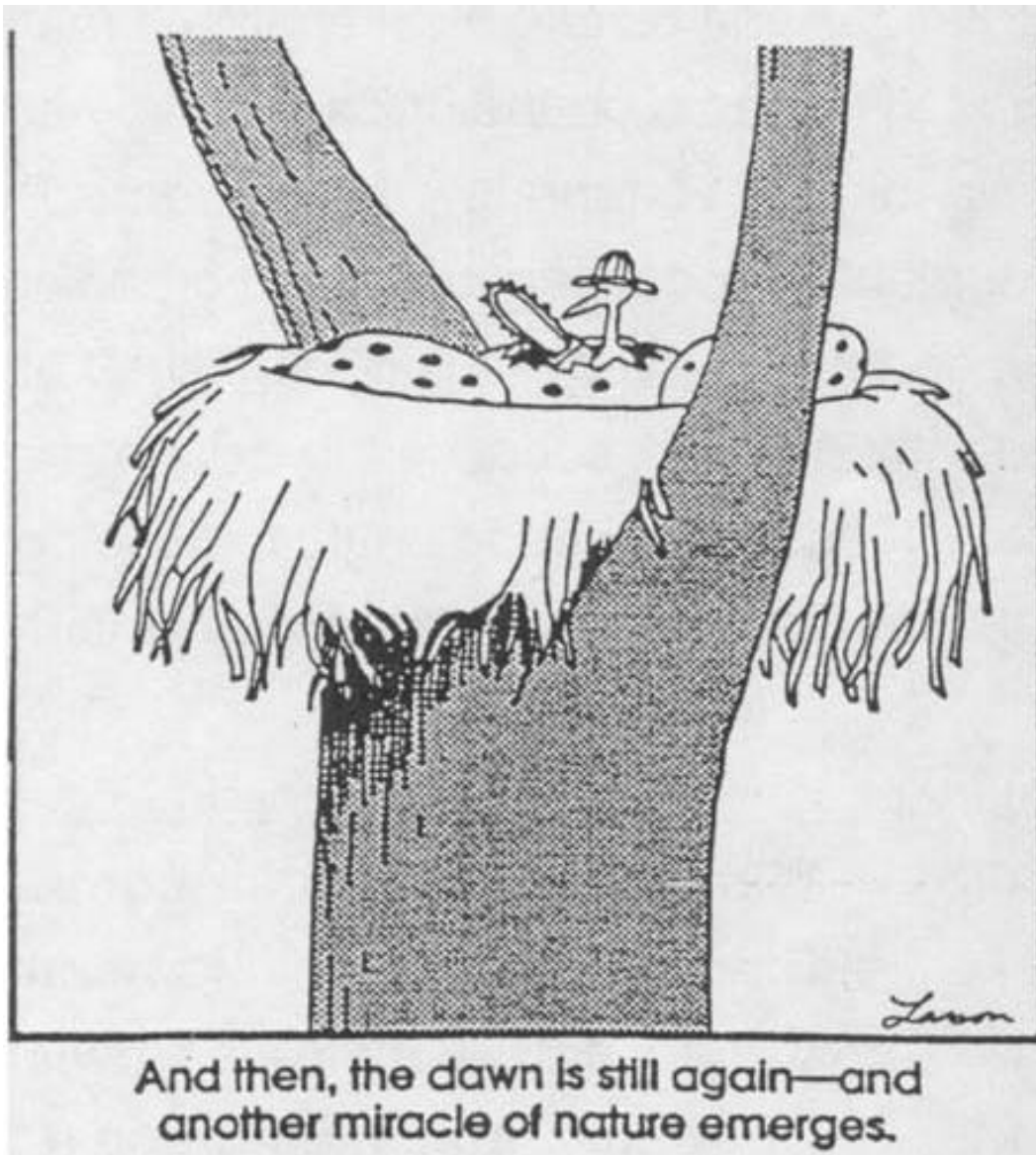


CHICK DEVELOPMENT



The chick embryo is one of the oldest vertebrate models for studying development. It continues to be an important developmental model for several reasons. First of all the morphological changes that occur during chick embryogenesis are very similar to those of other vertebrates. On the practical side, the large size of the embryo and *in ovo* development makes it accessible to surgical and biochemical manipulations and it is easy to observe embryos at different stages and follow them through developmental changes. Additionally, it is easy to obtain embryos year round.

