

## Chapter 4

# Developmental and Physiological Aspects of the Chicken Embryonic Heart

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## Introduction

The chicken embryo is a classic system used to illustrate the principles of basic vertebrate embryology primarily because it is easy to obtain and direct observation of a living embryo is possible even at the earliest stages of embryogenesis. One of the developmental systems which has been examined in the most detail by vertebrate embryology students has been the circulatory system. With the aid of a dissecting microscope, the early stages of heart development and the circulatory patterns can be observed even when the chicken embryo is still attached to the yolk. The other advantage to using the live chicken embryo to demonstrate the principles of heart development is that the heart can be removed from the embryo and maintained in a dish of warm saline for several hours; the beating of the heart, the flow of blood, and the differentiation of the four chambers are even more clearly visible. In addition, the heart is large enough for properly trained students to surgically isolate two or more of the heart chambers from the beating *in vitro* heart. Thus, students who work with this particular embryo in their development and/or physiology laboratories not only learn many principles of vertebrate morphogenesis, organogenesis, and cardiac physiology, but are also exposed to simple, useful micromanipulative techniques.

We designed this undergraduate laboratory to teach developmental/physiology students some of the basic principles of vertebrate heart embryology, blood flow, and electrical circuitry, using the chicken embryo as a model system. In addition, this lab requires the students to learn (or, hopefully, relearn) the scientific method; they must develop a hypothesis related to the effects of a reproductive toxin on heart rate, design a method for testing this hypothesis, carry out the experiment during the lab period, and write a lab report about their experiment and results. Finally, the students learn and utilize several micromanipulative techniques which exercise their fine motor control and develop their ability to work with live embryos. These particular dexterous manipulations allow many of those who aspire to become physicians to try their hands (no pun intended) at surgical technique.

### Materials

The number of items needed per student pair is in parentheses, unless otherwise indicated

72-hour old chicken embryos/eggs, in 37°C humidified incubator (7)

110 mm diameter glass dishes, lined with cotton (2)

Scotch Magic Tape (1)

20 G needle attached to a syringe (1)

Fine scissors (2)

Fine forceps (2)

65 mm Syracuse glass dishes (2)

Chick saline in 45°C water bath (100 ml)

Test tube rack in 45°C water bath

Filter paper "doughnuts" (8)

Microknife (Tyler, 1994) and/or iris microdissecting scissors (2)

Test tubes with caps (6)

Test tube rack on bench top (1)

1.5 ml dropping pipettes (6)

Embryo spoon (1)

Gooseneck lamp with 100 W bulb (2)

Large beaker for eggs/embryos after they have been examined

Dissecting microscope with lighting from above (2)

Labeling tape (1) and indelible ink pen (1)

**Recommended Atlas:** Mathews, W. W., and G. C. Schoenwolf. 1998. Atlas of descriptive embryology. Fifth edition. Prentice-Hall, Upper Saddle River, New Jersey, 266 pages. [ISBN 0-13-593740-X]

Stock solution of 3% caffeine, made with chick saline (500 ml per 24 students)

Stock solution of gin, made with chick saline (500 ml per 24 students)

Stop watch or clock with second hand

Heart models

### Notes to the Instructor

#### General Information

The lab that we have provided for this ABLE workshop is actually the second of a two-part laboratory experience. During the first three hour lab the students learn the fundamentals of 48 and 72-hour old chicken embryo anatomy (identification of the brain vesicles, somites, eyes, otic pit, pharyngeal pouches and clefts, limb buds, and heart), as well as two of the techniques they use in the following lab; thus, by the end of the first lab, they can window an egg, allowing them to observe the living embryo, and explant the live embryo into a dish of warm saline and maintain that embryo for at least an hour. It is possible to teach them these two techniques and complete the heart lab all in one 3 hour period. We believe, however, the students are more likely to

appreciate the beauty and dynamic nature of embryogenesis if they can concentrate on general anatomy and techniques in the first lab and the development of the heart in the following lab.

