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Frog labs are scheduled as the following: There are 4 frog TAs here to help you:
June 29   Tuesday   Frog   Andrea Wills – June 25 – July 6
June 30   Wednesday Frog/fish Chenbei Chang - June 25-July 6
July 1    Thursday   Frog/fish John Wallingford -
July 2    Friday     Frog/fish Ray Keller -
July 3    Saturday   Frog/Fish

Yes, you should take the time to read this whole info packet. But here are some important notes:

Make sure you use the appropriate solution:
- Embryos 1/3x MR
- Injections Ficoll to inject; replace with 1/3x MR after 1 h to culture ‘bros
- Animal Caps 3/4x NAM
- Gastrula tissue or graft healing 1x Steinberg’s or DFA

You can do just about anything to Xenopus embryos but then you have to TAKE CARE OF THEM. Xenopus embryos are yolky and MBL has a rich microbial diversity. A few dead/dying embryos can spoil the whole dish. Make sure to check on your experiments several times a day. Remove dead/dying embryos, wash your embryos frequently, and use antibiotic (100x ABC, in front of room on ice) in your culture media, especially when culturing explants.

You can culture Xenopus embryos at different temperatures (12°C, 15°C, 22°C/room temp, 25°C) to speed or slow development, but they are happiest at 15°C. Bacteria grow faster at the higher temperatures, so embryos grown at RT or 25°C must be watched carefully and rinsed often.

There are a limited number of microinjection stations (8). Take turns by signing up for (non-consecutive) 1 hour slots. Early cleavages are fairly rapid (30 minutes), so collaborate with your classmates if you want to do a big experiment. We will provide 2-4 cell embryos for microinjection throughout the afternoon and evening labs. Ask Chenbei or Andrea for other special requests.

Make sure to take some uninjected controls to compare to your experimental embryos! Similarly, if you inject embryos to use as donors in graft experiments, make sure to take some uninjected embryos for recipients. Make sure to keep some whole sibling embryos when making explants so you can accurately stage your explants.

Use an appropriate lineage tracer: mRNA (GFP, RFP, lacZ) for mRNA injections, small molecule (miniruby or control MO-Fl) for MO injections. The former will take 4+ hours to be visible, while the latter will be visible almost immediately. Look for the lineage tracer before analyzing your experiment to be sure your embryos are injected. GFP/RFP will not survive MeOH dehydration so use lacZ and perform X-gal staining if you want to see lineage tracer in stained embryos.

Embryos without vitelline membrane and explants are delicate. Exposure to air will cause them to explode. Watch for bubbles in your pipette and be careful in changing solutions.

Antibody staining takes 2-3 days following fixation of samples but requires only intermittent attention. Plan to start on July 3rd or 4th if you want to have TAs around to help you interpret/photograph results before Show and Tell. We have antibodies for neural and mesodermal tissue, reactive at tailbud and tadpole stages.
I. General Xenopus Protocols  

There have been entire books written about the practical uses of Xenopus and detailed methods of various kinds of experiments possible with Xenopus embryos. The most useful are Methods in Cell Biology Vol 36 (1991) "Xenopus laevis: practical uses in cell and molecular biology", edited by Brian Kay and Benjamin Peng and “Early Development of Xenopus laevis, a Laboratory Manual” (2000), edited by Hazel Sive, Robert Grainger and Richard Harland. However, the following brief notes, together with help from faculty and TA's, will suffice for you to master the kinds of experiment we are attempting here.

A. Obtaining and Caring for Embryos – Chenbei

Chenbei and Andrea will perform fertilizations every 2 hours during the afternoon and evening sections to provide a constant supply of embryos for microinjection. Fertilization times are on the whiteboard in the front of the room and on the lids of embryo dishes. If you have need of fertilizations at other times, or if you would like to try UV ventralization (see “Embryology” section below), please let us know. Also let us know if you would like to observe/participate in the fertilization process.

Isolating testes

Males are anesthetized by submersion in a solution of benzocaine (0.05%). To remove testes, expose the abdominal cavity and, using forceps, draw out the fat body. The testes lie at the base of the fat bodies. They are white oblong organs that are covered by a network of capillaries. Cut the testes free of the fat body with curved scissors. Testes can be maintained at 4° C in 1x MR for 7 days or OCM + antibiotic for 1-2 weeks.

Egg collection (“squeezing the frogs”)

Ovulation is induced in Xenopus females by injection of 500-1000 U (depending upon the size of the frog) of human chorionic gonadotrophin (stock solution of 1,000 U/ml sterile water) into the dorsal lymph sac, using a 25 gauge needle. The dorsal lymph sac lies just rostral to the hindlimbs beneath the lateral line “stitch” marks. The skin is very loose here and the needle should be inserted subcutaneously but not deep enough to penetrate the musculature. The frogs will lay eggs approximately 8 hours later if kept at room temperature. This time can be increased by lowering the temperature (e.g. about 17 hr at 16°C).

When the female is ready to lay, eggs collect in a sac near the cloaca, which will appear red and swollen. Eggs can be expelled into a Petri dish by gently applying lateral and vertical pressure simultaneously to this region (“squeezing”). Eggs obtained in this manner should be fertilized within 30 minutes. Eggs can also be collected passively by leaving females in 1x MR + high salt. Eggs will collect slowly over the course of the day and good quality eggs are viable for fertilization for up to 8 hrs.

Fertilization

Since Xenopus is a fresh-water frog, sperm is activated in low-salt conditions. There are several fertilization methods but all rely on this principle. To fertilize the eggs, cut off a small piece of testis (one quarter or less of a testis) and place in an Eppendorf tube filled with distilled water. Crush the testis with a pestle and pipet the slurry over the eggs. Alternatively, crush testes in 1x MR and pipet over the eggs, then flood the eggs with water or 1/3x MR to dilute the salt.

The first sign of a successful fertilization (within a few minutes) is a cortical contraction in the animal hemisphere. During this contraction, the pigmented portion of the egg is reduced in surface area from 1/3 to 1/4 of the egg. 15-30 minutes post fertilization the eggs will turn within their vitelline membranes and reorient according to gravity so that the pigmented animal poles face up. This is the result of release of the contents of
cortical granules into the space between the fertilized egg and the vitelline membrane, which provides a block to polyspermy. This is the most reliable sign of fertilization. Fertilized eggs are also much firmer and resistant to deformation than are unfertilized eggs.

Immediately after fertilization, the Xenopus embryo enters the first cell cycle, during which cortical rotation occurs on vegetal MT arrays. Cortical rotation is required for the formation of the dorsal tissues and should not be confused with the rotation of the egg within the vitelline membrane (“turning”), which occurs prior. Cortical rotation occurs within 55 minutes of fertilization at RT. During this time, embryos should not be disturbed if normal dorsal-ventral patterning is desired. Shaking embryos during this time can produce spontaneous secondary axes by reorientation of MTs, or embryos can be exposed to vegetal UV light to destroy MTs and ventralize the embryos.

**De-jellying**
Embryos are surrounded by layers of protective jelly which must be removed before injection or dissection. Unless you are doing UV ventralization, do not de-jelly eggs until after cortical rotation occurs (or do so very gently). To remove, swirl the embryos in 3% cysteine hydrochloride in H₂O pH 7.8-8.0 until the eggs pack closely together. The time required for this varies between batches of embryos but it will generally take 4-6 minutes. DO NOT over-treat. Wash the eggs 5-6 times with H₂O to remove the cysteine and then rinse several times in 1/3x MR. Embryos are reared in 1/3x MR.

**Rearing Embryos**
Grow Xenopus embryos in 1/3x MR. Explants and recombinants are cultured in other solutions (see “Embryology” section).

Xenopus embryos can be reared at a wide range of temperatures. We have incubators for 12°C and 25°C, the frog room is 15°C, and the lab is around 22°C. Xenopus are happiest at 15°C and slightly less so at 12°C. Incubation at higher temperatures will result in more spontaneous gastrulation defects and death due to bacterial contamination.

Make sure to check on your experiments several times a day. Remove dead/dying embryos (balls of white goo or embryos with cells sloughing off), decant most of the old culture solution, and then add fresh 1/3x MR. Repeat until culture medium is clear. If your dish is mostly dead, move live embryos to a new dish of fresh media. Use antibiotic (100x ABC, in front of room on ice) in your culture media, especially when culturing explants.

**Staging Embryos**
Embryos are staged according to internal and external morphology as described in the *Normal Table of Xenopus Laevis* by Nieuwkoop and Faber. Several copies are available in the lab. Condensed pictures of early stages, as well as developmental timetables, are attached at the end of this handout (Appendix II).

**B. Tools – John Demo**
For the experiments in this module you will need a set (2 Pairs) of #5 watchmakers forceps. You will need these for removing the vitelline membrane from embryos and other embryological microdissections. They are also used to break needles for microinjection. You will either have brought forceps with you, or purchased them from the MBL staff. Keep your forceps sharp by using the cover and make sure to put your name on them.

In addition to your forceps you will need tools for cutting embryos (eyebrow knife) and for moving embryos (hair loop). John will demonstrate how to make these tools.
To make an eyebrow knife simply dip the end of a drawn off pipette into melted paraplast or beeswax and apply the blunt end of an eyebrow hair to the tip. You will find that hairs from different people vary enormously in their usefulness as cutting tools, so shop around.

To make a hair loop, thread both ends of a longer hair into a drawn out pipette and seal the end with wax. Loops of different sizes can be made. They are useful for moving embryos around in the dish and for moving and placing small bits of dissected tissue.

Embryos and pieces of embryo are transferred from dish to dish using pasteur pipettes. Whole embryos are too big to fit in standard pipettes, so you will need to mark the end of a standard pipette with a diamond pen and break the end off cleanly. Pieces of embryo will fit easily into the end of a regular pasteur pipette. Allow explants to heal before moving with a pipette so they do not stick to the glass. Avoid air bubbles in your pipette when transferring explants or de-vitellinized embryos.

Sylgard-coated slides are useful mounting chambers for examining tailbud and tadpole stage embryos on a compound microscope, and are required for use with BB:BA, which is a strong solvent. Arrange slides in a foil tray, tightly packed. Make of Sylgard silicone elastomer according to manufactures’ instructions and pour over slides. The volume of Sylgard poured over the slides can be adjusted to produce slides of different thickness (1mm for gastrulae, thinner for tadpole). Cure slides overnight at RT or 2 hours at 60C. Cut apart with a razor blade and cut well(s) into Sylgard.

**C. Microinjection – Andrea Demo**

Reagents for manipulating gene expression (mRNAs, morpholinos, DNA, and even sometimes drugs and proteins), and for cell-labeling, are introduced to the embryos via microinjection. Embryos can be microinjected at the one cell stage or later, after they have been de-jellied. One advantage of the Xenopus embryo is the early cleavage fate map, allowing for precise localization of injected reagents (see Appendix III). For example, since the first cleavage divides left and right sides, injection into 1 blastomere at the 2-cell stage will give you a control side and an experimental side. At the 4-cell stage, in “good” batches of embryos, the dorsal side of the embryo can be distinguished from the ventral by lighter pigmentation on the animal hemisphere. Control of the location of injection comes in handy for reagents that have pleiotropic effects in the embryo. Look at the fate map included in this package to help you target your injection site, and choose embryos with good, regular cleavage planes for microinjection.

Make an “injection mix” of 5ul by diluting given reagents with RNase-free (DEPC-treated) water, in an RNase-free tube, using barrier tips. Keep RNA solution on ice. Heat MO solutions at 65C 5-10 minutes, vortex, and then spin at least 3 minutes in a centrifuge (max speed) to pellet insoluble crystals. MOs are somewhat insoluble and apt to clog microinjection needles.

We will be injecting approximately 10nl into a 1-8 cell X. laevis embryo (reduce volume for later stage embryos). The microinjection needle is attached to a pressurized picospritzer and you will use the “pulse” function (with the foot pedal) to deliver one droplet. The mRNA and MO stocks have been calibrated so that the effective dose is delivered in 10nl. Thus, you have to calibrate the microinjection needle so that a droplet of 10nl is delivered per pulse of the picospritzer. You will do this by breaking the tip of the needle with your forceps and by varying the time of the pulse. DO NOT ALTER THE INJECTION PRESSURE!! To get a good-sized needle (not too big to kill the embryo and not too small to clog), keep the time of the pulse at 200-500 msec.

The hardest part to learn about microinjection is breaking the needle, so be patient. We will provide you with a piece of paper with printed dots that corresponds to a 10nl droplet, as well as pulled needles of appropriate shape. The tapered end of the needles is sealed and very flimsy, so you must break the end to achieve a needle appropriate for injection. Back-load the needle with half of your injection solution using
a Seque-Pro tip (fits inside the capillary). Gently place it in the micromanipulator and tighten. Clip the end of the needle with your forceps and pulse, clipping more and varying the pulse time until you get a droplet of 10nl. We recommend using a medium to high magnification on the scope for this, as it is difficult to accurately compare the size of the droplet to the printed dot at low magnification.

Once you have calibrated your needle, injection will go fast. Place de-jellied embryos into meshed injection dishes filled with 2.5% Ficoll injection solution. The Ficoll collapses the vitelline space so that the embryo is not under pressure. Orient the embryos in the mesh in whatever direction you want (e.g. animal side up) with a hairloop. Then remove enough of the Ficoll (most of it) so the embryos do not wobble, but not so much that they are dry or under extreme pressure (explode when injected). We have set up the manipulators so that the needle will go into and out of the embryo at the correct angle (without shearing the membrane) by moving only one knob. So just stick the needle in the embryo, pulse, and go! It is a good idea to check at the end of each row of embryos to make sure that your needle is not clogged. If your embryos start leaking immediately, or exploding, then you are injecting too large a volume, or air. Take care with injection volume: even though the embryo is a big target, it is not as robust as you may think.

After you are done injecting all the embryos in a dish, flood them with Ficoll and pour them into a petri dish, leaving the mesh injection dish for the next user. The Ficoll will help the embryos to heal the injection wound. However, it will also promote bacterial growth and gastrulation defects. So, 

Rinse your injected embryos into 1/3x MR after about an hour.
Remember to take some uninjected controls as well!

