Chick Lab
MBL Embryology Course
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OVERVIEW OF POSSIBLE EXPERIMENTS

“Cut and paste” experiments
1. Transplant neural tube, notochord or somites to an ectopic location, or rotate them. Label afterwards with markers for neural tube cells, neural crest, muscle precursors, or somitic sclerotome. Notochord grafts next to the neural tube should give ectopic floor plate and motor neurons, while rotating somites or segmental plate mesoderm should alter the pattern of trunk neural crest migration and motor axon outgrowth.
2. Limb manipulations: remove or transplant the apical ectodermal ridge (AER) or zone of polarizing activity (ZPA); use molecular markers or cartilage preps to look for effects on limb outgrowth and polarity.
3. Transplant Hensen’s node (the chick organiser) ectopically and look for secondary axis formation.
4. Regulative development of the early chick embryo: cut the pre-primitive streak embryo in half, separating anterior and posterior halves, and grow them separately. Look for axis formation in the isolated anterior half.

Fate-mapping experiments
1. Microinject the fluorescent vital dye DiI, or electroporate GFP into the neural tube to label premigratory neural crest, motor axons, or other neural tube cells.
2. Label neurogenic placodes (cranial ectoderm) with DiI and look in vibratome sections at their contributions to the sense organs and cranial ganglia.
3. Label rostral intermediate mesoderm with DiI and follow migration of the pronephric duct.
4. Electroporate fluorescein-tagged control morpholinos in pre-streak embryos to follow cell movements (the embryos survive better after electroporating morpholinos than plasmids, for unknown reasons).

Manipulating signaling pathways using protein-soaked beads
Alter particular signaling pathways in your tissue of choice by implanting a bead soaked in your favorite protein. For example, you can alter segmentation of the presomitic mesoderm by implanting a bead coated with FGF8 protein, or you can implant an FGF8- or FGF4-soaked bead in the anterior part of pre-primitive streak embryo to cause ectopic axis formation. See list of available reagents.

Manipulating gene expression using electroporation
Electroporation is used to target DNA constructs to specific tissues at specific times. Once somites start to form, embryos can be electroporated in ovo. Younger embryos must be electroporated and cultured ex ovo: we will demonstrate this using New culture. Epithelial cells (neural tube, ectoderm, endoderm, epithelial somites) are electroporated more efficiently; paraxial mesoderm can be electroporated before gastrulation.
1. Electroporate DNA (GFP, or other constructs) into your tissue of choice (remembering that epithelia are easier to transfec), either in ovo (e.g. in the neural tube) or in pre-streak/streak-stage embryos ex ovo, in New culture.
2. Electroporate constructs to block or activate different signaling pathways (see list of available reagents) in your tissue of choice (either in ovo or in pre-streak/streak-stage embryos ex ovo, in New culture).

To analyze your results at the molecular level, you can use whole-mount in situ hybridization or antibody staining, and section the embryos if necessary using a vibratome. See list of available reagents.

Embryo stages: abbreviated staging tables (including photos) are at the back of the handout; full staging tables will be available in the lab. We will aim to have the following stages available on most days: stage 3 (for streak-stage experiments), stages 10-14 (for in ovo electroporation, cut-and-paste experiments, DiI labeling, etc), and stage 20 (for limb bud stage experiments). Unincubated eggs should also be available for pre-primitive streak stage embryos. Please bear in mind that we cannot guarantee that we will have embryos of exactly these stages on the day, though we will try our best!
# CONTENTS

**Introduction to the Chick Embryo**

**Experimental ideas based around particular developmental systems**

- Axis Formation
- Neural Patterning
- Neural Crest Cell Development
- Limb Development

**Preparation and handling of embryos**

1) Incubation:  
2) Opening the Egg and Exposing Embryos:  
3) Enhancing Visibility of the Operative Field  
4) Working with the Embryo  
5) Hydration  
6) For Further Development  
7) Collecting Embryos  
8) Fixing Embryos

**“Cut and paste” experiments**

1) Axis formation  
2) Neural Crest Ablation/Grafts  
3) Somite manipulations/removals  
4) Notochord grafts  
5) Notochord/neural tube cultures  
6) Limb bud manipulations

**Enzymatic isolation of chick tissues**

1) Isolating neural tube, notochord and somites  
2) Enzymatic separation of limb tissues

**Culture techniques**

1) Explant culture on filter paper  
2) Explant culture in collagen gels  
3) Micromass limb mesenchyme cell culture

**Fate-mapping experiments**

1) Dil Labeling  
2) Interspecies Grafts

**Manipulating gene expression**

1) *In ovo* electroporation  
   a) targeting left/right side of neural tube  
   b) targeting ventral neural tube  
2) *Ex ovo* electroporation (pre-streak and streak-stage embryos)

**Manipulating signaling pathways using beads**

1) Implanting Affigel blue beads or Heparin Acrylic beads  
2) Implanting ion-exchange beads

**Culture and manipulation of young embryos**

1) New Culture  
2) Whole Embryo EC Culture
Detecting apoptotic or dividing cells
Nile blue staining for apoptotic cells
BrdU labeling of dividing cells

Visualizing the vasculature - Indian Ink labeling

ASSAYING YOUR RESULTS

Wholemount in situ hybridization
Whole-mount immunohistochemistry
Vibratome sectioning
Immunocytochemistry on vibratome sections
Cartilage & bone staining using Alcian blue and Alizarin red

Image reference list

Appendix 1: Typical solutions for chick manipulation and care
Making Sylgard plates
Appendix 2: Staging chick embryos
INTRODUCTION TO THE CHICK EMBRYO

The chicken (*Gallus gallus*) embryo is an excellent model for the study of early vertebrate embryogenesis and later organogenesis. The embryo is encased within a hardened eggshell which provides a natural incubator or culture dish. Through a hole in the eggshell, the embryo can be visualized and easily manipulated with microsurgical tools or gene constructs, then allowed to continue development *in ovo* to determine the consequence of the experimental manipulation.

“Cut and paste” experiments have been classically and highly successfully used to determine which tissues talk to one another to control organ development and to test the ability of a specific molecule to mediate that interaction. These “cut and paste” techniques can be learned relatively easily, and combined with molecular gain and loss of function experiments to test both sufficiency and necessity for a molecule of interest. *In ovo* electroporation can be used to introduce plasmids containing a cDNA of interest into a particular tissue (neural tube, ectoderm, endoderm, etc) at a particular time; beads soaked in proteins/chemical inhibitors, and pellets of cultured cells secreting growth factors, can also be implanted in particular tissues at particular times. Moreover, the rapidity with which these experiments can be done, owing to the ease of manipulation and the large numbers of eggs that can be obtained, makes the chick embryo a powerful system to test gene function in a non-genetic way.

The genomic resources available for the chick have also increased rapidly. The chicken genome has been sequenced and there are collections of chicken ESTs readily available (for instance, UMIST/ARK-Genomics ([www.arkgenomics.org](http://www.arkgenomics.org)) and University of Delaware). Affymetrix has created chick DNA microarray chips for gene profiling, making gene discovery much easier. There are also public resources cataloguing the expression of genes in the embryo ([http://geisha.arizona.edu/geisha/](http://geisha.arizona.edu/geisha/)). The chicken genome is only $1 \times 10^9$ nucleotides, 1/3 the size of mouse or human; it is more compact owing to absence of some intronic sequences and smaller regulatory regions. The chicken evolved 85-90 million years ago from a galliform ancestor (van Tuinen M, Dyke GJ. *Mol Phylogenet Evol*. 2004, 30:74-86), yet the tissue and molecular interactions during gastrulation and organogenesis are highly conserved with mammals. Thus, genomic comparisons that include chicken sequence data aids tremendously in the search for evolutionarily conserved regulatory elements.

Fertilized chicken eggs are readily available anywhere in the world and the equipment needed is minimal – a humidified incubator (39°C, no CO$_2$ required), a dissecting microscope, microsurgical tools that can be prepared in the lab or purchased, and either a hand-held mouth pipette or a manufactured micromanipulator and picospritzer. The eggs can be stored (4-16°C) for at least 1 week before use. After the start of incubation, embryonic development can even be slowed down to allow the embryo to reach the appropriate stage at the time that you want to do the experiment. Following manipulation of the embryo, the egg can be sealed with tape and placed back in the incubator to continue development. The embryo heals very rapidly within a few hours.

The disadvantage of studies in the chick embryo is the relative lack of genetics. Although it has been possible to make transgenic chickens, the time to sexual maturity and the space required to maintain a flock of chickens places it beyond the normal capacity of a lab to rear their own experimental stock.
EXPERIMENTAL IDEAS BASED AROUND PARTICULAR DEVELOPMENTAL SYSTEMS

The experimental overview at the start of the handout (p. ii) gives you ideas for the general types of experiments you can do using the chick embryo. Here, we give you some ideas for experiments based around particular developmental systems. See later sections for detailed protocols.

Axis formation

The chick embryo is an extremely amenable system to study the mechanisms that set the initial embryonic polarity and result in the formation of the embryonic axis, or primitive streak. The early pre-gastrulating chick embryo is a flat one-layered thick blastodisc made of several thousand cells, called the epiblast. The central area (area pellucida) gives rise to the whole embryo. It is surrounded by two concentric areas, the marginal zone and the area opaca, which do not contribute to the embryo. Beneath the epiblast are scattered “islands” of cells, the precursors of the hypoblast (an extra-embryonic endoderm). The posterior edge of the area pellucida is marked by Koller’s sickle, a thickening of the epiblast at the area pellucida/marginal zone border. The islands of hypoblast coalesce to form the hypoblast, which gradually covers the area pellucida, in a posterior-to-anterior direction. Slightly afterwards, the axis starts forming from the posterior part of the embryo (Figure 1).