The four techniques the students utilize in this lab are: windowing a chicken egg, explantation of the living embryo into a dish of warmed saline, removal of the beating heart, and isolation of the heart chambers. The first two of these are very straightforward and the students can learn by observing our demonstrations. The latter two techniques, however, require us to demonstrate them while we are looking through a dissecting microscope and the students observe our manipulations. If you have a dissecting microscope with a camera attachment, the students can easily watch the monitor instead of you. Another approach would be to create a video of the procedures (using a camera attached to the microscope) and use this as the teaching tool. Currently, we are planning to produce such a video which we would then make available to any instructor.

## Chicken Eggs

As for the stars of the show, the chicken eggs, they can be bought from a local chicken farm or an agricultural school. It is easiest to get them at the correct age on the day that you need them; this way you do not have to worry about incubating them for a long period. With regard to egg incubation, you can purchase a Styrofoam egg incubator from Carolina Biological Supply (about \$44 US). The incubator will keep the eggs warm throughout the lab period and can also be used to raise the embryos over a long period of time, if needed. An automatic egg turner from Carolina Biological Supply (about \$49 US) will simulate the natural egg rotation necessary for proper development. Normally, about five eggs per student or student pair will suffice. It is recommended that you order at least 25% more eggs than you plan to use since many are infertile or abnormal.

## Solutions

There are only two or three solutions you need to make and the most crucial is the chick saline (a.k.a. Howard's Ringers). The caffeine and alcohol stock solutions are made with chick saline.

### *Recipe for Chick Saline*

#### Materials:

- 7.20 grams NaCl
- 0.23 grams  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$
- 0.37 grams KCl
- 1 liter of distilled water

- Combine all the above ingredients and adjust the pH to 7.2 - 7.3.

### *Recipe for Caffeine Stock Solution*

#### Materials for 2% Caffeine Stock:

- 200 ml of chick saline solution
- 4 grams of caffeine (Sigma)

- Combine all of the above ingredients and stir vigorously for 20 minutes.
- Slightly warming the solution aids dissolution and higher concentrations may precipitate out.
- Dilutions are made with chick saline solution.

### *Recipe for Alcohol Stock Solution*

#### Materials for 25% Gin Stock Solution:

- 200 ml of chick saline
- 50 ml of pure gin (94.6 proof or 47.3%)

- Combine the above ingredients.
- 100% Ethanol may be substituted for gin.
- Dilutions should be made with chick saline.

## Temperature, Temperature, Temperature

Have you ever heard the one about the three most important factors in choosing a house to buy? For this lab, the three most important factors are: temperature, temperature, and temperature. In order for the heart rate to stay normal and constant (about 100 beats per minute), the embryos must be maintained at 37°C. This can be easily controlled by having a steady supply of warm chick saline. To ensure that it is warm enough, we keep about six 200 ml bottles of the chick saline in a 45°C water bath. This temperature is a little higher than the normal body temperature (37°C) but since the bottles are continually being removed from the water bath, it probably all evens out. We encourage the students to use the saline and then promptly return it to the water bath. They probably shouldn't keep a bottle on their bench for more than five minutes. A test tube rack should also be placed in the water bath. It will be used to keep the test tubes of the students' drugs warm; they can store or remove their tubes as they perform their experiments.

The other important way in which to keep an embryo at optimal temperature is to place a lit gooseneck lamp close to, if not over, the embryo. Last year, one of us even discovered that the wattage of the bulb can make a huge difference; 60 watts was too low and 100 watts seemed ideal. The only drawback to this method is the *in vivo* windowed embryos will quickly dry out under the hot lamp unless the students periodically add drops of chick saline. Fortunately, the lab doesn't require the embryos to stay in this state for very long and most of the work is done *in vitro* in a dish of warm chick saline. Unfortunately, the heat created by the lamp could burn the students hands. We have not found this to be a problem, nonetheless students should be warned to be extra careful.