D. Microdissection – Ray Demo
Removal of the vitelline membrane is required before cells and tissues can be dissected. You may also want to remove the membrane from unhatched tailbud-stage embryos before fixing, for ease of imaging. Use one pair of forceps to grasp the vitelline membrane in a region of the embryo where any damage you cause will minimally impact your experiment. If you want the embryos to gastrulate, remove the membrane at the animal cap region as this area heals rapidly. Once you have grasped the membrane with one forceps, get a second hold with your other forceps and tear the membrane off. Intact embryos outside the vitelline membrane are fairly sturdy, but will rupture easily if exposed to the air-water interface. Wounded embryos are even more susceptible to explosion.

Embryos are dissected, and pieces of them cultured, on beds of agarose (2%) in a plastic dish. The agarose is made up in water, so always pre-equilibrate the agarose with the solution you are going to use. Dissections and grafting should be carried out on agarose pads or modeling clay in different solutions according to the experiment. While the outside (epithelium) of the embryo is happiest in low saline conditions (1/3MR), the deeper layers abut the blastocoel, which is filled with high saline fluid. Thus, explants of deeper tissue layers are cultured in other media that emulate the blastocoel fluid. For specific descriptions of various explants and grafts, see “Embryology” section. Ray will demo these operations.

- Animal caps: 3/4x NAM
- gastrula tissue or graft healing: 1x Steinberg’s or DFA

You will cut embryos using your eyebrow knife on agarose pads or in modeling clay and then let explants or grafts heal there for 30-60 minutes. Following operation and healing, explants can be transferred to and cultured in agarose dishes with wells in order to keep them from aggregating. Long term (>24 h) culturing on agarose is not recommended. Again, be careful to avoid the air-water interface when transferring pieces of tissue. Whole embryos with grafts should be reared in 1/3 MR following graft healing.
IMPORTANT!! When you are culturing embryos or tissues which have been micro-dissected, you should culture them in the presence of antibiotics and change the media at least twice a day.

E. Analyzing Experiments

Imaging Whole Embryos
Since Xenopus embryos are very large, whole embryo imaging is usually done on a dissecting scope. Magnification and lighting are important for achieving a good depth of image so you can get a pretty picture. Because the early embryo is yolky, live fluorescence may be dim and difficult to visualize through pigment. Use high magnification in a dark room and move the embryo around to see all sides. For fluorescent time-lapse imaging in whole embryos, you may want to use albinos. For fluorescent antibody staining, the pigment can be removed with bleach and the embryo cleared (in BB/BA) in order to aid visualization of the signal. Embryos can be photographed simply in a Petri dish filled with 1/3x MR (for live embryos) or PTw (for dead embryos). If you are having difficulty getting the embryo to stay in the desired orientation, use a small Petri dish filled with modeling clay and make appropriate indentations for your embryos with a fire-polished Pasteur pipet. Or, use a welled agarose dish. Or, use a mesh injection dish (live embryo only).

For imaging live later stage embryos, you may want to anaesthetize them. Transfer them to a Petri dish filled with a dilute benzocaine solution (0.005% in 1/3x MR). Tadpoles will survive for many hours in dilute benzocaine but will develop edema from long term culture (>24h). Tailbud and tadpole embryos can be imaged on a compound scope with a low magnification (2.5x) lens, since they are flatter than early embryos. Use a Sylgard slide of appropriate thickness with wells cut into it and cover with a coverslip.

Imaging Explants
Xenopus explants are smaller, and therefore can be imaged using the compound or confocal scopes. However, the tissue layers are still much thicker than the small marine embryos you have been working with. Use clay feet or a border of vacuum grease between the slide and the coverslip to avoid smashing your explant. For live (time-lapse) imaging, you will have to make a culture dish appropriate for the compound or confocal microscope. Ray will demonstrate this to you. A culture chamber for these explants can be made by boring a 20mm hole in a 60mm plastic dish and gluing a #1.5, substantially larger cover slip over the hole with a bead of silicone high vacuum grease. The tissue is placed with the inner or deep surface down on the cover slip, and restrained with a glass bridge (scored and broken coverslip), supported at each end with silicone high vacuum greased as described above. This allows high-resolution optics to be used with an inverted microscope or confocal. An upright microscope can be used by overfilling the dish with media and covering the top with a large glass slide, and then inverting it, such that the cover slip and the deep surface of the explant is at the top, facing the objective.

If you would like to label various cell components for imaging on the compound scope, you will have to inject less mRNA (e.g. membrane-GFP) that is required for visualization in the embryo. Similarly, if you want to observe accurate localization of a GFP-tagged protein (e.g. Dsh-GFP), you need to inject less than the phenotypic dose. For imaging of cell protrusive behavior, scatter labeling (graft or 32-cell injections) is recommended. See TA for help in making appropriate injection mix for such experiments.

Xenopus Embryo Fixation
Many of your experiments can be analyzed live simply by morphology, e.g. elongation of animal caps. But if you want to stain them, you have to fix them. Xenopus embryos are fixed in 1x MEMFA, a solution of 3.7% formaldehyde and salts (see Appendix I for recipe). For X-gal staining, fix 30 minutes at RT. For Ab staining, fix 1h at RT. For in situ hybridization, fix 2h RT or O/N at 4°C. Remember, GFP will survive fixation but not MeOH dehydration.
1. Rinse embryos to clean away any schmutz. If you will be doing immunohistochemistry you will need to remove the vitelline membranes from the embryos.
2. Transfer embryos to glass sample vials.
3. Add 1x MEMFA solution to fill vial and fix for 1 hour on nutator (OR 30 min for β-gal staining). There will be a bottle of 1xMEMFA ready for use in the hood in back hallway.
4. Remove MEMFA (discard in bottle marked FORMALDEHYDE waste).
5. Several 5 minute rinses with PTw (PBS + 0.1% Tween-20).
6. If you will be doing β-gal staining, go straight to β-gal protocol at this point. If not, do several rinses with 100% Methanol.

Methanol washes are important for permeabilizing the samples before Ab staining or in situ hybridization. Don’t skip this step. Again, do not put your embryos in methanol if you are doing β-gal staining. Methanol will KILL your β-gal reaction.

At this point, embryos can be processed for antibody staining (or in situ hybridization). Before Ab staining, you might want to bleach your embryos to remove pigment. Embryos can be stored in methanol at –20°C indefinitely.

X-gal staining reaction
If you have injected β-galactosidase mRNA, you can perform X-gal staining to lineage trace the injected cells. The colored precipitate (blue) will survive fixation and MeOH dehydration (unlike GFP) to show which cells have been injected. Useful for cell autonomy studies.
1. After 30 minute fix and PTw washes, remove PTw and add enough X-gal staining solution (see Appendix I) to cover embryos. Cover vial with foil and incubate, shaking, until desired color is achieved. Embryos can be incubated at 4°C, RT, or 37°C to slow or speed the reaction. The amount of time the embryos should be left in the stain will depend on the amount of β-galactosidase activity in the embryos.
2. Rinse embryos in 1x PTw several times and refix in 1x MEMFA for 1 hour.
3. Replace 1x MEMFA with several rinses of 100% Methanol. Embryos can be stored in methanol at –20°C indefinitely.

Antibody staining
1. Rehydrate step-wise with 5 minute washes to 1x PTw (75% MeOH, 50% MeOH, 25% MeOH).
2. Rinse 4x 5 minutes 1x PTw.
3. If desired, bleach embryos to remove pigment (see below for bleaching protocol).
4. Rinse embryos in 1x PBT (PBS + 0.1% TritonX100 + 2mg/ml BSA), 10 min.
5. Block in 1x PBT + 10% goat serum: 1 hour or more at room temp.
6. Incubate in primary antibody (diluted in blocking solution) overnight at 4°C.
7. Wash for 6 h, nutating/rotating, at least 3 solution changes, in 1x PBT.
8. Incubate in secondary antibody for 1 hour at room temp.
9. Wash as before. More washes = better.
10. Observe under fluorescence microscope. You may want to clear the embryos in BB:BA solution (2:1 benzyl benzoate:benzyl alcohol) to better observe internal structures.

Available primary antibodies – dilute in PBT + 10% goat serum
Tor 70 notochord (mouse IgM): dilute 1:5
12/101 muscle (mouse IgG): dilute 1:1
6F11 neural tissue (mouse IgG): dilute 1:5

Secondary antibodies – use at 1:200, dilute in PBT
Goat-Anti-Mouse-IgG-AlexaFluor 488 (green)
Goat-Anti-Mouse-IgG-AlexaFluor 555 (red)
Goat-Anti-Mouse-IgG-AlexaFluor 647 (far red)
Goat-Anti-Mouse-IgM-AlexaFluor 488 (green)

Bleaching
Pigment can be removed from the embryo following fixation, to aid in visualization of fluorescent signal. See Appendix I for bleaching solution recipe (reagents are in the cold room). Bleaching will be speeded by exposure to light. Following bleaching, rinse samples a few times through PTw before proceeding.

Clearing
Until tadpole stages (~st.42 on), Xenopus embryos are full of yolk and impenetrable to light. A solution of benzyl benzoate and benzyl alcohol (2:1) matches the reflective index of the yolk and makes the embryo clear, allowing for visualization of internal structures. BB:BA is highly toxic and will dissolve plastic dishes. Wear gloves when handling, and use small amount in glass depression dishes or Sylgard-coated slides for viewing embryos (see Richard for Sylgard slides). Make sure that embryos are thoroughly dehydrated in MeOH. Transfer to the depression slide. Draw off MeOH and replace with BB:BA (located in the hood). Once samples settle, replace with fresh BB:BA. Clearing happens in 5-10 min. If the solution is cloudy, or your embryos are not fully cleared, repeat the MeOH dehydration.

When you are done dispose of the specimens and BB:BA in waste container in hood.

II. Classical Embryology – Ray Demo
Before advances in molecular biology, developmental biologists used classical embryological techniques, including vital dye labeling, microdissection, and physical/chemical perturbations. With these techniques, embryologists could visualize the movements of cells in real time and find out which tissues they became (lineage/fate mapping). Then they could perform explants and transplants to test the timing of specification and commitment of cells to these fates, as well as their inductive ability. Later, proteins were isolated and genes cloned based on their biological activity in such classical experiments (e.g. FGF in mesoderm induction, Noggin in dorsal determination). These types of experiments form the basis of our knowledge about the Xenopus embryo. Several of the assays (e.g. animal cap) are still widely used today. You can reproduce these experiments in uninjected embryos or in conjunction with some of the many reagents we have for manipulating gene expression (See list of reagents in Section III).

Remember!!
- Use appropriate media, plus antibiotic. Clean explants frequently.
- When you transfer dissected tissues you must always keep them below the surface of the solution or they will lyse (watch out for bubbles and air/water interfaces inside the pipette).
- Be sure to keep some sibling embryos to stage your explants.

A. Explants
Explants are a good way to see what tissues will become in isolation and to be able to manipulate gene expression specifically (add protein or drug inhibitor to your explants and see what happens).

Animal Cap Assays
When removed from the embryo at stage 8 or 9 and cultured to stage 20+, the animal cap (the pigmented 1/3 to 1/4 of the blastula) will adopt an epidermal fate and a round (ball) morphology. Introduction of various molecules or co-culture with inductive tissues will cause the animal cap to adopt various other
fates (e.g. BMP antagonists induce neural, vegetal cells and FGF induce mesoderm). These new fates can be assayed by morphology (mesoderm will elongate) and/or immunohistochemistry.

Transfer several blastulae (st. 8 or 9) to a 60mm agarose dish containing 3/4x NAM. Remove the vitelline membrane from the vegetal side so you don’t damage the animal cap. Cut the animal cap (the central most 1/10 of the animal hemisphere) using your forceps or eyebrow knife. Alternatively, the blastocoel side of the animal cap can be exposed by opening the embryo from the vegetal pole and the cap can then be cut with an eyebrow knife.

After explanting an animal cap, move it to the side and remove the rest of the embryo. Cut at least 5-10 caps for each condition, and use a different agarose pad for each so you don’t mix them up! After the caps have healed (30 min – 1 h), transfer them carefully to the welled agarose dish containing 3/4x NAM for culturing to tailbud stages. If you are culturing with a protein, move your caps immediately into wells containing 3/4x NAM plus your protein of interest. Be careful not to dilute proteins with the transferred 3/4x NAM. If treating with activin, use stage 8, rather stage 9, caps.

**Keller explant/sandwich**

These explants are a great way to watch the cell behavior during gastrulation. The basic Keller explant is a rectangle of dorsal mesendoderm and ectoderm, approximately 60-90° wide, cut from an early gastrula embryo (st. 10) from the bottle cells to the animal pole. Two such explants stuck together (inner faces stuck together) make a Keller sandwich, which prevents the explant from curling up.

Transfer several gastrulae (st. 10+) to a 60mm agarose dish containing 1x Steinberg’s. Remove the vitelline membrane from the animal pole or the ventral vegetal side so you don’t damage the dorsal side. With the vegetal side up, use your knife to make two cuts, 60-90° around the blastopore lip, up to the animal cap. With the animal side up, make one cut through the animal cap to connect the sides of your rectangle. Peel back the dorsal tissues to the blastopore lip. (If necessary, use your knife to deepen the cleft formed by the involuting head mesoderm to allow peeling back to the lip). Flip the embryo over and make the final cut just below the bottle cells. With your hairloop/knife, remove any excess material below the bottle cells and head mesoderm on the inner side of the explant.

For an open face explant, transfer to a dish of DFA and lay the tissue flat. Place a dab of vacuum grease at each end of a coverslip and place the coverslip on the explant. Flatten but don’t squash.

For a Keller sandwich, quickly prepare two Keller explants and trim to the same size. Press inner surfaces together and allow to heal. After healing, transfer to a dish of DFA at treat as for open face explant.

**B. Grafts/Recombinants**

When cut, Xenopus tissues are fairly sticky. As they start to heal, they lose their adhesive property. So when doing grafts and recombinants, cut the pieces and stick them together right away (i.e. don’t cut a bunch of donor pieces and then try to stick them into hosts). Try not to move the samples too much while they are healing. To aid this, you can place the recipient embryo in modeling clay and use a glass bridge (scored and broken piece of coverslip), secure with vacuum grease, to press the tissue graft into place while healing.

