chick embryo."

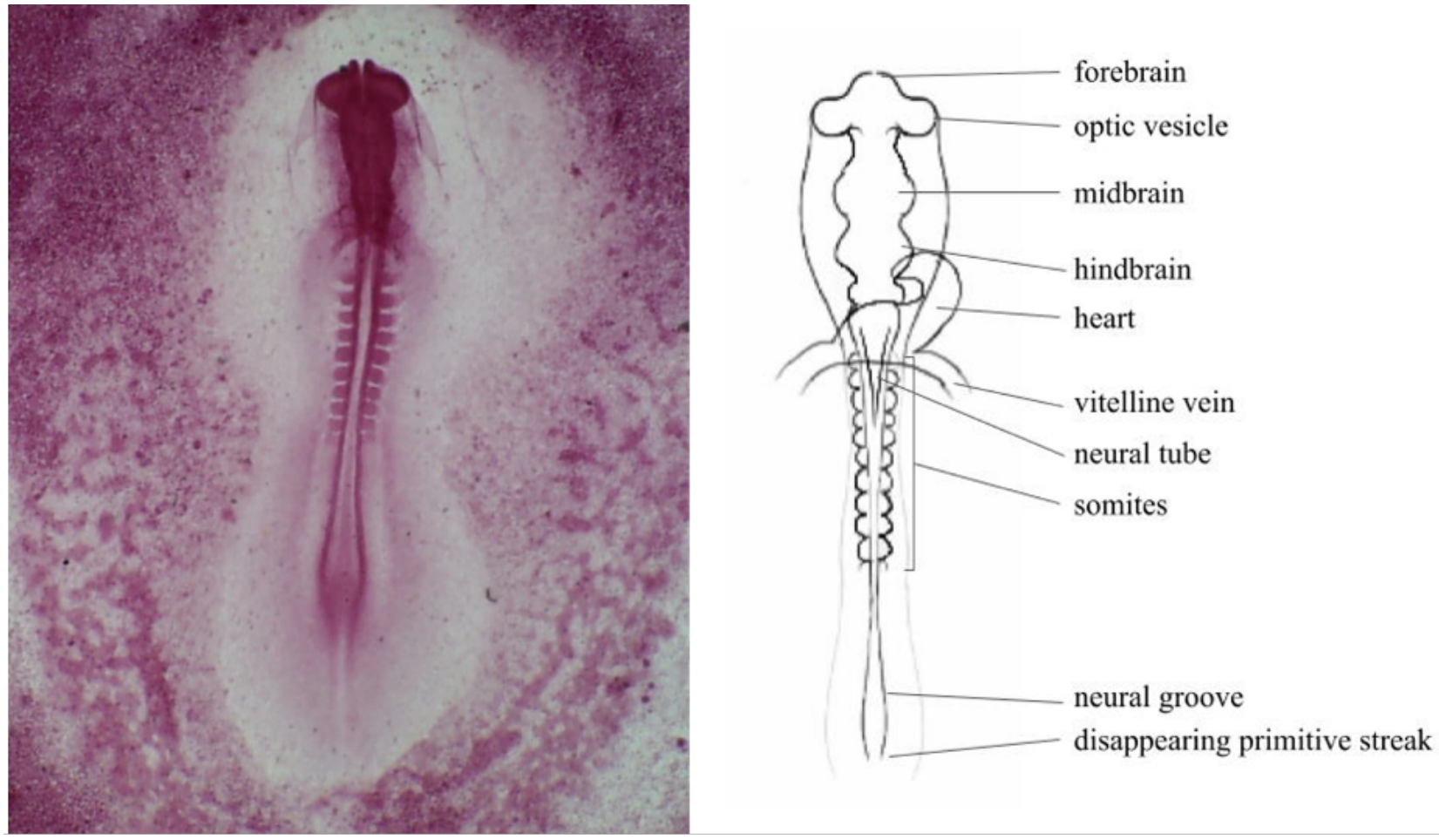


Figure 2-9. Normal chick embryo development (H&H stage 11). Chicken embryo after 2 days of incubation at 37-38°C; this is classically referred to as a "33-hour chick embryo."

References

Biroc, S., 1986. Developmental Biology, a Laboratory Course with Readings, Macmillan Publishing Company, New York.

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Cebra-Thomas, J. 2012. *In vitro* culture of early chick embryos. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Chick/agar_culture.html

Hamburger, V. and H. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *Journal of Morpholo*gy 88, 49-92.

Prep Checklist for 2-3:

For each group or pair of students:

- ☐ Dissecting microscope
- ☐ 60 mm and 100 mm plastic petri dishes
- ☐ Transfer pipets
- ☐ Kimwipes
- ☐ Bench paper sections
- ☐ 70% ethanol in squirt bottle
- ☐ Filter paper circles (sterile)
- ☐ Albumin-agar plates

Dissecting tools:

- ☐ Plastic spoon or Moria spoon
- ☐ 2 Dumont #5 fine forceps
- ☐ 1 Dumont #6 bent forceps
- ☐ 1 pair iris scissors (curved best)
- ☐ 1 half-curved iris forceps

For class:

- ☐ Howard Ringer’s solution
- ☐ 2-day eggs incubated at 37°C
- ☐ (Note: it helps to prevent the yolks from breaking if you allow them to cool at room temperature for 30 minutes before using)
- ☐ Copies of Hamburger & Hamilton staging series

Experiment 2-4. The Effect of Ethanol on the Patterns of Apoptosis

Objective:

The goal of this experiment is to investigate the effect of ethanol exposure on pharyngula-stage chicken embryo development and, in particular, its effect on the pattern and extent of apoptosis.

Experimental Procedure:

A. Ethanol Exposure

1. Incubate eggs at 37°C for 1 or 2 days.
2. Clean eggs with 70% ethanol. Create a small hole in the blunt end of the egg with forceps.
3. Using a syringe, inject eggs with 0.25 mL 5% – 10% ethanol. For controls, inject with 0.25 mL Howard Ringer's (HR) solution.
4. Seal hole with Scotch tape and return the eggs to the incubator at 37°C for an additional 1 or 2 days.

B. Embryo Isolation and Staining for Cell Death

1. Isolate 3-day control and treated embryos. Obtain an egg and clean scissors, forceps, and an embryo spoon. Gently rotate egg, clean with 70% ethanol and set down for a few minutes to allow the embryo to float to the top. Open up the blunt end of the shell and carefully peel back the shell membranes. Grab the outer ring of the blastodisc near embryo with fine forceps in your non-cutting hand. Quickly cut around embryo, slide embryo spoon below it, and transfer to a small petri dish with HR.
2. Use fine forceps to gently remove extra-embryonic membranes.
3. Transfer embryos to 2-3 mL of a 2 mg/mL propidium iodide (PI) solution in a small petri dish for 20 minutes at room temperature.
4. Transfer embryos to fresh HR to remove excess PI.
5. Examine under epifluorescence and photograph.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Cartwright, M.M. and Smith, S.M. 1995. Increased cell death and reduced neural crest cell numbers in ethanol-exposed embryos: Partial Basis for the Fetal Alcohol Syndrome Phenotype. *Alcohol, Clinical and Experimental Research* 19, 378-8

Prep Checklist for 2-4:

For each group or pair of students:

- ☐ Dissecting microscope
- ☐ 60 mm and 100 mm plastic petri dishes
- ☐ Transfer pipets
- ☐ Kimwipes
- ☐ Bench paper sections
- ☐ 70% ethanol in squirt bottle

Dissecting tools:

- ☐ Plastic spoon or Moria spoon
- ☐ 2 Dumont #5 fine forceps
- ☐ 1 Dumont #6 bent forceps
- ☐ 1 pair iris scissors (curved best)

For class:

- ☐ Howard Ringer’s solution
- ☐ 5%, 7% and 10% ethanol in Howard Ringer’s solution
- ☐ 3 mL syringes and 25 g needles (1.5 inch good) for injecting eggs
- ☐ Scotch tape
- ☐ Chicken eggs incubated at 37°C injected as described
- ☐ Propidium iodide stock (1 mg/mL); dilute to 2 mg/mL (1/500) in Howard Ringer’s for use
- ☐ Copies of Hamburger & Hamilton staging series

Experiment 2-5. *In Vitro* Culture of Embryonic Chicken Hearts

Objective:

The goals of this lab are to identify the anatomy of the developing chick heart, determine the direction of blood flow through the developing heart, and observe how the heart develops over time.

Experimental Procedure:

1. Prepare a clean working area. Wipe off tools with 70% ethanol. Obtain 1 large petri dish and PVC pipe section or finger bowl. Set up several small petri dishes with Howard Ringer's (HR).

2. Wipe off egg with 70% ethanol; rotate so that embryo moves freely. Set egg down on its side for several minutes; the embryo should float to the upper surface. Crack the 'lower' surface of the egg on the PVC pipe ring and open egg into large petri dish. Allow egg to sit for a few minutes to allow the albumin to flow off the top half of yolk.

3. Drop a filter paper disc around embryo (see Figure 2-7). Hold on to the filter paper with fine forceps and cut around the ring with sharp scissors. Gradually lift the paper disc with the forceps; the embryo should remain within the center of the disc. Transfer to warm HR. Dissect away the extra-embryonic membranes.

Note: The 3-day embryo can also be removed using a spoon. Puncture egg at wide end (above air space) using point of sharp forceps. Carefully remove shell above air space. Using forceps, peel back the shell membrane to expose the embryo. Keeping hold of the embryo with the forceps, cut around it with fine scissors. Pick up a spoon with your cutting hand and slide it under the embryo. Continue to hold onto the embryo, lift with the spoon and transfer to the petri dish filled with HR.

4. Using fine forceps, sever the trunk above and below the heart region (Figure 2-10). Then, carefully remove the dorsal region of the embryo, leaving the heart intact.

5. Identify the sinus venosus, atrium, and ventricle. Measure the heart rate and determine the direction of blood flow.

6. Transfer the heart to tissue culture media (2% FCS in DMEM) in 24-well plate. Check for a change in heartbeat rate or pattern.

7. Incubate in CO_2 incubator overnight. Check for beat and evidence of development the next day and thereafter.

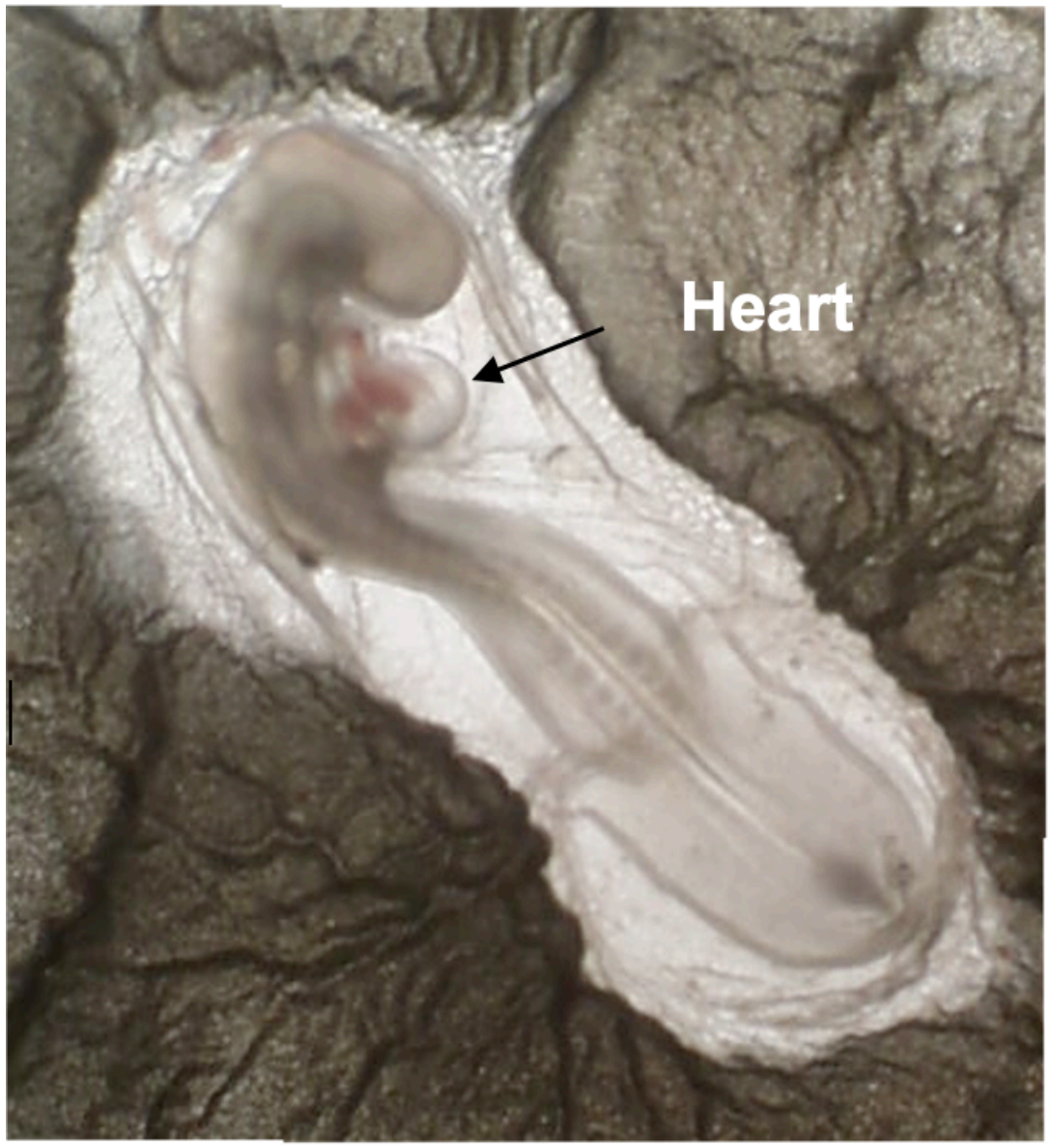

Figure 2-10. Chick embryo for isolation of heart. Three-day chicken embryo (HH Stage 15) isolated using filter paper ring.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Cebra-Thomas, J. 2012. *In vitro* culture of chicken heart. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Chick/Aaronhearlab.html

Patten, Bradley M., *Early Embryology of the Chick*, 4th edition, McGraw-Hill, New York, 1951.