## Tools

The most challenging procedure is the removal of the heart from the live explanted embryo. With the correct tools and some simple rules, anyone can do it. The two tools that we have used for heart explantation are iris microdissecting scissors from Carolina Biological Supply (about \$20 US a piece) and microknives (about \$0.02 US a piece). Both have proven to be effective but some students prefer one over the other. If you decide to make the microknives yourself, you can follow Tyler's (1994) directions which are similar to the following:

### *Directions For Making Microknives (modified from Tyler, 1994)*

#### Materials:

Fresh single edge razor blades  
 Wooden applicator sticks (about 2 mm diameter and 100-150 mm long)  
 Super glue  
 Pair of pliers or tin snips

- Break the razor blade into small fragments with the pliers or tin snips, making sure that each fragment has the cutting edge on it. Avoid damaging this edge. The cutting edge should be up to 2 mm long and the fragment should be between 5 and 10 mm long.
- Soak one end of the wooden applicator stick in water for about 5 minutes; this will soften the wood so that the tip can be split.
- Make a 5 mm split down the center of the moistened end of the stick with a fresh razor blade. Do not splay out the two sides of the split; just create a crack in the wood large enough for the razor blade fragment to fit into.
- Carefully insert the rough edge of the razor blade fragment into the split. You can't use the pliers to do this because they will damage the cutting edge. Thus, you must use your fingers. Be careful! The cutting edge of the blade should not be embedded in the wood at all and should be oriented

## 90 Chicken Embryonic Heart

about 45 degrees to the axis of the stick. You should be able to hold the stick comfortably like a pencil and have the razor's edge almost flat with a desk surface. Let the wood completely dry.

- Paint the split edges with super glue so the razor blade fragment will be held in place.
- Store the microknives so their edges are protected. One method is to stick Styrofoam peanuts on the razor edge.

**Note:** The microknives can only be used for one lab period. They dull quickly.

Another useful tool to have for emergencies is a small plastic ice cream spoon that has holes in it. These are great for rescuing an embryo that has sunk down into the yolk during explantation. Like the microknives, these are cheap (and often free) to make. Most ice cream stores will willingly donate a few in the name of science.

### *Directions For Making an Embryo Spoon (Tyler, 1994)*

Materials:

Small, sample size, plastic (not wooden) ice cream spoon  
Insect or dissecting pin  
Bunsen burner or alcohol flame  
220 and 400 grit sandpaper

2. Warm the pin over the flame and use it to melt a small hole in the back of the end of the spoon.
3. Repeat this process so there are about a dozen holes.
4. Use the sandpaper to remove the sharp plastic edges.

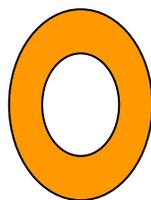
One of the techniques that is used to explant chicken embryos requires filter paper donuts.

### *Directions For Making Filter Paper Donuts*

Materials:

Whatman #1 filter paper (3.2 cm)  
Fine scissors

1. Fold paper in half, cut out a semi-circle.
2. Cut out a smaller semi-circle in center of larger one. The end result should be an oval shaped donut.



**0.5 cm**

**Figure 4.1 Oval shaped donut.**

## **Techniques for Heart Removal**

The prospect of surgically manipulating such a tiny embryo usually strikes great doubt in the students - "There is no way that I can cut out that heart AND keep it beating!" Fortunately, it is not as hard as it seems because the chicken embryo is a very resilient subject; you can put considerable tension on its tissues and it will not be destroyed.

There are a couple of tips that make this procedure easier. First, you need the correct lighting; each student should spend some time changing the angle of the light to achieve maximum clarity under their microscope. Secondly, the fact that the heart is beating makes it very easy to keep track of,

even when it is tangled up with the extraembryonic membranes and vitelline envelop. This, again, reveals why it is so important to keep the *in vitro* embryo's heart beating at the normally high rate.