You can graft and recombine any tissues you can dissect out. Here are some common experiments:

**Spemann-Mangold (organizer) grafts**

The dorsal marginal zone (DMZ) is also called “organizer” tissue or Spemann’s organizer. It is fated to become prechordal plate and notochord, but can organize surrounding tissues to form a dorsal axis. For a Spemann graft, the organizer is placed in the ventral region of an intact embryo to form a secondary dorsal axis. However, the organizer can also make a secondary axis when placed in the blastocoel (Einsteck graft) and induce neural tissue in recombinant with an animal cap. The Spemann graft is
technically more challenging than Einstecks. To be successful, you must get your graft to heal seamlessly into the host embryo so that gastrulation movements can proceed normally.

On an agarose-padded dish filled with 1x Steinberg’s, carefully remove the vitelline membrane from stage 10-10+ host embryos, from the animal pole so that any wounds may heal. Now remove the vitelline membrane from st. 10.5 donor embryos (again from the animal side but you don’t have to be as careful with these – you only want the DMZ). Place donor embryos vegetal side up and use your knife to make a radial cut from either side of the blastopore lip to the blastocoel. Flip embryo animal side up and cut the blastocoel in between your two radial cuts. Peel back the DMZ to the bottle cells. Flip the embryo over and spread the DMZ out flat, then cut just below the bottle cells. Trim away animal tissue to make a square. This is your organizer graft.

As soon as you see the bottle cells on the dorsal side of your host embryo, use your hair knife/forceps to make a small wound on the ventral side (opposite). Press the graft into this wound MAINTAINING THE SAME ORIENTATION it had on the donor embryo. Trim any overhanging tissue. Let the graft heal for at least 30 min in 1X Steinberg’s and then transfer to 1/3XMR to grow your two-headed tadpole!

* To win the coveted Organizer Graft Award, your organizer graft must be lineage-traced. The most complete secondary axis wins!

**Einstecks**

Einstecks refer to a procedure for placing a piece of tissue into the blastocoel of an early gastrula, to test the inductive potential of the donor tissue. This is a quick and dirty way to do a Spemann-type experiment to get a secondary axis, or you can also use it to test the inductive abilities of other tissues.

Transfer several embryos at stage 10 (host) and several donor embryos to an agarose dish filled with 1x Steinberg’s. Remove the vitelline membrane from host and donor embryos. Dissect tissue of interest from donor embryos (for a secondary axis, dissect the organizer from st. 10.5 embryo as described above). Now make a small hole in the roof of the blastocoel (the middle of the animal cap) in your donor (st. 10) embryo, and push the donor plug of tissue inside the blastocoel. Culture the host embryo for 30 min in 1x Steinbergs to allow it to heal, then transfer it to 1/3x MR to develop.

**Animal Cap/Vegetal Endoderm (Nieuwkoop) Recombinants**

The vegetal cell mass (presumptive endoderm) can induce mesodermal tissue in animal cap cells (which normally become epidermis). This experiment, first performed by Nieuwkoop, is the basis for the animal caps assays used so extensively today. Several molecules (Nodals, activin, FGFs) can also induce mesoderm in animal caps.

Transfer blastulae (st. 8-9) to agarose dish filled with 1x Steinbergs and remove vitelline membrane. Isolate vegetal cells from dorsal, ventral, or central (cap) region. Remove the animal cap from another embryo. Gently push together inner surfaces of both tissues and allow the recombinant to heal for at least 30 min. Transfer to welled agarose dish and culture in 1/3x MR. Examine the recombinants the next day to see if the animal caps have elongated in response to the vegetal masses.

**C. Dorsalization/Ventralization**

**UV ventralization**

UV light applied vegetally will destroy the microtubule arrays that are required for cortical rotation and localization of dorsal determinants.

- Fertilize embryos and dejelly after they turn (15-20 min).
- Put embryos on dishes with a Saran Wrap bottom and remove most of the liquid to immobilize.
- Subject embryos to UV light (set at energy of ~1200 μjoules x 100) for 30-90 seconds, within 40 minutes of fertilization time. You may have to do different doses/exposure times, as different batches of embryos have different sensitivities to UV).
- Try to avoid shaking embryos too much, as this may facilitate gravity-mediated cortical rotation. UV embryos can be rescued by manual rotation of the egg 90° with respect to the animal-vegetal axis. This is also useful for manually establishing and marking the dorsal side of a batch of embryos. Do this in 5% ficoll/0.5x MR in an agarose dish in which you have made shallow wells to support the embryo. You can mark the dorsal side by making nile blue precipitate (mix a drop of 1% nile blue sulfate with a drop of 100mm Na2CO3). Use a drawn out pipette to collect flecks of the ppt and apply to the embryo. Score by comparing with the dorsoanterior index (DAI) in Appendix IV.

**LiCl dorsalization**
LiCl inhibits GSK-3β, leading to β-catenin activation, which is required for dorsal fates.
- Immerse embryos in 0.3 M LiCl in 1/3x MR for 5-7 minutes at the 32-cell stage.
- Wash extensively (5 washes with 1/3x MR). Do several batches and different lengths of time to observe a range of phenotypes.
Score by comparing with the dorsoanterior index (DAI) in Appendix IV. Embryos that are first ventralized by UV can be more radially dorsalized by Li ion than normal embryos.

**D. Cell Lineage Analysis**

**Microinjection of mRNA and fluorescent dyes**
We have a variety of lineage tracers available for microinjection into early cleavage stage embryos (Section III). You can use these alone to perform early cleavage fate maps or label donor tissues for grafting experiments, or you can co-inject them with other reagents to lineage trace the injected cells. Perform the injections as described in “Microinjection” section.
Be aware that mRNA and small molecule dyes diffuse very differently in the early Xenopus embryo because it is so yolky. Small molecules will diffuse quickly to fill the entire blastomere, while mRNAs will remain somewhat localized. For this reason, when co-injecting, use mRNA tracer with mRNA, small molecule dye with MOs. Keep in mind that while all our reagents are good vital dyes, only a few lineage tracers will survive the fixation and immunohistochemistry protocol. Plan your experiment accordingly.

**DiI/DiD labelling**
Use this method for labelling small clones of cells (e.g. migrating muscles or neural crest) after cleavage stages.
- Dissolve DiI or DiD powder in pure 100% Ethanol (or dimethylformamide, DMF) at 0.1% and store with tight parafilm in −20° or −80°C.
- Dilute 1:10 in 0.3 M sucrose for working solution and heat at 37°C to improve solubility.
- Load 5ul into a microinjection needle and inject a 10nl drop in the area of interest. Repeated injections may be required.

**III. Manipulating Gene Expression**
We have many reagents to affect a wide variety of signaling pathways. The mRNA and MO stocks are diluted to give phenotypes with proper microinjection technique (10nl/cell). Go crazy thinking of ways to combine these reagents with classical embryological techniques!

**Guidelines:**
- Keep mRNA on ice and use RNAse-free (RNF) tips, tubes, and water to reduce risk of RNAse contamination. For this reason, also keep the lids closed on RNA solutions and tips.
• MOs are fairly insoluble and tend to clog needles. Heat the working solution 5-10 minutes at 65°C and spin at least 3 minutes to pellet insoluble crystals. Check needle often for cloggage.
• Use an appropriate lineage tracer. mRNA for mRNA, small molecule for morpholinos. These molecules diffuse very differently in the embryo so do not accurately reflect each other’s distributions when co-injected. When labeling tissues for grafting, we recommend using one of the small molecule dyes so you don’t need to worry about targeted injections.
• Think carefully about your experiment. Do you want a cell-autonomous reagent or a secreted protein? Do you have to inject both sides of the embryo to get a phenotype?
• Remember appropriate controls. Uninjected is often acceptable, but don’t forget a solvent (DMSO) control for drug experiments.
• Embryos are pretty sensitive to RNA injections (up to 5 ng, depending on the RNA). The less you can inject the better. If you are trying out your own RNAs, we recommend trying several doses, ranging from 50 pg to 1 ng.

List of Reagents:

1. Lineage tracers

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>MOs</th>
<th>other</th>
</tr>
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<tbody>
<tr>
<td>mem-RFP</td>
<td>control MO-fluorescein</td>
<td>Dil (fixable)</td>
</tr>
<tr>
<td>mem-GFP</td>
<td>miniruby (fixable)</td>
<td>DiO</td>
</tr>
<tr>
<td>mem-cherry</td>
<td></td>
<td>DiD</td>
</tr>
<tr>
<td>histone-GFP</td>
<td></td>
<td>Phenol red (to see injection solution)</td>
</tr>
<tr>
<td>tau-GFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-galactosidase</td>
<td></td>
<td>(fixable)</td>
</tr>
<tr>
<td>nuc-β-galactosidase</td>
<td></td>
<td>(fixable)</td>
</tr>
<tr>
<td>Kaede (GFP, becomes RFP following UV light exposure)</td>
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2. Markers

<table>
<thead>
<tr>
<th>mRNAs</th>
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<tbody>
<tr>
<td>mem-RFP</td>
<td>cell membrane</td>
</tr>
<tr>
<td>mem-GFP</td>
<td>cell membrane</td>
</tr>
<tr>
<td>mem-cherry</td>
<td>cell membrane</td>
</tr>
<tr>
<td>histone-GFP</td>
<td>chromosomes</td>
</tr>
<tr>
<td>tau-GFP</td>
<td>MTs</td>
</tr>
<tr>
<td>moesin-GFP</td>
<td>actin</td>
</tr>
<tr>
<td>centrin-RFP</td>
<td>basal bodies (centrosomes)</td>
</tr>
<tr>
<td>clamp-GFP</td>
<td>ciliary rootlet; midbody of dividing cells</td>
</tr>
<tr>
<td>galT-RFP</td>
<td>golgi</td>
</tr>
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</table>

3. TGFβ superfamily

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>MOs</th>
<th>other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin</td>
<td>activinB</td>
<td>activin protein</td>
</tr>
<tr>
<td>BMP4</td>
<td>follistatin</td>
<td>SB431542 (inhibitor)</td>
</tr>
<tr>
<td>chordin</td>
<td>Chd1, Chd2</td>
<td></td>
</tr>
<tr>
<td>noggin</td>
<td>noggin</td>
<td></td>
</tr>
<tr>
<td>lefty</td>
<td>lefty1, lefty2</td>
<td></td>
</tr>
<tr>
<td>Xnr3</td>
<td>Xnr3</td>
<td></td>
</tr>
<tr>
<td>Smad7</td>
<td>Cerberus</td>
<td></td>
</tr>
<tr>
<td>Smad6</td>
<td>ALK4 (Nodal receptor)</td>
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<tr>
<td>Smad2</td>
<td>ALK2, ALK3, ALK6 (BMP receptors)</td>
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### 3. TGFβ superfamily (cont’d)

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<tr>
<td>Smad4</td>
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<tr>
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<tr>
<td>GFP-Smad2</td>
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<td>GR-Smad1</td>
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<td></td>
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<tr>
<td>GR-Smad2</td>
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<td></td>
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<tr>
<td>GR-Smad2Δ (activated)</td>
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<tr>
<td>Ski</td>
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### 4. FGF

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<td>Fgf8a</td>
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<td>SU5402 (inhibitor)</td>
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<tr>
<td>Fgf8b</td>
<td>Fgf8spd</td>
<td>bFGF protein</td>
</tr>
<tr>
<td>Fgf4</td>
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<td></td>
</tr>
<tr>
<td>XFD (DN-receptor)</td>
<td>FgfR4</td>
<td></td>
</tr>
<tr>
<td>Sprouty</td>
<td>Sprouty</td>
<td></td>
</tr>
<tr>
<td>Spred</td>
<td>Spred</td>
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</tr>
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### 5. Wnt (canonical)

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<tr>
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<th>other</th>
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<tbody>
<tr>
<td>active β-catenin</td>
<td>β- catenin-Fl</td>
<td>LiCl (activator)</td>
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<tr>
<td>β- catenin</td>
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<td></td>
</tr>
<tr>
<td>Dkk1</td>
<td>Dkk1</td>
<td></td>
</tr>
<tr>
<td>Wnt8</td>
<td>Cerberus</td>
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</tr>
<tr>
<td>LRP5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gsk3</td>
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</table>

### 6. MAPK and PI3K

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<th>other</th>
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<tr>
<td>MEK* (activated)</td>
<td></td>
<td>PD98059 (MAPKKK inhibitor)</td>
</tr>
<tr>
<td>p110CAAX (activated PI3K)</td>
<td>U0126 (MAPKK inhibitor)</td>
<td>LY294002 (PI3K inhibitor)</td>
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### 7. Planar cell polarity and morphogenesis

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<tbody>
<tr>
<td>Wnt11</td>
<td>Vangl2/Van Gogh/Strabismus</td>
<td></td>
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<tr>
<td>Xdd1 (DN-Dsh)</td>
<td>Inturned</td>
<td></td>
</tr>
<tr>
<td>Dsh-GFP</td>
<td>Par1</td>
<td></td>
</tr>
<tr>
<td>Moesin-GFP</td>
<td>p130cas/BCAR1 (stress response)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MyosinHa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myosin IIb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fmn1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IFT172</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IFT52</td>
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### 8. Neural Crest Development

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>MOs</th>
<th>other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sox9</td>
<td>AP2</td>
<td></td>
</tr>
</tbody>
</table>
8. Neural Crest Development (cont’d)
mRNAs | MOs | other
--- | --- | ---
Slug | Slug | Snail
Snail | FoxD3 | Pax3-F1
Zic1 |

IV. Suggested Projects
We have collected a wide variety of reagents for gain- and loss-of-function experiments in many signaling pathways, as well as given you the knowledge of what questions classical embryology techniques can answer. We hope you think of new and amazing things to do with these. We’re happy to help you design an experiment. In case you’re stuck, here are a few ideas to get you started. Pick a few experiments from a few areas or collaborate with classmates to do all the experiments in one area.