Prep Checklist for 2-5:

For each group or pair of students:

- ☐ Dissecting microscope
- ☐ 60 mm and 100 mm plastic petri dishes
- ☐ Transfer pipets
- ☐ Kimwipes
- ☐ Bench paper sections
- ☐ 70% ethanol in squirt bottle

Dissecting tools:

- ☐ Plastic spoon or Moria spoon
- ☐ 2 Dumont #5 fine forceps
- ☐ 1 Dumont #6 bent forceps
- ☐ 1 pair iris scissors (curved best)

For class:

- ☐ Howard Ringer’s solution (sterile) with 50 ug/mL gentamicin
- ☐ Dulbecco's Modified Eagles Medium (DMEM), 10% fetal calf serum, 50 ug/mL gentamicin
- ☐ 24-Well tissue culture plates
- ☐ 3-day eggs incubated at 37°C
- ☐ CO_2 incubator
- ☐ Copies of Hamburger & Hamilton staging series

Experiment 2-6. Development of Feather Buds in Culture

Objective:

The goals of this lab are to practice microdissection skills and observe organogenesis *in vitro*. The lab can be modified to examine the effects of growth factor inhibitors.

Experimental Procedure:

1. Open blunt end of 7-day chick egg. Transfer contents into large (100 mm) petri dish.

2. Transfer embryo to clean small (60 mm) petri dish with sterile Howard Ringer's (HR) solution. Remove head close to shoulders and discard.

3. Use fine forceps like scissors to slice skin along flanks, beginning at the neck (Figure 2-11). Try to cut only through the outer layer (skin). Using fine forceps, gently peel back skin. Connect cuts behind hind limbs. Try to remove a large intact piece. Store skin explants in HR until ready to set up the cultures.

Figure 2-11. Six-day chicken embryo with dorsal skin removed for explant culture

4. Set Transwell™ tissue culture filter inserts in wells of a six well plate and underlay with 1.8 ml culture media each. (Note: do this in sterile hood!).

5. Cut off the end of a sterile transfer pipet to make a large opening. Suck up explants and transfer to top of filter inserts. Explants of dorsal skin should be cultured with the mesenchyme side down. Looking under dissecting microscope, grab one edge of explant and gently pull over filter until explant is flat with dorsal (ectodermal) side up. Generally, the ectodermal surface looks smoother.

6. Remove any media on top of filters. The explants should be cultured at the air-fluid interface, surrounded by a thin film of media that passes through the filter. Incubate at 37°C in a humidified incubator with 5% CO_2.

7. Optional: to investigate the requirement for signaling molecules implicated in feather bud initiation or outgrowth, inhibitors can be included. Cyclopamine (SHH inhibitor) can be applied directly to the explants. LDN-193189 (BMP inhibitor) and SU5402 (FGF and VEGF inhibitor) can be dissolved in the culture media. Control cultures should be treated with vehicle alone.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Cebra-Thomas, J. 2012. *C*ulturing skin from eight day chicken embryos: The development of feather buds. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Student/katy/chickskinpage.html

Chuong, C.-M. and R. B. Widelitz. 1998. "Feather morphogenesis: A model of the formation of epithelial appendages." *Molecular Basis of Epithelial Appendage Morphogenesis*. R.G. Landes Company. Austin, pp. 57-74.

Prep Checklist for 2-6:

For each group or pair of students:

- ☐ Dissecting microscope
- ☐ 60 mm and 100 mm plastic petri dishes
- ☐ Transfer pipets, sterile
- ☐ Kimwipes
- ☐ Bench paper sections
- ☐ 70% ethanol in squirt bottle

Dissecting tools:

- ☐ Plastic spoon or Moria spoon
- ☐ 2 Dumont #5 fine forceps
- ☐ 1 Dumont #6 bent forceps
- ☐ 1 pair iris scissors (curved best)

For class:

- ☐ Howard Ringer’s solution (sterile) with 50 ug/mL gentamicin
- ☐ Dulbecco's Modified Eagles Medium (DMEM), 10% fetal calf serum, 50 ug/mL gentamicin
- ☐ Transwell™ 3452 (Costar, 24 mm in diameter, 3.0 mm pore size) tissue culture inserts
- ☐ 6-well tissue culture plate (Costar)
- ☐ 7-day eggs incubated at 37°C
- ☐ CO_2 incubator

Chapter 3 Zebrafish Development

Zebrafish (*Danio rerio*) Development

In order for you to repeat a published experiment or have someone else repeat yours, it is important to use the same materials. For developmental studies, that means knowing the precise point in development that the embryos have reached. The time of incubation gives only an approximation of the developmental age. For this reason, staging series based on morphologic criteria have been prepared.

Zebrafish embryos are laid over approximately an hour and develop very rapidly. A given batch will contain embryos at a range of developmental stages. To obtain a reproducible group of embryos for experimentation, sort embryos after collection and maintain at a constant temperature. Zebrafish embryos are staged using a series prepared by M. Westerfield, *The Zebrafish Book*, or Kimmel et al., 1995.

The zebrafish egg is surrounded by a chorion with an opening (the micropyle) over the animal pole to allow the entry of sperm. After fertilization, the non-yolky embryonic cytoplasm streams to the animal pole, and the chorion swells away from the zygote. The early embryonic divisions are meroblastic (incomplete) and discoidal. The cleavage furrows initiate at the animal pole and divide the embryonic cytoplasm, but not the yolky portion, leaving the blastomeres open to the yolk cell. Each of the cleavage furrows in cycles 2-5 form at right angles to the previous one, eventually resulting in the 32-cell stage with 4 rows of 8 cells forming a blastodisc on top of the yolk. The 6th cleavage is horizontal, creating a second layer of blastomeres (surrounded by a complete cell membrane). As the cleavage divisions continue, the embryonic cells become smaller and form a mound at the animal pole. Even those on the bottom layer are sealed off from the yolk, leaving only a ring of meroblastic cells connected to the yolk around the perimeter. At the 512-cell stage, these marginal cells fuse with the yolk cell to form a ring of nuclei in the yolk syncytial layer (YSL). By the 1000-cell stage, the outermost layer of blastodisc cells establish connections with each other to form the enveloping cell layer (EVL). The majority of the epiblast cells (the deep cells) are not connected by cell junctions and can change positions due to external forces. The deep cells begin to undergo the mid-blastula transition, and the cleavage divisions become less regular. At the 2000-cell stage (or high stage), at the end of the cleavage divisions, the embryonic deep cells form a mound at the animal pole with a constriction around the base where the EVL cells connect to the yolk cell above the YSL nuclei.

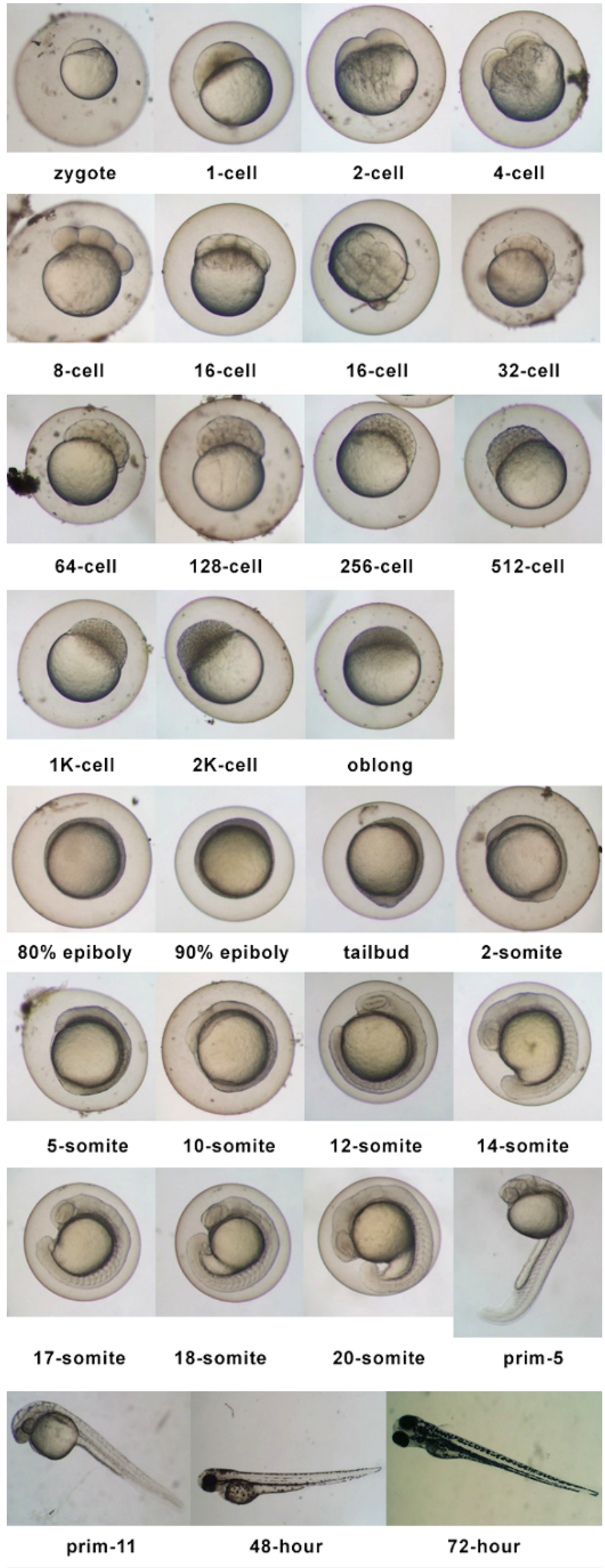

Figure 3-1. Normal development of zebrafish.

Gastrulation begins with the YSL nuclei moving towards the vegetal pole, pulling down on the EVL. This shortens the animal-vegetal axis and flattens the deep cells over the animal pole portion of the embryo until the overall shape becomes spherical. As the YSL nuclei continue to pull towards the vegetal pole, the EVL and deep cells are pulled with them. This eventually causes the yolk cell to bulge upwards in the center, with the epiblast cells forming a dome over the top portion of the embryo. This is known as “epiboly” as the epiblast cell layers are becoming thinner and spreading over the yolk cell. Once the epiblast cells cover the top 50% of the yolk cell (50% epiboly), the deep cells form an epiblast of uniform thickness over the animal half of the embryo. After this stage, the blastoderm cells at the bottom edge begin to turn under (involute) as they are being pulled downwards, forming a new lower layer of cells just above the yolk cell (the hypoblast). This thickened double layer of cells forms a band (the germ ring) around the circumference of the embryo. The hypoblast cells become the mesoderm and endoderm of the embryo while the cells that remain in the epiblast become the embryonic ectoderm. The extent of involution is greatest on the future dorsal side of the embryo where a locally thickened region known as the embryonic shield forms. The shield is the source of dorsalizing signals and acts as the embryonic organizer. The hypoblast cells that involute through the shield region and migrate back towards the animal pole become the pharyngeal endoderm and notochord mesoderm.