There are two general methods for removing the heart using the iris microdissecting scissors or microknife. One is to simply cut the heart out of the embryo's chest without removing any other tissues beforehand. The other method is to remove the head and then the trunk below the heart; you are left with the spine right behind the heart and the heart itself. Then, you can hold on to the spine and cut out the heart. In either case, you are usually left with a heart that needs to be separated from the various membranes. This is where the students need some patience. They need to unwrap the membranes from around the heart using forceps (two pair helps the process), and then use their cutting tools to cut away these membranes. It is okay to stretch the tissues, the embryos can handle it.

## Student Outline

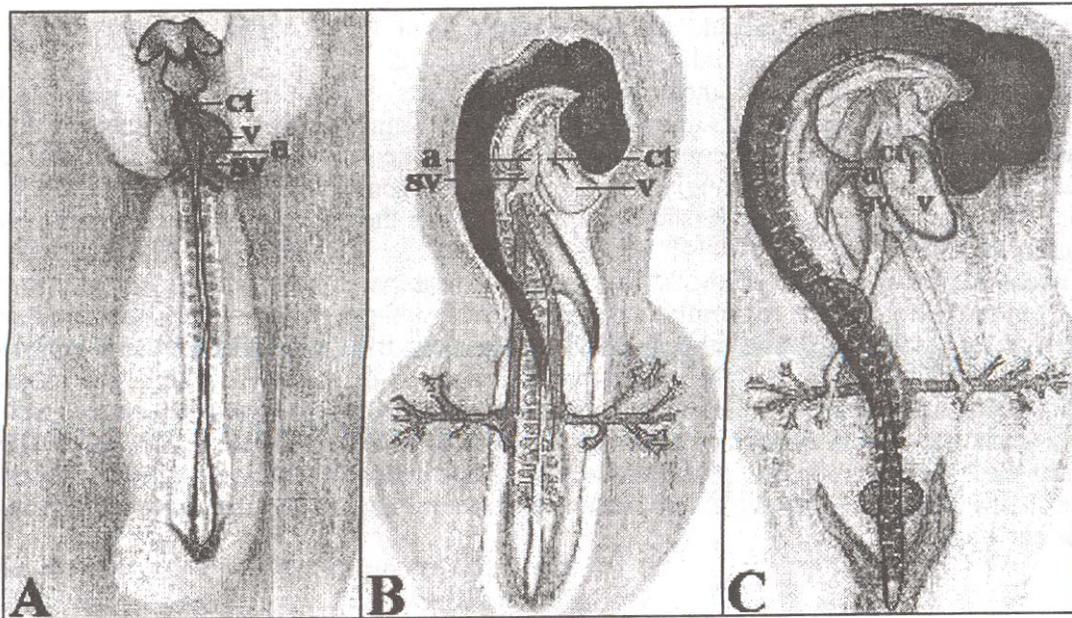
### Introduction

The development of the heart involves a multitude of morphogenetic mechanisms, including a series of cellular migrations, fusions, and specific differentiations. The heart of the chicken embryo develops from the fusion of paired precardiac mesodermal tubes located on either side of the developing foregut, on the ventral surface. Between 25 and 30 hours of incubation, the paired heart vesicles begin to fuse at the anterior (head) end and continue to fuse posteriorly to form one continuous tube. After fusion is complete, the heart tube is ventral to the foregut and has four distinct regions that can be identified from anterior to posterior: *conotruncus*, *ventricle*, *atrium*, and *sinus venosus*. Blood flows anteriorly, from the sinus venosus to the conotruncus. At approximately 33 hours the heart tube bends to form an "S" shape, with the prominent ventricle bulging to the right (Figure 4.2A).

By 48 hours, 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 conotruncus. The ventricle is U-shaped and in the medial ventral position. The blood flows posteriorly and then makes a sharp turn to flow anteriorly (Figure 4.2B).