A. Gastrulation and mesendodermal cell behaviors
Ray has lectured on the roles of myosin II in convergent thickening and extension, as well as the role of integrin/fibronectin in mesodermal cell behaviors. Recent studies suggest that non-canonical Wnt signalling components in the planar cell polarity, or PCP, pathway play essential roles in convergent extension movements of dorsal midline cells. FGF signaling has also been implicated in convergent extension, through a non-MAPK pathway. We have reagents to perturb all of these pathways!
- Disrupt these pathways and observe the morphological phenotype in whole embryos.
- Disrupt these pathways and scatter label cells at 32-cell stage with a membrane marker. Make Keller explants and time-lapse cell behavior with membrane-GFP/RFP, moesin-GFP or Dsh-GFP.
- Compare the intrinsic cell movements of dissociated cells from various parts of the embryo (wildtype and/or manipulated) on a fibronectin substrate.

B. Axis Induction
Everybody likes a two-headed tadpole! There are many ways to get one:
- Classical Embryology: Einsteck or Spemann graft; mechanical disruption of cortical rotation
- Molecular Biology: Injection of dorsal determinants on the ventral side of the embryo
You can also ablate the dorsal axis (UV treatment) and inject various molecules to see if they rescue the dorsal axis. This is how noggin was cloned! Perform the reverse experiment and dorsalize the embryo and try to recover ventral fates by microinjection.

C. Growth Factor Signaling in Embryonic Patterning
In addition to axis induction, mesoderm and neural induction are other popular areas of study in the early embryo. In lecture you have learned about the embryological experiments that showed what parts of the embryo induce mesoderm (vegetal – Nieuwkoop) and neural tissue (organizer – Spemann/Einsteck). You have also learned about the molecules involved (Nodal/Activin, FGFs, Wnts, BMP antagonists).
- Combine gain- and loss-of-function approaches in whole embryos to determine the role of a signaling pathway on embryonic patterning. When possible, use chemicals to dissect temporal roles of pathways (e.g. FGF early induces mesoderm, but later affects convergent extension; Wnt early determines dorsal, but later Wnt inhibition is required for head induction).
- Test the ability of various molecules to induce mesoderm or neural tissue in animal caps. Analyze with morphology and immunohistochemistry. You can also use GFP-Smad1 or 2 to monitor active BMP or Nodal signaling.
- Test the requirement of various signaling pathways in classical conjugate experiments (Spemann, Nieuwkoop, Einsteck) with MOs and drug inhibitors.
D. Neural Crest Induction and Migration

The neural crest arises from the dorsal portion of the neural tube, at the border between neural and non-neural ectoderm. It is induced by the concerted actions of different signals, including BMPs, Wnt, FGFs, and Notch/Delta, and expresses several transcription factors for which we have MOs and mRNA. In embryological experiments, the dorsolateral marginal zone (DLMZ), adjacent to the organizer, can induce neural crest markers. The neural crest is a migratory population; the most impressive migration in Xenopus is of the cranial crest into the branchial arches of the head. The neural crest contributes to many cell types, the most easily distinguishable being melanophores.

- Test the requirement of different signaling pathways/transcription factors for melanophore induction in whole embryos or DLMZ/AC conjugates. *Melanophore induction takes 3-4 days.
- Label the neural crest in Xenopus and/or Abystoma with DiI/O/D and time-lapse. Combine with MO/mRNA injections (in Xenopus) to test the effect of various genes on migration.
- Knock-out neural crest with the Slug MO. Graft wildtype (labeled) neural fold tissue into the neural fold and see if migration/melanophore specification is cell-autonomous. Perform the reverse experiment. Or try other perturbations of donor tissue and host embryo.
- Remove neural fold from wildtype or injected embryos and plate them on fibronectin-coated dishes. Neural crest cells will emigrate and form a sheet of cells. Test requirements of various genes in migration by microinjection of donor embryos or protein/drugs in the culture medium.

E. Axolotl experiments

The Mexican Salamander (*Ambystoma mexicanum*) lays enormous eggs and the embryos have beautifully defined anatomy. The large size of the embryos facilitates embryological manipulations. The well-defined anatomy, particularly the neural folds, really enhances observations of development in action.

- Label the neural folds with DiI/DiD and time lapse neural crest migration
- Organizer grafts or Einsteck experiments. Label donor tissue with Nile blue.
- Animal cap experiments: We have several proteins you can treat animal caps with to see if they induce neural or mesoderm tissue. Also, Barth (1941) showed spontaneous neural induction in axolotl animal caps cultured in saline solution, or even with sand! Try to repeat this and see if various chemical inhibitors can prevent the neural induction.

F. Planar polarization of ciliary beating:

**Basic visualization:**
1. Cilia beat with a highly organized pattern on the *Xenopus* tailbud epidermis beginning around stage 25. This pattern of beat can be easily assessed by placing colored latex beads in the media and watching through a stereoscope. If the embryos are injected with mem-GFP, mem-RFP, or tau-GFP (microtubule label) these cilia can be directly imaged using the Zeiss 5LIVE or a spinning disk confocal (Park et al., 2008).

2. The regular orientation of these beating cilia stems from the orientation of the basal bodies. Planar polarity of basal bodies can be imaged by co-injection of centrin-RFP to mark the basal body and CLAMP-GFP to mark the rootlet (Mitchell et al., 2009; Park et al., 2008). The rootlet in motile cilia always points away from the effective stroke (so here it points anterodorsally).
3. The ciliated cells are born deep in the embryo and intercalate outward to join the surface layer (Drysdale and Elinson, 1992) (Stubbs et al., 2006). Thus, transplants of surface layer can be used to preferentially label ciliated cells and non-ciliated (mucus-secreting) cells. Transplant the surface layer from the animal-ventral side at stage 10 from a mem-RFP labelled donor to the equivalent region of a mem-GFP labelled host. Culture to stage 25 and you should have green ciliated cells in an otherwise red epidermis. This can be used to make time-lapse movies of the intercalation event itself or to for experiments (see below).

Note there is also a population of smaller, non-ciliated intercalating cells called ionocytes.

Experiments:
A. Planar cell polarity in this tissue is under the control of the PCP pathway. Thus expression of dominant-negative Dvl (Xdd1) or overexpression of Vangl2 mRNA will cell-autonomously cause defects in basal body alignment resulting in defects in ciliary beat direction and fluid flow (Park et al., 2008).

B. We have MOs for two IFT genes. Knockdowns should lead to cilia defects. Inject and examine with alpha-tubulin or acetylated tubulin immunostaining (or by live imaging of tau-GFP or even mem-RFP).

C. The PCP pathway also acts non-cell autonomously. So if you use the transplanation technique above you can get wild-type ciliated cells in a sheet of mucus cells that are expressing Xdd1 or Vangl2 overexpression of MO. In this case, the basal bodies will be aligned within the cell, but each cell will be randomly oriented with respect to the a/p axis (Mitchell et al., 2009).

D. When is planar polarity entrained? Well, see (Konig and Hausen, 1993) or see for yourself. Remove small patches of epidermis and rotate them at various stages to determine the point at which the can no longer re-orient.

E. Flow is required to orient cilia in this tissue (Mitchell et al., 2007). Using transplantaion, make large clone of Xint or IFT52 morphant cells. Assess fluid flow, polarity of basal bodies, or organization of ciliary beating in wild-type cells just downstream (just posterio-ventral) from the clone.

G. Developmental control of basic cell behaviors:

1. Cell division: Cells “typically” divide such that the separating chromosomes separate in anaphase to the geometric center of the daughter cells and cytokinesis initiates as anaphase proceeds. This is exactly what happens in the posterior tailbud epidermis in Xenopus (Kieserman et al., 2008) (Kieserman and Wallingford, 2009). This is very easy to image in time-lapse using any of several labels (memRFP/memGFP for cell outlines, Tau-GFP for the mitotic spindle, H2B-GFP for the chromosomes, INCENP-GFP for 1st: the nuclear envelope, 2nd, the centromeres, an third the central spindle).

Interestingly, cells in the Xenopus neural plate divide quite differently: Chromosomes separate all the way to the cell cortex and cytokinesis onset is delayed until anaphase is complete
(Kieserman et al., 2008). Imaging the neural plate is just a bit harder than the epidermis, as some care is needed in mounting the embryos. See attached imaging protocol.

It’s not known if the Axolotl has the same behaviors.

2. **Nuclear morphology:** We’ve noticed that nuclei are very oddly shaped in the tailbud epidermis. Certainly, these epidermal nuclei do not look like the standard, expected “round” nuclei one see, for example, in neural plate cells. As near as we can tell, this has not been studied. However, there is an entire spectrum of human diseases linked to defective nuclear lamina and changes in nuclear shape are a hallmark of malignancy in many cancers.

The odd morphology of *Xenopus* tailbud epidermal nuclear shape is a wide-open research area. The nuclei can be easily imaged with H2B-GFP/RFP to label chromosomes.

Some basic questions include:

i) When does this odd morphology arise? Animal cap ectoderm at gastrula stages has, I think, fairly normal-looking nuclei.

ii) Is nuclear position planar polarized in the epidermis? The whole epidermis is planar polarized in order to control directional ciliary beating, so maybe the nuclear position is as well.

iii) Are there other tissues with odd nuclear morphologies?

iv) Is the same thing true in axolotls?

If anyone makes progress on this, maybe we can collaborate!

3. **Golgi morphology:** We’ve also noticed that Golgi morphology varies by tissue type, differing between neural and epidermal tissues. This can be imaged with GalT-GFP.

H. **Migration of primitive immune cells and response to wounding:**

Early embryos develop a population of primitive myeloid cells that patrol the early embryo and respond to wounds and infections. These can be easily studied in *Xenopus* by transplanting fluorescently labelled anterior ventral blood island into an unlabelled host around stage 14-16 (Chen et al., 2009; Costa et al., 2008). Migration will commence around stage 19-20 and can easily be followed by confocal microscopy. Low-mag imaging will reveal patterns of migration. Hi-mag imaging will reveal cell behaviors associated with this migration.

If the epidermis is wounded, these myeloid cells will quickly migrate to and accumulate at the wound site.

References:


V. Appendices

Appendix I – Solutions
For all solutions, final concentrations are on left, recipe on right made with common stock solutions.

Dejelly solution (make fresh daily)
3% L-Cysteine 3 g pure solid
water or 1/3X MR 100 ml
pH to 7.8-8.0 with 10 M NaOH or pellets.

Modified Frog Ringers (MR)
100x MR salts (autoclave):
180 mM KCl 13.4 g
200 mM CaCl₂•2H₂O 29.4 g
100 mM MgCl₂•6H₂O 20.3 g
1L (w/water)

1/3x MR:
1/3x salts 3.3 ml 100x MR salts
1.67 mM HEPES, pH 7.6 1.67 ml 1 M (23.8g in 100 ml)
33.3 mM NaCl 1.95 g
1 L (w/water)
**Injection Solution**

- 2.5% Ficoll-400  2.5 g
- 1/3x MR  100 ml

**Normal Amphibia Media (NAM)**

**10x NAM salts** *(autoclave):*

- 1.1 M NaCl  64.3 g
- 20 mM KCl  1.5 g
- 10 mM Ca(NO₃)₂•4H₂O  2.4 g
- 10 mM MgSO₄•7H₂O  2.5 g
- 1 mM EDTA (disodium salt dihydrate)  0.37 g
- 1 L (w/water)

**100 mM phosphate buffer:**

- Na₂HPO₄ anhydrous  11 g
- NaH₂PO₄•H₂O  3.1 g
- 1 L (w/water), adjust pH to 7.4 with phosphoric acid

**3/4X NAM** *(make fresh weekly):*

- 3/4x salts  75 ml 10x NAM salts
- 1.5 mM sodium phosphate, pH 7.4  15 ml 100 mM phosphate buffer
- 0.75 mM NaHCO₃  7.5 ml 100 mM (0.84 g /10 mL water – make fresh)
- 1x antibiotic  10 ml 100x stock
- 1 L (w/water)

**1x Steinberg’s**

- 60 mM NaCl  15 mL 4 M
- 0.67 mM KCl  670 μl 1 M
- 0.34 mM Ca(NO₃)₂  340 μl 1 M
- 0.83 mM MgSO₄  830 μl 1 M
- 10 mM HEPES, pH 7.4  10 ml 1 M
- 1 L (w/water)

**Danilchik’s for Amy (DFA)**

- 49.5 mM NaCl  12.38 ml 4 M
- 36.5 mM gluconic acid, sodium salt  7.95 g
- 5 mM Na₂CO₃  0.53 g
- 4.5 mM KCl  2.25 ml 2 M
- 1 mM CaCl₂  1 ml 1 M
- 1 mM MgSO₄  1 ml 1 M
- 1 x antibiotic  10 ml 100x
- 0.1% BSA  1 g
- 1 L with 6 mM HEPES, to pH 8.1

Filter sterilize and freeze in 50 ml aliquots.