The young chick embryo can be explanted, manipulated and grown in New culture. Manipulations include experiments to assess the regulative capacity of the embryo (embryo cut in half, anterior half cultured separately from the posterior, and analysis of the anterior half behavior, e.g. axis formation), analysis of the inducing properties of a particular portion of the embryo via ectopic graft experiments (e.g.. posterior marginal zone ectopic graft), or of inhibitory properties via removal of the region of interest (e.g. hypoblast removal). Signaling pathways can be altered via graft of protein-soaked beads or pellets of COS cells transfected with cDNA encoding the factor of interest. Alternatively, electroporation in the epiblast of pre-primitive streak embryo can be performed in order to deliver a specific DNA (gain of function or loss of function) or morpholino (loss of function) in a precise area of the embryo.

Figure: Diagram of early stages of chick embryo development. Roman numbers indicate pre-primitive streak embryonic stages. Sagittal sections of stage X and XIII are shown below the respective stage, anterior and posterior to the left and right, respectively. Ventral view, posterior to the bottom.
Neural Patterning
The chick embryo has provided a wealth of information as to the cell-cell and cell-tissue interactions and the molecular players that regulate patterning of the neural tissues. Studies in chick and frog embryos have provided much of our knowledge related to neural induction. Dye-labeling of cells within Hensen’s node (the chick organizer) and of the neighboring cells has revealed the movement of cells into and out of the node and their contribution to different tissues. Grafting Hensen’s node to ectopic locations induces the formation of a second axis. The chick embryo is a readily accessible organism in which to watch the process of closure of the neural tube and this can be combined with visualization of dye-labeled cells. Notochord grafts adjacent to the dorsal neural tube revealed how the notochord communicates with the neural ectoderm in the formation of the floor plate and motor neurons. This was followed by functional identification of Sonic Hedgehog as key in this communication and in patterning the ventral cells of the neural tube. Retinoid and Bmp signaling are also implicated in neural patterning. The effects of these signaling molecules can be studied by the implantation of beads or cells that alter signaling followed by analysis of neural cell types using in situ hybridization or immunohistochemistry.

Neural Crest Cell Development
Neural crest cells were discovered in the chick embryo in 1868 and the chick is still a very important system for the analysis of the mechanisms and molecules underlying neural crest cell induction, migration and differentiation. Neural crest cells delaminate from the dorsal neural tube in a rostrocaudal wave and migrate through the embryo along characteristic migration pathways. Cranial neural crest cells migrate in distinct streams into the pharyngeal arches, while trunk neural crest cells migrate in a segmented fashion only through the rostral sclerotome. Neural crest cells give rise to all peripheral glia, all peripheral autonomic neurons (postganglionic sympathetic and parasympathetic neurons; enteric neurons), all sensory neurons in the trunk, and some cranial sensory neurons, together with many non-neural derivatives such as pigment cells, endocrine cells, facial cartilage and bone, teeth, and smooth muscle.

In the late 1960’s, Nicole Le Douarin discovered that quail nuclei could be distinguished histologically from chick nuclei, and developed the quail-chick chimera technique. This involves grafting sections of neural tube (or other tissues of interest) from quail to chick embryos, either isotopically (to analyse normal migration pathways and fate) or heterotopically (to test commitment and potential). Quail neural crest cells migrate out of the grafted neural tube into the surrounding host chick tissue, and their ultimate fate can be determined in sections (originally by using a nuclear stain, today by using an anti-quail antibody, QCPN). The use of quail-chick chimeras led to an explosion of data on the normal fate, migration pathways and potential of neural crest cells arising from different axial levels of the neural tube.

Vital fluorescent dyes, such as the lipophilic fluorescent dyes Dil and DiO, can also be used to label neural crest cells so that they can be followed (a) without invasive microsurgery and (b) using time-lapse microscopy. Prior to neural crest emigration, the neural tube is only one-cell thick, so filling the lumen of the neural tube with Dil labels all neural crest precursors. Once they migrate into the surrounding unlabelled periphery, the Dil-labelled neural crest cells are easily identified.

Finally, in ovo electroporation can be used to introduce DNA constructs into neural crest precursors or other tissues of interest. For neural crest cells, the DNA construct is injected into the neural tube prior to neural crest emigration; electrodes are placed on either side of the embryo and several square-wave pulses of electricity passed across it. The negatively-charged DNA moves towards the positive electrode, so the DNA incorporates into cells on only one side of the neural tube, leaving the other side as an internal control. In this way, for example, GFP can be electroporated into neural crest cells so they can be followed using time-lapse video microscopy, or specific genes (including dominant negative constructs) can be overexpressed, or functional knockdown can be attempted using morpholinos.
Studying neural crest cell migration using a range of different techniques (grafting, Dil injection, electroporation, bead implants) will give you a good sense of the kinds of experiments that are possible in the chick. We do not have quail embryos in the lab, but you will be shown a movie of quail-chick neural tube grafts (courtesy of Dr Alan Burns, Institute of Child Health, London). If you would like to try your hand at neural tube grafts, e.g., if you wish to see the effect of moving neural crest cells from one axial level to another, you could inject Dil into the lumen of the neural tube in one embryo, graft the labelled neural tube into a second host embryo (in the same region, or heterotopically into a different region), and look one or two days later for labelled neural crest cells. You could label the neural tube with Dil and then perform unilateral or bilateral ablations of different-sized regions, to see how well neural crest cells emigrating from the contralateral side or adjacent regions can compensate for the missing section. You could follow neural crest cell migration by injecting Dil or electroporating GFP into the neural tube. We will provide a range of electroporation constructs (and proteins in which beads can be soaked) with which you can manipulate different signalling pathways; these could be introduced into the neural tube to look for effects on neural crest cell migration. For example, neural crest cell emigration from the neural tube requires BMP2/4 signalling. So, you could label the neural tube with Dil and implant Noggin-soaked beads next to the neural tube, or electroporate constructs encoding Noggin or a dominant negative BMP receptor into the neural tube itself, with GFP, and look one or two days later at the effect on neural crest cell migration. There are lots of possibilities!

Limb Development

The embryonic limb has provided a classical model system to study many questions in developmental biology such as pattern formation, skeletal formation, cell fate, and cell and molecular interactions. Limb development can be perturbed without affecting the development of other tissues or the animal itself and hence provides a simple system to test the function of genes that may be required more generally during embryonic development. Much of our understanding of limb development has come from studies utilizing the chick embryo due to the accessibility of the developing limb, its relative large size, and the ease of experimental and molecular manipulation.

Some techniques for carrying out experiments on the chick limb bud will be illustrated in the laboratory by means of videotaped films (courtesy of Dr. John Saunders). These films show grafts of the limb bud to various ectopic sites; excision of the Apical Ectodermal Ridge; reorientation of the wing-bud apex; exchange of ectodermal jackets between the mesodermal limb-bud cores; and grafts of the Zone of Polarizing Activity. Fate-mapping can be done using dye labels (Dil/DiO). Gene activity can be manipulated in a variety of ways using dominant-negative constructs, ectopic expression, or RNAi knock-down. However, it is relatively difficult to transfect a large number of cells in the limb except by infection of the early limb with retroviral constructs. More discrete transfections can be done by in ovo electroporation.

The formation of the vertebrate skeleton reflects the culmination of a well-orchestrated and intricately controlled multi-step process whereby mesenchymal cells progressively differentiate to form numerous precisely shaped cartilage elements. Initially, individual mesenchymal cells must interact and aggregate to form mesenchymal condensations that prefigure the future skeletal elements. These cells then differentiate into chondrocytes that form the template, or anlagen, of the future bone. In the vertebrate limb, a complex array of skeletal elements is present: a single proximal long bone within the stylopod (humerus; femur), followed by two long bones within the zeugopod segment (radius, ulna; tibia, fibula) and then the distal autopod segment comprised of wrist or ankle, and digits. The limb skeletal elements are first prefigured by mesenchymal condensations, which are progressively established along the proximal to distal (P-D) axis.

Analysis of the skeletal pattern is an easy way to begin to understand the effect of loss or manipulation of key genes that regulate limb development. For example, effects of microsurgery on the generation of the skeletal pattern of the limb can be demonstrated by fixing embryos 8-10 days after the start of egg incubation and staining for cartilage using Alcian Blue dye. The embryonic
limb skeleton can be visualized at early stages using molecular markers such as Sox9 or Collagen type IIa in whole-mount RNA in situ hybridization.

Cartilage formation can also be analyzed ex ovo in micromass cultures. In the micromass system, limb mesenchyme is dissociated into single cells and plated at high-density and over a three day period these cells form cartilage nodules that accurately recapitulate the formation and maturation of the cartilage anlagen characteristic of embryonic skeletal development. Various factors can be added to these cultures to determine their effect on chondrogenesis as a means to understand gene function.
PREPARATION AND HANDLING OF EMBRYOS

1) Incubation:
Fertilized eggs are best held at ~13-16ºC prior to incubation (but 4ºC is OK). Incubate at 38ºC-39ºC to the desired stage in a humidified incubator with the eggs placed on their side (horizontal). For long-term post-operative survival, it is best that the eggs be left in the incubator until experimental manipulation. However, eggs can be removed from the incubator and held at room temperature to slow development.

2) Opening the Egg and Exposing Embryos:
To visualize the embryo, a "window" is made in the shell to expose the embryo. Several methods for windowing the egg are in common use.

Eggs to be operated on should be removed from the incubator and placed on a "nest," or platform to support the egg under the dissecting microscope. A nest can be cotton batting in a glass dish, or a moulded piece of modelling clay in a Petri dish. A piece of cardboard egg-carton works equally well, as does a piece of styrofoam with a depression. In eggs incubated in a properly humidified chamber, the embryo usually floats uppermost under the shell. The position of the embryo can be localized by "candling," that is, by transillumination from the side or below.

Windowing an egg
Before opening the egg, swab the shell with 70% ethanol. The embryo should be floating immediately under the shell, so to avoid the chances of cutting into the embryo or blood vessels, it must be lowered away from the shell. This is usually done by inserting a 3ml syringe fitted with an 18-gauge needle through the shell at either the blunt or acute end of the egg, and withdrawing up to 3ml of the watery albumen from the lowest part of the egg. The hole thus made can be used to start cutting a window with scissors (hold the scissor blades flat to avoid cutting into the yolk). Placing scotch tape over the shell prior to opening can help keep eggshell pieces from falling into the embryo, but this can make it harder to make a good seal when you cover the window afterwards.