Summary of schedule for chick experiments (February 5-9)

Monday

Basic overview of Vertebrate development

Demo: Window eggs/stain for contrast

Examine normal development at different stages

Tues./ Wed

Labeling of embryonic cells with Dil/DiO

Microsurgeries 2 and 3-day embryos

Demo: Chorioallantoic membrane (CAM) grafts.

Bead Implants

Thus/Fri.

Fix and Image Dil injections

Microsurgeries 2-5 day embryos

Bead implantation at limb bud and somites (RA or FGF)

Optional: CAM grafts (alcian green staining)

THE BASICS: WORKING WITH CHICK EMBRYOS

You will need to learn how to open eggs without disturbing the embryo, and how to visualize the embryo once you have access to it. Here are some general considerations for working with chick embryos:

1. **KEEP IT STERILE.** It is very easy to contaminate the eggs. To avoid contamination of the egg, keep your hands, tools, scope and egg as clean and sterile as possible. Before you start, wash your hands with antibacterial soap, then swab your hands, egg holder and stage of scope with 70% ethanol. Keep your tools in container of 70% ethanol and flame glass rods and needles each before use. Swab the surface of the egg with 70% ethanol before opening it, and let dry completely before cutting into the shell.
2. **KEEP IT WARM.** All of the eggs should be kept in the incubator when you are not working on them. Check to see that incubator is at the correct temperature and that there is water in the bottom to maintain the proper humidity. Try to minimize the time the incubator is open and avoid altering the temperature controls. Remove one egg at a time and when you are finished, return it quickly to the incubator.
3. **KEEP IT MOIST.** After you have windowed an egg or manipulated it, drop a few drops of sterile Tyrode's with pen/strep (antibiotics) into the shell, towards the edge, not directly on top of the embryo. This will also help inhibit contamination, but is not foolproof, so use good sterile technique. Be sure tape covers the window to keep it from drying out.
4. **KEEP IT LABELED.** If you plan to keep a windowed egg, you must write IN PENCIL on the egg. Make sure your eggs are clearly labeled so that you know WHAT you did to the egg, WHEN you did it and WHO did it. Make the labels easy to read without turning the egg. Each time you remove an egg from the incubator to examine it, double-check to make sure it is YOUR egg. Be VERY CAREFUL with removing and returning eggs to the incubators because it is easy to cause the windowed eggs to roll over and ruin someone else's experiment!
5. **DISPOSE OF IT PROPERLY.** Please use the egg waste buckets, so we can avoid rotting eggs in the lab room. Freeze older embryos before disposing of them.
6. **BE CAREFUL.** Chickens carry pathogens that can infect humans like *Salmonella*. The eggs we receive are lot-tested to be free of pathogens, but you should wash your hands before leaving the lab or eating. Keep all food and drinks away from the lab benches!

GETTING TO THE CHICK EMBRYO

I. Candling

Before you open an egg, you need to determine the position of the embryo and the air space. In eggs that have been sitting in the same position for at least 30' the embryo should float to the top side. If you are careful not to turn the eggs when you take them from the incubator you can be reasonably confident that is where the embryo will be. You will need to know the position of the air space. Typically it is found at the flat end of the egg. To confirm the location of the embryo and air space you will "candle" your eggs by holding them up to a bright light.

1. Use a fiber optic arm from your dissecting scope as a light source.
2. Remove an older egg (e.g., 3 day) that you are going to window from the incubator.
3. Hold the egg over the light. Move the light source around the egg, starting at the top of the egg. First you will see the yolk as a big blob. Then look more closely for the embryo, which should be on top of the yolk (at 24 hrs, it will be difficult to see the embryo, but when the embryo develops blood vessels it will be a dark shadow on top of the yolk).
4. USING A PENCIL (never write on the shell with anything but a pencil), draw a small circle around the location of the embryo.
5. To locate the air space, hold the egg obliquely to the light. Move the light around until you can see a dark circle around the blunt end (air space). Make a dot with a pencil in the middle of that circle.