As epiboly continues until the yolk cell is completely covered, the embryonic epiblast and hypoblast cells converge towards the dorsal side of the embryo. The embryonic axis is initially C-shaped with the future head at the animal pole and the tailbud at the vegetable pole, but the converging cells drive the extension of the embryo, pushing both the head and tail around the embryo. At the same time, organogenesis begins with the formation of the eyes in the head, the heart below the head adjacent to the yolk cell and the somites organizing along the body. As the yolk is harvested, the yolk cell shrinks and the trunk and tail detach, leaving the yolk below the head by the second day after fertilization.

During the second day, cells such as those of the retina, myoblasts and melanocytes begin to differentiate, and the fins initiate as mounds. The heart beats and blood vessels extend over the yolk cell.

References

Cebra-Thomas, J. 2001. Zebrafish staging. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Fish/fish_stage.html

Kimmel, C.B, Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. *Developmental Dynamics* 203, 253-310

Stages of Zebrafish Embryo Development. n.d. *Developmental Dynamics*. www.anatomy.org. [accessed 2025 Jun 15]. https://anatomypubs.onlinelibrary.wiley.com/pb-assets/hub-assets/anatomypubs/posters/Zebrafish_stage_poster-1575648621470.pdf

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Experiment 3-1. Zebrafish Fertilization and Early Development

Objective:

The objective of the experiment is to become familiar with the breeding and early embryonic development of zebrafish.

Background:

Zebrafish (*Danio rerio*) are a relatively new model organism used to study embryonic development. They are teleost fish, originally from southeast Asia, but now popular aquarium pets around the world. Zebrafish are relatively small and easy to care for. They produce large numbers of fertilized eggs that develop rapidly at warm room temperature. The eggs and embryos are optically transparent, and they can be observed from the zygote through organogenesis all the way to the feeding larval stages. Alterations in development can be easily observed using a stereo dissecting microscope.

Experimental Procedure:

1. **The night before**: Clean algae and other debris out of tank with adult fish by vacuuming the tank with a siphon. After cleaning, place a single layer of washed marbles across the bottom of the tank.

2. **One to three hours after the fish have awakened** (at the appearance of light, e.g., 'dawn'): Use the siphon to collect the embryos from between the marbles into a mesh filter or fry net placed over a bucket. Wash embryos from filter into a dish of tank water. Remove marbles from tank to large sieve and rinse thoroughly with hot chlorinated water; allow to air-dry before next use.

3. Eggs and embryos will look like small glass marbles. Transfer away from debris using wide-mouth Pasteur pipets to glass petri dishes containing filtered tank water or Zebrafish Embryo Medium (ZFEM) solution.

4. Examine using a dissecting microscope and sort into groups based on their developmental stage (see Figure 3-1). Photograph or sketch representative examples.

5. One day later, observe embryos and determine their developmental stage. After 24 hours, the embryos have reached the pharyngula stage (Figure 3-2). Note the development of their somites, notochord and nervous system, tailbud, and organs such as the eye and inner ear.

6. Continue to observe the embryos and make a note of when they begin to move, hatch from the chorion and develop pigment cells.

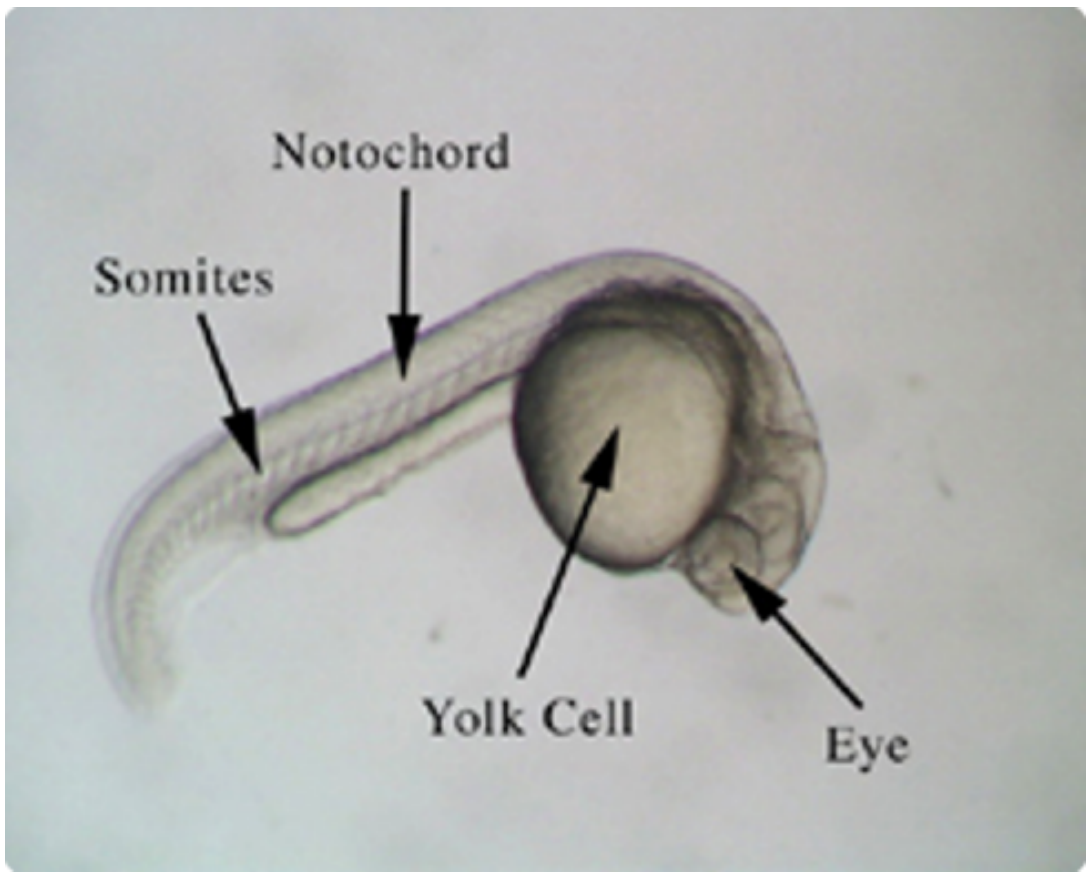

Figure 3- 2. Zebrafish embryos at the pharyngula stage of development (prim-5).

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. 1995. Stages of embryonic development of the zebrafish. *Developmental Dynamics* 203, 253-310.

Rubinstein, A.L. 2006. Zebrafish assays for drug toxicity screening, *Expert Opinion on Drug Metabolism Toxicology* 2, 231-240.

Westerfield, M. 1993 *The Zebrafish Book*, University of Oregon.

Prep Checklist for 3-1:

- ☐ Mature zebrafish
- ☐ Marbles for tank
- ☐ Large bore plastic tubing for syphon
- ☐ Fine mesh filter (e.g. fry net)
- ☐ Bucket
- ☐ Large glass dish (e.g. Pyrex baking dish)
- ☐ Zebrafish Embryo Medium (ZFEM) or filtered tank water
- ☐ 60 mm glass petri dishes
- ☐ Wide-mouth glass Pasteur pipets and bulbs
- ☐ Incubator (28°C)
- ☐ Dissecting microscope and camera

Experiment 3-2. The Effects of Different Concentrations of Lithium Chloride on the Development of Zebrafish Embryos

Objective:

The purpose of this experiment is to monitor the effects of different concentrations of LiCl on anterior development in zebrafish embryos. This will be carried out by assessing the degree to which certain amounts of the lithium chloride teratogen affect the morphology of anterior structures such as the eye.

Experimental Procedure:

1. Obtain 10-15 zebrafish embryos at the sphere/dome-stage of development (Figure 3-3) at approximately four hours post-fertilization for each of four groups (the control and three concentrations of lithium chloride). Therefore, about 40-60 sphere/dome-stage embryos must be isolated in total.

2. Transfer each population of embryos from the embryo medium into four separate petri dishes, each containing 10 mL of ZFEM or the embryo medium containing 0.45 M, 0.30 M, or 0.15 M LiCl using a wide-mouth pipette, and immerse them in solution for 10 minutes.

3. After 10 minutes, rinse the embryos by placing each group in a separate dish containing 10 mL of embryo medium and then transferring them again into fresh embryo medium. Be sure to label the dishes with the corresponding amount of LiCl to which they were exposed.

4. Incubate embryos at 28°C overnight. Photograph the embryos after 24 hours to observe anterior development, carefully noting deviations in anterior patterning and formation from the control population.

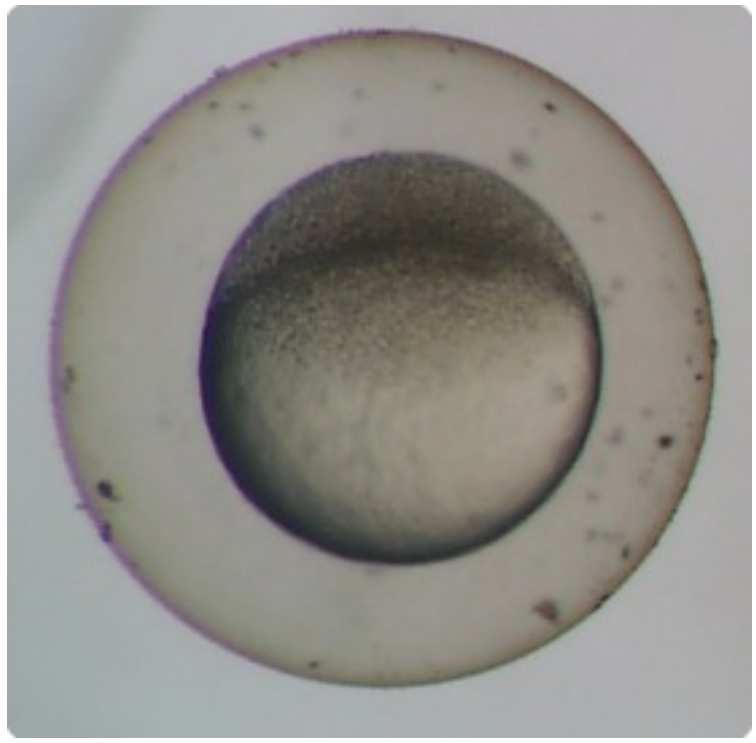

Figure 3-3. Zebrafish embryo at the sphere stage of development.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

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Prep Checklist for 3-2:

- ☐ *Danio rerio* embryos at the sphere/dome-stage of development
- ☐ Zebrafish Embryo Medium (ZFEM) or filtered tank water
- ☐ LiCl (0.15 M, 0.30 M, and 0.45 M) in ZFEM
- ☐ 60 mm glass petri dishes
- ☐ Wide-mouth glass Pasteur pipets and bulbs
- ☐ Siphon and fine fry net or mesh filter
- ☐ Incubator (28°C)
- ☐ Dissecting microscope and camera

LiCl Solutions

Formula weight	Final concentration (M)	Conversion factor	Amount LiCl added
42.39 g/ 1 mole	0.45 M/ L	.01 L/ 10 mL	0.19 g/ 10 mL
42.39 g/ 1 mole	0.30 M/ L	.01 L/ 10 mL	0.13 g/10 mL
42.39 g/ 1 mole	0.15 M/ L	.01 L/ 10 mL	0.06 g/ 10 mL

Experiment 3-3. Retinoic Acid-Induced Truncation of Zebrafish Embryos

Objective:

The purpose of this experiment is to examine the effects of treating zebrafish (*Danio rerio*) embryos with varying concentrations of retinoic acid.

Experimental Procedure:

1. Obtain dome/30% epiboly stage zebrafish embryos (Figure 3-4) at 4-5 hours post-fertilization at room temperature.

2. Dilute 10^{-4} M retinoic acid stock using ZFEM:

    10^{-8} M (1.0 µL stock solution in 10 mL ZFEM)

    10^{-9} M (1 mL 10^{-8} M solution in 10 mL ZFEM)

3. Place approximately 5 mL of each solution into an appropriately labeled petri dish. For the control, use 5 mL of ZFEM.

4. Transfer 10-15 zebrafish embryos into each petri dish.

5. Incubate embryos at 28°C overnight.

6. Observe embryos for malformations using the dissecting microscope. The embryos may need to be dechorionated, which may be done under the dissecting microscope with clean fine forceps by gently tearing the chorion and allowing the embryo to pass though.