At later stages (3 days and older), eggs can be entered simply by using a pair of blunt tweezers ("chick beaks") to gradually remove the eggshell and eggshell membrane over an avascular area near the embryo (after locating the embryo by candling). Once the eggshell membrane is penetrated, gentle tapping of the egg allows the embryo to fall away from the shell to prevent inadvertent damage. You can then cut a window using scissors as above.

3) Enhancing Visibility of the Operative Field (Seeing what you are doing!)
The contrast between the white embryo and the underlying yolk is very poor. Contrast may be enhanced in several ways:

a) For early embryos (up to ~stage 14, i.e., 22-23 somites), inject India ink (Pelikan Drawing Ink A) diluted 1:10 in Ringer’s solution, using a 1ml syringe equipped with a 26-gauge needle (it helps to bend the needle ~30 degrees, bevel up). Puncture the vitelline membrane outside the blastoderm with the tip of the needle and bring the needle up very carefully just beneath the embryo. Inject a small bolus of ink solution and shake the egg gently to disperse the ink, if necessary. (Alternatively, use an angled pulled pasteur pipette to mouth pipet a small amount of ink.)
b) For older embryos, or embryos exposed via smaller windows, or for which it is not desired or necessary to inject India ink, a vital dye may be used to visualize more easily the parts of the embryo to be manipulated. Nile Blue Sulfate dye (0.5%) may be applied by means of a glass needle whose balled tip has been coated with agar and impregnated with the dye, or by putting a few µl into a pipetman and ejecting it above the embryo, moving your pipetman tip around as you do so. Nile blue is also a good method for revealing the distribution of apoptotic cells.

c) Visibility of organs or parts (e.g. limbs) of older embryos (3 to 4 days of incubation) can be enhanced by inserting a small piece of aluminum foil or dark paper beneath the structure to be operated on. Reflected light may then provide useful optical contrast.

4) Working with the Embryo
Before operating on embryos 2 days or older, you may have to remove a bit of the vitelline membrane from over the embryo. This can be done by placing a drop of Ringer’s solution over the embryo and then tearing a small hole in the vitelline membrane using a sharpened tungsten needle or a pair of finely sharpened watchmakers' forceps (No. 5). If the vitelline membrane is torn properly, the embryo should sink when another drop of Ringer's is added.

5) Hydration
During manipulation of the embryo, it is important to keep the embryo hydrated with Ringer’s solution. Seal the window with parafilm, or cover it with a petri dish lid when not manipulating the embryo. NB: for some grafting experiments it is better not to have too much liquid over the host embryo because the graft can float away. However, the longer the embryo is exposed to the air, the more likely it is that survival will be reduced. The bottom line is that embryos don't like to dry out!

6) For Further Development
After carrying out your experiment, gently ease one or two drops of Ringer’s solution (all Ringer’s used in this module has penicillin/streptomycin added) onto the embryo (though note this is best avoided after grafts, since the graft may float away), and re-seal the shell with parafilm (or scotch tape) to prevent dehydration. Return the embryo to the humidified incubator for development and ensure it doesn't fall over, as the embryo may stick to the tape.

7) Collecting Embryos
Following your experiment, carefully remove the parafilm. There are two methods for removing the embryo from the yolk for fixation. Care must be taken especially with operated embryos due to their fragility.

a) Place a small "lifesaver" of filter paper onto the vitelline membrane such that the embryo lies beneath the hole in the paper. Using scissors, cut around the edge of the filter paper, lift the filter paper and adhering embryo with forceps and transfer to a dish of saline. Remove the vitelline membrane before fixing.

Alternatively, a spoon or plastic pipet can be used to remove the embryo from the egg. These approaches are especially useful for collecting HH stage 4-12 embryos.

b) for older embryos, it is easier to hold the vitelline arteries with forceps, cut the membranes around the embryo, and then spoon out the embryo.
8) Fixing embryos
Here we will use 4% Paraformaldehyde in PBS. (Note: 10x10ml aqueous 16% PFA can be bought from Electron Microscopy Sciences, catalog number 15710.)
For *in situ* hybridization, embryos of any stage may be fixed overnight at 4°C; alternatively, for embryos after New culture or up to stage 19, fix for 1 hour at room temperature; for embryos at stage 20 or older, fix for 3 hours at room temperature.
For antibody staining: antigens are often sensitive to over-fixing and all PFA must be washed out. For embryos at stage 20 or less (3-3.5 days of development), fix for an hour on ice. You can fix the embryo “flat” by placing it on a Petri dish lid in a drop of Ringer’s, gently pulling on the membranes till the embryo is flat, removing the Ringer’s (carefully!), and adding PFA dropwise. After 5 minutes or so you can remove the now-flattened embryo into more PFA to continue fixing. Wash 3x5 minutes in PBS and then leave in PBS for 4 hours on ice or overnight in the fridge. For older embryos, fix for 2 hours on ice and wash as above.
“CUT AND PASTE” EXPERIMENTS

1) Axis formation: assessing the regulative properties of the early chick embryo

   The pre-primitive streak chick embryo (stage X-XIII) is cut in half along the equator. The anterior and posterior halves are separated. The cut side of each half is sealed with the outer piece of the area opaca from the anterior portion of another embryo (without this operation the half embryo will stretch and die). The two isolated halves are grown in New culture. While the posterior half grows an axis as expected, the anterior half will regulate and form an axis as well, with a delay of 8-9 hours. This regulative anterior half can be used to analyze the molecular events underlining axis formation.

2) Neural crest ablations/grafts

   Segments of the premigratory neural crest either alone or in combination with the neural tube are removed with glass needles or sharpened tungsten wire. Glass capillaries are pulled to generate a sharp pointed tip. Incisions are made perpendicular to the long axis of the neural tube at the rostral and caudal edges of the site to be ablated. A longitudinal cut then is made on both sides of the boundary between the epidermis and the neural folds or in the neural tube proper, and additional cuts are made horizontally at the rostral and caudal borders of the ablation. Ablated tissue is removed by suction through a micropipette, broken off to approximately 30 μm outer diameter. This can be grafted from e.g. a donor quail or GFP-electroporated chick neural tube to the same region (isotopic graft) or to a different region (heterotopic graft) of a chick host.

3) Somite manipulations/removals

   To study the time in which the somites are specified (ie. when is the anterior-posterior or medial-lateral polarity established) or when are the cells committed to a particular fate (heterochronic transplants), the somites from a particular stage and anteroposterior level can be removed and grafted to a host embryo. The caudal region of the embryo including the presomitic mesoderm can also be cultured in explant cultures as described further below.

   Window the egg and tear away the vitelline membrane superficial to the operation site. Using a very fine sharpened tungsten needle (.004 inches diameter), poke a line of holes in the ectoderm just lateral to the somites to be removed and superficial to the mesomere (mesonephros). Separate the medial side of the somites (with overlying ectoderm) from the neural tube. To do this, insert the needle until it just sticks into the underlying endoderm. Push the needle slightly rostrally and pull up, gently separating the somites from the neural tube. It is best to do a series of these movements, working from the caudal to the rostral end. Be careful not to cut through the endoderm! After these cuts, make 2 cuts across the rostral and caudal ends of the somites to be removed. It is best to make these cuts from medial to lateral. Now recut the endoderm just lateral to the somites and superficial to the mesomere. Start at the rostral end of the cut and work caudally. Finally, gently tease the rostral end of the somites (with the overlying ectoderm) up and away from the underlying endoderm. Working towards the caudal end of the graft, lift the somites up and tease away from the underlying endoderm. The somites can be transferred to another embryo with either a Spemann pipette or a micro-curette.

4) Notochord grafts

   Isolate 150-300 μm (roughly the length of 2-3 somites) segments of notochord using 1-2 mg/ml Dispase treatment as described in section “Enzymatic Isolation of Chick Tissue”. Prepare host by windowing the egg, injecting ink and tearing a hole in the vitelline membrane as described above. Using a glass or tungsten needle, make a 150-300 μm long incision along the edge of the neural plate or neural tube and separate the neural tissue from more lateral tissues by moving the needle back and forth in the space of the incision. Transfer a notochord into the field using a pulled mouth pipette or a P20 pipetman. Careful not to lose sight of your notochord bit or to get it stuck on the end of the pipette. Guide the notochord into the incision using your needles. Notochords pushed too deep into the incision will end up to close to the host notochord or near the gut so its best to just place the
notochord in the incision and gently push the surrounding tissues against it using your needles. You want the notochord to stay in the incision but to stay close to the surface so it ends up dorsolateral to the neural tube and/or somite. Gently seal the egg completely and place it back in the incubator.

5) Notochord/neural tube cultures

Culture the notochord (or cells expressing Shh) next to a naïve neural tube explant (ie, the “center” portion of the neuroectoderm taken from ~stage 9 embryo in the caudal region where the neural tube has not closed). These tissues can be placed close to one another in a collagen gel explant. In both cases, you can look for the induction of floorplate and motoneuron markers.

6) Limb bud manipulations

Some techniques for carrying out experiments on the chick limb bud will be illustrated in the laboratory by means of videotaped films (courtesy of John Saunders). These films show grafts of the limb bud to various ectopic sites; excision of the Apical Ectodermal Ridge; reorientation of the wing-bud apex; exchange of ectodermal jackets between the mesodermal limb-bud cores (see later for a detailed protocol); and grafts of the Zone of Polarizing Activity.

Effects of microsurgery on the generation of the skeletal pattern of the limb can be demonstrated by fixing embryos at 9-10 days after operation and staining for cartilage using Alcian Green or Alcian Blue.
Enzymatic Isolation of Chick Tissues

Enzymatic treatment loosens connections between the various structures, allowing for tissue isolation. These methods can be used to isolate a tissue or population of cells to study their development in isolation (explant or collagen gel) or to study their influence on the development of a tissue or organ in vivo following transplantation of the isolated tissue to a host embryo.