II. Windowing

Now you are ready to open an egg in a process called windowing. Not every egg will be viable, so we will try to help you spot "duds" as you are windowing. Fertility rates vary from batch to batch of eggs, so keep track of the total number of eggs you window in your notebook. Sterility is very important for successfully following up experiments. DON'T FORGET TO KEEP IT

STERILE. There is an alternative method that we will demonstrate that you may find much easier and faster (we do).

1. Take one egg from the incubator and swab with ethanol. Let it dry completely.
2. Use a STERILE dissecting needle to CAREFULLY make a small hole just barely through the shell) at the location of the air space (your pencil marking on the blunt end). If anything comes oozing out of the hole you are not in the air space!
3. Place the egg in an eggcup.
4. To lower the embryo in the shell, put a STERILE 20g needle on a 5cc syringe and insert the needle into the air space hole. Make sure you keep the needle pointed downward to avoid damaging the embryo. Withdraw 1-2 mls of albumen and empty it into the waste bucket. Don't take out any more than 2mls, if you lower the embryo too far it will be difficult to image or inject your embryos. Conserve needles by carefully placing it in a location where it is unlikely to stick someone and where it will remain sterile. **CAUTION: AT THIS POINT YOU ARE USING SHARPS AND A POTENTIAL SOURCE OF HUMAN PATHOGENS. DO NOT RECAP NEEDLES. DO DISPOSE OF THEM IN THE SHARPS WASTE. DO BE EXTREMELY CAREFUL AND REPORT ANY NEEDLE STICKS!**
5. Put a piece of scotch tape over the circle you drew to mark the embryo. (The shell you cut should stick to this tape rather than fall down onto the embryo).
6. Using one point of your STERILE scissors or dissecting needle, make a small opening in the shell within the circle.
7. Very carefully, insert your scissors into the opening and cut around the perimeter of the circle (a 2 cm circle is a good size to start with). Cut through the shell and shell membranes, but keep your scissors at a shallow angle at all times so that you do not disturb the embryo. Do NOT make complete cuts with the scissors because this increases the chances you will crack the shell. If you do crack the shell, you may be

able to salvage the egg by placing a piece of tape over the crack. After you are done cutting, lift off the piece of shell.

8. The embryo should be ready for viewing under the dissecting microscope. To enhance the contrast between the embryo and the underlying yolk, we will stain the embryo using a small amount of dye (see staining protocol).

9. If this is an egg you need to keep for injecting or surgery proceed immediately with the manipulation OR prepare the egg for the incubator. Do so by dropping a few STERILE drops of Tyrode's with pen/strep added in the shell, but not directly on top of the embryo. Seal the opening with two pieces of scotch tape at right angles to one another, label your egg, and return it to the incubator.

III. Visualizing the embryo

1) Staining for contrast

Staining chick embryos with a dye like neutral red or Nile blue will increase the contrast of the embryo to enable you to see morphological features better. Note that neutral red is photo-toxic to your embryos, so use it sparingly and limit light exposure to your embryos. Green tape over the light source eliminates the toxic effects but makes viewing difficult.

- a. Position the windowed egg (sitting in egg cup) under the scope. Use lower light to illuminate because the Neutral Red dye is photo-toxic to the embryo.
- b. Dip the STERILE glass tool tip 1-2X into the small tube of dye. You just want a thin coating of dye on the glass.
- c. To begin staining, gently stroke the embryo (or area where you think the embryo should be) with the tip containing the dye. **Wait 1-2 minutes to see how much staining has occurred.** This is important, because it takes a little while to see the contrast between the dye and the embryo. **If** the embryo is still hard to see, re-treat, but try not to overdo the staining (especially if you intend to keep the embryo alive).

2) India ink injection

Another means of increasing contrast of embryos is to use India ink to provide a black background. This is done by injecting a small pool of ink directly under the embryo. As long as

the ink is sterile, and you do not drastically rip the membranes, this method works very well for somite stages and earlier and does not have the problems of phototoxicity.