**Calcium/Magnesium-Free Medium** *(make fresh weekly)* will not dissociate outer layer of animal cap.

- 88 mM NaCl  5.14 g
- 1 mM KCl  1 ml 1 M
- 2.4 mM NaHCO₃  24 ml 100 mM (8.4 g /100 mL water – make fresh)
7.5 mM Tris Base, pH7.6 \[ \frac{908 \text{ g}}{1 \text{ L (w/water)}} \]

**PhoNaK Buffer** – will completely dissociate embryos

50 mM NaH₂PO₄ \[ 7.1 \text{ g} \]
35 mM NaCl \[ 2.0 \text{ g} \]
1 mM KCl \[ \frac{1 \text{ ml 1 M}}{1 \text{ L (w/water)}} \]

**MEMFA**

10x MEM salts (autoclave):

1M MOPS \[ 209 \text{ g} \]
20 mM EGTA \[ 7.6 \text{ g} \]
10 mM MgSO₄ \[ 2.5 \text{ g} \]

\[ \frac{1 \text{ L (w/water)}}{} \] – pH to 7.4 with NaOH pellets

1x MEMFA:

3.7% formaldehyde \[ 10 \text{ ml 37\%} \]
1x MEM salts \[ 10 \text{ ml 10x MEM salts} \]
water \[ 80 \text{ ml} \]

**Phosphate-buffered Saline (PBS)**

10x PBS:

1.4 M NaCl \[ 80 \text{ g} \]
26.8 mM KCl \[ 2.0 \text{ g} \]
0.1 M Na₂HPO₄ \[ 14.4 \text{ g} \]
17.6 mM KH₂PO₄ \[ 2.4 \text{ g} \]

\[ \frac{1 \text{ L (w/water)}}{} \], pH 7.4 with HCl

1x PBS-Tween (PTw):

1x PBS \[ 100 \text{ ml 10x PBS} \]
0.1% Tween-20 \[ \frac{1 \text{ ml}}{1 \text{ L (w/water)}} \]

1x PBS-BSA-Triton (PBT):

1x PBS \[ 100 \text{ ml 10x PBS} \]
2 mg/ml BSA \[ 2 \text{ g} \]
0.1% Triton X-100 \[ \frac{1 \text{ ml}}{1 \text{ L (w/water)}} \]

**Bleaching Solution** (make fresh)

1% H₂O₂ \[ 333 \mu \text{l 30\%} \]
5% formamide \[ 500 \mu \text{l} \]
0.5x SSC \[ 250 \mu \text{l 20x SSC} \]

10 ml (w/water)

**Standard Saline Citrate (SSC)**

20x SSC:

3M NaCl \[ 175.3 \text{ g} \]
0.3 M Na citrate dihydrate \[ 88.2 \text{ g} \]

\[ \frac{1 \text{ L (w/water)}}{} \], pH 7.0 with HCl
**X-gal staining solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM MgCl₂</td>
<td>10 µl 1M</td>
</tr>
<tr>
<td>5 mM K₃(Fe(CN)₆)</td>
<td>250 µl 100 mM</td>
</tr>
<tr>
<td>5 mM K₄(Fe(CN)₆)</td>
<td>250 µl 100 mM</td>
</tr>
<tr>
<td>1 mg/ml X-gal</td>
<td>250 µl 2% solution (in DMF)</td>
</tr>
<tr>
<td></td>
<td>5 ml (w/PTw), add enough to cover embryos</td>
</tr>
</tbody>
</table>
Appendix II – Developmental Stages and Timetable
See Normal Table (several copies in lab) for detailed descriptions of internal and external staging criteria, as well as a systematic description of the internal development of Xenopus laevis.
Appendix III. Fate Maps

Figure 9.1. Summary of the fates of the blastomeres of the 16-cell-stage embryo. Structures written all in capital letters receive major contributions from the cell in which that structure is written. Structures starting with a capital letter receive small contributions from the cell in which that structure is written. Structures written all in lowercase letters receive contributions from the cell in which that structure is written in only 50% of the embryos studied. Structures that contained labeled cells in fewer than 50% of the embryos studied were not included in these summary diagrams. (Reprinted, with permission, from Moody 1987a.)
FIGURE 9.2. Summary of the fates of the blastomeres of the 32-cell-stage embryo. For explanation of frequency of formation of different tissues by given blastomeres, see Figure 9.1 legend. (Reprinted, with permission, from Moody 1987b.)
Figure 9.3. Prospective regions for each tissue at the 32-cell stage. In each diagram, the animal pole is up and the dorsal side on the right. Percentages indicate the proportion of each blastomere that contributes to named tissues. (Reprinted, with permission from Dale and Slack 1987 [© Company of Biologists Ltd.].)
Figure IV. Dorsoanterior Index (DAI)
Manipulating and Imaging The Xenopus Embryo
MBL Embryology 07

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Instruments for Handling Embryos and for Microsurgery

Using the Stereomicroscope:

The stereomicroscope, often called the dissecting microscope, is designed for low and intermediate magnifications, usually less than 100x. The most important feature, beyond supplying this level of magnification, is that it preserves our stereovision, so we can perceive depth. This is done by providing two optical axes at some small angle with respect to one another, giving each eye a different view of the object. In contrast, compound microscopes usually have only one optical axis up to the oculars or eyepieces, where two are usually provided in order to minimize eyestrain. Note that having two eyepieces does not mean that a microscope offers stereovision. Depth perception is very important in manipulating embryos, so you should take some care in setting up your microscope. The following are basic but important considerations that are often overlooked.

Posture: Make sure that your chair is adjusted in relation to the bench top and microscope oculars such that your eyes fall naturally to a position just above the oculars. You should not have to stretch your back and neck, or be bent over the scope to see into the oculars. Also note that many recent stereomicroscopes have gotten increasingly large and clumsy, and some are ergonomic disasters despite the fact, and often because of the fact, that they have many different capabilities, many of which just get in the way when manipulating embryos. Often it is useful to buy a simpler, agile microscope with good optics and ergonomics for embryo manipulation and another for capturing and recording images.

Trans-illumination. Trans-illumination is rarely useful for frog embryos, unless they are fixed and cleared for photography. If the trans-illuminator base is very thick and hard to work with, see if it can be removed. Generally, embryos and pieces thereof are easiest to work with if your wrists rest on a flat surface in the same plane as the embryo. We mount most of our stereoscopes on large aluminum plates (about 18 x 30 inches). Their temperature is regulated by a re-circulating water bath that feeds water channels inside the plate, thus allowing us to change the rate of development. This gives us a large work surface in the same plane as the embryo, and thus we can move dishes in and out of the field just by sliding them, thereby minimizing the chance of disturbing our preparations. We can send you the plans for these plates.

Epi-illumination. Epi-illuminators may be built in to the scope, and most provide very even, high-angle illumination. But often low angle, shadowing illumination is much more useful for raising the contrast necessary to see particular embryonic features. Therefore a separate, two-armed fiber optic illuminator is usually the best, as it allows the angle of illumination to be adjusted. Epi-fluorescence is useful if one is making grafts or explants with embryos injected with fluorescent markers or transgenics expressing fluorescent markers in particular places. We pick stereoscopes with convenient sliders for fluorescence. We also have our standard epi-illumination controlled by a foot switch, and when doing grafts with fluorescent tissues, we set the level such that the fluorescence and the unlabeled parts of the embryo can both be seen for manipulation. If we want to
check the fluorescence without background illumination, we just hit the foot switch to toggle back and forth.

**Eye-point, Inter-ocular Distance, and Parfocal Adjustment.** To operate the microscope without strain, you want to look *through* the microscope, not *into* the microscope, and both eyes must be in a state of relaxed accommodation. If the image is not in focus for both eyes, they try to accommodate for the mis-focus, causing eye strain. Focus on a forceps or other high contrast object such that the “eye-point”, which is distance your eyes are from the ocular surface when the image is in focus, is a comfortable distance from the ocular surface. The oculars supplied are “high eyepoint” oculars, and you should not have your eyes pressed down against their surfaces. If you wear glasses or contacts, you may continue to wear them with the microscope. If you chose not to wear them, you may have trouble, depending on what kind of vision defect your glasses correct; you can adjust the oculars for the focus of each eye independently (see below) but you cannot adjust them for axially asymmetric or toric differences between your eyes. Thus depending on the type of correction they provide, your glasses may be necessary for stereovision and to avoid eye-strain. If you do not use glasses, the rubber eye-cups on the oculars are probably best turned up; if you use glasses, roll them down the down.

Both eyes must be focused on the specimen at the same time. To do this, adjust the inter-ocular distance such that you see the same object with both eyes. Close the eye *opposite* the eyepiece that has no diopter adjustment; if both have diopter adjustments, set one to the middle or “0” setting, and use it. Bring a high contrast object into sharp focus in the open eye. Now close this eye and open the other, and without moving your head, grasp the diopter adjustment ring on the second ocular and turn it with respect to the barrel of the ocular until the image of the object comes into sharp focus with second eye. You have now adjusted the focus of each ocular such that both eyes are focused at the same specimen plane, and should now be able to switch from one eye to the other without the object changing focus. If it does, you may have let one eye accommodate and its level of focus. If so, try use a card in front of the eye instead of closing it.

**Depth Perception:** Check that you have depth perception by looking at 3 dimensional objects. Take two disposable pipets or forceps and practice making the tips meet in the center of the field. If you often pass one tip above or below the other, you do not have depth perception; notify the instructor.

**Practice Focusing, Changing Magnification, Finding the Field:** Practice focusing on large and small objects and changing magnification. Become familiar with which knob does what, and which way to turn each to get the desired result. Practice bringing objects within the field of view without taking your eyes off the oculars. One of the initial difficulties in using a stereomicroscope is being able to reliably place your instruments within the field of the microscope without dinging the side of the dish, the microscope, your other hand, and so on. These movements should become committed to “muscle memory” and done without thinking about them.

**Magnification, Iris Diaphram, Contrast, Resolution, Depth of Field:** Many microscopes are continuous zoom and can be set for smooth zoom, or with click-stops at designated magnifications. Others have discrete magnifications determined by separate sets of objectives. Some microscopes have a zoom magnification, but also have a turret containing several objectives. Note when magnification goes up, depth of field goes down; when the iris diaphram is opened, resolution
increased and contrast and depth of field decrease; when the diaphragm is closed, resolution goes down and contrast and depth of field go up. Objectives differ in design according to their intended use and vary greatly in working distance, depth of field, contrast and resolving power. For manipulations of frog embryos, depth of field and contrast are more important than flatness of field and resolving power. Thus large, expensive Panapochromat objectives of excellent color correction, high aperture, and flat field also usually have a very shallow depth of field and not at all what you need for making explants or grafts.

**TOOLS TO MANIPULATE EMBRYOS:**

The following describes how to make several tools used in handling amphibian embryos.

*Dumont #5 Forceps:* Many styles of forceps are available but the favorite of most embryologists is the Dumont #5. They come in stainless steel, tool steel and titanium versions, the first having the advantage of not corroding, the second of taking a finer point, and the third of being light and expensive and thus impressing some folks; they offer little or no advantage in embryological work, since the weight of the forceps is seldom a problem.

Inevitably the tips of the forceps will become damaged, since the tips are quite fine. They can be sharpened on a fine-grained, hard Arkansas oil stone. First, straighten the tips with a pliers if they are bent; this is done by grasping the points, one at time, along the bend and straightening it (Fig. 1a). If one tip much shorter than the other, cut the long one off even with the shorter with wire cutters. Begin sharpening by holding the tips together and grinding both down to matched shapes (Fig. 1b). Raise the forceps perpendicular to the stone and grind the tips to exactly the same length (Fig. 1b). Then true up the shape and fineness again. The tips should meet first at the tip and be tips should be exactly even when they close (Fig. 1c). Various shapes are useful for different tasks (Fig. 1d-f). You can also separate the tips and grind each separately to different shapes for special applications.

*Pipets:* Two types of pipets are in current use. The first is a plastic disposable pipet with a stepped-down bore size toward the open end. Cutting off the pipet at different levels with a razor blade yields different bores for different sizes of embryos. The second is the traditional, disposable glass pipet, which comes in 7 and 9 inch versions. Use a diamond pencil to score the tapered section at a point where the inside diameter is just above that of the embryos, approximately 1.2 mm–1.3 mm for *Xenopus laevis* (Fig. 2a)

If the main objective of the pipet is to transfer parts of embryos ( explants), a smaller bore is much better. Heat and pull a glass pipet to the desired inside diameter (it takes practice). Score and break at the narrow part (Fig. 2b-c); breaking is best done by ticking the pulled end, causing it to vibrate and flex. Heat in a small flame to soften the glass until it droops to 120 degree angle (Fig. 2K). Use a large, soft rubber bulb with the pipet, or a mouth pipet (a practice no longer approved most places, but offers much finer control). This pipet can be used to transfer cells, even individual cells, and small explants. These can be fire-polished if desired, but the need for this is often over-emphasized. The viscosity of the water and flow pattern at the tip of the pipet prevents most contact with the edge.
**Hair-loops:** The hair loop is made by pulling a glass disposable pipet to a relatively fine diameter, no more than 6 or 7 times the diameter of a hair (Fig. 2a-b); after cooling, which occurs in a few seconds, score and break the pipet (Fig. 2c). Insert half the length of a hair, at least 4 inches long, and preferably longer, into the end of the pipet, using your forceps (Fig. 2d). Then grasp the other end of the hair and insert it, pushing it in until a small loop, about 1-2 mm in diameter is achieved (Fig. 2e). The reason for using a longer hair is that the friction on the sides of the glass prevents the loop from expanding, and thus the more friction, the smaller the loop can be made. When the desired loop is formed, dip the end in molten paraffin (Fig. 2g). Then tear off a small bit of Kimwipe tissue, about an inch square, and lay it under your stereoscope; heat the handle end of your forceps in a flame and lay it on the stage of your microscope; quickly lay the tissue across the forceps handle and touch the hair loop to the tissue until the wax melts and flows into the tissue (Fig. 2h); do not hold it there so long that the retaining wax in the pipet barrel melts. The ideal shape and size will come to you, as you gain experience. Baby hair is best for its fineness; otherwise use hair as fine as you can find.

**Eyebrow Knives:** The eyebrow knife is ideal for cutting amphibian embryos at early, pre-tailbud stages. It is sharp, does not stick to the cells of the embryo, and is inexpensive. Pull gently on your eyebrows with apposed thumb and forefinger, and place any eyebrow hairs removed under your stereoscope. Search for ones that are sharply tapered to a good point (Fig. 2i). Pull a pipet as described above and insert the eyebrow hair into the barrel. The tip should be at an angle, about 120 degrees, with respect to the pipet (Fig. 2i). Seal with wax and clean off the excess wax as described above. Make 5 or 6 of different designs. As you gain experience, you will settle in on an ideal shape.
Glass Ball Tip: Pull out a disposable pipet to a diameter of about 0.5 mm. Score and break near the base. Heat the tip in an alcohol lamp until a glass ball, about 1 mm diameter, is formed (Fig. 2l).
These are used to make depressions in clay-bottomed dishes or in agarose bottomed dishes (see below). These depressions are useful to hold embryos in a given orientation for microsurgery.

**CLAY-BOTTOMED DISHES: ORIENTING EMBRYOS FOR GRAFTING:** These are used to hold embryos in specific orientations for grafting. Take a 60 mm plastic dish and push modeling clay, designed for children’s play (and therefore non-toxic), into the bottom of the dish with your fingers. Smooth the clay by pressing on it with a flat, smooth surface, such as the top of the scintillation vial, to remove fingerprints. Make sure the layer of clay is no more than a quarter the depth of the dish, because you will want to add medium to a depth of at least 3 mm to avoid exposing embryos and explants to the air-water interface. If the medium comes too near the top, it will wick over the edge of the dish, between the lid and the dish, and out on your hands. To sterilize flood the dish for 10-15 seconds with 70% ethanol and rinse 4 times in sterile distilled water or medium. Replace the lid. When needed, add medium to the proper depth and add the embryos.