1) Isolation of neural tube, notochord and somites
Tissues for grafting can be easily isolated by the judicious use of enzymes. Typically, embryos are incubated for about 1.5-2 days (HH stage 10-14; 10-18 som) for isolation of neural tubes, notochord and somites.

a) Collect embryos as described above into a dish of Ringer’s; remove bits of yolk floating in the Ringer’s with glass pipette, and add fresh Ringer’s as needed.

b) Using a tungsten or glass needle, or for manual dissections a 30.5-gauge needle attached to a 1ml syringe with the plunger removed, isolate the region of the embryo surrounding the tissue that you are interested in and place it in a separate small dish containing Ringer’s. For instance, to obtain notochords, remove the trunk region of each embryo comprising the last 6-8 newly formed somites.

c) Once all of the embryos have been trimmed, remove the Ringer’s solution and replace with 1-2mg/ml dispase in ice-cold 20mM Hepes-buffered DMEM. Leave for 20 minutes on ice and then 15 minutes at 37°C.

d) During the incubation, rinse a separate recovery dish and fill it with recovery medium: 10% serum in DMEM (any medium, even Ringer’s solution, should be fine). Keep the dish on ice, and covered as much as possible to avoid getting dust in with your grafts: tissues are very sticky after enzymatic isolation and hairs and dust will stick to them with great affinity.

e) After the enzyme digestion, transfer the tissue to the recovery dish and allow the tissue to recover on ice for at least 15 minutes.

f) Carefully aspirate and expel the tissue through a bent pasteur pipet. This will generate shearing forces which will separate the tissues from each other. Pry the tissues apart under the microscope, isolating the bit that you are interested in. You may find this easiest using a pair of 1ml syringes (plungers removed) with 30.5-gauge needles attached. Rinse tissue in Ringer’s before grafting. You could also label the tissue pieces with Dil prior to grafting.

For reference: to prepare Hepes-buffered DMEM, make 1M Hepes, pH to 7.5, autoclave. Take a bottle of 500ml DMEM and add 5ml Hepes. Aliquot into 50 ml tubes and freeze. To make 1.5 mg/ml dispase in Hepes-buffered DMEM, put 75mg dispase into a 50ml tube of Hepes-buffered DMEM, vortex and make 1ml and 5 ml aliquots. Store in freezer.
2) Enzymatic Separation of Limb Tissues

To prepare an intact ectodermal jacket of a young avian limb bud:

a) Cut off the limb bud and rinse in 2 or more changes of calcium-and-magnesium free (CMF) PBS.

b) Either:
   
   (i) Place in a solution of 2- 2.5 % trypsin at 4°C for about 30 min to several hours depending on the age of the limb. When the ectoderm begins to curl up near the cut edges, this indicates that the ectodermal layer is loosening from the mesoderm. Test that the ectoderm is pulling away from the mesoderm. Transfer the limb to a cold mixture of 10% serum in Ringer’s. By means of fine tweezers, gently slide the ectoderm from its mesodermal core. Hold the ectoderm in the cold mixture for further use. If the mixture is allowed to warm, the ectoderm will shrink into a configuration useless for further manipulation.
   
   Or:
   
   (ii) After treatment with CMF, incubate the isolated limb at 37°C in a solution of 2% trypsin plus 1% pancreatin for about 30 minutes. This should suffice to loosen the ectoderm. Remove the ectoderm after placing the limb in cold PBS:10% serum.

c) These procedures leave the mesoderm a bit soft. If you want a nice, firm, mesodermal core to receive a covering of foreign ectoderm, for example, provide a mesodermal donor by incubating an isolated, CMF-treated limb bud in 1% EDTA (ethylene diamine tetracetate) at 37°C for about 30 minutes. The ectoderm can then be removed, often in patches, by means of fine needles, and the mesoderm then removed to serum-containing medium for further manipulation. For example, a wing-bud mesoderm may receive a covering of inside-out wing-bud ectoderm, the combination being done in the cold (4°C). Mesodermal cores isolated by either of the trypsin methods can be processed into single-cell (or almost) suspensions, by flushing repeatedly through small-bore pipettes after being removed to Tyrode’s solution or to other suitable medium. Such suspensions can be pelleted centrifugally and stuffed into ectodermal jackets, and grafted to a host embryo, or they can be used for micromass cultures.

Making a mouse-chick recombinant limb
Culture Techniques

1) Explant culture on Millicell-CM culture plate insert (Biopore membrane)
   Prepare culture chamber by equilibrating medium (DMEM/F12 medium with Glutamax, 15% fetal calf serum, 1:1000 pen/strep) and Millcell-CM culture plate insert at 37°C with 5% CO₂. Use 2ml medium per well of 6-well plate. Excise tissue and rinse in Ringer’s solution or culture medium prior to transfer to culture dish (use forceps or a pipetman to move the tissue). Filter insert should be completely wet but organ explant tissues should not be submerged. Place in tissue culture incubator (37°C with 5% CO₂) and culture for desired period of time.

2) Explant culture in collagen gels
   a) In an eppendorf, add: 90 ml collagen (type I rat tail; Collaborative Research)
      10 ml 10xDMEM
      Vortex briefly. Then add 4.5 – 5.0 ml 7.5% sodium bicarbonate (store at 4°C)
      Vortex for 20 seconds and store on ice.
      Solution should be straw yellow with a pink meniscus. If solution is pink, the pH is low and the collagen will set quickly.

   b) Pipette 10 ml collagen onto the bottom of a tissue culture well (4-well dish). Spread it around a little with the pipette tip to flatten it. Allow to set for 10 minutes at 38°C, 5% CO₂ (this helps prevent dehydration) or for 20 minutes at room temperature.

   c) Transfer tissue to surface of gel by mouth pipette or in 3ml Ringer’s solution (Gilson). Aspirate as much liquid as possible so that gel surface is dry (easiest by mouth pipette).

   d) Cover tissue with 5ml collagen mix. Use pulled glass capillary needle to make sure tissue is below the surface of the collagen, and to orient the tissue pieces as necessary.

   e) Allow to set for 10 minutes at 38°C, 5% CO₂ or for 15 minutes at room temperature.

   f) Cover gel with 0.5ml DMEM/F12 or other desired medium, with 5% serum if necessary. Incubate at 38°C with 5% CO₂ overnight or as long as desired.

   g) For immunostaining fix in 4% PFA for 25 min on ice, wash 3 times in ice cold PBS and wash in PBS for 4 hours to overnight on ice. For in situ analysis fix in 4% PFA for 3 hours at room temperature and wash once in PBS.
3) Micromass limb mesenchyme cell culture

Chick (or mouse) limb buds are removed from embryos and transferred to Hank’s balanced salt solution, Ca++ and Mg++ free, containing 0.2% trypsin-0.1% collagenase, and incubated at 37°C for 30 minutes. A Pasteur pipette is used to disperse cell aggregates into approx. single cell suspension. Cells are placed in D-MEM/F-12 without phenol red with 10% FBS, pelleted, washed, and resuspended in ~100ul DMEM + FBS. Fifty microliters of cells are plated in a small drop in the center of a 35mm tissue culture dish. Cells are allowed to attach to the plate for 60 minutes and then 3 ml DMEM + FBS is added. Within 3 days of culture, cartilage nodules will be visible by Alcian blue staining. Muscle cells can also be detected with MF20 antibody.
FATE-MAPPING EXPERIMENTS

Dye-labeling or the transplantation of cells from quail or GFP-labeled embryos (transgenic or electroporated tissue) to a host chick embryo allows one to fate-map tissues, to follow the movements of migratory cell populations or to mark a particular population for transplantation studies. This is a useful technique to explore questions of cell fate, specificity and commitment.

1) Dil labeling
   a) The vital dye, Dil (1,1-Dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate; Molecular Probes) is fluorescent and lipophilic. Dil can be injected into the lumen of the neural tube to label premigratory neural crest or growing motor axons, or to the somites to label limb muscle precursors, or to regions of the gastrulating embryo to follow cell movements and to determine the tissue to which the labeled cells will contribute. In this course we generally use Cell Tracker CM-DiI (Molecular Probes, C-7000), which is retained better by the tissue throughout fixation, as well as after in situ hybridization. Add 10μl 100% ethanol to a single premeasured ampule (50μg) for stock solution. Use undiluted stock for focal injections. For larger injections, add 90μl of 10% sucrose in distilled water to the stock. The Dil can be diluted up to 300-400μl with 10% sucrose. It is important to centrifuge down the Dil crystals prior to use to avoid clogging of the micropipette tip. NOTE: Freshly made Dil works the best (within one-two weeks of use).
   b) Back-fill micropipettes pulled from thin-walled borosilicate or aluminosilicate with the Dil solution. Use forceps to remove the very end of the tip under the dissecting microscope. To expel the dye from the needle, attach the needle to a picospritzer or similar pressure injection system, or simply use a mouth pipette.
   c) In most cases, the labeled cells appear slightly pink under a dissecting microscope. In addition, labeled cells can be viewed briefly using fluorescence optics (rhodamine filter).
   d) Allow embryo to develop to desired stage. You can view the embryo immediately using fluorescence optics (rhodamine filter), or fix in 4% paraformaldehyde in PBS for 1-2 hours at room temperature/overnight at 4°C before viewing.