- a. Prepare a solution of Pelikan india ink, 1 part to 9 parts with sterile Ringer's. Load into a 3 ml syringe equipped with a 27 gauge needle with the tip bent at a 45 degree angle.
- b. Approach the embryo at a shallow angle, with the bevel of the needle pointed up. Pierce the membrane just outside the *area opaca*. When the opening of the needle is directly under the embryo, inject a small amount of ink. Examine the embryo. See III below.
- c. After you are finished and if you want to save the egg, add tyrodes and antibiotic, tape the egg and place it back into the incubator.
- d. Clean tools with ethanol. Wipe down your bench with ethanol and wash your hands.

Embryo Fixation

We will be fixing embryos at appropriate time intervals in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). So that you will be comfortable with this procedure when it is time to fix your "experimental" embryos, you should practice on some spare eggs. We will demonstrate the filter paper method: older embryos can be pulled out of the egg with forceps by their membranes

Filter paper method In order not to damage embryos younger than stages 18 or 19, it is very effective to provide a "frame"- filter paper cut in small discs with the center cut out (donuts). The membranes stick to the filter paper with the embryo suspended in the middle of the hole.

1. Use forceps to pick up the donut and place it over the embryo. The embryo should be centered in the hole and not covered by filter paper.
2. Use your scissors to cut into the membranes immediately surrounding the filter paper ring -just cut around the perimeter of the filter paper).
3. Use your forceps to pick up the filter paper, the embryo should stick to the filter paper. Since the membranes are cut you should be able to lift out the embryo.
4. Place the embryo and filter paper combination into a small Petri dish containing Tyrode's or saline solution for rinsing yolk and observation. Then, go to the hood, remove the saline solution and replace it with 4% paraformaldehyde. WEAR GLOVES, so you do not fix your skin.
5. After fixing the embryo carefully move the dish with the fixed embryo into the refrigerator until the next class.

III. LOOKING AT NORMAL DEVELOPMENT

Now that you know the basics of handling chick embryos we will look at several stages of chick embryogenesis. Some of the experiments we will do in the next few days involve perturbing normal development. In order to become familiar with normal developmental events, it is important that you begin each section looking carefully and recording in your notebook embryonic stages of development. We can help you identify structures, but we also expect you to use the available resources (prepared slides, hand-out figures, books, software, etc.) to do some exploring on your own. We will have available on the computer the classical Hamburger and Hamilton (H H) staging series for chick embryos from J of Morph (1951) 88:49-92.

What do you see?

We have eggs that are at 24, 36, 48, and 72 hours of development. Examine them using the hand-outs, HH staging, and textbooks as references for the names of structures. For a photographic record you can record an image using the Spot camera mounted to a dissecting scope. You may also wish to record the same embryo over a period of days to record its development, just keep it sterile, warm and moist. TIP: when collecting images, be sure to keep a log in your notebook so you will know what you collected and when.

The simplest way to record accessible information is make DRAWINGS in your notebook. Don't worry if you are not artistic.

The following is a list of structures that you should identify.

blastoderm	area pellucida	area opaca
primitive streak	Hensen's node	somites
neural tube	neural groove	notochord
head fold	heart	foregut
limb buds	forebrain	midbrain
hindbrain	optic cup	vitelline veins
chorio-allantoic membrane		allantois

Dil/DiO Labeling of Embryonic Cells

Background and materials:

Embryos are extremely dynamic. Cells move from one place to another, cell phenotypes change, cells divide, cells organize into new arrangements. How can these dynamic changes be studied? One way is to use vital dyes to label a cell at a particular time point and position. By looking at the dye at a later time in development it is possible to determine where that cell has moved and to what tissue it is making a contribution. This method, some times referred to as fate mapping, is important for tracing lineage histories, and it is useful for understanding how the morphology of the older embryos and adult organisms are derived.

In this lab you will be labeling a populations of cells with the fluorescent dyes, called Dil and DiO (pronounced like dye-eye and dye-oh). Dil/DiO are members of a large family of carbocyanine dyes. These are lipophilic and can be used to label cell membranes in lineage tracing experiments. The fluorochrome consists of a pair of conjugated rings that are each attached to special side groups in a specific position (Dil has an isopropyl group, DiO has an oxygen in this particular location). The two rings are linked together by an odd number of carbons. The length of the carbon chain connecting the two rings and the specific group attached to each of the rings determines the dye's excitation and emission properties. The conjugated rings are in turn linked to two long alkyl chains whose length determines the dye's affinity for the membrane. For Dil, excitation and emission spectra are similar to the rhodamine optical filter sets. For DiO, use the fluorescein filter sets.