Use the ball tip to make depressions. De-vitelline the embryos, and roll them in to wells. Orient them by using the tips of your forceps (carefully), and when satisfied, quickly use the forceps tip to push nibs of clay against the sides of the embryo, thus holding it in place with the area of interest oriented most favorably for what is to be done. To remove the embryo, pull the clay nibs away in reverse fashion. You may want to prepare the graft site first and then roll the embryo into the well and orient, if the operation you are doing requires complex needle angles.

**DIAMOND PENCIL:** A diamond pencil should be at hand at all times for scoring glass pipets and for cutting cover slips.

**COVER SLIPS:** Keep a stock of cover slips at hand. Large ones (40mm on a side or larger, are preferable, as they can be cut to make large culture chambers for high resolution imaging. It is preferable to use #1.5 (0.17 mm) thickness, since this is what most microscope objectives are designed to use.

**GLASS BRIDGES:** Glass bridges are used to hold grafts in place and to constraint explants. To make glass bridges, use a diamond pencil to score a large cover slip in a grid pattern by first scoring parallel lines parallel to the long axis of the cover slip. This is best done on a very flat, hard surface. Place a glass microscope slide on the cover slip and press down tightly, and score by passing the
diamond point along its edge. Score perpendicular across these lines in the same fashion, spacing the lines to give the desired size. Then tap the cover slip gently with the handle of a forceps or diamond pencil to break up the cover slip into small rectangles. Usually, the cover slip will break along some of the score lines during the first scoring; if so, push them all together again and use the glass slide to hold them down tightly, and make the cross wise scores. Slide them into a 60 mm dishes, sorting by size (Fig. 3).

**Silicone Grease:** Silicone grease has almost as many uses in microsurgery as duct tape dose in the rest of life. The first is to position and support glass bridges. Place a small dab of silicone high vacuum grease in the center of a small (35 or 60 mm) plastic petri dish and cover. For holding explants down to hard surfaces, take a glass bridge in your forceps and drag first one end and then the other across this silicone grease to get a small dab on both ends. Place the glass bridge silicone grease side down, across the explant, and then tap with forceps until it is apposed to the surface of the explant with the desired pressure. Make sure the bridge is long enough to place the silicone grease at its ends will away from the explant, and that there is enough grease to support the bridge well above the explant on initial placement, before tapping.

The second important use of silicone grease is to make a bead of grease to seal imaging and culture chambers. Pull the plunger from a small plastic syringe, and fill it with silicone grease from the back (plunger) side, using a spatula. Try to avoid bubbles. Replace the plunger, and press for a bead of silicone. For large beads, use the syringe as is; for smaller ones, fit the syringe with a pipet tip can cut it off at the appropriate diameter.

**Instrument Sterilizer:** Wad up two lab tissues and stuff them into the bottom of a 50 mm beaker. Fill the beaker to the 3/4 level with 70% ethanol and cover with a piece of aluminum foil. Dip your forceps tips and hair tools into the ethanol and flick dry.

**Solutions and Culture Media.**

There are several saline solutions commonly used to culture amphibian embryos and embryonic tissues. They differ but not in ways that are critical for most purposes. Our favorites are modified Barth’s solution (MBS), a general purpose saline (Gurdon, 1977), and modified Danilchik’s solution (DFA) (Sater et al, 1993), which is a saline specialized for supporting the normal motility and behavior of deep, nonepithelial cells (see Shih and Keller, 1992a, b).

**MBS (Modified Barth’s Solution, from Gurdon (1977):**

<table>
<thead>
<tr>
<th>STOCK</th>
<th>per 1 liter</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M NaCl</td>
<td>22.0 ml</td>
<td>88.0</td>
</tr>
<tr>
<td>50mM MgSO₄</td>
<td>16.4 ml</td>
<td>0.82</td>
</tr>
</tbody>
</table>
0.8M NaHCO\textsubscript{3} & 3.0 ml & 2.40 \\
0.1M KCl & 10.0 ml & 0.01 \\
33mM Ca(NO\textsubscript{3})\textsubscript{2} & 10.0 ml & 0.03 \\
1M CaCl\textsubscript{2} & 0.41 ml & 0.41 \\
Hepes & 2.38 gm/l & 5.00 \\

Adjust pH to 7.4 with NaOH

**DFA (“Danilchik’s for Amy”, from Sater and others (1993))**:

<table>
<thead>
<tr>
<th>STOCK</th>
<th>in 500 ml</th>
<th>in 1l</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M NaCl</td>
<td>6.625 ml</td>
<td>13.25 ml</td>
<td>53.0 mM</td>
</tr>
<tr>
<td>1M Na\textsubscript{2}CO\textsubscript{3}</td>
<td>2.5 ml</td>
<td>5.0 ml</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>K gluconate</td>
<td>0.525 gm</td>
<td>1.05 gm</td>
<td>4.5 mM</td>
</tr>
<tr>
<td>Na gluconate</td>
<td>3.49 gm</td>
<td>6.93 gm</td>
<td>32.0 mM</td>
</tr>
<tr>
<td>1M CaCl\textsubscript{2}</td>
<td>0.5 ml</td>
<td>1.0 ml</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>1M MgSO\textsubscript{4}</td>
<td>0.5 ml</td>
<td>1.0 ml</td>
<td>1.0 mM</td>
</tr>
</tbody>
</table>

pH to 8.3 with 1M Bicine

The original Danilchik's, as well as subsequent versions, were patterned after the ionic composition of the blastocoel fluid of *Xenopus laevis* (see Shih and Keller, 1992a, b; Sater et al., 1993).

**Use of Solutions**: Intact embryos are kept in one third to one tenth (low) strength saline, and then transferred to full (high) strength saline when the outer epithelial sheet is broken during microsurgery. Normally the outside of the embryo is facing low strength saline (pond water, stream water, equivalent to about one tenth to one hundredth of full strength saline), but the interior of the embryo has a salinity roughly equivalent to the media described above. This internal environment is maintained by the epithelium covering the surface of the embryo. An epithelium is a sheet of cells whose margins are tightly connected all way around with a junctional complex, forming a selectively permeable barrier to the passage of ions and large molecules, and even small molecules and water, in some cases. Ion pumps in the epithelium maintain this difference between inside and outside, and one of the first things an embryo does is form this epithelium; thereafter it can control its internal environment. The epithelium is unstable in high salt; the outside surface of the epithelium is expecting low strength saline and may break down when exposed to high salt or high pH. Thus in
your experiments, the embryo should be maintained in relatively low strength saline as long as the epithelium is intact. But if the epithelial sheet is cut and can no longer maintain the high salinity of the internal embryonic environment, the embryo should be put in full-strength saline. The deep cells and the internal ends of the epithelial cells will take up water, swell, behave badly, and even die in lower strength saline.

If an “open-faced” explant, which is one having exposed deep cells is to be made, the best choice is DFA, which allows more normal behavior of the deep, non-epithelial cells (see Shih and Keller, 1992a, b). However, DFA interferes with some functions of the superficial epithelium. For example, the neural folds often will not fuse to form a tube in DFA. For all other culture, MBS or an equivalent should be used for microsurgery; after healing the embryos should be transferred to third or tenth strength saline. Our full strength salines are made in two versions, one plain and one with 0.1% bovine serum albumin (BSA) added, which reduces adhesion of the cells to the surfaces of the dish and cover slips. Embryos should be transferred to plain saline before fixation, or the BSA may be fixed or precipitated on the surface of the specimens, making them appear quite dirty in a number of staining procedures. Solutions are filtered through a 0.22 mm Millipore filter and aliquotted into 50 ml plastic centrifuge tubes and frozen at -20°C until needed. We also freeze 1.0 ml aliquots of 100x antibiotic/antimycotic solution (10,000 U penicillin, 10 mg streptomycin, 25 mg amphotericin B, per ml, in 0.9% NaCl, Sigma Chemical, St. Louis, Catalogue #A9909) at -20°C. These are thawed and added at 0.5 ml/50 ml tube of solution at the time of use. Culture solutions are replaced with fresh every 12 hr of culture. Embryos are kept at 16 to 24°C. We try to avoid temperatures below or above this range, although two more degrees lower is commonly used, and perhaps with good spawnings, it probably makes no difference, but often the embryos do not look right or cut right when opened.

MANIPULATIONS:

LAYOUT: You should have the items shown in Fig. 4 laid out around your stereoscope in preparation for manipulating the embryos. The details of right and left and exact position are not important and should be tailored to your desires. Keep in mind where your hands will move when you move them from one operation to another, or away from the microscope; a common problem is dragging things off the table with one’s sleeves, breaking tips of needles or forceps by focusing with instrument in hand.

PIPETING: Pipeting embryos, and particularly explants, from dish to dish and requires practice, as this is where most are lost by either dropping them on the table or getting them in the air-water interface where surface tension spreads them into a lipid film. Chose a pipet that is just larger than the object to be pipeted; if it is too small and the object will hang up, and if too large, it will not be easy to into the pipet or to hold there. Squeeze the pipet bulb, place the tip in the solution away from the specimen, draw in solution until the bulb is relaxed. Place the pipet tip near the object to be moved, keeping the pipet at a low angle, about 30 degrees with respect to the table top. Squeeze the bulb a bit, move the tip near the object, and gently release. If the object does not roll into the pipet some distance, repeat the operation, squeezing the bulb more, initially, so that more fluid is pulled into the end when it is released; also if the embryo does not move, place the tip closer to the embryo and release faster. The amount the pipet bulb is squeezed initially determines roughly how far into
the pipet the object will be pulled when the bulb is released. Before the embryo or explant can roll out of the tip, move the pipet abruptly through the surface of the medium, move it to the desired location, and plunge it beneath the surface of the receiving dish, keeping the pipet at the same angle with respect to the table top. Squeeze the bulb gently and expel the specimen. Do not draw an air bubble into the end of a large pipet, as it may move up the bore, passing the specimen as it goes. If you want to imagine what will happen, picture yourself passing a large bull in a tunnel that is about the same size as the bull.

**Cutting Tissues:** Long straight cuts through the entire embryo can be made by pressing a long, straight, and stiff eyebrow hair through the tissue until the substratum is reached. Then quickly flick the eyebrow hair laterally (the “Harris Flick” or “Harris Flip”, so-named for Bill Harris, Cambridge, UK) Explants of the embryo can be made and trimmed to size with the same type of movement (Fig. 5a).

To remove a superficial section of the embryo without getting into deeper tissues, use a “stitching motion”. Insert the eyebrow hair into the wall of the embryo at an angle of about 45 degrees with the surface, until the tip is at the desired depth; the hair loop is then placed next to the eyebrow hair, along the line of cut on the surface of the embryo, and the tissue is cut by quickly raising the tip of the eyebrow hair (Fig. 5b); the operation is repeated very fast in a stitching motion, advancing the eyebrow hair and retaining hair loop only a small distance at each cycle, never cutting through more than a few cells at a time, to avoid undue stress on the tissue (Fig. 5b). Any depth of the gastrula or neurula can be cut this way, ranging from the epithelial layer alone (about 10-15 microns thick) or the epithelial and deep region together (about 25 to 100 micrometers, depending on the stage and region), just by varying the depth of insertion of the eyebrow hair. A small, sharp-tipped eyebrow hair is best for this operation.

An alternative method is to insert a long, stiff eyebrow hair along the desired line to be cut, taking care not to go deeper than the cut is to be made. Then the hair loop is rubbed back and forth on the outside, cutting the tissue between the hair loop and eyebrow hair. This method produces straight
cuts, but often that is not what one wants, and with this method, it is difficult to cut a long distance on a curved embryo and maintain a constant depth, or to control depth at all, for that matter.

**Peeling Layers Apart:** Layers of tissue can be peeled from one another, as, for example, removing the epithelium from the underlying deep cells (Shih and Keller, 1992c), or removing the outer wall of the gastrula from the underlying involuted cells (see below). The “peel” is begun by probing with the tip of an eyebrow hair for the interface between the tissues to be separated; the interface will reveal itself as a line of easier separation. Once this is discovered, the background tissue is held with the side of the hairloop, and the tissue to be peeled off is pulled away by running the tip of the eyebrow hair along the interface and angling the butt or heel of the eyebrow hair against the surface of the tissue, to push it away (Fig. 5c). Initiation of a peel at the right interface is difficult if the cut at the edge of the peel has gone too deep, because the area of the underlying tissue beneath the peel will then be more likely to come along.

![Figure 5](image-url)
**Shearing Cells Off Layers:** Related to peeling is the necessity of shearing unwanted cells off layers that should have been cleanly peeled from others. For example, mesodermal cells will adhere to the inner surface of the blastocoel wall when the latter is peeled off the embryo (see below). The best way to remove them is to turn the explant with the deep surface uppermost. Then the eyebrow hair is turned such that a considerable length of its tip lies parallel and just above the explant, tip forward, at an angle of about 20-30 degrees off a line through the top and bottom of the field of view, and to the right edge of the area of contamination (Fig. 5d). The hair loop is then placed against the near edge of the explant, just to the left of the eyebrow hair; this will prevent movement of the whole explant toward the operator. The eyebrow hair is then brought down just touching the inner surface of the explant and brought rapidly back toward the experimenter but kept in its original plane. This is repeated very fast and lightly, moving the restraining hair loop slowly to the left, and following with the eyebrow hair, shearing off any recalcitrant, adherent cells. It is important to keep the angle of the eyebrow hair steep, since a low angle of attack shears much better without exerting large forces on the explant (Fig. 5d). If one prefers, of course, the eyebrow hair and hair loop can be switched right to left and the process began at the other edge of the explant.