2) Interspecies grafts

Quail-chick chimeras can also be used to fate map tissues and label migrating cell populations. Quail cells are recognized by the QCPN monoclonal antibody (Developmental Studies Hybridoma Bank). See Le Douarin et.al., 1996, Chick Quail transplantations in "Methods in Avian Embryology", editor Marianne Bronner-Fraser Academic Press. Or, Le Douarin and Weston, 1963, Dev Biol 6: 279-310. We do not have quail embryos in the course, but you can try grafts using donor tissue labelled with Dil or GFP electroporation (see next section).
MANIPULATING GENE EXPRESSION

1) In ovo electroporation:

In ovo electroporation can easily be used to misexpress genes of interest or morpholinos in the neural tube, somites, and limb mesoderm (Swartz et al., 2001). A square-wave low-voltage pulse across the embryo allows DNA to enter particular tissues (epithelia are most efficiently electroporated); the negatively-charged DNA moves towards the positive electrode. Recent studies have shown that this approach also works to knock down particular protein products, using shRNA (Sahin et al., 2005) or morpholinos. In principle, both approaches work in a similar manner: plasmid DNA encoding the gene of interest is transfected using low voltages into particular embryonic tissues. If one requires short-term knockdown (1-2 days), another alternative is to purchase siRNAs directly (Dharmacon).

Note: the first few times you try electroporation, you may wish to use fluorescein-tagged control morpholino, so you can see immediately whether or not it has worked! Depending on what you are introducing, you could also mix the fluorescein control morpholino with your DNA.

a) Targeting one side of the neural tube (left or right):
This is a basic protocol similar to that reported by Krull, 2004, Dev. Dynamics, 229:433-439. Use clean plasmid DNA (see Krull, 2004 about preparation).

i) For mis/overexpression in the neural tube, we use embryos at HH stages 10-13 (10-17 somites).

ii) For young embryos, inject DNA into the neural tube by placing the pipet through the vitelline membrane (leaving the vitelline as intact as possible). Fast green (2.5%) can be added to the DNA for visualization. The DNA should be 0.5 – 5 μg/μl. IMPORTANT: you should test which concentration of DNA gives you efficient transfection. The appropriate DNA concentration often depends on the size of your insert and plasmid. The larger the insert and plasmid, the higher DNA concentration required.

iii) Place a few drops of Ringer’s onto the vitelline membrane, position the electrodes straddling the embryo (see figure on next page), and pass current. DNA will move into the cells on the side of the neural tube nearest the positive electrode, leaving the unelectroporated side as an internal control. The pulses should be 50ms square pulses, typically 3 pulses at 25 volts. For younger embryos, the voltage and/or number of pulses can be decreased to help reduce mortality. Older embryos can tolerate higher voltages. Assess the voltage requirements for each tissue you are transfecting, as they will vary.

iv) Add a few drops of Ringer’s to your embryos. Tape the eggs and return them to the incubator. Expression begins at different times depending on the construct used as well as cell proliferation and the promoter of the plasmid you are using. Strong expression is usually observed 24 hours post-electroporation, but GFP can be detected 4-6 hours post-electroporation.

b) Targeting motor neurons (ventral neural tube only):
Motor neurons are generated in the ventral neural tube. At limb levels, motor neurons in the lateral motor column (LMC) extend their axons to the limb, to innervate particular dorsal or ventral muscles. The motor neurons in the LMC innervate dorsal versus ventral muscle, forming a precise topographic map: motor neurons in the lateral LMC innervate dorsal muscle, whereas motor neurons in the medial LMC innervate ventral muscle. For many experiments, one would like to target ventral cells in the neural tube, leaving dorsal cells (including neural crest cells) unperturbed. To transfect motor neurons at the level of the hindlimb with in ovo electroporation, there are a few modifications to the basic procedure described above.
i) For transfecting motor neurons at hindlimb levels, use embryos at stage 15-17 of development. If you transfect stage 15 embryos using *in ovo* electroporation, you will label motor neurons in the medial part of the lateral motor column (LMC) primarily. If you transfect stage 17 embryos, you will label motor neurons in both the medial and lateral LMC. Tear off carefully the vitelline membrane that overlies the hindlimb neural tube using your tungsten needle and forceps, prior to injecting your DNA into the neural tube.

ii) After DNA microinjection, place your negative electrode dorsal and slightly to the left side of the neural tube; insert carefully your positive electrode under the embryo, preferably through the hole that you injected ink. Place the positive electrode below the neural tube, to the right. Carefully lift the electrode towards the embryo, so you can see its position. Be careful not to make a hole in the embryo. Keep your hands steady. Pass current, as described above. Carefully remove the positive electrode from below the embryo, keeping the spillover of yolk to a minimum. Prepare the embryo for reincubation as described above.

**Chick neural tube electroporation**

![Diagram of Chick neural tube electroporation](image)
In ovo electroporation

"Parallel" electroporation

1. Inject plasmid DNA
2. Apply electrodes and pass current

Targeting Neural Crest Cells

Place negative electrode under the embryo via the hole of the inking needle
Targeting the ventral neural tube

In ovo electroporation targeting limb mesoderm

sop = somatopleur
spl = splanchnopleur
2) **Ex ovo electroporation in pre-gastrulation and early streak-stage chick embryos**

The epiblast (upper layer) of the early chick embryo can be electroporated to deliver DNA or fluorescein-tagged morpholino in a specific region of the embryo. Embryos are explanted into an appropriately-designed electroporation chamber (see Figure below, from Voiculescu et al., 2008, Nature Protocols 3, 419-426) and submerged in Tyrode's or Pannett-Compton saline.

Electroporation conditions are described in Voiculescu et al., 2008, Nature Protocols 3, 419-426.

Briefly: 0.7-2.0 mg/ml DNA or 1-3 mM fluorescein-tagged morpholino is electroporated in a solution containing 1/10 volume of 60% (wt/vol) sucrose and 1/10 volume of 0.4% (wt/vol) Fast Green.

Pre-primitive streak (stage X-XIII) and early streak embryos (stages 2-3): use 5 pulses of 4.0 volts, 50 ms each, at 500 ms intervals.

Primitive streak embryos (stages 3+ to 7): use 5.6-6 volts, 50 ms each, at 500ms intervals.

After electroporation, embryos are transferred to New culture for incubation to the desired stage.

Fluorescein-labeled control morpholinos allow visualization of the electroporated cells.

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**Figure 1** | Electroporation chamber. (a) Top view of the chamber. (b) Cross-section through the middle of the chamber, along the central canal (positive electrode). (c) Close-up of the electroporation setup, indicating the position of the embryo, application of electroporation mixture and the optimum distance of the negative electrode above the embryo.
Electroporation at pre-primitive (A, B) and primitive streak stage (C, D).

Large (A, C) and focal (B, D) electroporations of fluorescein-coupled control morpholino (A, B), and plasmid (DsRed-Express) (C, D).
1) Implanting Heparin Acrylic or Affigel blue beads (for growth factors)

Heparin/Acrylic beads: we use routinely for FGFs and BMPs (anything that may bind heparin)
Sigma: H-5263 (large range in bead size).

Affigel Blue beads: these can be used for a variety of proteins (even FGFs work).
Bio-rad: 153-7301 (75-100μm diameter); Bio-rad: 153-7302 (100-200μm diameter)

Each type has advantages and disadvantages. Affigel blue beads tend to be stickier than heparin beads so they stick in tissue better, but are squishy so sometimes harder to work with. Heparin beads are easier to manipulate; also, you can chip off a small piece at the edge of the bead (before soaking) in protein the chipped region tends to hold well in the tissue.

Select appropriate size beads (we use forceps to pick up) and collect in a 100 microliter drop of PBS in 35mm petri dish. Prepare a second 35mm petri dish – place many (~15) 8 microliter drops of PBS throughout the dish, excluding the center (this is for humidification). In the center, place a 1-3 microliter drop of protein. Protein dilutions in 0.1% acetylated BSA and PBS or as directed by manufacturer.

Pick up bead and transfer to protein drop. Make sure that you do not transfer PBS solution, which will dilute the protein. To get rid of PBS that is sucked up by capillary action along with the bead, work back and forth between dry area on plate and 8 microliter drop until you can pick up the bead dry. For instance - drop bead + PBS onto dry portion of plate, hold forceps together close to bead and suck up PBS, discard PBS, continue until bead is no longer sitting in puddle of PBS, pick up bead on one tip of forcep and place in protein.

You can put >20 100 micron beads in a 1 microliter drop of protein. Soak the beads in protein for 2 hrs at room temp or overnight at 4°C. Rinse beads in PBS just prior to grafting etc.

How to implant...that really depends on where you want to put it. It is easy to stick a bead into a tube or cavity (pick up with forceps and push it in). Solid tissue is harder because when the tissue heals it tends to force the bead out (though cells love FGF so they tend to grow around an FGF bead). You can make a pocket in the AER (figure). Following AER removal, we staple the bead at the edge of the mesenchyme using very fine (0.025mm) platinum wire bent to form a staple. Often it just takes some playing and practice.

Heparin bead implanted beneath pocket made in AER at anterior edge of wing bud. Blue staining is Nile Blue sulfate (helps visualise tissue; also shows apoptotic cells, so AER is highlighted).
2) Implanting ion exchange beads (for retinoic acid, chemical inhibitors)

Ion exchange beads (AG1-X2) (for retinoic acid or SU5402 or Mek inhibitor)

Bio-rad: 140-1231 (50-100 mesh)
These beads come in chloride form and to use them for retinoic acid you must first re-derivatize them (see below). The beads used in the course are already re-derivatized

Retinoic acid (dissolved in DMSO). RA is light sensitive. RA will diffuse out of the beads so prepare and use when needed.

Place a 100 ul drop of RA on a piece of parafilm
Pick up formate derivatized AG1-X2 bead with forceps and place at the bottom of the RA drop (the beads will swell to ~2x the dry size so select smaller beads than will be required).
Soak in RA for 20 minutes
Prepare a strip of parafilm with 2 rows of 100 ul drops of media (equal number of drops relative to number of beads)
Place each bead in first row of drops (the beads will take up the phenol red dye making them easier to visualize)
Move each bead to the second row of drops.
Beads are now ready for use.

To formate derivate AG1-X2:
- run 3 bed volumes of 0.5-1M formic acid over over column (Bio-rad #731-1550)
- wash with dH2O until pH ~5 (test with pH paper)
- swish beads up and down with pasteur pipets to get out of column
- put in 100ml petri dish
- dry in 37°C incubator overnight with lid slightly ajar (do not over-dry or you will not get efficient exchange)
- transfer to an eppendorf tube and seal with parafilm. Store at room temp.