Figure 3-4: Zebrafish embryo at 30% epiboly.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Cebra-Thomas, J. 2012. Testing the effects of retinoic acid on tail formation of developing zebrafish embryos. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Fish/ZF_RA.html

Cebra-Thomas, J. 2012. Retinoic acid-induced truncation of zebrafish (*Danio rerio*) embryos. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Fish/Fish%20RA_2/fish_RA1.html

Prep Checklist for 3-3:

- ☐ *Danio rerio* embryos at the dome/30% epiboly stage of development
- ☐ Zebrafish Embryo Medium (ZFEM)
- ☐ All-*trans*-retinoic acid (Sigma R2625), stock concentration 10^{-4} M in DMSO
- ☐ DMSO
- ☐ 60 mm glass petri dishes
- ☐ Wide-mouth glass Pasteur pipets and bulbs
- ☐ Siphon and fine fry net or mesh filter
- ☐ Incubator (28°C)
- ☐ Dissecting microscope and camera

Experiment 3-4. The Effects of Differing Ethanol Concentrations on Zebrafish Embryos

Objective:

The objective of the experiment is to determine the effects of ethanol exposure on the embryonic development of zebrafish through observation of physical deformities.

Experimental Procedure:

1. Clean algae and other debris out of tank with adult fish by vacuuming the tank with a siphon. After cleaning, place a single layer of washed marbles across the bottom of the tank. One to two hours after 'dawn,' use the siphon to collect the embryos from the marbles into a mesh filter. Place the embryos on the mesh filter into a glass dish of tank water and examine using a dissecting microscope.

2. Select embryos that are at the dome/30% epiboly stage (Figure 3-4).

3. Prepare five separate petri dishes that contain either normal ZFEM, or 1%, 2%, 2.5% or 3% ethanol in ZFEM solution.

4. Place at least 10 of the selected embryos into each of the petri dishes and let them sit for 3 hours.

5. After 3 hours have passed, transfer embryos into petri dishes with normal zebrafish embryo solution. (Note: this step can be omitted if timing does not permit).

6. One day later, observe embryos for abnormalities and photograph them.

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Blader, P. and Strähle, U. 1998. Ethanol impairs migration of prechordal plate in the zebrafish embryo. *Developmental Biology* 201, 185-201.

Cebra-Thomas, J. 2012. The effects of ethanol on zebrafish development. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Fish/Ethanol_1.html

Cebra-Thomas, J. 2012. Effect of ethanol on development of *Danio rerio* . *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Fish/zebra%20webpage/page1.html

Prep Checklist for 3-4:

- ☐ *Danio rerio* embryos at the dome/30% epiboly stage of development
- ☐ Zebrafish Embryo Medium (ZFEM)
- ☐ 1%, 2%, 2.5% and 3% ethanol in ZFEM
- ☐ 60 mm glass petri dishes
- ☐ Wide-mouth glass Pasteur pipets and bulbs
- ☐ Siphon and fine fry net or mesh filter
- ☐ Incubator (28°C)
- ☐ Dissecting microscope and camera

Experiment 3-5. The Effects of Valproic Acid on Somite Development in Zebrafish

Objective:

The purpose of this study is to examine the effects of valproic acid exposure on somite patterning and cartilage formation in zebrafish embryos. Valproic acid affects the expression of pax-1, a gene product that regulates the differentiation of cartilage precursors in somite-derived cells. This suggests the potential for valproic acid to disrupt somite organization as well as early development of the vertebrae. Embryos will be treated with 0.1 M and 0.05 M valproic acid and scored for visible malformations.

Experimental Procedure:

1. Stage the embryos and allow to develop to the time of first somite formation (Figure 3- 5). This is approximately eleven hours after fertilization (Kimmel et al., 1995).

2. Prepare solutions of 0.1M and 0.05M valproic acid (VA) in ZFEM. Use ZFEM without VA as a control. Pour solutions into two labeled dishes each.

3. Transfer 10-15 embryos into each of the six dishes. Incubate embryos at 28°C overnight.

4. Transfer to ZFEM. Observe.

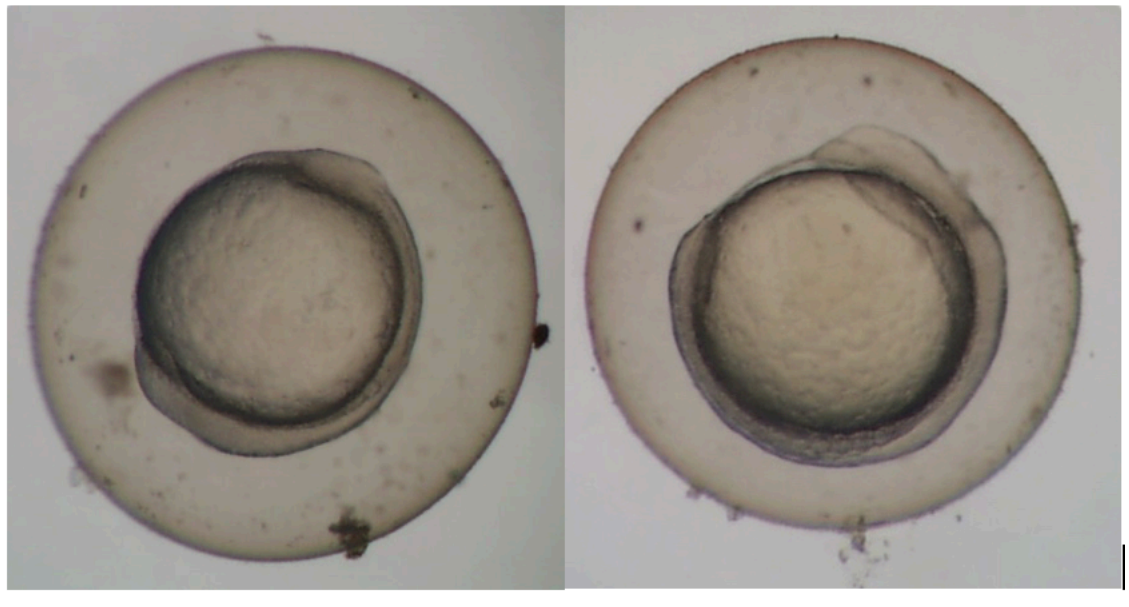

Figure 3-5. Zebrafish embryo at 2-somite (left) and 4-somite (right) stages. Head is at the top of embryos.

References

Barnes, G. L., Hsu, C. W., Mariana, B. D., and Tuan, R. S. 1996. Valproic acid-induced somite teratogenesis in the chick embryo: Relationship with Pax-1 gene expression. *Teratology* 54, 93-102.

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Cebra-Thomas, J. 2001. Effects of valproic acid on the somitic development of *Danio rerio*. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Student/VPA/fish_vpa01.html

Cebra-Thomas, J. 2014. Effects of valproic acid on the somite development and chondrogenesis of *Danio rerio*. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Fish/FISH_valproic1.HTML

Prep Checklist for 3-5:

- ☐ *Danio rerio* embryos at early somite-stage of development
- ☐ Valproic acid (2-propylpentanoic acid, Sigma P-4543)
    - Prepare 0.1 M VA; 0.83g/50 ml ZFEM
    - Dilute 1:1 with ZFEM to prepare 0.05 M VA
- ☐ Zebrafish Embryo Medium
- ☐ 60 mm glass petri dishes (soap-free)
- ☐ Wide-mouth glass Pasteur pipets (Fisher 13-678-30) and bulbs
- ☐ Incubator @ 28°C

Experiment 3-6. The Ototoxic Effects of Antibiotics on Hair Cells of Zebrafish

Objective:

The purpose of this study is to investigate the effects of ototoxic antibiotics, such as gentamicin and neomycin, on hair cells of zebrafish. The experiment involves exposing 5-dpf zebrafish embryos to antibiotics at a range of concentrations. To evaluate the damage to the hair cells, the zebrafish embryos will be treated with fluorescent dye that stains the mechanosensory hair cells of the lateral line. After exposure, the zebrafish will be observed under epifluorescence and compared to a control group.

Experimental Procedure:

A. Gentamicin Treatment

1. Prepare the stock solution of 200μM gentamicin using 95.5μL of Gentamicin (50 mg/mL) and bring the total volume to 50 mL with tank water, then dilute the stock for the other concentrations desired.
2. Soak the zebrafish larvae at 5 dpf (Figure 3-6) in glass petri dishes filled with varying concentrations of gentamicin (10 mL of 0μM, 25μM, 50μM, 100μM and 200μM) for 6 hours.
3. Transfer larvae into 10ml of fresh tank water for 1 hour of recovery.
4. Stain the larvae as specified by the general protocol.
5. Analyze the larvae using a stereomicroscope with epifluorescence.

B. Neomycin Treatment

1. Prepare the stock solution of 200uM neomycin using 615μL of neomycin stock (10mg/mL) and bring the total volume to 50mL with tank water, then dilute the stock for the other concentrations desired.
2. Soak the zebrafish larvae in glass petri dishes filled with varying concentrations of neomycin (10ml of 0μM, 25μM, 50μM, 100μM and 200μM) for 30 minutes.

3. Transfer larvae into 10mL of fresh tank water for 1 hour of recovery.

4. Stain the larvae as specified by the general protocol.

5. Analyze the larvae using epifluorescence.

C. DASPEI for Hair Cell Staining

1. Create the stock staining solution.

   - Dissolve 12 mg DASPEI stain in 5 mL of tank water in 15 mL centrifuge tube to make a stock concentration of 2.4g/L.

   - Wrap centrifuge tube in foil.

2. Add 1 mL of the stock staining solution into a petri dish filled with 10mL of tank water and the zebrafish larvae.

3. Swirl gently to make sure the stain is evenly distributed and wait 15 minutes.

4. Wash the larvae 3 times by pipetting out as much of the tank water from the petri dish as possible and replacing it with fresh tank water.

D. Anesthetize the Embryos

1. Add a few crystals of Tricaine mesylate to each petri dish (just enough to see on the end of a microspatula).

2. Swirl the petri dish to dissolve and distribute the Tricaine.

3. Verify that the larvae are anesthetized by swirling the petri dish; if the larvae move with the current and not against it then they are ready for analysis.

E. Analysis

1. Using a fluorescence stereomicroscope, photograph the larvae on a depression slide under bright field and then with the appropriate filter to visualize green fluorescence.

2. With the control, locate staining on the head and in pairs down the dorsal sides of the embryos (see Figure 3-6).

F. Hair Cell Regeneration

1. a.Place control and treatment groups of larvae left from the ototoxic treatments in new glass petri dishes in fresh water in the 28°C incubator for 48 hours.

2. Expose both the control and treatment group to the staining solution (see general protocol for staining procedure).

3. Observe both groups under the fluorescence microscope and capture the intensity of fluorescence in photographs.

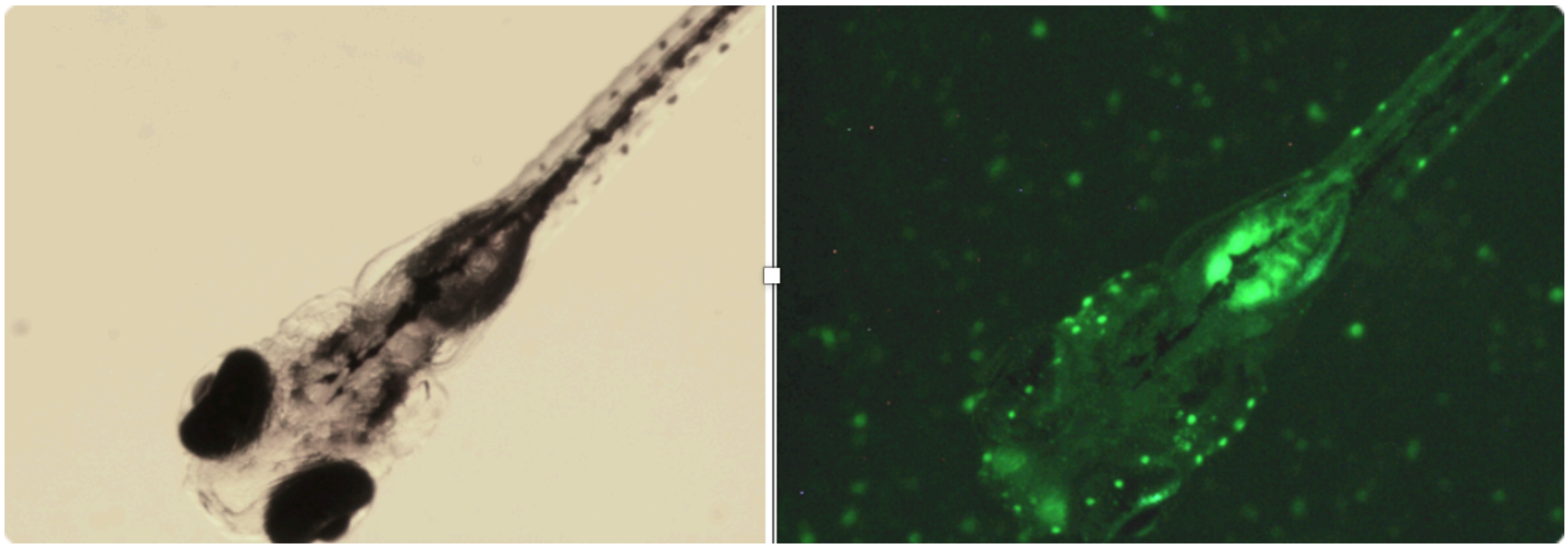

Figure 3-6. Zebrafish hair cell neuromasts. Zebrafish larva at 5 days post fertilization (dpf) stained with DASPEI (right) and visualized under epifluorescence to label hair cells in the neuromasts located in the head and along the trunk (the lateral line).