**Open-Faced Explants:** “Open-faced” explants are ones having the inner, deep cells exposed to the medium, which allow imaging of their motility (Shih and Keller, 1992a, b), and thus Danilchik’s medium is used. These explants are often made in chambers consisting of milled plastic with bottom consisting of a cover slip glued on with high vacuum grease (see below, p.   ). The design allows easy use in automated, multi-position stages, which are used to make movies of many individual experiments at once, using image acquisition programs such as Velocity (Open Lab) or Metamorph (Universal Imaging). However, an inexpensive culture chamber for these explants can made by drilling a 20 mm hole in a 60 mm plastic dish and gluing a #1.5, substantially larger cover slip over the hole with a bead of silicone high vacuum grease (Dow Corning, Midland, Michigan 48640) (see below, p. ). The tissue is placed with the inner or deep surface down on the cover slip, and restrained with a glass bridge, supported at each end with silicone high vacuum grease as described above. This allows high-resolution optics to be used with an inverted microscope or confocal. An upright microscope can be used by overfilling the dish with media and covering the top with a large glass slide, and then inverting it, such that the cover slip and the deep surface of the explant is at the top, facing the objective.

**Sandwich Explants:** Sandwiches consist of abutting the deep, inner surfaces of to identical tissues together; such that the epithelial sheets covering each half heal across the exposed edges, forming a physiological barrier surrounding the deep cells. The two components are excised and their inner surfaces apposed, the quicker the better, since delay will result in curling of the two halves, which makes apposition difficult. Dabs of silicone high vacuum grease are placed on both ends of a pre-cut rectangular coverslip, considerably larger than the explant, and the coverslip is rested on the grease, straddling the explant and some distance above it. Then the coverslip is tapped with a forceps, bringing it down on top of the sandwich, pressing the two components together lightly. Healing should occur in 15 to 20 minutes and then the explant should be removed. The high vacuum grease does not appear to be overtly toxic, but the less time the explant spends near it, the better.

**Grafting Tissues:** The easiest method of grafting from embryo to embryo is to use a clay-bottomed dish. A hole the size of the embryo is made in black modeling clay with a ball tip, formed at the end of a disposable pipet by holding it in a flame until the correct size molten ball is formed. The host
embryo is placed in the hole, graft site uppermost, if possible. If the embryo rotates, small nibs of clay can be pushed against it with forceps, holding it in position. The donor embryo is placed along side the host, also in a depression (necessary only if one wants to keep the same levels of both embryos in focus at higher magnifications). The graft is cut out of the donor and the graft site cut in the host; the most time-consuming operation should be done first and the preparation that gives the most trouble when left alone for a time should be done last. For example, in grafting, speed is very important, since the graft will tend to curl, and the hole made to accept it will first gape and then heal. Usually curling of the graft causes more difficulty than healing of the hole, so it is usually best to make the hole first and cut the graft second. Close matching of the boundaries of graft and graft site is important, since healing will occur fastest under these conditions. If the graft is slightly larger than the site, it is important to tuck the edges down into the graft site. If the edges of the graft overlap the host epithelium, healing will be delayed; the graft epithelium will turn back on itself and ultimately find the edge of the host epithelium, but this will take a while.

To hold the graft in place, two long ridges of clay are pushed up on each side of the embryo, some distance (2 to 4 mm) away with forceps tips, and a glass bridge is bridged across the two ridges, above the embryo. Then the glass bridge is pushed down on the graft with forceps tips, taking care to align the surface of the glass exactly perpendicular to the graft surface, pushing it directly into the graft site. If the embryo or graft moves one way or the other, as pressure is applied, the glass bridge can be tilted to counter these movements. Healing should occur in 15 to 20 minutes, at which time the glass bridge should be removed and embryo should be removed from the clay. Most batches of clay appear to be more deleterious to the embryo than the silicone high vacuum grease, although embryos from many spawnings will develop normally to advanced stages on clay. We prefer black clay, for contrast, but other colors can be used according to the experimenter’s taste, but some colors appear to affect the embryos more than others.

The above is the easiest but not necessarily the best way to graft tissues. With practice, one can make grafts between free, unconstrained embryos lying in a dish. This is the least disruptive and yields the best results, but the edges of grafts must be mated precisely, the grafts must be relatively small, and the grafts must stick in the graft site without external pressure, all of which require much practice.

Tricks: There are a number of tricks that have been used to facilitate grafting and explanting embryonic tissues. Many have special advantages that may be useful in some situations, but usually these advantages come with liabilities. High pH and, or low calcium makes tissues easier to separate or cut, but also retards healing in many cases. Hypertonicity and hypotonicity can both make layers of cells easier to separate from one another, by shrinking or swelling the cells, but often retard healing, and may kill or damage cells. When one cuts through the epithelial surface of the embryo, either the epithelial or deep cell populations will be at a disadvantage, depending on what solution is used. The deep cells and basolateral surfaces of the epithelial cells require high salt, whereas the outer surface of the epithelium is normally exposed to low salt. External solutions of high pH and/or high salt cause lesions in the epithelial layer and increased cell motility at these lesions, particularly in the case of high pH. Conversely, low pH and low salt cause sluggish or abnormal motility or swelling of deep cells, respectively. These facts, which have been known since the classic work of Holtfreter, make any grafting or explantation operation less than ideal for the health and function of one cell population or the other.
**Handling Albinos:** It is an advantage to use albino embryos for experiments involving whole mount, RNA *in situ* hybridization, as well as for some methods of antibody staining of tissues. However, these embryos are difficult to stage and orient, because they lack of pigment, making landmarks for staging and orientation difficult to see. These can be staged and oriented more easily if they are stained a pale, “baby” blue by soaking the embryos for a few minutes in Nile blue, made by adding drops or two of 1.0% solution of Nile blue in distilled water to third strength medium in the culture dish to make the medium pale blue. Transfer the embryos when they take on a very pale color, as the color will deepen with time, even after you remove them from the stain, and it is easy to overstain them. With this light background stain, the emerging morphogenic features, such as the blastopore and neural folds, have higher contrast, making staging and orientation possible, although perhaps not quite as easy as in normally pigmented embryos.

**Tipping and Marking:** For injecting dyes, RNAs, plasmids, morpholinos or other reagents into identified regions of early cleavage stage embryos, or for any manipulation requiring indentification of the dorsal, lateral or ventral side of the embryo prior to gastrulation, it is advantageous, particularly in the case of albinos, if the embryos, are “tipped and marked” (Kay and Peng, 1991). This procedure makes use of the fact that movement of the cortex of the egg relative to the deep cytoplasm in the first cell cycle determines where the dorsal side will form. If the equator at any meridian is rotated uppermost early in the first cell cycle and left there, the dorsal side will form at that meridian, over-riding the influence of the sperm entry site on specifying the dorsal side (see Gerhart et al., 1981). The embryos are first placed on a Nitex grid (about 1mm mesh size) in 6% Ficoll, made up in one third MBS. The equator is then tilted uppermost within 20 min of fertilization and left that way until after first cleavage. The site that is uppermost is marked with a wand bearing vital dye. The wand is made by pulling a disposable glass pipet to a small diameter, and then melting a small glass ball at the end. Dye is precipitated by mixing on a microscope slide a bit of Nile blue sulfate (1% in water) and 100 mM sodium carbonate with the wand. A precipitate of dye will form, which is then sticks on the tip of the wand. These crystals are placed against the embryo for a couple of seconds. Since the dye appears darker later than it does initially, and it is easy to overstain. The dorsal side will develop at or within a few degrees of the stained site.

**Literature Cited:**


High Resolution Imaging of Cell Behavior

The cell motile behavior underlying the various regional morphogenic movements can be visualized with high resolution, comparable to cell culture, in a number of ways, most involving explants in order to gain access to deep tissues of the nearly opaque embryo. This has the disadvantage that behavior might not be normal in absence of the internal physiological environment, or without normal tissue interactions, or a different version of an endogenous movement might even be displayed in absence of specific tissue interactions. Conversely, the advantage is that the tissues are in mechanical isolation, whereas in whole embryos one often never knows if one is looking movements resulting from local force-generating events, or passive responses to global mechanical interactions. *Xenopus* also has the advantage of being physically robust. Small explants of many regions robustly display the movement that appears to mimic what happens in vivo, and it is possible to compare control and molecularly manipulated embryos in terms of details of cell behavior and cell polarity, and also measure the scaling up of local force-generating processes to the level of forces and mechanical properties of the tissues. In short, it is a system in which one can go from molecule to cell behavior to the forces that actually shape the embryonic form.

Exposing the deep cells to the external environment requires that that environment be suitable for cell behavior. In the late 70s and early 80s, we made various attempts at getting a culture solution that would support deep cell behavior, and success, at least partial success, came as a result of Gillespie’s publication of the ionic content of the blastocoel of *Xenopus* and the Mexican axolotl, *Ambystoma mexicanum* (Gillespie, 1983), which we used as a guide for the original Danilchik’s solution, and two succeeding versions, Danilchik’s for (John) Shih (DFS) and finally the current Danilchik’s for Amy (Sater) (DFA) (see above p. 9). These media are characterized by high pH, moderate calcium, and low chloride, the high level of which seems to be the problem with many commonly used media. All versions work, but the latter is the easiest to make up and most accurately matches the measured small ions in blastocoel fluid. We include BSA to coat the glass surfaces next to the explant to minimize its adhesions to the substrate in cases we are assaying movements generated by endogenous forces rather than traction on exterior surfaces. The explants will stick anyway, to a degree, and so agarose coatings are used to eliminate all substrate adhesions if that is desired. In other cases, adhesion to the substrate is desired, and for that one can use the matrix of one’s choice, fibronectin for example (see Davidson et al., 2004), or the cadherin ectodomain (Zhong and Gumbiner, 1999) for the substrate.

To gain high contrast images of cells, we inject fluorescent dextrans, or inject RNAs encoding cytoplasmic GFP, membrane targeted GFP, or color variants thereof. Uniform labeling of cells in the same color often does not allow resolution of protrusive activity, however, for lack of contrast with background. Confocal microscopy helps somewhat, but there several additional ways of enhancing contrast and resolution. First, target relatively small blastomeres at the 64 or 128 cell stage in the region of interest. This usually yields a “scattered” array of labeled cells on a dark background. Targeting adjacent blastomeres of any size with two different colors is another method of accomplishing the same goal. Finally, injection of experimental reagents along with one color generates mosaics that can be used to assay cell interactions and cell autonomy. One can also graft regions between embryos and then make explants to generate specific, whole tissue interactions. These can be guided by using transgenic lines with markers for particular tissues. Use a fluorescent microscope to screen and graft or assay the amount and location of fluorescent tags. Use of a foot
switch for normal illumination and a microscope with a handy slider for fluorescence greatly simplifies doing these operations without over-exposing and damaging the embryo, even before you get the prep made.

To use high numerical aperture, high-resolution objectives, make a chamber with a cover slip bottom. We have rectangular, bottom-less chambers, about 5 mm thick, and containing one or more wells, milled from plastic by the Dept of Physics shop (Fig. 1A, left). We then encircle the base with a bead of silicone grease and press a #1.5 cover slip to this surface, which forms an optically desirable floor (Fig. 1A, right). The explants are then placed on this surface and held down by glass bridges resting on nibs of silicone grease. These chambers can be used on any microscope and are convenient for mounting on our multi-position, automated stage, which allows us to make movies of many explants at once. These can be covered on both sides, allowing them to be flipped upside down, and used alternately on inverted and upright compound scopes or on confocals of either design.

Fig. 1
To make a cheap, and in some ways more versatile version of the same thing, heat a cork borer (ask somebody who is over 50 if you don’t know what it is) in a burner and melt a clean hole in the bottom of a 60 mm plastic dish (Fig. 1B). Scratch the berm of melted plastic off the OUTSIDE of the dish with a razor blade to give a flat surface. Run out a bead of silicone grease around the hole, and press a cover slip over the hole to form the bottom (Fig. 1C). Cut the cover slip substantially larger than the hole, and press it down firmly but carefully, without bending or breaking it; you do not want unrelieved stresses in the cover slip, since it may “creep” during your experiment. Note also that the amount of grease and the size of the cover slip should be such that the grease is not squeezed out around the edges of the cover slip. If it does, it will smear the optical surface as you move the chamber around, and ruin your image, as it is very hard to remove all of it. The same applies to the milled chambers.

References:


Gillespie, J. I. 1983 The distribution of small ions during the early development of Xenopus laevis and Ambystoma mexicanum embryos. *J Physiol (Lond)* 344, 359-77.

Fate Maps of Blastula Stage Xenopus Embryos

Fate maps are used to understand what cells or regions of the embryo at early stages form specific parts of later stages, and conversely if one has identified a particular tissue of interest at later stages, one may want to know what blastomeres or regions of the early embryo formed this area. Xenopus has a reasonably stereotyped cleavage pattern and knowing the fates of the blastomeres allows targeting of reagents and labels to specific regions. The 32 cell embryo is commonly used to target experimental perturbations to specific tissues in this manner. It offers a fate map of reasonable resolution for targeting reagents to the major tissue types and region specific behavioral domains of morphogenesis, and yet the size of blastomeres to be injected or otherwise manipulated is not excessively small. Smaller tissue areas and more specific manipulations can be affected by using later, 64 cell or even 128 cell stage embryos, and larger areas and more general effects can be had by injecting 2 to 16 cell stages. Provided it cannot pass through gap junctions, material injected into one of the early blastomeres will be confine to that blastomere, provided that the cytokinetic furrow is or is about to close when the injection occurred. Occasionally, a dye injected into one blastomere results in strongly labeled cells and more weakly labeled cells at later stages, probably because the furrow had not closed at the time of injection, and some of the label diffused into the adjacent blastomere.

Keep in mind that a fate map reflects where cells end up in normal development, and it does not mean that they must go there in all cases nor does it mean that they cannot make something else if give the opportunity. A specific region of the embryo, or a specific blastomere, is said to have a “presumptive” or “prospective” fate, meaning that this is what it is likely to make in normal development. Note that large regions or blastomeres may have heterogeneous fates; that is, they may contribute to several or many different tissues.

It should also be noted that fate maps made by labeling blastomeres have resolution no greater than the size of the blastomere, and the smaller the blastomere, the more accurate the fate map. Note also that fate maps made by labeling small blocks of tissue or cells at later stages have much finer resolution than those made early by labeling large blastomeres at earlier (see below). Also, early blastomeres and their progeny twist and turn with respect to one another in ways that varies from embryo to embryo, and thus no two give exactly the same fate map. This is less true from the early gastrula onward, because at that point, highly constrained patterns of cell movements begin.