The advantages of these dyes as lineage tracers include brightness and photo-stability. Because of the lipophilic nature of the compound there is no need to inject directly into cells. Focal injections will label small numbers of cells to be detected later in development. Cell morphology and cell processes can be clearly seen in freshly labeled cells. Dil can also be used retrograde label neurons and has been used to label a variety of cell types and organisms. There are also disadvantages. The dye becomes internalized into vesicles over time, some forms cannot be fixed easily, high concentrations are toxic, and some claim that dyes with shorter alkyl chains can 'flipflop' between membranes and transfer to label neighboring cells.

Before lab you should read about neural tube formation and neural crest migration. Neural crest cells originate from the dorsal region of the neural tube and migrate throughout the embryo to produce a wide range of differentiated cells types (such as neurons, pigment cells, endocrine cells, cartilage, bone and connective tissue).

We will show you how to inject using a mouth pipette and capillary needle. We will also demonstrate the micro-manipulator/picospritzer method of injection.

1. To label **neural crest cells** before they migrate in developing chick embryos, we will use eggs that have been incubated for about 48 hrs and have approximately 16-22 somites (HH stage 12-14).
2. To track changes in the position of cells in the **Lateral Plate Mesoderm (LPM)**, relative to the main body axis we will inject small foci of dye into the LPM at pre limb bud stages

(12-14). One dye will be used to mark a particular somite, and the other to label the adjacent LPM.

Read up on these two cell populations before class.

Injection procedures

Window several eggs (do one at a time and then return them to the incubator). To insure that your micro-injected embryo survives, remember to do the following:

- a. Use sterile technique. Wipe egg with 70% ethanol and sterilize tools using ethanol.
- b. Try to window the eggs quickly. Keep the egg out of the incubator for less than 15 minutes.
- c. Only remove a small amount of albumin (<2 ml) using the syringe. It is easier to inject the embryo if it is closer to the surface of the egg.
- d. Make a small window. You can enlarge it later if necessary.
- e. Stain the embryo LIGHTLY with Neutral Red or use ink injection.

Once the embryo is labeled:

- f. Place 2 drops of Tyrode's with pen-strep onto the yolk.
- g. Label your egg with pencil. Seal with tape and return it to the incubator. Make sure that the tape used to cover the window seals tightly.

Mouth pipette injection: Inject the closed neural tube with DiI. We will demonstrate how to use the mouth-pipette to fill the ventricular space, and label the pre-migratory neural crest cells, or the LPM.

Picospritzer injections: When it is your turn to inject, bring your egg to the injection station.

Backfill a fine-tipped filament-bearing needle with some of the diluted DiI solution. The needles will be provided and the method of filling will be demonstrated.

Mount the needle into the micro-manipulator holder. The needle will be connected to a 'picospritzer' (a machine which delivers a small measured puff of air under a defined pressure).

Bring the needle into position using the manipulator. Once the needle is inserted into the embryo, pump the foot peddle once to deliver the dye. You should see the dye spreading through the neural tube if your needle is in the correct location. (It is best if you don't overload the neural tube with dye so only inject a small amount.) If the dye is in the wrong place, withdraw the needle and try again. Keep in mind repeated injections will reduce the chances of embryo survival.

Embryo Fixation

Approximately 24 hours after the Dil injection you will need to remove the injected embryo from the egg and place it in fixative (4% paraformaldehyde (PFA) in phosphate buffered saline).

Examining your embryos

1. To examine embryo, remove the fixative in the hood and dispose of it in designated waste containers.
2. Replace the fixative with saline (PBS).
3. Lift the embryo out of the saline and place it onto a glass slide - filter paper and all.
4. Make a footed cover slip to prevent the cover slip from crushing the embryo. To do this scrape the four corners of a cover slip on modeling clay to make 'feet'. Gently place the cover slip on top of the embryo. Blot excess liquid from the slide.
5. Now examine the embryo using fluorescence microscopy. You may have a fairly "thick mount", so be careful with higher power objectives. You will not need high magnification to see the labeled cells. What are you looking for? What do you see? Do the labeled neural crest cells follow a predictable path? Are the labeled LPM cells at the same somite level as when injected or have they moved?