References

Cruz I, Kappedal R, Mackenzie S, Hailey D, Hoffman T, Schilling T & Raible D. 2015. Robust regeneration of adult zebrafish lateral line hair cells reflects continued precursor pool maintenance. *Developmental Biology.* 402, 229-238.

Ernest S, Rauch GJ, Haffter P, Geisler R, Petit C & Nicolson T. 2000. Mariner is defective in myosin VIIA: a zebrafish model for human hereditary deafness. *Human Molecular Genetics* 9, 2189-2196.

Harris JA, Cheng AG, Cunningham LL, MacDonald G, Raible DW & Rubel EW. 2003. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish. *Journal of the Association for Research in Otolaryngology* 4, 219-234.

Ou HC, Santos F, Raible DW, Simon JA & Rubel EW. 2010. Drug screening for hearing loss: Using the zebrafish lateral line to screen for drugs that prevent and cause hearing loss. *Drug Discovery Today* 15, 265-271.

Whitfield TT. 2002. Zebrafish as a model for hearing and deafness. *Journal of Neurobiology* 53, 157-171.

Prep Checklist for 3-6:

- ☐ *Danio rerio* larvae at 5 days post-fertilization (5 dpf)
- ☐ Gentamicin stock (50 mg/mL) (Invitrogen)
- ☐ Neomycin stock solution (10 mg/mL) (Sigma, Product N1142)
- ☐ DASPEI vital dye (Sigma, Product 280135)
- ☐ Tricaine mesylate (Argent Chemical Laboratories)
- ☐ Micropipette P1000 and tips
- ☐ Micropipette P20/P100 and tips
- ☐ 15mL and 50mL sterile centrifuge tubes
- ☐ 60 mm Glass petri dishes
- ☐ Plastic disposable transfer pipets or wide-mouth glass Pasteur pipets and bulbs
- ☐ Microscope with epifluorescence or NIGHTSEA adaptor and camera

Appendix A: Instructors' Notes for Sea Urchin Protocols

Solutions

ASW: Artificial Sea Water (Woods Hole MBL) (Make 8 L)

NaCl	24.72 g
KCl	0.67 g
$CaCl_2.2H_2O$	1.36 g
$MgCl_2.6H_2O$	4.66 g
$MgSO_4.7H_2O$	6.29 g
$NaHCO_3$	0.18 g
dH_2O	to 1L

0.5 M KCl To Induce Gamete Shedding (Make 100 mL)

KCl	3.73 g
dH_2O	to 100 mL

Sulfate-Free Sea Water (Make 2 L)

NaCl	27.72 g
KCl	0.67 g
$CaCl_2.2H_2O$	1.36 g
$MgCl_2$	9.32 g
$NaHCO_3$	0.18 g
dH_2O	to 1 L

60 mM LiCl (Make 500 mL)

Prepare 60 mM LiCl in ASW, dilute with ASW for use (15 mM and 30 mM final).

Alkaline Phosphatase (AP) Substrate Buffer (Make 100 mL)

100 mM NaCl	2 mL 5 M NaCl
100 mM Tris	5 mL 2 M Tris, pH 9.5
50 mM $MgCl_2$	1 mL 4.9 M $MgCl_2$
dH_2O (ultrapure)	to 100 mL

Alkaline Phosphatase Substrate (Make Fresh)

Nitro blue tetrazolium (NBT)	22.5 mL
BCIP	16.5 mL
AP substrate buffer	to 5 mL

*Note: Western Blue (Fisher) or BM-Purple (Sigma) are ready to use AP substrates and can be substituted for NBT & BCIP

Animals

Gravid animals can be obtained virtually year-round, depending on the species. *Lytechinus pictus* or *Strongylocentrotus purpuratus* (from the Pacific coast), *Arbacia punctulata* (from the Atlantic), or *Lytechinus variegates* (from Florida to the mid-Atlantic) can be used for these experiments. Sand dollars (*Dendraster* or *Echinarachnius parma*) can also be used, but I do not have any personal experience with them.

I have had the best luck with *L. pictus*, *L. variegatus*, and *S. purpuratus* but only variable luck with *A. punctulata*. The *Lytechinus* species are preferable because their eggs and embryos contain much less pigment, allowing the internal structures of the embryos to be seen more easily. Gametes and embryos from Atlantic urchins can be kept at room temperature, but eggs and embryos from Pacific urchins must be kept at approximately 15 °C. An e-ice bucket or circulating cooling water bath would be needed to maintain the cooler temperature.

There are many reliable suppliers:

Gulf Specimen Marine Laboratories,
P.O. Box 237, Panacea, FL 32346

(850) 984-5297
L. variegatus are available from summer through September.

Carolina Biological Supply Company,
(800) 335-5551; try to confirm availability with the Living Material department.
L. variegatus are available from February through September; also *Eucidaris tribuloides.*

Marinus Scientific,
1500 Pier C. St., Long Beach, CA 90813
(714) 901-9700
L. pictus are usually available from May until October and *S. purpuratus* in the fall to spring.

Note: Marinus now has a commercial license, so you no longer need a collector's license from the State of California (as previous).

Marine Biological Laboratory (Resources Department),
Woods Hole, MA
(508) 289-7375 or -7477
Arbacia punctulata and *Echinarachnius parma*.

Ward's Science (I have not used this supplier)
(800) 962-2660

If class times allow it, animals can be used the day that they arrive; otherwise and for use in multiple classes, they will need to be maintained in a saltwater tank (see below). *Lytechinus* and *Arbacia* adults are shipped in bags of sea water; *S. purpuratus* can be shipped in wet newspaper and will survive for a few days if kept in a cold room.

Animal Maintenance

The following is written to help non-marine biologists use sea urchins occasionally in teaching; disregard if you have running sea water tanks in your lab!

Gravid animals can be obtained virtually year-round. Animals can be ordered to arrive on the morning required. This assures that they will be fresh but can lead to nervously waiting around for their delivery. Also, any animals not used are wasted. I prefer to maintain the animals in tanks of Instant Ocean until needed. I have had the best luck with *Lytechinus pictus*, *Lytechinus variegatus*, and

Arbacia punctulata. *Strongylocentrotus purpuratus* are large and difficult to keep in an aquarium but can be stored in a cold room, wrapped in wet paper for a few days.

If you plan to maintain adults, set up your tanks several weeks in advance. Introduce some marine organisms into the tank such as algae or slimy rocks from the ocean. Established marine tanks are ideal. Alternately, dried bacteria for conditioning marine tanks, available from fish stores, can be added. Ask your supplier for the temperature and salinity that your animals will be accustomed to and adjust your tank conditions if necessary. Salinity can be adjusted with a refractometer or hydrometer. *L. pictus* and *S. purpuratus* need to be maintained at a lower temperature (12–15 °C); *L. variegatus* and *A. punctulata* can be kept at room temperature. It is important to plan on lots of room per urchin, especially for the larger species; I will put 20 *L. pictus* per 20-gallon tank, but only 6–8 *L. variegatus*, depending on their size. It is also important to have adequate filtration and aeration; I use an external filter unit, a bubble filter or protein skimmer, and numerous air stones. Also, illuminate the tank using aquarium lights or fluorescent lights for 12–14 hours per day.

When the animals arrive, gradually acclimate them from their shipping containers into your tank water. Allow urchins to equilibrate to the tank temperature by floating the bags in the tank; then make a small hole and gradually allow the water to mix. If any animals look as if they may be spawning, DO NOT put them into the main tank. They can encourage others to do the same. Instead, harvest the gametes for future use or pilot experiments. For the first few days, watch the animals carefully for signs of ill health: happy urchins have erect, motile spines, move about, and climb the walls, while unhappy ones sit around with drooping spines. REMOVE any that appear unhealthy. Dying urchins will release gametes and encourage others to do the same. You can collect gametes from questionable animals and store them. If you see signs of gamete release in the tank (water will be very cloudy), remove any sick animals, change the water, and hope. Dead animals should be bagged and stored in a freezer until disposal.

If you keep the animals for more than a few days, they will need to eat. The best solution is to request algae or kelp from your supplier (Marinus and Gulf Specimen are very cooperative about this). With live plants, it is even more important to light the tank; otherwise the plants will die. The second best option is to clip sheets of dried seaweed to the side of the tank. I have not had much luck with carrots or lettuce. Change the filters frequently and vacuum the debris from the bottom by siphoning and replacing about 10% of the water every week.

With care, *L. pictus* animals can be kept healthy and gravid for months; it’s best to plan on using other, larger urchins within a week or two.

Storing Sperm and Eggs

(Based on Protocol From Epel Lab)

Store excess sperm "dry" in microfuge tubes at 15 °C (Atlantic urchins) or 4 °C (Pacific urchins). The sperm should remain viable for one to two weeks.

Eggs can be stored to provide material for multiple lab sections or for several weeks of experiments.

ASW cannot be autoclaved; to sterilize, filter through a 0.2 mm filter unit. Rinse the urchin with filtered ASW when she begins to spawn; collect eggs into sterile beakers with filtered ASW and antibiotics (containing 200 mg/L sulfamethoxazole and 10 mg/L trimethoprim). Remove any spines or excrement that falls into the beaker with a pipet. Wash the eggs three times by gravity sedimentation. Dilute to approximately 0.2% (v/v) in ASW with antibiotics. Transfer to tissue culture flasks and store flat in a 15 °C incubator (Pacific urchins) or at ~20 °C (Atlantic urchins) in the dark for one week.

Storing sperm and eggs works extremely well with *L. pictus*, and acceptable results can be obtained with *L. variegatus* and *S. purpuratus*.

Sulfamethoxazole, Sigma S-7507

Trimethoprim, Sigma T-7883

Additional Information on Sea Urchins

Contaminants on glassware (bottles, finger bowls, tubes) including (especially) soap can be a big problem—make sure all glassware is new or very well rinsed! I keep separate glassware just for urchin labs.

Valuable information can be obtained from the Stanford University Sea Urchin Embryology website. In addition, sea urchins are covered very thoroughly in Leland Johnson's *Johnson & Volpe's Pattern and Experiments in Developmental Biology*, 3rd edition, McGraw Hill, 2001.

For additional ideas for labs or student projects, see:

DevBio_Lab: Urchin Advanced Experiments

Online Resources

- **Sea Urchins and Tunicates**
- **Sea Urchin Development**

Additional Background Information

Experiment 1-2. Inhibition of the Fast & Slow Blocks to Polyspermy

Sea urchin eggs release components of the egg jelly that attract sperm and induce the acrosome reaction (Barresi and Gilbert, 2020). In the laboratory, a single egg may be covered with dozens or even hundreds of sperm burrowing through the egg jelly (Figure 1-6B). Once a sperm contacts the vitelline envelope, it binds to a receptor protein that triggers the events of fertilization. Eventually, the plasma membranes fuse, and the sperm nucleus and centriole enter the egg. However, if a single egg is fertilized by multiple sperm (polyspermy), additional centrioles and sets of chromosomes are brought inside.

Two mechanisms have evolved to reduce the occurrence of polyspermy. Sperm binding initially triggers an influx of Na^+ ions leading to depolarization, which transiently blocks the binding of additional sperm; this is known as the fast block to polyspermy. This depolarization can be blocked by the addition of nicotine (Jaffe, 1980). In addition, sperm binding also stimulates the release of Ca^{2+} ions from the endoplasmic reticulum, leading to the fusion of a series of exocytic vesicles (the cortical granules) with the plasma membrane. This releases their contents, including a trypsin-like protease that degrades the sperm-binding sites on the vitelline envelope and detaches it from the plasma membrane (Vacquier et al., 1973). Water rushes into the space, causing the vitelline envelope to rise and become the fertilization envelope (Figure 1-6C). This produces a more permanent block to polyspermy (the slow block). Polyspermy can cause defects at the first cleavage division (Figure 1-6D-F), as the chromosomes are segregated randomly between multiple spindles (Just, 1919; Barresi and Gilbert, 2020).