These beads will swell to ~2x the dry size so select smaller beads for soaking.
CULTURE AND MANIPULATION OF YOUNG EMBRYOS

1) New Culture
To study early stages of embryonic development (ie. gastrulation, somitogenesis), embryos can be removed from the egg for greater ease of visualization and manipulation. The early embryo is cultured using a technique called New Culture. This is used for young embryos (before 2 days of incubation) for up to 36hr (e.g. stage 3.5 to stage 13).


Material needed:
Pyrex backing dish about 8cm deep
Pannett-Compton saline solution (2 liters)
2 pairs of blunt fine forceps (watch makers No.4)
1 pair of coarse forceps, 1 pair of small scissors
1 Pasteur pipette, flamed to remove sharp edges; rubber bulb
1 aspiration tube
35mm plastic dishes with lid
watch glasses (2 or 3)
ring glasses (27mm outer diameter, 24mm inner diameter)
25 ml beaker
plastic bag for egg waste
plastic box with lid to use as humid chamber for culture incubation

Clean the working area (including microscope) with water and then ethanol. Put freshly made Pannett-Compton (PC) saline solution to fill up the basin to _.

Crack open the egg by knocking it with the coarse forceps at its equator. Pour out the liquid albumin in the 25 ml beaker. Using the coarse forceps, remove all the thick albumin from the yolk and place the yolk in the basin containing PC saline. Important: if there is any thick albumin still attached to the yolk, remove it while in the basin.

Gently turn the yolk so that the blastodisc is facing upward. Cut the vitelline membrane below the equator of the yolk. With the coarse forceps hold one edge of the vitelline membrane and lift it. Gently separate the vitelline membrane from the yolk by slowly and with an even speed pulling the yolk away from the membrane with the help of the fine forceps.

Move the vitelline membrane away to a clean area of the basin, leaving the outer surface of the membrane facing the bottom of the basin. The embryo will remain attached to the vitelline membrane (for embryos st 3+ or older, younger embryos will stay attached to the yolk). The vitelline membrane will be facing down, towards the basin, while the ventral side of the embryo will be facing up, towards you.

Hold the vitelline membrane with the coarse forceps and with circular movements place a glass ring on top of it. The circular movements will flatten the vitelline membrane and the embryo will be placed in the centre of the glass ring. Be careful to not touch the embryo at any stage, nor stretch the membrane too much, the embryo will easily detached from the membrane. Wrap the edges of the vitelline membrane around the glass ring (edges towards the centre) so that the membrane is even and flat but not tight. With the coarse forceps, hold the membrane around the ring and move it slowly to place it in a watch glass.
Carefully take the watch glass out of the basin and put it under the microscope. Wrap all the edges of the vitelline membrane around the ring. With the flamed Pasteur pipette, suck out all the liquid in the ring, being careful not to poke the membrane, and to suck from the meniscus formed where the glass ring touches the membrane.

When there is no liquid in the glass ring, lift the ring (together with the membrane) from one side and suck all the liquid that may be between the membrane and the watch glass. Now tighten the membrane around the glass ring. It is important that the membrane is tight: this will generate the tension that allows the embryo to expand and grow in the membrane.

Cut the edges of the membrane so that it does not touch the embryo or the membrane next to it. The ventral part of the embryo is facing you (don’t forget!).

Slide the glass ring with the membrane out of the watch glass, and place it in a 35mm dish that has been previously _ filled with thin albumin. Suck out all the remaining liquid inside the membrane. Embryos need to be dry to grow well.

Any embryonic manipulations can be performed at this point.

Wet the lid of the dish with thin albumen to seal it. Place the dish in the plastic box container together with a piece of wet tissue. Seal the box and place in the incubator (37°C with CO2).

**Pannett-Compton saline:**
Solution A: for one liter, 121g NaCl, 15.5g KCl, 10.42g CaCl$_2$.2H$_2$O, 12.7g MgCl$_2$.6H$_2$O
Solution B: for one liter, 2.365g Na$_2$HPO$_4$.2H$_2$O, 0.188g NaH$_2$PO$_4$.2H$_2$O
To 900ml of H$_2$O, add 40ml of solution A and 60ml of solution B. Do not mix A and B together without water as it will precipitate!!!.
2) Whole Embryo EC Culture

From Chapman *et al* 2001 Developmental Dynamics 220: 284-289

Things you will need:
EC plates
Petri dishes
Filter papers (circular)
PBS (sterile with PEST in)

1) Prepare the filter paper by punching 4 holes in a clover motif in the centre.
2) Pour the yolk and albumin into a Petri dish (Figure A).
3) Using a tissue, carefully remove as much albumin as possible by wiping the surface of the yolk working from the centre out. (embryos can be centred by gentle stroking towards the centre, the filter paper will not stick adequately if too much albumin is left).
4) Using tweezers place the filter paper on the yolk with the embryo showing through the hole (Figure B and C). Gently press down on the filter paper helps it stick to the yolk membrane.
5) Using the scissors, puncture the yolk membrane at the corner of the paper, then complete cutting the membrane completely around the filter paper (Figure D + E).
6) Using fine dissection tweezers clamp onto the filter paper and attached membrane (Figure F).
7) Very gently peal the filter paper and membrane with the embryo attached from the yolk with one slow continuous movement towards you (Figure G).
8) Place this into a Petri dish of Normal saline with the yolky side up.
9) Gently remove as much of the yolk as possible from the surrounding paper – do not try to remove yolk from over the embryo at this stage.
10) Using the tweezers, place the filter paper with membrane and embryo attached on a EC culture plate.
11) While holding the plate at a gentle angle, wash normal saline over the surface of the embryo to remove remaining yolk.
12) Place lid on the plate, and place in a humidified chamber (box with wet paper towel in).
13) Incubate at 37°C

**EC plates**
a) Prepare Normal Saline, 1L distilled water and 7.19g NaCl and autoclave
b) Prepare 0.6% bacto-agar in Normal saline (1.5g bacto-agar in 250ml normal saline) and autoclave
c) Collect 100 ml thin albumin in sterile falcon tubes
e) Equilibrate 100ml thin albumin and 100ml bacto-agar in the 49C water bath
f) Add the albumin to agar and 1 x Gentamycin to the agar and mix by swirling.
h) Using a sterile pipette pour 2.5ml to 35mm plate.
i) Replace the lids of the plates, allow to dry at room temp for a couple of hours and then store the plates in the fridge for up to 2 weeks.
DETECTING APOPTOTIC OR DIVIDING CELLS

Nile blue staining for apoptotic cells in wholemount specimens
[Alternatively, use the anti-cleaved caspase 3 antibody (see “Whole-mount antibody staining”).]

Nile Blue (Sigma N5632) 1.5% in water; Culture media e.g. DMEM or M199 (does not require serum)
1. Prior to dissection, set up 35mm dish with 2ml culture media + 2-5 μl of Nile blue stock (should be visibly blue). Put dish at 37°C (does not require CO2 incubator)
2. Dissect embryos in PBS (chick stage ~31-33)
3. Put embryos into Nile Blue solution and incubate at 37°C for ~30 minutes (usually 30-45 min).
Monitor under microscope every 15 minutes. Embryo will have faint blue background staining and dark blue staining in regions of cell death. Avoid stopping reaction too quickly as cell death staining will become more intense whereas background staining will not increase.
4. Stop by putting embryo into PBS. Place at 4°C for 4 hours to overnight (perhaps 1 hour if wish to perform in situ hybridization) background staining will fade significantly.
TAKE PICTURES - cell death staining will be lost if left longer than 24 hours.
Embryo can be fixed now for in situ hybridization. Nile blue stain will be lost during in situ steps.

BrdU labeling of dividing cells
[Alternatively, use the anti-phosphohistone H3 antibody (see “Whole-mount antibody staining”).]

1. Add BrdU to the chick embryo, 10mM concentration.
   - Early stage (up to stage 14) add 50 μl on top of embryo with pipette
   - Later stages use syringe and add 250 μl into amniotic cavity (30G1 gauge needle)
   - For tissue culture or pieces of tissue add BrdU to a final concentration of 50μM (5μl/ml of media)
2. Put back in incubator for 1.5-2 hours
3. Fix embryos in 4% PFA (2 hours room temp or 4°C overnight)
4. Wash embryos twice for 10 min in PBS + 0.5% TritonX-100.
5. Block for 1 hour at RT in PBS + 0.5% TritonX-100 + 2% BSA.
6. Add primary antibody (anti-BrdU, 1:200 dilution) overnight at 4°C (in PBS + 0.5% TritonX-100 + 2% BSA).
7. Wash 3x 1 hour in PBS + 0.5% TritonX-100
8. Add secondary antibody (fluorescent) and incubate overnight at 4°C.
9. Wash 3x 1 hour in PBS + 0.5% TritonX-100
10. Examine under epifluorescence.

VISUALIZING THE VASCULATURE

Indian Ink Labelling
Indian ink is diluted 1:1 with PBS, sonicated and filtered with a 45μm syringe filter. A finely drawn glass capillary needle is attached to a 100μl Hamilton syringe via Tygone tubing, back filled with mineral oil and then filled with Indian ink. The needle is inserted into an extraembryonic yolk sac artery, and ink gently expelled from the needle. Once ink has reached all blood vessels, embryos are fixed in 5% Trichloroacetic acid (TCA) for several hours, dehydrated and cleared in methyl salicylate.
ASSAYING THE RESULTS OF YOUR MANIPULATION

Wholemount in situ hybridization
As modified from Domingos Henrique and David Ish-Horowicz. Note: Wear gloves for all of the RNase-free steps (fixation through hybridization). (If you want more details, see http://www.anat.ucl.ac.uk/research/sternlab/INSITU.htm, where there is also a probe synthesis protocol.)