In the 72-hour chick embryo, the atrium has begun to expand to the left in preparation for 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 is via the right side of the atrium (Figure 4.2C). This is the first step towards the sinus venosus becoming part of the future right atrium. The conotruncus will eventually give rise to the aorta.

The heart begins to beat just after the paired heart rudiments begin to fuse, immediately before the conotruncus forms. Once the heart tubes have completely fused, the sinus venosus becomes the *embryonic pacemaker*. Eventually, when the atrium and ventricle each divide into a pair of chambers, and a typical four-chambered heart is present, the sinus venosus is incorporated into the right atrium where it gives rise to the sinoatrial node, *the mature pacemaker*.



**Figure 4.2** Chicken heart development. A. Dorsal view of a 33-hour chicken embryo. The conotruncus (ct), ventricle (v), atrium (a), and sinus venosus (sv) are evident within the S-shaped heart. B. Right side of a 48-hour chicken embryo. The heart is now U-shaped, with the ventricle (v) and sinus venosus (sv) lying most posterior, while the atrium (a) and conotruncus (ct) are anterior. C. Right side of a 72-hour chicken embryo. The atrium is now expanded on the right and left sides.

### Objectives

*This lab has six goals:*

1. To identify the anatomy of the developing chicken heart.
2. To determine if the heart muscle has an intrinsic ability to contract by surgically removing a beating heart from the living embryo.
3. To determine which region of the chicken heart controls the heart beat by isolating *the atrium*, *ventricle*, and *sinus venosus* and then observing which regions continue to beat while in isolation.
4. To determine the direction of blood flow through the developing heart.
5. To show how *in vivo* and *in vitro* micromanipulative techniques can be used to study developmental and physiological processes.
6. To observe the effects of various concentrations of caffeine and/or gin on an embryonic heart. Your written lab report will be based on this component.

For the last component, you and your lab partner must design an experimental protocol which you will bring to lab. You should consider the following: how you will administer these drugs to an explanted chicken embryo, what concentrations you will use (you must have at least three concentrations), what will be your control, what kind of data you will collect, how you will collect the data, how many repetitions you will carry out, and how you will represent these data in their final form. Finally, you must have a clearly stated hypothesis. At the beginning of the lab your protocol will be reviewed.

**Materials per Student Pair**

72-hour old chicken embryos/eggs, in 37°C humidified incubator (7)  
 110 mm diameter glass dish, lined with cotton (2)  
 Scotch Magic Tape (1)  
 20 G needle attached to a syringe (1)  
 Fine scissors (2)  
 Fine forceps (2)  
 65 mm Syracuse glass dishes (2)  
 Chick saline in 45°C water bath (100 ml)  
 Test tube rack in 45°C water bath  
 Filter paper "doughnuts" (8)  
 Microknife (Tyler, 1994) and/or iris microdissecting scissors (2)  
 Test tubes with caps (6)  
 Test tube rack on bench top (1)  
 1.5 ml dropping pipettes (6)  
 Embryo spoon (1)  
 Gooseneck lamp with 100 W bulb (2)  
 Large beaker for eggs/embryos after they have been examined  
 Dissecting microscope with lighting from above (2)  
 Labeling tape and indelible ink pen (1)  
 Atlas  
 Stock solution of 3% caffeine, made with chick saline (500 ml per 24 students)  
 Stock solution of gin, made with chick saline (500 ml per 24 students)  
 Stop watch or clock with second hand  
 Heart models