Manipulating signaling molecules using bead implantation

Tiny beads can be used to apply biologically active compounds to the embryo to test the effects of these compounds on gene expression and morphogenesis *in vivo*. We will have available two types of beads carrying different morphogenetically active compounds- retinoic acid on ion exchange beads and fibroblast growth factors on heparin acrylic beads (diameter = 200-250 μ m).

Below are tried-and true suggestions for experiments, but feel free to research and design your own.

Effects of retinoic acid on limb development

The posterior margin of an early limb bud contains a signalling center known as the Zone of Polarizing Activity (ZPA). If the ZPA is grafted onto the anterior margin of a host limb bud, a limb with duplicated digits develops. These duplicated digits are mirror images of the normally produced digits. ZPA transplantation experiments suggest that the ZPA is a source of some type of positional signaling activity that specifies the anterior-posterior axis of the developing limb. Some of the signaling molecules originating in the ZPA have now been identified (*i.e.* *sonic hedgehog*). Retinoic acid (RA), a naturally occurring compound, also has polarizing activity, but is very difficult to detect in embryos. When beads containing retinoic acid or *shh* protein are transplanted to the anterior margin of a host limb bud, the same digit duplications seen with ZPA transplantation were created.

We will use beads soaked in RA to alter limb development in 3.5 -5 day embryos.

Effects of fibroblast growth factor on development

Fibroblast growth factors (FGF) form a large family of heparin-binding proteins that stimulate the proliferation and activation of cells that express FGF receptors. FGFs are probably active in all aspects of development, and we will experiment with two systems, **A]** somitogenesis and **B]** limb development.

A] During stages of somitogenesis, FGF-8 is expressed in posterior presomitic mesoderm (PMS) and plays some role in segmentation. We will place FGF 8 soaked beads in the PMS to examine the effect of this protein on somite formation. For somitogenesis experiments, eggs should be incubated 2-3 days and embryos should have 12- 18 somites-

B] FGF-8 (and FGF-2) are expressed in the thickening of the ectoderm at the apex of the limb bud known as the apical ectodermal ridge (AER), and appear to be important for the proliferation of limb bud cells.. FGF-8 has been shown to induce ectopic limbs. We will attempt to induce ectopic limbs in the mid flank of chick embryos by implanting FGF-8 soaked beads. For limb bud experiments, eggs should be incubated 3.5 -5 days.

Bead protocols:

Bead Prep

1. Ion exchange beads and RA:

- a) rinse in 0.5-1 M formic acid in 15 ml tube
- b) rinse in H₂O to pH 5.5
- c) dry on petri dish at 37°.- store dry
- d) soak in 10⁻² (3 mg/ml) RA in 100% EtOH. NO MORE THAN 20 min.
- e) rinse in DME media - will turn pink

2. Heparin acrylic beads and FGF:

- a) rinse beads in PBS
- b) place drop of 1mg/ml FGF in PBS (~20 beads in 2µl drop in petri dish)
- c) soak at 4° for 1 hr or more (no max)

Implantation

1.Window an egg of appropriate stage.

- a) 2-3 day for somitogenesis,
- b) 3-5 day for limb bud

2. Using a scapel or tungsten needle, make a small, shallow slit as follows:
 - a) at the lateral edge of the PSM, approximately halfway between the last formed somite and the tail bud.
 - b) at the anterior margin of a developing wing bud (opposite somites 16-17).
3. Using fine forceps get a bead soaked in RA or FGF. With the use of forceps and needle, insert the bead into the slit. Take care to make sure the bead stays in place- this can be the hardest part.
4. You may want to image your surgery, then reseal your egg and return it to the incubator.

What should be used as a control?

You will need to wait 2-3 days before examining the results of your experiments.