References

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Jaffe, L. A. 1980. Electrical polyspermy block in sea urchins: Nicotine and low sodium experiments. *Development, growth & differentiation*, 22, 503–507.

Just, E.E. 1919. The fertilization reaction in *Echinarachinus parma*. *The Biological Bulletin*, 36, 1–10.

Vacquier, V. D., Tegner, M. J., and Epel, D. 1973. Protease release from sea urchin eggs at fertilization alters the vitelline layer and aids in preventing polyspermy. *Experimental Cell Research*, 80, 111–119.

Experiment 1-3. Gastrulation & Cell Differentiation in Sea Urchin Embryos

Gastrulation is a process involving extensive cell rearrangement; cells undergo dramatic movements and change relative positions. From this ordered movement, layers of cells are created. The cells that will form the endodermal and mesodermal layers, and subsequently organs, are brought inside the embryo, while the cells that will form the skin and nervous system spread over the outside surface. The three germ layers—outer ectoderm, inner endoderm, and interstitial mesoderm—are produced during gastrulation (Barresi and Gilbert, 2020). The endoderm (which will form the digestive tract) comes from the vegetal plate that invaginates and then elongates to form the archenteron (Figure 1-7). The primary mesenchyme cells ingress into the blastocoel, migrate midway up towards the equator, cluster, and lay down the calcium phosphate spicules to form the larval skeleton (Figure 1-8). As the endoderm cells differentiate, they begin to express tissue-specific genes, including the enzyme alkaline phosphatase, which can be detected by histochemical staining (Drawbridge, 2003; Figure 1-8).

References

Barresi, M. and S. Gilbert. 2023. *Developmental Biology*, 13th edition, Oxford University Press.

Cebra-Thomas, J. 2006. Histochemical staining of sea urchin embryos for alkaline phosphatase (AP) enzyme activity. *DevBio_Lab website.* Millersville University. [accessed 2025 Jun 15]. https://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Urchin/Urchin_AP.html

Drawbridge, J. 2003. The color purple: analyzing alkaline phosphatase expression in experimentally manipulated sea urchin embryos in an undergraduate developmental biology course. *The International Journal of Developmental Biology*, 47, 161–164.

Experiment 1-4. Perturbation of Gastrulation in Sea Urchin Embryos

Gastrulation is a process involving extensive cell rearrangement; cells undergo dramatic movements and change relative positions. From this ordered movement, layers of cells are created. The cells that will form the endodermal and mesodermal layers, and subsequently organs, are brought inside the embryo, while the cells that will form the skin and nervous system spread over the outside surface. The three germ layers—outer ectoderm, inner endoderm, and interstitial mesoderm—are produced during gastrulation (Barresi and Gilbert, 2020).

The fate of the cells that will form the three germ layers in sea urchin embryos is established very early. The fourth cell division is unequal and produces three different types of cells: the small **micromeres** and the large **macromeres** above them are both formed from the vegetal pole half of the embryos, while the intermediate-sized **mesomeres** are formed from the animal pole half of the embryo (Figure 1-9A). After the sixth cleavage division in normal sea urchin embryos, the size difference is no longer apparent, but the cells maintain their relative positions (Figure 1-9B).

After the sixth cleavage division in normal sea urchin embryos, two tiers of eight cells each are formed in the vegetal half of the embryo. The top tier is termed veg1, and the bottom tier is termed veg2. Veg1 lineages have been shown to contribute portions of the definite hindgut, midgut, and ectoderm that surrounds the blastopore at the completion of gastrulation (Cameron, 1997). Veg2 and micromere lineages normally contribute to the secondary mesenchyme, the coelomic sacs, as well as most of the archenteron (Cameron, 1997). As the cells continue to divide, tight junctions are formed, converting the embryo into a hollow ball of epithelium (the blastula). At the **midblastula transition**, the genome is activated, and the cells grow a cilium and produce an enzyme that digests a hole in the fertilization envelope, allowing them to “hatch.” The cells in the vegetal half of the embryo thicken (Figure 1-9C) to form the vegetal plate.

Gastrulation in sea urchin embryos progresses in a predictable and easily observable way (Barresi and Gilbert, 2020). The vegetal plate thickens and primary mesenchyme cells ingress and form the spicules that constitute the urchin skeleton (Figure 1-10). Then, the vegetal plate invaginates, forming the archenteron, and this archenteron extends up the embryo’s blastocoel wall by convergent extension, with the help of the secondary mesenchyme cells (Figure 1-10). The initial cell buckling that leads to vegetal plate invagination depends on

the secretion of chondroitin-sulfate proteoglycan (Lane et al., 1993). The migration of the archenteron also depends on extracellular materials that have been produced by the developing organism. Karp and Solursh (1974) have suggested that secondary mesenchyme cells, which form filopodia at the tip of the developing archenteron (primary gut), attach to sulfated proteoglycans in the extracellular matrix within the blastocoel of a developing sea urchin. In both cases, sea urchin embryos incorporate sulfate from the environment into their extracellular matrices. The extracellular matrix contains acid mucopolysaccharide that, when bound to sulfated proteoglycans, forms a textured surface (Karp and Solursh, 1974). The secondary mesenchyme cells attach to the extracellular matrix and pull the archenteron up along the blastocoel cavity. If sulfate is not present, the archenteron does not form. However, the vegetal plate cells may still be capable of differentiating into intestinal epithelial cells that can be detected by staining for alkaline phosphatase.

Prior to the onset of gastrulation, b-catenin localizes to the nucleus in the cells in the vegetal half of the embryo; the highest levels are found in the descendants of the micromeres that will form the primary mesenchyme cells (Miller and McClay, 1997). If embryos are treated with LiCl, the region of cells expressing nuclear b-catenin expands (Gilbert, 2014). Treatment with LiCl has also been shown to perturb cell differentiation, resulting in an excess of cells committed to the endodermal fate and a condition known as **exogastrulation** where the archenteron protrudes outward. LiCl inhibits GSK-3, a b-catenin regulating molecule, leading to higher b-catenin levels (Logan et al., 1999), suggesting that nuclear b-catenin is involved in cell fate specification.

Pigment-containing cells (**echinophores**) are first observed in the ectoderm surrounding the blastopore during early gastrulation while the veg2 cells are invaginating. As development proceeds, they become dispersed through the ectoderm, forming the outer body wall (Figure 1-10). Dye-marking experiments have suggested that the echinophores derive from the veg1 layer (Young, 1958), which is affected by LiCl treatment (Cameron and Davidson, 1997).

In this study, you will examine the effects of sulfate-deprivation or exposure to LiCl on gastrulation and the specification of vegetal cell fates.

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Appendix B: Instructors' Notes on Chicken Protocols

Solutions

Howard Ringer's (HR) solution

Reagent	Amount
NaCl	14.4 g
$CaCl_2.2H_2O$	0.46g
KCl	0.74g
dH_2O	to 2 liters

Albumin-Agar Plates

1. Transfer 50 ml "Just Whites" Eggbeaters™ or equivalent to sterile 50 ml tube. Warm to 45°C in water bath.

2. Autoclave 0.65 g agar, 0.65 g glucose, and 16.5 mL Howard Ringer's solution in 100 mL flask for 15 minutes. Cool to 45°C in water bath.

3. Combine agar and egg whites with stirring. Transfer between flask and beaker to aid combination. Turn water bath up to 48°C to keep agar from solidifying while pouring plates.

4. Cut off tip of transfer pipet. Transfer approximately 3 ml to 35 mm petri dishes. Avoid transferring bubbles. Allow to cool overnight.

5. Store in bags or sealed with parafilm in refrigerator. Use within 2 weeks

Fertile Eggs and Egg Incubation

Fertile chicken eggs can be most economically obtained from a local farm or hatchery if there are any nearby. Eggs can be stored at room temperature for a week before being incubated; for longer-term storage, keep the eggs in humidified cold storage at approximately 10°C (e.g. in a small wine cooler). Colder temperatures (e.g., in the refrigerator) will adversely affect viability. Eggs can also be ordered from online suppliers including Sargent Welch and Texas A&M, but I do not have any experience with this. Finally, fertile eggs can be ordered from AVS Bio, a subsidiary of Charles River Laboratories. This is an

expensive option, but the fertile eggs from this supplier can be held for several weeks at cold temperatures without losing viability.

Incubators need to maintain temperature (37-38°C) and humidity to preserve viability of the embryos. Incubators that are able to move the eggs periodically and circulate the air also improve the rate of successful development. Egg incubators are available at a variety of prices, from classic cedar wood incubators (if you are lucky enough to inherit one) to inexpensive ones available on the web. I've had good luck with Hovabator incubators. Chicken embryos up to day 15 are considered too young to perceive pain and usually are not considered to require IACUC oversight. These embryos can be sacrificed by hypothermia by placing them in the freezer. Chicken embryos that have reached day 16 of incubation should be sacrificed by decapitation and may require IACUC oversight. Take care to monitor students' incubation of eggs to prevent unexpected hatching of chicks.

Online Resources

- **Chicken Embryo Development**
- **For Developmental Studies, Windowing Chicken Eggs**
- **How Birds Get Oxygen Inside Their Eggs**
- **Virtual Chicken: Part 1: The Female Reproductive Tract**
- **FLIGHT: The Genius of Birds - Embryonic development**
- **Chicken Development**

Supplies

- DMEM (Fisher MT-17-204-CI)
- Fetal calf serum (Fisher BW14507E)
- Costar 24 well plates (Fisher 09-761-146)
- Costar 6 well plates (Fisher 07-200-83)
- Costar Transwell™ Clear, 3 µm pore, 24 mm (Fisher 07-200-171)
- Gentamicin sulfate, 10 mg/mL (Fisher 50841714; Teknova G3625) or 50 mg/mL (Invitrogen, 15750060)
- Cyclopamine (Tocris 1623; LC Laboratories C-8700)
- SU5402 (Tocris 3300; Selleckchem S7667, 10)
- LDN-193189 (Stemgent 04-0074; Selleckchem S2618)
- (2-Hydroxypropyl)-β-cyclodextrin (SIGMA H107)
- Dimethyl Sulfoxide (SIGMA D2650)

Special Instructions

Experiment 2-1

Slides with resin-embedded chicken embryos can be obtained from Carolina Biological Supply.

Experiment 2-3

Albumin-agar plates can be made with fresh egg whites, but it is more difficult to get a uniform solution. Plates can be poured in 35 mm petri dishes (e.g., Falcon 351008). The plates with embryos should then be incubated in a sealed container to reduce evaporation. Alternately, but more costly, the albumin-agar can be added to the center well of center-well organ culture dishes (e.g., Falcon 353037). If using organ culture dishes, Howard Ringer's solution can be added to the outside ring to keep the embryos hydrated.

Experiment 2-4

The sensitivity to ethanol exposure may vary with the strain of chicken used. I have had the best success with 0.25 mL of 5% ethanol in Howard Ringer's solution injected after 24 hours of incubation (e.g., during gastrulation). Cell death can also be measured using acridine orange or neutral red staining. Nile blue sulfate has been reported to stain dead cells, but I have not had any success with several batches of stain. More specific methods of staining for apoptotic cells (e.g., TUNEL) would also be expected to be successful.

Experiment 2-5

1. Fine forceps (e.g. Dumont #5) are very valuable for microdissection.

2. Tissue culture supplies are needed, which make this lab more expensive. Many reagents are shared with chick skin culture lab.

3. Pen/strep can be substituted for gentamicin sulfate.

4. Culture of hearts at loop stage is more technically difficult for the students, but in some cases evidence of organogenesis can be observed.

Experiment 2-6

1. Fine forceps (e.g. Dumont #5) are very valuable for skin microdissection.

2. Tissue culture supplies are needed, which make this lab more expensive. Many reagents are shared with chick heart culture lab.

3. Additional 6 well plates allow you to transfer only the Transwell™ inserts needed (usually 1 per group, which will hold 3-4 skin explants each).

4. Pen/strep could be substituted for gentamicin sulfate.

5. Apply 10 µL of Cyclopamine (1 mg/mL in 45% hydroxypropylcyclodextrin [HPDC]) directly to explants. SU5402 (3 mg/mL stock in DMSO) should be diluted in culture media to a final concentration of 3 µg/mL. LDN-193189 (1 mg/mL in DMSO or 45% HPDC) is added to culture media to a final concentration of 1 µg/mL.