Dissect embryos in PBS removing extraembryonic membranes (except for embryos < stage 9). In young embryos the extraembryonic membranes help decrease damage to the embryos during the procedure. Young embryos also tend to curl up so you may want to fix them flat on a filter disc or pinned on a Sylgard dish. For later stage embryos (H&H stage 16 and older), dissect away the extraembryonic membranes. For all stages after neural tube closure, puncture the roof plate of the brain tearing small holes to avoid buffer and probe trapping. For embryos older than stage 14, also puncture the anterior ventral forebrain and the pericardium around the heart (also big sources of probe trapping). At very old stages, getting rid of what you don't want always helps to decrease background.

NB: Do your fixing and in situ in either 2ml siliconized tubes or glass scintillation vials.

**DAY 1: RNase free (gloves!)**
1. Fix in 4% Paraformaldehyde 1-2h@RT (or overnight at 4°C)
2. PBT RNAse free 2x5'
3. MeOH 100% RNAse free 2x5'. **Leave at -20°C for as long as you want.**
4. 1x5' 50% MeOH: 50% PBT RNAse-free
5. 1x5' 25% MeOH: 75% PBT RNAse-free
6. 2x5' PBT RNAse-free
7. Proteinase K treatment (1:1000 in PBT; final conc. = 10µg/ml).
   Up to st 3+ no PK. After st 3+, use the same number of minutes as the stage (eg for stage 20, do 20min.)
8. Rinse with PBT
9. 1x20' post-fix in 4% Paraformaldehyde + 0.1% Glutaraldehyde in PBT RNAse-free
10. Hybridization buffer leave 1-6hrs at 65°C (normally 2-3hrs)
11. Add probe overnight at 65°C (a mixture of dig+fluoro probe if doing double in situ; final probe concentration 1µg/ml)

**DAY 2 Post-hybridization washes**
12. Recover the probe, it will last for ever.
13. 2x30’ at 65°C Hybridization buffer
14. 1x10’ at 65°C 1:1 Hybridization buffer: TBST
15. 3x10’ TBST (room temperature)
16. 2x15’ TBST
17. Block 2-3hrs in blocking buffer (5% heat inactivated serum + 1 mg/ml BSA in TBST)
18. Antibody either overnight at 4°C (anti-dig: 1:5000, anti-Fluo 1:10000 in blocking buffer), or 6hr at room temperature and wash overnight with TBST at 4°C.

**DAY 3: Post-antibody washes**
19. 6x45’ TBST (or more times and longer; better if it can be left overnight at 4°C after a few washes at room temperature)
20. 2x10’ Alkaline Phosphatase buffer (NTMT buffer)
21. Develop in 3.5ul BCIP+4.5ul NBT per 1ml. If the probe is slow to develop or gives background, develop at room temperature for a few hours until you see some colour and leave
in TBST at 4°C overnight, then redevelop during the day at RT, leave at 4°C overnight, etc, for as long as needed or until background appears.

22. Fix in 4% Paraformaldehyde or Formaldehyde in TBST.

FOR DOUBLE IN SITUS (DIG + FLUO)

23. 3x5’ TBST
24. 2x10’ MeOH 100%
25. 1x30’ to 1 hr MeOH 100% at 65°C (keep an eye on them: the colour can dissolve)
26. 1x10’ 50% MeOH:TBST
27. 1x5’ 25% MeOH:TBST
28. 2x5’ TBST
29. Overnight anti-body at 4°C (anti-dig: 1:5000, anti-Fluo 1:10000 in blocking buffer)
30. 6x45’ TBST (or more times and longer, it can be left O/N at 4°C after a few times at RT, is better)
31. 2x10’ Alkaline Phosphatase buffer
32. Develop 3.5ul/ml INT/BCIP or 7ul/ml BCIP
33. Fix in 4% Paraformaldehyde or formaldehyde in TBST.

Wholemount in situ
using a Sonic Hedgehog probe.
### SOLUTIONS:

**PBT**: 1xPBS+0.1% Tween

**Hybridization buffer (once made, store at -20°C)**

<table>
<thead>
<tr>
<th>Component (stock conc.)</th>
<th>Final conc.</th>
<th>Add</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>50%</td>
<td>25 ml</td>
</tr>
<tr>
<td>SSC (20x, pH 5.3 adjusted with citric acid)</td>
<td>1.3x SSC</td>
<td>3.25 ml</td>
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<tr>
<td>EDTA (0.5M, pH 8.0)</td>
<td>5mM</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Yeast RNA (20mg/ml)</td>
<td>50µg/ml</td>
<td>125 µl</td>
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<tr>
<td>Tween-20</td>
<td>0.002%</td>
<td>100 µl</td>
</tr>
<tr>
<td>CHAPS (10%)</td>
<td>0.005%</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Heparin (50 mg/ml)</td>
<td>100µg/ml</td>
<td>100 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>~18.4 ml</td>
<td></td>
</tr>
<tr>
<td><strong>Total:</strong></td>
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**TBST 10x:**

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>1M Tris-HCl pH 7.5</td>
<td>25 ml</td>
</tr>
<tr>
<td>Tween-20</td>
<td>11 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>~64 ml</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td>100 ml</td>
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</tbody>
</table>

**Alkaline Phosphatase Buffer (NTMT)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>5M NaCl</td>
<td>1 ml</td>
</tr>
<tr>
<td>2M Tris HCl (pH 9.5)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>2M MgCl₂</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>10% Tween-20</td>
<td>5 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>44.75 ml</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td>50 ml</td>
</tr>
</tbody>
</table>
**Whole-mount immunohistochemistry**

1. **Fix embryos in 4% paraformaldehyde**

   Antigens are often sensitive to over-fixing and all PFA must be washed out. For embryos at stage 20 or less (3-3.5 days of development), fix for an hour on ice. Wash 3x5 minutes in PBS and then leave in PBS for 4 hours on ice or overnight in the fridge. For older embryos, fix for 2 hours on ice and wash as above.

2. **Wash 2-3 times in PBS with 0.1% Tween-20 for 5 minutes each.**

   [Note: fluorescent antibody staining works fine up to at least stage 25 (E5) in wholemount, but for embryos at this stage and older you should next do a methanol dehydration to help penetration: 25%, 50%, 75% and 100% MeOH in PBS with 0.1% Tween-20; store at –20°C at least overnight, then rehydrate through the same methanol/PBT series into PBS with 0.1% Tween-20.]

3. **Block overnight at 4 degrees in 10% serum in wash solution (PBS with 0.1% bovine serum albumin, 0.1% Triton X-100).**

4. **Add primary antibody solution in wash solution, and incubate at least overnight at 4°C (2 nights is also fine, especially for embryos older than E3). Wash three x 1-hour at room temperature.**

5. **Add secondary antibody solution in wash solution (keep samples in the dark as much as possible), and incubate overnight at 4°C. Wash three x 1-hour at room temperature.**

6. **Fluorescent-conjugated secondary antibodies can be visualized immediately. Deeper staining may not be visible without some clearing in glycerol (30-50%).**
Vibratome sectioning

Some labeled cells are difficult to visualize in whole mount embryos because they are deep. Some axonal projections travel in and out of the plane of section, making it difficult to view them in thin sectioned material. Vibratome sections are ideal for dealing with these two problematic issues. Typically, 100 to 125 um vibratome sections are cut, followed by immunocytochemistry or in situ hybridization. The following protocol applies to generating transverse vibratome sections.

a) After collecting embryos, fix them for 2 hours at room temperature or overnight in the refrigerator (this depends primarily on your antibodies). Wash 3X 15 minutes in PBS.

b) Make 7% low melting temp agarose (Sigma) in water. First, bring water to near boil in microwave. Add agarose and stir to bring into solution. Reheat in microwave for a few seconds if needed. Agarose solution should be warm to use, but not boiling. Let it cool for a few minutes. If cooled too long, it will become solid.

c) Remove one embryo from PBS (remove excess structures ahead of time), place on parafilm. Use Kimwipe to pull off the excess liquid around embryo. Pour warmed agarose solution into metal mold (or foil shaped into small square); place embryo on and pour additional agarose over. Orient embryo in agarose using wire probes (with birch handles) under dissecting microscope. Allow agarose to become solid; place in refrigerator or freezer for 2 minutes.

d) Pop agarose block with embryo out of metal mold or foil. Trim under dissecting microscope.

e) Add one drop of crazy glue to mounting chuck on vibratome and apply trimmed agarose block. Allow to airdry for 5 minutes.

f) Fill vibratome bath with PBS. Putting mounting chuck on vibratome. Add 1/2 razor blade to cutting device. Adjust lighting (it often helps to turn off lights to eliminate aberrations) and adjust settings on vibratome for cutting to 3 (you can increase or decrease these, depending on tissue age).

g) Cut 100 ul thick sections and collect using paint brush. Place each section into a shell vial or 12 well TC dish with PBS. If you want to collect serial sections, you must do this in a 12 well dish.
Immunocytochemistry on vibratome sections

After cutting vibratome sections, you often will apply antibodies to discern different cell types. A general protocol for staining vibratome sections follows. The only item that you have to revise is the primary antibody concentration.

Check vibratome sections using a dissecting microscope or upright microscope to determine which sections to save (correct axial level; contains structures you want) and which to discard (poorly cut, missing structures). Place saved sections in PBS in shell vials or in 12 well TC dishes, in preparation for immunocytochemistry.

a) Remove PBS and place sections in permeabilization solution for 25 minutes, at RT, with gentle rocking on orbital shaker.
   Permeabilization solution:
   0.5% Triton X in PBS.

b) Remove permeabilization solution and add blocking buffer to sections for 30 minutes, at RT, with gentle rocking.
   Blocking solution:
   4% goat serum
   0.1% Tween 20
   PBS

c) Remove blocking buffer. Place in primary antibody solution (consists of primary diluted in blocking solution), overnight in refrigerator, gently rocking on orbital shaker. You want flow of antibody around sections. Avoid piling up of sections as antibody penetration will be poorer. Yet, you do not want so much flow that tissue sections are damaged.

d) Remove primary antibody solution and wash with blocking solution, 3X 15 minutes each. Then place in secondary antibody solution (Alexa fluor secondary antibody, 1:500, in blocking solution) for 45 minutes to 2 hours at room temperature, gently rocking. Cover with foil, to prevent quenching of fluorescence. Remove secondary antibody and wash 3X 15 minutes with PBS. View labeling using microscope equipped with fluorescence optics. To acquire exceptional images, view on confocal microscope.
Cartilage & Bone Staining Using Alcian Blue and Alizarin Red

Check which stages are suitable for the cartilage staining you are interested in. The earliest stage at which it’s worth looking is 5 days but better results are obtained on days 8-9. Bone is not detectable until at least E10: we may not have embryos old enough for this, but in this module we use the mouse protocol in which the stain combines both Alcian Blue (for cartilage) and Alizarin Red (for bone).