OPTIONAL EXERCISES:

Chorioallantoic membrane (CAM) grafts

Rationale:

This experiment is an example of an embryological manipulation where you can ask whether a tissue at a particular stage relies upon adjacent tissues for patterning information and whether or when it is competent to fully pattern itself. To do this you have to keep the isolated tissue alive by organ culture. There are many methods of organ culture used for different developing organisms. In this lab you will use the heavily vascularized chorioallantoic membrane (CAM) of a "host" chick to grow a limb transplanted from a younger "donor" chick. The CAM provides nutrients, waste removal and gas exchange for the grafted tissue. **NOTE:** some optional exercises are listed at the end of this section-you may wish to try those in addition to the standard CAM graft.

Two important points to think about before you come to class: how are you going to record an image of your graft so you will have a "before and after" image of your experiment? what will be your **control** so that you will be able to document normal limb development (or development of whatever structure you have grafted?) Before this lab you will want to read in the text about limb development. It may give you ideas for additional experiments.

1. **Host embryos**

Figure 12.1 shows an embryo after 9-10 days of incubation. The diagram shows the chorioallantoic membrane. It is a heavily vascularized extra-embryonic membrane that grows as the embryo grows. Its derivation is a fusion of the allantois (a derivative of the primitive urinary

bladder) with the serosa (the original extra-embryonic ectoderm and mesoderm). This combination provides two extra-embryonic epithelial layers (ectoderm on the outside and endoderm on the inside) with mesoderm in between carrying blood vessels that will provide the vascular bed for the graft. Trace the allantoic stalk back to the embryo and you will see that it originates in the site where the umbilicus is derived. In mammals the same chorioallantoic membrane is absent but the vascular remnants of the chorioallantoic membrane provide the major vascular bed in the fetal placenta.

2. Preparation of host eggs

After 72 hours of incubation, we windowed several dozen eggs for you to use as transplant hosts. They should now be 10 days old. Normally at this stage the CAM is close to the inside of the shell above the embryo. Windowing then threatens to damage the membrane irreversibly, which is why we did the windowing for you at 72 hours.

3. Donor limb buds

Window an embryo that has been incubated for two or three days (three will be easier to start with). Gently tear away the membranes above the embryo. Avoid puncturing the yolk sac because this will cause death of the embryo. Find the limb buds on the exposed embryo. (See Figure 1 1.1 b) If you are careful, a single embryo can provide enough limb buds for several grafts. You will have the greatest likelihood of success if you perform the surgery as quickly and cleanly as possible; the longer you muck around with an embryo, the more likely you will kill it and/or damage the limb bud.

The traditional method of removing a limb bud uses a glass or tungsten needle that has a sharp bend near the tip to sever the limb. If you hook the sharp tip of the needle under the limb bud and very gradually tear it away from the embryo, you will probably be OK. However, it is very easy to mangle the limb bud to the point that it will not "take" when placed on to a host CAM. It is essential that the grafted limb bud form a connection with and become vascularized by the CAM within 24 hours. Another method you can use to remove limb buds is to simply "clip" them off with a pair of microscissors. This is much quicker and leaves a cleaner edge on the limb bud that can be grafted onto the host CAM.

Once you have severed a limb bud carefully pick it up with a glass pipette or forceps and transfer it to the donor egg. **If** the host is not ready you can temporarily transfer the donor limb to a small dish of sterile Tyrode's solution. If you have been extremely careful in your surgery and the chick is still alive you can keep the donor egg to also see whether the chick will replace the removed limb. Alternatively you can remove all the fore- and hindlimbs from this donor.

4. Preparing the host site (do this just before you are ready to make the graft)

With your limb ready, locate the chorioallantoic membrane. It should be just beneath the window and will be highly vascularized. (See Figure 12.1) To prepare the donor site on the CAM you will need to slightly injure the membrane and cause it to ooze a little bit of blood. This can be done by scratching the surface of the CAM with a sharp tungsten needle. Place the cut side of the

graft over the wounded area/blood, hold it there for a few seconds until it sticks. Then close the window and return the egg to the incubator.

Your finished graft will now be incubated for 7 days until the host is 14-16 days into development and the graft is about 10 days into development. You should take a peek at the graft during the next lab period to see if it is alive. If it did not survive or is contaminated, DISPOSE OF IT IMMEDIATELY.