Additional Ideas for Labs or Student Projects

- **DevBio_Lab: Chick Advanced Experiments**

Additional Background Information

Experiment 2-4. The Effect of Ethanol on the Patterns of Apoptosis

Fetal alcohol syndrome (FAS) is one of the most common causes of developmental disabilities. Animal models, such as the chicken embryo, can be used for the exploration of the teratogenic effects of alcohol. Ethanol exposure can cause stunted growth, facial abnormalities, and dysfunction of the brain and heart (Kiran, 2010). Exposure to ethanol during embryogenesis disrupts the development of the neural crest cells in the craniofacial region. It causes elevated levels of apoptosis, resulting in a decrease in the number of neural crest cells in the frontonasal prominence and first pharyngeal arch. This provides a possible explanation for the observed craniofacial malformations (Cartwright, 1995).

Ethanol, like other toxins, affects only certain areas within the embryo, and there are particular developmental stages where the embryos are more susceptible to these toxins than others (Smith, 1997). These windows of vulnerability provide insight into the mechanisms by which the toxin causes its effects (Smith, 1997). The regions affected by FAS (the bones of the upper and lower jaw, forehead and orbitals) are derived from the same cellular lineage, the neural crest cells**.** Hence, when alcohol is present and causes damage to these neural crest cells, multiple areas of the embryo are affected (Smith, 1997). Exposure during late gastrulation and early pharyngula stages leads to facial deformities in the developing embryo.

Apoptosis, or programmed cell death, is a normal part of development, and it occurs at specific stages of development in multicellular animals, such as chickens. Apoptosis is a cellular suicide mechanism that differs in several ways from “necrosis,” which typically results from cellular injury (Maccabe & Noveroske, 1997). Apoptosis is characterized by damage to the cellular repair systems, chromatin condensation, DNA fragmentation, and cellular breakdown into membrane-enclosed, metabolically active cell fragments (Smith, 1997). Apoptosis serves multiple functions during development, including the removal of damaged, misplaced, abnormal or excess cells; sculpting structures during morphogenesis; removal of structures as during metamorphosis; controlling cell number; and removing cells that establish inappropriate connections or that are improperly induced (Hirata & Hill, 2000). Propidium iodide is a ‘vital dye’ that is able to penetrate only dead cells. It binds to DNA by intercalating between the

stacked bases of the double helix and fluoresces with an emission maximum of 617 nm (red).

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Experiment 2-5. *In Vitro* Culture of Embryonic Chicken Hearts

The chicken is a classic organism used to illustrate the principles of basic embryology. One of the developmental systems that has been examined in great detail is the circulatory system. In the developing embryo, the circulatory system is the first functional unit, and the heart is the first functional organ. The embryonic chick (and mammalian) heart first forms as a single loop (Figure 2-5) on the outside of the embryo. This development process enables us to clearly observe the ongoing contraction and relaxation of the heart.

The heart of the chicken embryo develops from the fusion of paired precardiac mesodermal tubes, forming a straight anterior to posterior ventricular tube. After fusion is complete, the heart tube has four distinct regions: bulbus cordis, ventricle, atrium, and sinus venosus. Pulsations in the heart start while the paired primordial cells fuse. The sinus venosus is the pacemaker of these initial contractions (Barresi & Gilbert, 2023). After approximately 33 hours, the heart tube bends to form an "S" shape structure with a single atrium and a single ventricle. By 2 days, the heart has folded upon itself, forming a single loop. This moves the sinus venosus and atrium to a position anterior and dorsal to the ventricle and the bulbus cordis. In 3-day chick embryos, the atrium has begun to expand to the left in preparation of the division into the right and left atria. Although the heart still has two chambers at this time, communication between the sinus venosus and the atrium occurs through the right side of the atrium. Times of development may vary. Eventually, when the atrium and ventricle divide to develop a typical four-chambered heart, the sinus venosus will be incorporated into the right atrium. The bulbus cordis will eventually give rise to the aorta. In this experiment, we will remove hearts from 3-day chick embryos and maintain them under specific conditions that allow for their development. By this time the two-chambered heart should be visible as well as the blood flow entering the lower chamber and being pumped out through the aorta.

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Experiment 2-6. Development of Feather Buds in Cultured Embryonic Chick Skin

Many organs are formed through the interaction between two distinct tissue layers: the epithelium, a sheet of cells that are tightly linked together, and the mesenchyme, a more loosely associated group of cells. The cells of the epithelium and mesenchyme are adjacent but are not directly connected. They communicate with each other through the production of signalling molecules—secreted proteins produced by one cell that bind to receptors on the other. This communication is essential for the proper formation of organs and the differentiation of the specialized cells within them.

The developing chick feather bud presents a good model system in which to study organ formation (Chuong and Widelitz, 1998). The outer, epithelial layer of skin, the epidermis, is derived from the ectoderm. Under the epidermis lies the dermis, which is derived from the trunk somites (Olivera-Martinez et al., 2001) and forms a loosely packed mesenchyme. The epidermis initially can form either skin or feathers. The underlying dermis produces and secretes signalling molecules, such as FGF10 (Mandler and Neubüser, 2004; Barresi & Gilbert, 2023). These proteins bind to receptors on the epithelial cells, causing them to divide and differentiate. The epithelium, in turn, produces sonic hedgehog and BMPs through which it signals back to the mesenchyme. The mesenchyme then condenses under the newly formed feather bud (Chuong and Widelitz, 1998). As a result of this molecular dialogue, the feather bud grows out.

Feather formation initiates at Hamburger & Hamilton (1951) stage 28 (between days 6 & 7 of incubation). The earliest feather bud placodes form along the dorsal midline, above the neural tube that may provide a source of Wnt proteins (Chuong and Widelitz, 1998; Olivera-Martinez et al., 2001). Feather bud induction then progresses laterally to form evenly spaced tracts of feathers. After initiation, feather bud development can progress in culture to the long bud stage (Chuong and Widelitz, 1998), suggesting that interactions between the dermis and epidermis are sufficient.

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Appendix C: Instructors' Notes for Zebrafish Protocols

Solutions

Hank's Stock #1

NaCl	4.0 g
KCl	0.2 g
ddH_2O	to 50 ml

Hank's Stock #2

$Na_2HPO_4*7H_2O$	0.36 g
KH_2PO_4	0.30 g
ddH_2O	to 50 ml

Hank's Stock #4

$CaCl_2*2H_2O$	0.96g
ddH_2O	to 50 ml

Hank's Stock #5

$MgSO_4*7H_2O$	1.23 g
ddH_2O	to 50 ml

Zebrafish Embryo Medium

Stock #1	0.5 ml
Stock #2	0.05 ml
Stock #4	0.5 ml
ddH_2O	48.3 ml
Stock #5	0.5 ml
$NaHCO_3$	0.18 g

Add about 10 drops of 1 M NaOH to bring to pH 7.

(from Westerfield, M. 1993 *The Zebrafish Book*, University of Oregon.)

Animals

Adult fish can be obtained from most pet stores; these are usually young and require at least 1 month of intensive feeding to reach breeding form. Alternately, fish can be obtained from Carolina Biologicals (800-334-5551), although I have received fish of variable age from Carolina. Adult zebrafish are vertebrate animals and may require approval from an Animal Care and Use Committee (ACUC) at your institution. Zebrafish embryos and larvae up to 6 days post-fertilization are generally not considered to require institutional ACUC oversight.

Setting Up Fish Tanks

Fish tanks, especially brand-new ones, require conditioning before and during the introduction of fish to promote the establishment of a biological filtration system. This is especially important since the bottom of the tank will need to be bare of gravel to allow harvesting of the eggs once they have been laid. Sponge filters with aerators and floating BioBalls™ sold for pond filters both provide additional surface area for beneficial bacteria to colonize and aid in breaking down nitrogenous waste. Set up your tanks several weeks before you will need them and seed with a source of beneficial bacteria (such as Stress ZYME™); circulate the water but do not use a charcoal-containing filter. After a week or so, gradually introduce fish a few at a time, dosing with additional bacteria each time. Once fish are added, you can add a charcoal-containing easily changeable filter system to the tank. A ten-gallon tank can comfortably accommodate a school of 12-20 adults, but you will need to build up to that number gradually. Adding too many fish before the biological filtration has built up its capacity will result in the fish developing ‘new tank syndrome,’ which can be fatal. You will need at least one tank of fish per lab section; I tend to maintain two tanks of breeding fish for a class with one section, along with a third tank that can be used for emergencies.

Care and Maintenance of Zebrafish Adults

Zebrafish are common aquarium fish and are relatively easy to maintain. They breed best from about 6 to 18 months of age. Fish should be fed 2 or 3 times a day, no more than they can consume in 10 minutes. One of these feedings can be a rich protein source, such as live brine shrimp, although this does not appear to be necessary. I feed a variety of TetraMin™ flakes (all-purpose, shrimp, vegetable-based). I also supplement their diet by the addition of Spawn Aid (a mixture of L-Lysine and L-Ornithine amino acids) to the tank water several times a week while the fish are breeding. Decrease feeding frequency to once a day if

fish are not to be bred for a while. Fish should be kept in tanks bare of gravel and with filters that will not pick up eggs when laid, such as sponge type biofilters. Clean tanks by siphoning debris from the bottom and replacing approximately a quarter of the water once a week. In most cases, fish will thrive in dechlorinated tap water; alternately, tank water can be constructed from deionized water by adding R/O Right™ as instructed or 60 mg Instant Ocean per liter. If the tank pH is too low, add pH Stable™ to buffer. Use a heater to maintain the temperature at approximately 28°C (80-82°F) and a light with a timer to give a 14-hour light:10-hour dark cycle.

Working With GLOfish

GLOfish are transgenic organisms that express various fluorescent proteins. They come in a variety of colors, but the most useful ones for developmental biology are the green and red varieties because they can be observed using the FITC and Rhodmine/Texas Red filter sets that are most common on fluorescent microscopes. GLOfish can often be obtained from pet stores, from Carolina and from internet sources including the GloFish company. They behave like wild-type zebrafish, and breed well, but tend to be on the smaller side, so I recommend getting them and starting to introduce them while the wild-type fish are small if you are trying to establish a mixed school. GLOfish females lay fluorescent eggs, but the fluorescence is restricted to the yolk. The embryo does not become fluorescent until the transgene is activated. The initial gene expression is in the somites and is clearly visible after 24-48 hours. In a mixed school, embryos will suddenly express the transgene from zygotes that were not initially fluorescent. This is a nice visual demonstration of differential gene expression. If you have the ability to examine fluorescent embryos, I recommend including GLOfish in Laboratory 3-1.

Background: “GloFish”, Wikipedia article

Isolation of Zebrafish Embryos

Zebrafish will mate and deposit fertilized eggs on the bottom of the tank at 'dawn' (e.g., when the lights turn on). They can be accustomed to lay at any convenient time by keeping the room dark and lighting the tank with a timer. The maximum embryo production is obtained with a ratio of 1 male for every 2 females, with a group of ~12-20 fish in a 10-gallon tank. The night before embryo collection, vacuum the tank well with a siphon to remove debris several hours after the last feeding. Deposit a layer of washed marbles to cover the entire bottom surface of the tank. Do not disturb for the first 30 minutes after 'dawn' to allow fish to mate (see below). Embryos can be collected once a week, with best results from tanks kept on a regular schedule. Collect embryos and fertilized eggs from between the marbles with a siphon and collect with a fine fry net or mesh filter. Transfer the net to a glass dish full of tank water and examine the embryos with a dissecting microscope at low magnification. Transfer healthy embryos to a clean glass petri dish containing embryo media with large-bore glass Pasteur pipets. Discard any that are cloudy or ruptured. Keep embryos at 28°C. An inexpensive incubator is good for this. Embryos can be reared in ZFEM or filtered tank water.

Equipment

Working with zebrafish embryos doesn’t require a lot of specialized equipment, but it is important to have a dedicated set of glassware as the embryos are very sensitive to soap and other pollutants (so make sure that the Petri dishes, Pasteur pipets and larger glass bowls/pans are not washed with soap!). I generally just use hot/warm chlorinated tap water to rinse after use and autoclave the 60 mm dishes and pipets before reuse. The marbles used during laying should also be washed only with hot chlorinated water and allowed to air dry in a colander. The developing embryos can be observed and photographed in glass dishes using a dissecting microscope. A dissecting microscope equipped with epifluorescence is required for visualizing embryos stained using immunofluorescence, but embryos stained with DASPEI or expressing fluorescent transgenes (e.g., GLOfish) can be visualized using a less expensive system such as the NIGHTSEA™ viewing system that can be added to a basic dissecting microscope.