**Making a Windowed Egg (Modified from Cruz {1993})**

1. Pick up an egg from the incubator, maintaining its horizontal position as you carry it back to your bench. Place the egg, in that horizontal position, in the glass dish that is lined with cotton.
2. Place Scotch Magic tape along the long axis of the egg, so that it covers most of the "top" of the egg. Place 2 more pieces of tape on the egg, on either side of the center piece.
3. Cover the rounded end of the egg with a small piece of Magic tape.
4. Puncture the rounded end of the egg that is covered by the tape with a 20 G needle. Insert the needle into the egg so that the needle is pointing down. Be careful of your fingers!
5. Withdraw 1-2 ml of albumen. This allows the embryo (if there is one) to move away from the upper surface of the egg, where you will be cutting out the window. Save the syringe and the albumin.
6. **Carefully** puncture the taped-covered top of the egg with the tip of your scissors. (Again, be careful of your fingers.) The location of the puncture should be about a half an inch off center.
7. Proceed to cut out an oval opening, pulling up with your scissors so that you are keeping them as far away from the embryo and vitelline envelope as possible.
8. The size of the opening depends on the size of the egg; it should be about the size of a quarter. With forceps, remove the shell cap, exposing the window.
9. If you are going to observe the embryo for more than a couple of minutes while it is still in the egg, you will need to prevent dehydration. Add several drops of the chick saline solution to the

surface of the embryo. If you are going to explant the embryo immediately, do not add any saline. It will prevent the filter paper doughnut from adhering to the vitelline envelop.

10. **Determine the in vivo heart rate, the number of beats per minute. Record this information in Table 1. Then draw and label a picture of the in vivo embryo (Appendix A).**

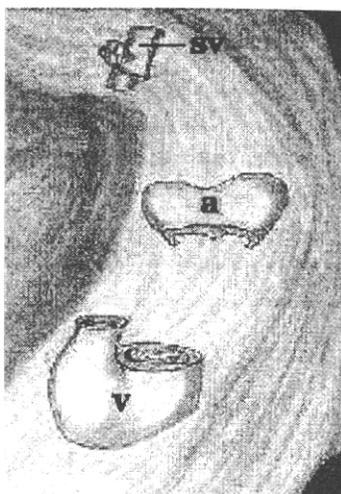
### **Explantation of a Chicken Embryo (Modified from Cruz {1993})**

1. Explantation means an embryo or tissue is removed from its normal environment, in this case the egg, and placed in another location, in this case a dish of saline. This is a useful method because it is much easier to manipulate or operate on an embryo when it is in a dish, rather than in an egg.
2. With the forceps, gently pick off any lumps of albumen on the egg's surface until it appears almost dry.
3. Place a filter paper doughnut on the blastoderm so it frames the embryo. The filter paper will stick to the vitelline envelop, holding the embryo in the center. Cut the filter paper off the surface of the egg, lifting the embryo with it. When you finally free the filter paper, the embryo should be framed within the filter paper. Transfer the embryo to the dish of warm saline solution.
  - With forceps, gently position a filter paper doughnut on top of the vitelline envelope, framing the embryo. Gently pat the doughnut in place with the tips of the forceps. Wait a minute to allow the vitelline envelope to adhere to the filter paper.
  - While waiting, fill one of the small dishes with about a quarter of an inch of warm chick saline solution and place the dish on the stage of your dissecting microscope. Angle the gooseneck lamp so that it is as close as possible to the dish; this keeps the saline solution warm. ***This is a critical step since the chicken embryo's normal body temperature is close to 37°C. Why do you think temperature is such a critical factor?***
  - Hold one edge of the doughnut with the tips of the forceps and cut the vitelline envelope along the edge of the doughnut with a pair of scissors. Slowly work your way around the rim of the doughnut, carefully checking to see that the vitelline envelope adheres to it.
  - Gradually lift the doughnut with the forceps - the embryo should remain within the center of the doughnut. Quickly transfer the explant to the small dish with the warmed chick saline solution. You can keep the embryo "upright" so the right side is facing up, or you can flip over the explant so the surface that was facing the yolk (the left side) is now facing upwards.
  - If the embryo doesn't stay attached to the filter paper use your embryo spoon. Remember to change your saline solution several times since much yolk will be transferred using this process.
4. Place the dish on the stage of the dissecting microscope.
5. To keep the embryo alive as long as possible, place the illuminated gooseneck lamp close to the dish to keep the embryo's body temperature close to 37°C. Periodically add fresh, warm chick saline solution.
6. **Draw and label a picture of the in vitro embryo (Appendix A). In your diagram indicate the direction of the blood flow through the heart.**
7. **Determine the in vitro heart rate, the number of beats per minute. Record this information in Table 1 at the end of the chapter. How does this heart rate compare with the in vivo heart rate?**