5. Recovering the graft (after 7-10 days)

After a week open the window over the host embryos and look for the graft. If the host embryo is alive and you do not immediately see the graft it may have been surrounded by the CAM and moved below the surface. So, you may need to transfer the embryo out of the egg into a dish in order to search for the graft.

6. Image the graft.

Cut the graft free of the CAM and wash it in saline to clean it up. Then place it in a dish of saline under the imaging dissecting microscope and record your success. As a comparison, record images of your control. Do you get a wing from a forelimb and a leg from a hindlimb? Is there any difference between the experimental and control limb? Are there differences between grafts from two and three day donors? What do we mean when we say that the limb develops autonomously?

If you have a successful limb graft, you may want to fix, clear and stain it using a cartilage-specific stain (alcian blue). Protocol is at the end of this section.

Clearing and Staining to Visualize Skeletal Elements

Rapid Method with Alcian Green

1. Fix embryos in 10 formalin or 4% PFA. o/n at 4 degrees, or minimum of 6 hours at RT.
2. Rinse in PBS, 2 x 5 minutes
3. 5% trichloroacetic acid- 3hr.s
4. Wash in dH₂O
5. 70% EtOH 10 minutes
6. 70% acid EtOH 10 minutes (1ml HCl/100 mls EtOH)
7. 0.1% Alcian Green 2GX in Acid EtOH. 3 hr.s
8. 70% acid EtOH O/N
9. 70% EtOH 10 minutes
10. Abs. EtOH 30. min. x 2
11. Methyl Salicylate in GLASS

Cell Death and Digit Formation

This lab procedure should allow you to examine areas of cell death in developing hind limbs. Programmed cell death causes the disappearance of the inter-digital tissue (webbing between the toes) so that each digit becomes separate from the others. This is a quick experiment.

1. Start with 5 day old eggs. You should also try older embryos (6-7 days) which have more developed limbs.
2. Window the egg. Make your window fairly large.
3. Make a large hole in the vitelline membrane and a smaller hole in the amnion. You must do this so that the stain can reach the embryo. Tear these membranes carefully so that you don't damage the embryo.
4. Add one drop of the vital dye Trypan Blue (0.4%) to the embryo.
5. Wait 2-3 min until you can see that the embryo is taking up the stain.

Continue slowly adding stain, one drop at a time, until discrete areas are visible, these are the areas of cell death.

6. Record the results in your notebook or capture images on video.
7. Discard the egg and embryo once you are done.

Transplants and Surgical Ablations:

The following experiments will give you some basic experience with micro-surgery in chicks. Using spin-offs of this technique you may want to design other experiments. The following questions may help give you some ideas:

1. Are plans for wings and leg established at the same time?
2. Are the limb axes established at the time of grafting?
3. Can the graft regulate for missing pieces of the limb bud at this early stage?
4. Can other tissues be grafted?
5. Can a chick regenerate a limb, somites?

HINTS: (These are even more challenging surgeries,

Switch a forelimb bud and hindlimb bud.

Remove a limb bud, flip it over and graft it back to the same location.

Remove a portion of a limb bud being used in a graft.

Graft other tissues like eyes (2 days), posterior portion (2 days), dorsal tissue makes a good skin graft (5-6 days) for CAMS, bone (3-4 days)

An interesting set of experiments can be done to examine the function of the **apical epidermal ridge** (AER).

"The apical epidermal ridge runs along the distal margin of the limb bud and will become a major signaling center for the developing limb. Its roles include 1) maintaining the mesoderm beneath it in a plastic, proliferating phase that enables the linear (proximaldistal) growth of the limb 2) maintaining the expression of those molecules that generate the anterior-posterior (thumb-pinky) axis and 3) interacting with the proteins specifying the anterior-posterior and dorsal-ventral axes so that each cell is given the instructions on how to differentiate." -from Gilbert, Developmental Biology

It is possible to gently tear the AER off of a limb bud. The use of trypsin makes tissue removal easier.

What happens to limb development if the AER is removed?

FGF's can rescue the development of limbs after the AER is removed. Try bead implants to demonstrate this.

If you get really good at removing the AER, try performing the surgery on embryos of different stages (best to use stages 18-21 or 70-84 hrs). Be sure to perform appropriate controls. As before with other surgeries, tape, label and return eggs to the incubators.