Additional Information on Zebrafish

The 'bible' for all things zebrafish is Monte Westerfield, *The Zebrafish Book*, University of Oregon, 1993. More specialized methods can be found in H.W. Detrich, M. Westerfield and L.I. Zon, *The Zebrafish: Biology, Methods in Cell Biology*, Vol. 59, Academic Press, 1999.

For additional ideas for labs or student projects, see:

DevBio_Lab: Zebrafish Advanced Experiments

Online Resources

- **Zebrafish Development**
- **Zebrafish**
- **Zebrafish Development**
- **Annotated Zebrafish Development Timelapse**
- **Zebrafish Embryo Development Animation**
- **Dechorionating Zebrafish Embryos**

Additional Background Information

Experiment 3-2. The Effects of Different Concentrations of Lithium Chloride on the Development of Zebrafish Embryos

Lithium chloride, a teratogen, alters the development of a variety of organisms including sea urchins, *Xenopus laevis* (frogs), and *Danio rerio* (zebrafish). In sea urchin embryos, lithium chloride causes the accumulation of nuclear beta-catenin in an expanded region of vegetal cells and transforms presumptive ectoderm into endoderm (Barresi & Gilbert, 2023). Lithium exposure in cleavage-stage embryos of *Xenopus* inhibits dorsal/ventral axis specification and results in radially symmetric, dorsal-anteriorized embryos (Stewart & Gerhart, 1990). Stachel and colleagues (1993) report that lithium induction of pre-midblastula stage zebrafish prevents normal dorsal/ventral axis patterning by acting as an inhibitor to the phosphoinositol pathway. This results in an expansion of the region of *goosecoid* and *noggin* expression beyond the region of the presumptive embryonic shield.

Stachel and colleagues have also shown that the development of anterior structures is dependent on WNT signaling, especially the transcription of *goosecoid*, which codes for a dorsalizing protein necessary for normal anterior development (Stachel et. al., 2003). The increase in expression of organizer-specific proteins such as *Goosecoid* in the presumptive ventral regions of the organism produces different phenotypic results depending on the stage in development at which the induction occurs (Stachel et. al., 1993). For instance, the exposure of embryos to LiCl before the midblastula transition results in hyperdorsalization and the inhibition of normal dorsal/ventral axis patterning (Deitrich, 1999). In contrast, embryos exposed to LiCl four hours after the midblastula transition experienced normal dorsal/ventral axis specification but perturbed development of anterior structures such as the eyes (Stachel et. al., 1993).

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Experiment 3-3. Retinoic Acid-Induced Truncation of Zebrafish (*Danio Rerio*) Embryos

Retinoic acid (RA) is a highly teratogenic derivative of vitamin A that is known to influence Hox gene expression. RA-mediated gene activation is important for normal vertebrate development; RA acts as a posteriorizing signal in many developing systems, including mammals, and is also involved in limb formation (Barresi & Gilbert, 2023).

When embryonic exposure is higher than normal, however, developmental anomalies occur. Exposure of the human fetus to 13-cis-retinoic acid results in a characteristic pattern of anomalies, including absent or defective ears, absent or small jaws, cleft palate, aortic arch abnormalities, thymic deficiencies, and abnormalities of the central nervous system. Similar anomalies are observed in other vertebrates. In mice, for example, embryonic exposure to retinoic acid results in axial truncation and causes a dramatic reduction in the sizes of the first and second pharyngeal arches, which normally form the jaw, ear, and other facial bones (Barresi & Gilbert, 2023). The truncated embryo exhibits a posterior region having the characteristics of the anterior region of an embryo that had developed normally, including a posterior extension of the ribcage. At very high concentrations, the cells do not differentiate to form the posterior of the embryo at all (Modak et al., 1993).

Retinoic acid disrupts development by altering the expression of Hox genes, causing the re-specification of the anterior-posterior axis and inhibition of neural crest cell migration from the cranial region of the neural tube (Barresi & Gilbert, 2023). Retinoic acid binds to specific retinoic acid receptors (RAR; Hyatt et al. 1992). After binding, the receptor becomes an active transcription factor. The retinoic acid-bound RARs have at least two modes of action, one of which is to bind to their DNA enhancer sequences and activate particular genes that are not usually activated in these cells. These genes include certain homeotic genes that specify the anterior-posterior position along the body axis. In this way, they can cause homeotic transformations, generally converting anterior structures into more posterior structures (Barresi & Gilbert, 2023).

Zebrafish embryos treated with retinoic acid have been shown to exhibit truncation similar to that observed in mammalian embryos (Holder & Hill, 1991). To further examine the effect of retinoic acid on zebrafish embryos and to determine whether the magnitude of such effects is concentration dependent, you will need to treat the embryos with different concentrations of retinoic acid and allow them to continue development.

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Experiment 3-4. The Effects of Differing Ethanol Concentrations on Zebrafish Embryos

Exposure of prenatal humans to alcohol can produce Fetal Alcohol Syndrome (FAS), which results in characteristic craniofacial defects, poor muscle tone, underdeveloped fetuses and heart defects. As a model organism, zebrafish (*Danio rerio*) have been used to investigate teratogens, including alcohol exposure, in part because they develop most of the major organ systems present in mammals in less than a week (Rubinstein, 2006).

Embryonic development of zebrafish is affected by ethanol in a manner similar to higher vertebrates (Blader and Strähle, 1998). Exposure of zebrafish embryos to ethanol causes cyclopia and craniofacial abnormalities, alters gene expression in the ventral aspects of the fore and midbrain, induces developmental abnormalities of the notochord and spinal cord, and causes malformation of the body trunk (Blader and Strahle, 1998). Reimers et al. (2004) also observed adverse developmental effects including cyclopia, under-developed mid, fore and hindbrains, and pericardial edema.

An understanding of the origin of the deformities requires knowing how embryonic cells of zebrafish normally migrate during gastrulation. During gastrulation, blastoderm cells epibolize over the yolk. When about half the yolk cell becomes covered with blastoderm cells, the margin of epibolizing blastoderm cells form the epiblast and the hypoblast. The organizer, known as the embryonic shield, forms on the dorsal side of the embryo (Barresi & Gilbert, 2023). Hypoblast cells of the embryonic shield converge, extend anteriorly, and narrow along the dorsal midline of the hypoblast. Some of these cells eventually form the prechordal plate and the notochord (Barresi & Gilbert, 2023).

The prechordal plate cells of zebrafish embryos exposed to ethanol form at an ectopic position (Blader and Strähle, 1998). It is, therefore, believed that ethanol is responsible for the abnormal migration of prechordal plate cells that ultimately causes cyclopia and other deformities. The prechordal plate cells express genes like *goosecoid* and *islet-1*, which control cell differentiation in the anterior region of the embryo (Blader and Strähle, 1998). Hence, prechordal-specific genes are expressed ectopically to bring about the observed deformities.

In addition to the deformities, ethanol appears to cause abnormal cell death (Sulik *et al*., 1988). Ethanol exposure at early development stages results in significant death among the cells destined to give rise to facial structures (Sulik *et al*., 1988). Ethanol seems to achieve apoptosis by activating the cells' self-

destruction machineries (Sulik *et al*., 1988). Cell death has also been proposed as a potential explanation for ethanol-dependent toxicity in developing embryos (Reimers et al., 2004). The link between cell death and ethanol in early embryos can be partially explained by an increase in oxidative stress, leading to oxidative tissue damage and, in higher doses, death.

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Experiment 3-5. The Effects of Valproic Acid on Somite Development in *Danio Rerio*

Valproic acid was first used as an anticonvulsant drug to treat epilepsy in the late 1960s. It has been shown to be a means of controlling seizures. However, valproic acid is able to cross the placenta and has been linked to human birth defects (Polifka and Friedman, 2002), including facial and skeletal malformations. Other vertebrates show similar birth defects after exposure. For example, pregnant rats treated with valproic acid produced embryos with fused ribs, missing vertebrae and kinky tails (Voorhees, 1987).

The teratogenic activity of valproic acid is thought to stem from its down-regulation of the *pax-1* gene thereby leading to skeletal malformations (Lammer et al., 1987). *Pax-1* is a transcription factor that functions in the development of chondrocytes from the scelerotome portion of somites (Barresi & Gilbert, 2023). These chondrocytes later give rise to portions of the axial skeleton, including vertebrae and portions of the ribs (Smith and Tuan, 1995). *Pax-1* expression is induced by Sonic Hedgehog protein produced by the notochord and is expressed in the ventral portions (the sclerotome) of the somites. *Pax-1* protein is thought to be a necessary ventral fate patterning molecule required for the proper formation of the vertebrae (Koseki et al., 1993). *Pax-1* null mutant mice have little to no formation of the ventral portions of their vertebrae and vertebral discs (Wallin et al., 1994). Similar malformations are induced by treatment with valproic acid. Chicken embryos exposed to valproic acid also showed significant somite disorganization and malformations accompanied by a decreased level of *pax-1* expression (Barnes et al., 1996).

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Experiment 3-6. Ototoxic Effects of Antibiotics on Hair Cells of Zebrafish

Zebrafish (*Danio rerio*) possess a lateral line system composed of neuromasts containing a group of hair cells (Harris et al., 2003). The neuromasts are distributed in small groups of cells along particular lines across the body of the embryo. Each neuromast is composed of a group of mechanosensory hair cells and supporting cells arranged in rosette fashion that bulge through openings in the periderm (Whitfield, 2002).

From an anatomical perspective, the zebrafish does not seem like an ideal model since it lacks outer and middle ears (Whitfield, 2002); however, physiologically, and even structurally for the most part, the zebrafish hair cells conserve the same major proteins, signaling pathways (Ernest et al., 2000), and genetic mechanisms of otic development as the mammalian inner ear (Whitfield, 2002). However, contrary to mammals, zebrafish are able to regenerate hair cells to their original number after they have been damaged (Thomas et al., 2015). Considering all this, the zebrafish is a solid model for human deafness.

The lateral line hair cells in their larva are easily accessed and transparent, making zebrafish a powerful model organism for screening drugs that can cause or prevent hair cell death (Ou et al., 2010). Hair cells in the zebrafish lateral line are considered to be homologous to sensory hair cells in the inner ear of mammalians, showing similar responses to ototoxic drugs (Harris et al., 2003). Aminoglycoside antibiotics —for example, gentamicin and neomycin —are capable of ototoxic effects. This means that exposure to aminoglycoside antibiotics can cause the deaths of sensory hair cells. As zebrafish are a model organism, they are key to researching the ototoxic effects of many groups of chemicals (Coffin et al., 2013). Research suggests "activation of an 'acute' cell death mechanism is shared by both neomycin and gentamicin, while a 'slow' mechanism is specific to gentamicin-induced damage" (Coffin et al., 2013). The extent of damage caused by neomycin is dependent on the dosage, while the effects of gentamicin depend on both dosage and length of exposure (Owens et al., 2009).

The advantage of zebrafish and non-vertebrates is the ability to regenerate hair cells immediately after damage. Several studies conducted on the lateral line of the zebrafish have demonstrated that mitotic proliferation of supporting cells is the probable source of regeneration after direct damage by chemicals or other damaging treatments, particularly aminoglycoside- or copper-induced hair cell damage (Brignull et al., 2009). Ou and colleagues (2010) reported that hair cells of the lateral line in zebrafish have been known to regenerate within 24 hours of

hair cell injury. However, zebrafish cells not only regenerate after damage to the hair cells but also regenerate in the absence of damage as they have continuous hair cell turnover. The lateral hair cells have two support cell types that aid in the maintenance and regeneration of the hair cells (Cruz et al., 2015). Hair cell regeneration in the auditory system of birds after induced damage has also been observed, which opened the door to the identification of the progenitor cell identity of hair cells and the hope of cell regeneration for mammals such as humans (Brignull et al., 2009).

Several compounds that protect against hair cell loss from drug-induced damage in zebrafish have been identified. PROTO1, a benzothiophene carboxamides, provided significant protection from hair cell death caused by neomycin. PROTO1 and PROTO2 do not inhibit aminoglycoside uptake but rather act intracellularly to reduce the degree of damage to the hair cells (Coffin et al., 2010). Other protective compounds have been identified that reduce drug-induced damage. These compounds could be used individually or in combination to prevent mammalian inner ear damage, for example, in dire situations where infants may need a high dose of antibiotics (Coffin et al., 2010).

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