Important note: We have removed the 1%/2% KOH step present in the mouse module handout as the chick embryos are too small to withstand this.

The quickest way to kill the embryo is decapitation, but if you want a whole skeleton, we suggest putting the egg (without opening it) at -20°C for 20-30 minutes, to kill the embryo before opening the egg. Widen the window over the embryo by means of scissors or by the use of blunt forceps. (If decapitating the embryo, i.e., without having put the egg in the freezer, hook beneath the neck of the embryo with curved forceps and sever the head.) Place embryo in a fresh bath of PBS; rinse the embryo in PBS and place the body in 4% PFA at room temperature for 2-4 hours, or overnight at 4°C.

1. Dissect out embryos, rinse in 1XPBS, and place in ice-cold 95% ETOH for 1 hour. Change to fresh 95% ETOH and rock overnight at RT. Change 95% ETOH and store at 4°C until ready to do stain.

2. Stain overnight with rocking at RT in freshly prepared stain solution (see recipe below).

3. Very briefly rinse in water.

4. Rinse in 0.25% KOH for 30 mins.

5. Clear in 20% glycerol/0.25% KOH for 1 hr.
   Clear in 33% glycerol/0.25% KOH for 1 hr.
   Clear in 50% glycerol/0.25% KOH overnight.

6. Change solution to fresh 50% glycerol/0.25% KOH for further clearing and storage.

Recipe for stain solution:

- 5 ml 0.4% Alcian Blue 8 GX in 70% EtOH
- 5 ml glacial acetic acid
- 70 ml 95% EtOH
- 20 ml water

final volume is 100ml

When ready to do stain add 100ul of 0.5% Alizarin red to 10 ml of stain solution.

0.4% Alcian Blue/70% EtOH:
Alcian Blue does not readily dissolve in 70% EtOH, but does dissolve in 50% EtOH. To make 100 ml of 0.4% Alcian Blue stock, add 0.4g Alcian Blue to 10 ml 50% EtOH in a 250 ml bottle. Swirl and place in 37°C water bath. Swirl occasionally. When dissolved, add 25 ml water and 65 ml 95% EtOH.

0.5% Alizarin Red S/ water:
Add 0.5g alizarin red S to 100ml water. Swirl to dissolve.
Image Reference List:
- Egg windowing and lifting the embryo from http://www.biology.clc.uc.edu/fankhauser/labs
- Dil labeling of chick limb bud from Vargesson et al., 1997 Development 124, p. 1909
- Chick/Mouse limb recombination from Kuhlman & Niswander, 1997 Development 124, p. 133
- Stage 12/14 chick embryos originally from Hamburger & Hamilton
- Bead in AER from Lee Niswander (Woods Hole Embryology Course, 2008)
- Sonic hedgehog in situ from Ros et al., 2003 Development 130 p527
- Alcian blue staining in chick hindlimb from Pizette & Niswander 2000 Developmental Biology 219, p.237
APPENDIX 1: Typical Solutions for Chick Manipulation and Care.

1. Ink
1ml Pelikan Drawing ink A
9ml Howard’s Ringer’s

2. Howard Ringer’s
NaCl 7.20g
CaCl₂ (2H₂O) 0.17g
KCl 0.37g
H₂O 1000ml

3. 10x Tyrode’s saline (calcium-magnesium free)
   80 g NaCl
   2 g KCl
   0.5 g NaH₂PO₄·2H₂O
   10 g glucose
   Distilled water to one liter; autoclave.
Dilute to 1x with sterile distilled water on day of use. Store in fridge once opened. Bicarbonate is used as a buffer in the original recipe (1 g NaHCO₃ per liter) but solutions to which it has been added cannot then be autoclaved.

3. 10x Phosphate Buffered Saline (10xPBS) for New culture and general use:
Na₂HPO₄ 11.5 g
NaCl 80.0 g
KH₂PO₄ 2.0 g
KCl 2.0 g
in 1 litre dH₂O. Add salt slowly. Adjust pH to 7.2 - 7.3 and autoclave.

Making Sylgard plates
Sylgard 184 (Dow Corning) is clear silicone rubber polymerized by mixing two components (9 parts rubber solution to 1 part of accelerator/catalyst). Mix the two well and pour to the desired depth (2-5 mm) into the plastic Petri dish. Allow the dishes to stand for about 1 h at room temperature for air bubbles to leave, then cure at about 55°C until polymerised (3 h to overnight). The dishes can be stored indefinitely.
APPENDIX 2: STAGING CHICK EMBRYOS

Before Laying
Early cleavage (3.5-4.5 hr) Shell membrane of egg formed in isthmus of oviduct
During cleavage: Germ wall formed from marginal periblast
Late cleavage (4.5-24.0 hr) Shell of egg formed in uterus

After Laying:

Eyal-Giladi & Kochav stages (abbreviated)
X one layer of cells (epiblast) and scattered island of hypoblast ventrally
XI hypoblast starts forming in the posterior part
XII hypoblast reaches the half of the area pellucida length
XIII hypoblast covers the area pellucida entirely
XIV bridge visible in the posterior part of the epiblast, where the primitive streak is about to form

Hamburger Hamilton Stages (abbreviated)
(Full tables available: http://www.ncbi.nlm.nih.gov/pubmed/1304820)
2 6-7 hr: Initial primitive streak, 0.3-0.5 mm long
3 12-13 hr: Intermediate primitive streak
4 18-19 hr: Definitive primitive streak, ±1.88 mm long
5 19-22 hr: Head process (notochord)
6 23-25 hr: Head fold
7 23-26 hr: 1 somite; neural folds
7 to 8-, ca. 23-26 hr: 1-3 somites; coelom
8 26-29 hr: 4 somites; blood islands
9 29-33 hr: 7 somites; primary optic vesicles
9+ to 10-, ca. 33 hr: 8-9 somites; anterior amniotic fold
10 33-38 hr: 10 somites; 3 primary brain vesicles
11 40-45 hr: 13 somites; 5 neuromeres of hindbrain
12 45-49 hr: 16 somites; telencephalon
13 48-52 hr: 19 somites; atrioventricular canal
13+ to 14-, ca. 50-52 hr: 20-21 somites; tail bud
14 50-53 hr: 22 somites; trunk flexure; visceral arches I and II, clefts 1 and 2
14+ to 15-, ca. 50-54 hr: 23 somites; premandibular head cavities
15 50-55 hr: 24-27 somites; visceral arch III, cleft 3
16 51-56 hr: 26-28 somites; wing bud; posterior amniotic fold
17 52-64 hr: 29-32 somites; leg bud; epiphysis
18 65-69 hr: 30-36 somites extending beyond level of leg bud; allantois
19 68-72 hr: 37-40 somites extending into tail; maxillary process
20 70-72 hr: 40-43 somites; rotation completed; eye pigment faint grey
21 3.5 da: 43-44 somites; pharyngeal arch IV, pharyngeal cleft 4; eye pigment faint
22 3.5 da: Somites extend to tip of tail; eye pigmentation distinct
23 3.5-4 da: Dorsal contour from hindbrain to tail is a curved line
24 4 da: Toe plate in leg-bud distinct; 4th pharyngeal cleft reduced to small pit
25 4.5 da: Elbow and knee joints; 3rd & 4th pharyngeal clefts reduced to small pits
26 4.5-5 da: 1st 3 toes; 3rd and 4th pharyngeal clefts no longer visible
27 5 da: Beak just barely recognisable
28 5.5 da: 3 digits, 4 toes; beak outgrowth distinct in profile
29 6 da: Rudiment of 5th toe; beak more prominent; no egg tooth
30 6.5 da: Feather germs; scleral papillae; egg tooth
7 da: Web between 1st and 2nd digits; feather germs dorsally continuous from brachial to lumbosacral level

7.5 da: Anterior tip of mandible has reached beak

7.5-8.0 da: Web on radial margin of wing and 1st digit

8 da: Nictitating membrane

8 - 9 da: Phalanges in toes

10 da: Length of 3rd toe from tip to middle of metatarsal joint = 5.4±0.3mm; length of beak from anterior angle of nostril to tip of bill = 2.5mm; primordium of comb; labial groove; uropygial gland

11 da: Length of 3rd toe = 7.4±0.3mm; length of beak = 3.0 mm

12 da: Length of 3rd toe = 8.4±0.3 mm; length of beak = 3.1 mm

13 da: Length of 3rd toe = 9.8±0.3 mm; length of beak = 3.5 mm

14 da: Length of beak = 4.0 mm; length of 3rd toe = 12.7±0.5 mm

15 da: Length of beak from anterior angle of nostril to tip of upper bill = 4.5 mm; length of 3rd toe = 14.9±0.8 mm

16 da: Length of beak = 4.8 mm; length of 3rd toe = 16.7±0.8 mm

17 da: Length of beak = 5.0 mm; length of 3rd toe = 18.6±0.8 mm

18 da: Length of beak = 5.7 mm; length of 3rd toe = 20.4±0.8 mm

19-20 da: Yolk sac half enclosed in body cavity; chorio-allantoic membrane contains less blood and is "sticky" in living embryo

20-21 da: Newly-hatched chick