### **Examining the Electrophysiology of the Embryonic Heart**

1. The 72-hour old chick explant should be oriented so the right side is facing upwards; this allows you to access the beating heart. Add some fresh, warm chick saline solution.
2. **Determine the heart rate, beats per minute, of the in vitro heart. Record this information in Table 1. How does this rate compare to the in vivo heart rate?**
3. Fill a clean small dish about a quarter of an inch deep with warm chick saline solution.

4. Surgically remove the beating heart by cutting it above the conotruncus and below the atrium or sinus venosus.
5. **Observe the beating of the excised heart and record the following information in your lab notebook and in Table 1.**
  - *Where does the beating begin and end?*
  - *Determine the heart rate, number of beats per minute and record in Table 1. Is it similar to or different from the heart beat in the explanted, in vitro embryo?*
  - *Draw and label a picture of the explanted heart, labeling each region (Appendix A).*
6. If your explanted heart stops beating, change the saline solution and place the dish closer to the gooseneck lamp.
7. Use the microknife or iris microdissecting scissors to isolate each of the two or three regions of the heart. If you can not easily identify the sinus venosus and conotruncus, just cut between the atrium and ventricle (Figure 4.3).



**Figure 4.3.** Explanted 72-hour chicken heart showing isolated heart chambers: sinus venosus (sv), atrium (a) and ventricle (v).

8. **Observe each isolated tissue and record the following information in your lab notebook and Table 1.**
  - *Does each have an intrinsic heart beat? If so, are they synchronous?*
  - *What is the heart rate for each region? Do they beat at the same rate as the in vivo or in vitro heart?*
  - *Draw a properly labeled picture of the isolated chambers (Appendix A).*

### Determining the Effects of Caffeine and/or Alcohol on Heart Rate

1. Prepare the dilutions of the drug with which you chose to work. Make each dilution in one of the test tubes and use the warm chick saline solution to create the dilutions. Store your labeled test tubes in the test tube rack in the 45°C water bath.

2. Design a table similar to Table 1 wherein you recorded all of your previous data. Remember to include both *in vivo* and *in vitro* embryonic heart rates before you expose your embryos to the drugs.
3. Explant an embryo into a clean dish of saline solution. Remember the temperature of the solution that surrounds the embryo has a significant effect on the heart rate. Keep the embryo and your solutions as close to 37°C as possible.
4. Be sure to determine the baseline heart rate for each embryo before exposing the embryo to the drug.
5. Carry out your experiment!

**Table 4.1. Record of observed heart rates (beats per minute)**

Embryo #	<i>In vivo</i>	<i>In vitro</i>	Explanted heart	Isolated heart chambers	
	Embryo	Embryo		Atrium	Ventricle

### Clean Up

1. Put all yolk, albumen, and embryos in a beaker.
2. Throw the egg shells and cotton in the trash.
3. Put the syringes in the red "Sharps" receptacles.
4. Clean forceps, scissors and microknife with a wet paper towel. Be sure to remove all traces of albumen and yolk. Thoroughly dry them with paper towels.
5. Clean the bowls, empty and clean the test tubes, and let them air dry.
6. WASH YOUR HANDS WITH SOAP AND WATER!

### Acknowledgments

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**Appendix A: Student Sketches and Data**

***In vivo* 72-hour Chicken Embryo**

***In vitro* 72-hour Chicken Embryo**

**72-hour Chicken Explanted Heart**

**Isolated Chambers of 72-hour Chicken Heart**