Fates are mapped retrospectively on to the early stages. For example, the presumptive ectoderm, endoderm and mesoderm are by convention, defined as the outer, inner, and middle layers of the body at the pharyngula stage (early tailbud) and mapped back to the gastrula or blastula as presumptive ectoderm, endoderm, and mesoderm, without regard to their actual location on the blastula, which varies widely from species to species. An alternate and unfortunate convention of naming layers by actual position in the blastula or gastrula, has occasionally appeared. It makes no sense, is confusing, and even comical in its result.

A conventional blastomere nomenclature is used for the Xenopus (Fig. 1). The animal view of the 16-cell embryo shows the lighter pigment of the future dorsal side (Fig. 1, dorsal is to the right in all figures). This pigment difference results from the rotation of the cortex of the fertilized egg across
the animal pole toward the point of sperm entry, which becomes the darker ventral side, leaving the lighter, dorsal side to form the dorsal side. The first cleavage in Xenopus occurs about 90-100 min after fertilization at room temperature and subsequent cleavages occur every half hour, the first cleavage occurring meridionally, and often, but not always corresponding to the plane of bilateral symmetry. The second is meridional and perpendicular to the first, dividing the egg into 4 cells, and the third is para-equatorial, displaced somewhat to the animal pole end of the egg (Fig. 2). The pigment pattern is not always an accurate predictor of dorsal-ventral axes, however. Use the tipping and marking technique, described above (p. ) for greater accuracy. At the 32 cell stage the blastomeres are named according to position with the rows describing dorsal-ventral position indicated by numbers from 1 to 4 with 1 being dorsal-most and 4 being ventral most. The tiers that describe animal-vegetal position are indicated by letters with A being animal-most and D being vegetal-most (Fig. 1, middle and right).

Nomenclature of Blastomeres of the 32 cell stage:  
(dorsal is to the right)

Figure 1: Diagrams show the conventional nomenclature of the Xenopus blastula. The presumptive dorsal side is to the right in all diagrams. The fates of the blastomeres at the 32 cell stage are summarized in Table I, which is based on one of the earliest and best fate maps of this stage by Nakamura and Kishiyama. For additional information, see Bauer and others (1994) and Vodicka and Gerhart (1995).
Fig. 2. The pattern of cleavage of the Xenopus embryo is shown from the lateral view, with dorsal to the right. Animal tiers are shaded. Note that this does not reflect pigment patterns in the embryo.

The fates of the 32 cell stage were traced by marking the blastomeres with dye and tracing their progeny to the pharynula (tailbud) stage by several investigators. One of the best of these fate maps is by Nakamura and Kishiyama (1971) (Table I). This table is a convenient guide for targeting tissues. Note that this table indicates the major derivatives of each blastomere; there will be considerable or extensive overlap in many cases. Also, note that, in general, the overlap is great on the dorsal side where convergent extension elongates the axis, such that A1 often contributes to what are usually B1 tissues; for example, sometimes A1 will label the entire posterior notochord and archenteron roof. Likewise, B1 often overlaps C1. Note also that because convergent extension greatly stretches out the embryonic anterior-posterior axis, one blastomere, the B1, can label the entire nervous system and underlying notochord. The overlap of A1, B1 and C1 is illustrated in Vodicka and Gerhart, 1995. See their color photos of A1 labeled in fluorescein, B1 with rhodamine, and C1 with cascade blue dextrans (Fig. 2) and summary diagram (Fig. 3).

Table I: Fates of 32 Cell Blastomeres of Xenopus laevis
(modified from Nakamura and Kishiyama, 1971)

<table>
<thead>
<tr>
<th>Blasto-mere</th>
<th>Ectoderm</th>
<th>Mesoderm</th>
<th>Endoderm</th>
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<tbody>
<tr>
<td></td>
<td>Epidermis</td>
<td>Neural Plate</td>
<td>Prechord Plate</td>
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<tr>
<td>A1</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>A3</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>++</td>
<td></td>
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<tr>
<td>B1</td>
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<td>B2</td>
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<td>B3</td>
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<tr>
<td>C4</td>
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<td>D1</td>
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<td>D3</td>
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<td>D4</td>
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</table>

There are several reasons for the variable fates of blastomeres labeled at the early cleavage stages. First, the large size of the blastomeres limits the resolution of the fate map that can be made at a particular stage. Second, there is large variation in cleavage patterns of the early embryo. For example, labeling the A1 blastomere at the 32 cell stage could label only the head epidermis, forebrain, and eye, if the jostling about of the blastomeres during subsequent cleavages is such that
the progeny of A1 remain towards the animal pole. However, if during cleavage the blastomeres twist around such that some of them are located more vegetally, the A1 may label the posterior neural region or even the posterior mesoderm. In addition to variation in movements of blastomeres, the cleavage pattern itself is not always regular, and, in fact, a minority of embryos actually adhering to the stereotyped pattern illustrated above. For example, the first cleavage plane sometimes predicts the location of the dorsal midline and the axis of bilateral symmetry, but some times it does not (see Figure 3).

Figure 3: Diagrams from Vodicka and Gerhart (1995) show the 32 cell stage blastomeres (A), a midsagittal sectional view of the blastula at the 32 cell stage (B) and corresponding fluorescence micrograph, with labeled blastomeres (C), with labeled blastomeres A1 (green), B1 (red), and C1 (blue). Also shown are derivatives at the early gastrula (D), the late gastrula (E), and at the tailbud stage (F).
Figure 4. Patterns of cell mixing from early blastula to gastrulation are shown (Bauer et al., 1994)

Black and Vincent, 1988). Note the relationship between the injected blastomeres and the scattering of the label as cleavage and gastrulation occurs (Fig. 3 and 4).

Microinjection can be done with a number of different injection machines and micromanipulators, and the detailed instructions for variation machines should be consulted.

Injection is done in 5-6% Ficoll in third strength MBS. There are several ways of handling the embryos for injection. Some investigators make long channels or grooves in clay-bottomed dishes and line up the embryos in these grooves, which restrain their movement. One of the most popular methods of restraint is to glue a piece of Nitex mesh to the bottom of a culture dish with the grid size being such the embryo is partially settled into the depressions formed by the grid. One of the fastest and most precise methods is to push the embryo near the needle with forceps tips, spin it to the proper orientation, brace it with the forceps tips, and stick it (Fig. 5). Leave the embryos in the Ficoll solution for a half hour to an hour after injection, and transfer to third strength MBS.

Consult the fate maps of the 32 cell stage made by Bauer and Moody (1994) and Vodicka and Gerhart (1995) and compare with the tabular information on blastomere fates at the 32 cell stage by Nakamura and Kishiyama (1971) (Table I). Note the relationship between the injected blastomeres and the scattering of the label as cleavage and gastrulation occurs (Fig. 3 and 4).
Figure 5: Injection of a blastomere, using a blunt forceps (left) as a orienting and bracing device.

This diagram of tadpole anatomy is useful for interpretation of fate mapping.

Figure 5: The anatomy of the Xenopus tadpole at approximately stage 34 is shown in lateral view (top) and in cross section (bottom).
Fig. 6: Template for stage 34.

References


Map of Cell Fate and Tissue Movements In The *Xenopus* Gastrula

Fig. 1. Map of fates and movements of Xenopus laevis, revised and updated from Keller, 1991 and Keller and Shook, 2004. Shown are the right side surface of the embryo (left column), the mid-sagittal section (second column), the deep mesenchymal layer of the involuting marginal zone (the mesodermal mantle) (third column), and the superficial epithelial layer of the IMZ (right column) just prior to gastrulation (top row), the early gastrula (second row), the late gastrula (third row), and mid neurula (last row), with dorsal to the left in all figures. The arrows indicate movements. The presumptive tissues are: epidermis (Ep), light blue; forebrain (Fb), blue; hindbrain/spinal cord (Hb/Sc), dark blue; notochord, magenta; somitic mesoderm, red;
posterior somitic mesoderm, dark red; lateral plate, orange; superficially derived notochord, light magenta; superficially derived somitic mesoderm, pink; endoderm from the IMZ, yellow, including bottle cell derived, bright green; vegetal endoderm; light green. Blc- blastocoel; Bp- blastopore; Hd- head mesoderm; Ht- heart mesoderm; IMZ- involuting marginal zone; Lat. Vent.-lateral ventral mesoderm; LI- limit of involution; NIMZ- non-involuting marginal zone. Based on Keller, 1975, 1976, 1991; Shook and Keller, 2004; Winklbauer and Schurfeld, 1999. Also see Lane et al., 2000, 2002, 2006.

Keller, R. E. 1975 Vital dye mapping of the gastrula and neurula of Xenopus laevis. I. Prospective areas and morphogenetic movements of the superficial layer. Developmental Biology 42(2), 222-41.
Keller, R. E. 1976 Vital dye mapping of the gastrula and neurula of Xenopus laevis. II. Prospective areas and morphogenetic movements of the deep layer. Developmental Biology 51(1), 118-37.
Nieuwkoop and Faber Staging Series (Nieuwkoop and Faber, 1967)
Manipulating Embryonic Axis Formation In *Xenopus*.

**A. Materials and Solutions:**

- 35 mm petri dishes (some 1% agarose coated)
- 60 mm petri dishes
- Saran wrap
- PVC pipe rings (transverse sections of PVC plumbing pipe, ~1 cm x 5 cm)
- UV trans-illuminator
- Stimulated Xenopus females
- Testes: Sperm
- LiCl in 1/3X MBS (0.20, 0.25M, 0.3M, 0.35M, 0.4M)
- 2% cysteine, pH 8
- 1/3X MBS

**B. UV Ventralization of Xenopus Development:**

1. Shortly after fertilization, de-jelly eggs with the cysteine solution and wash thoroughly (7-10x)

2. Place the 4 rings covered with Saran wrap provided (used as bowls for embryos) on the top of the UV transilluminator at evenly spaced intervals.

4. Fill each ring with 1/3X MBS

5. After the eggs have rotated within the vitelline envelope, but before 20 minutes have passed, transfer the “1-4 Earlys” to the wells of 1/3 MBS on the Saran wrap (USE PLASTIC PIPETS TRANSFER PIPETS)

6. CLOSE THE UV SHIELD (THE LID)

7. Irradiate for 1.5 min, stop, open lid and remove the first, low dose batch and return them to their 60 mm dish.

8. CLOSE THE SHIELD, irradiate for 1.5 min, stop, open lid and remove the second batch

9. CLOSE THE SHIELD, irradiate for 1.5 min, stop, open lid and remove the third batch

10. CLOSE THE SHIELD, irradiate for 1.5 min, stop, open lid and remove the last batch

11. Return all embryos to their plastic petri dishes and add to the label the CUMMULATIVE time of irradiation. Suggested times may change depending on the lamp used.

12. Use the Dorsoanterior Index (DAI) (see Index) developed by of Kao and Elinson (1988) to evaluate the degree of ventralization.
13. Fix your embryos in MEMFA (see Index) at tailbud stages.

C. Dorsalization of embryos with Lithium chloride:

1. Record the time of fertilization, and de-jellying of eggs with cysteine.
2. A graded series of LiCl concentrations, all in 1/3X MBS, are provided
3. Pipet the embryos into the LiCl solution and leave them for 8-12 min
5. Pipet them into a large petri dish of 1/3X MBS and gently circulate to wash
6. Return them to fresh 1/3XMBS in 35 mm petri dishes
7. Be sure to label all dishes
8. Observe the embryos and record images on subsequent days
9. Record images of your results

Analysis of Results:

Use the DAI (Kao and Elinson, 1988) to quantify the amount of hyperdorsalization or ventralization. Place the embryos in the various categories according to this scale. Papers are provided describing LiCl effect on axis formation (see references).
DAI (Dorsoanterior Index)  
(Kao and Elinson, 1988); see Sive, et al., 2000).

Figure 7.1. The Dorsoanterior Index (DAI). Numbers in parentheses refer to the index of axis deficiency (IAD) used previously for dorsoanterior-deficient embryos. Designations for various DAI's are indicated in Table 7.1. (Reprinted, with permission, from Kao and Elinson 1988.)

Table 1.

<table>
<thead>
<tr>
<th>DAI no.</th>
<th>Designation</th>
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<tbody>
<tr>
<td>0</td>
<td>(IAD 5) no somites present; trace of tail mesenchyme occasionally seen</td>
</tr>
<tr>
<td>1</td>
<td>(IAD 4) no otic vesicle(s) present; somites present in trunk or portion thereof</td>
</tr>
<tr>
<td>2</td>
<td>(IAD 3) no visible eye pigment; otic vesicles or single vesicle still visible</td>
</tr>
<tr>
<td>2.5</td>
<td>cement gland present, but no eye pigment visible</td>
</tr>
<tr>
<td>3</td>
<td>(IAD 2) eyes fused or cyclopic, but at least some eye pigment visible; cement gland present</td>
</tr>
<tr>
<td>4</td>
<td>(IAD 1) reduced forehead; eyes smaller than normal and sometimes joined</td>
</tr>
<tr>
<td>5</td>
<td>(IAD 0) normal in all externally visible respects</td>
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<tr>
<td>6</td>
<td>mildest effect; embryos show a slight bend in the axis, visible at stage 28</td>
</tr>
<tr>
<td>7</td>
<td>severely reduced trunk, ranging from a truncated tail to a small, vestigial axis closely attached to the head; somites visible</td>
</tr>
<tr>
<td>8</td>
<td>complete lack of trunk but relatively normal looking face, although the eyes may be enlarged; no somites visible but muscle tissue can be seen sparsely in histological section; the notochord appears enlarged and irregularly shaped</td>
</tr>
<tr>
<td>9</td>
<td>multiple eyes and cement glands</td>
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<tr>
<td>10</td>
<td>radially symmetric embryos which have two forms</td>
</tr>
<tr>
<td></td>
<td>• embryos either exhibit radial eye (retinal) pigment and cement gland</td>
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<tr>
<td></td>
<td>• no eyes/cement gland visible; neural and notochordal tissue develops into a large progbosics extending either inward or outward at the end of gastrulation</td>
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The DAI scale is given in whole numbers. Averaging the DAI relative to the number of embryos scored in an experiment will usually give a fractional DAI. This is not meaningful and should be avoided.

Data from Scharf and Gerhart (1983), Kao and Elinson (1988), and Sive et al. (1990).
References:


