SEA URCHIN EMBRYOLOGY - 2009

This collection of phenomena and techniques is compiled and updated each year to give access to the many experimental approaches that have made the sea urchin embryo a good model for embryonic studies. A number of references have been added to guide you to original sources. The manual is biased in that many of the experimental approaches described were developed or used in the McClay lab, and the Davidson lab over the years. There are many other techniques that have not been included simply because they are less likely to be adapted for general use. The purpose of this manual is to provide a series of launching ideas for manipulating the embryo. With the many molecular probes and technologies now available, the combination of experimental embryology and molecular biology provides tremendous potential for the experimentalist.

Dave McClay Jenifer Croce, June, 09

Contents

| Obtaining gametes                        | 2 |
| Eliminate jelly coats                   | 3 |
| Eliminate fertilization membranes       | 3 |
|   PABA method                            |   |
|   Trypsin method                         |   |
|   DTT method                             |   |
| Acid arrest of the fertilization membrane | 4 |
| Elimination of the hyaline membrane     | 4 |
|   The Kane method                       |   |
|   The McClay method                     |   |
| Centrifuged eggs and merogone preparation | 4 |
| Hybridization between species           | 4 |
| Embryo dissociation                     | 5 |
| Cell aggregation                        | 5 |
| Cell labeling methods                   | 5 |
|   Radioactive methods                   |   |
|   Rhodamine                             |   |
|   DiI                                   |   |
| Micromere isolation                     | 7 |
| Primary mesenchyme cell isolation       | 7 |
| Skeleton isolation                      | 7 |
| Ectoderm isolation                      | 7 |
| Endoderm isolation                      | 8 |
| Deciliates embryos                      | 8 |
| Release cortical granules from plasma membranes | 8 |
| Immunofluorescence of whole mounts     | 8 |
|   Fixed eggs - permeabilized            |   |
|   Fixed eggs - alternate protocol       |   |
| Basal laminar bag isolation             | 9 |
I. Handling of sea urchins
   A. Shipping:
      The several species used in the inland laboratory can be shipped although often by
different methods. Most species are shipped in containers with sea water. Either
tupperware containers half full of sea water (with plenty of air above), or double layers of sealed
baggies (with half sea water and half air) are good for transporting Lytechinus variagatus,
Lytechinus pictus, Paracentrotus lividus and Strongylocentrotus purpuratus. Containers are
placed in a cooler and bags of ice are added to keep the inside of the cooler from heating up.
Arbacia punctulata is shipped between layers of (seawater) wet newspaper. On top of the
alternating layers of newspaper and urchins, place a baggie of ice to keep the urchins cool during
shipping.

   B. Husbandry:
      Adult animals of the several species have been maintained in recirculating
acquaria for extended periods of time. Three conditions are crucial. First, animals must be kept
within a temperature range that is appropriate for that species. S. purpuratus and L. pictus (4-
16°C); L. variegatus (18-26°C); Arbacia (15-20°C); Paracentrotus (14-19°C).

1. Gametes: For most species injection of 0.5M KCl is sufficient. Usually up to one ml of KCl
   is injected into the soft tissue just lateral to Aristotle's lantern, followed by a second injection on
the opposite side of the mouth if the animal is reluctant. After several seconds to a minute, if the
animal is ripe, gametes will emerge from one or more of the five gonopores on the top of the
animal. The animals are dioecious (separate sexes) so if you see white sperm emerge first from
the gonopores, place the males, gonopores down on a dry petri dish and collect what is called
“dry sperm” (undiluted seminal secretion) into an eppendorf tube. That tube of undiluted sperm
can be stored at 4°C for up to a week – as long as the sperm have not been diluted into seawater.
Alternatively, remove the sperm directly from the male with a glass pasteur pipette and place in
an eppendorf tube. If the secreted cells that emerge from the gonopores are yellowish or red,
they are eggs and you should now place these shedding females upside down on a beaker filled
with sea water so the eggs fall through the SW into the beaker. Hints for belligerent urchins:
Some animals give more gametes if they are shaken after injection. Others can take more KCl in
the initial injections. Still others will give more gametes with a third injection of KCl. Finally, there are methods of electrical stimulation that allow one to collect gametes from some species and to reuse the same female many times. Gametes from Paracentrotus are obtained by cutting the test, dissecting the gonads and if female, dropping them in SW. Male gonads are placed into 15 ml tubes where dry sperm will exude from the testes. Eggs will leave the ovaries in SW so to separate the eggs from the remaining ovaries simply filter the eggs through 102 mesh Nitex.

2. **Eliminate jelly coats:** Three simple methods: (1) Pour twice through clean cheesecloth into a beaker. The jelly will remain in the cheesecloth. This works for Lytechinus. (2) Filter through Nitex that is slightly larger than the eggs. For Lytechinus use 102 micron Nitex, For Arbacia use 73 micron. (3) For Paracentrotus and for Strongylocentrotus bring the eggs to pH 5.4 with SW containing 0.1 N HCl. Stir the eggs for 1 min at that pH. Return the pH to 8 with SW containing 0.1 M Tris-HCl at pH 8. This procedure is done with citrate buffered SW in some labs. Wash the eggs into SW or ASW

3. **Eliminate Fertilization membranes:**

   a. **PABA method:** Make up a solution of 10mM Paraminobenzoic acid in SW. You either can make it fresh, or dilute from 1 M PABA – which is stable for months in the refrigerator. This simple treatment keeps fertilization envelopes from covalently crosslinking in Lytechinus, Strongylocentrotus, and Arbacia. For Paracentrotus use 10 mM PABA + 10 mM Amino triazole (ATA – dilute from a 2 M stock for use).

   Fertilize the eggs in the PABA solution and monitor the elevation of the fertilization membrane. The PABA prevents hardening (covalent crosslinking) of the fertilization envelope so that you can remove it with Nitex, starting about a minute after fertilization. Flush the zygotes through a Nitex filter several times to shear off the fertilization envelope. Egg sizes vary so use: 102 mesh for Lytechinus, 73 mesh Nitex for Arbacia, Strongylocentrotus and Paracentrotus. Strongylocentrotus and arbacia have very low fertilization envelopes making complete removal more of a challenge than for Paracentrotus or Lytechinus.

   Passing embryos through nitex can sometimes cause embryos to cleave irregularly. Usually the cause is a dirty nitex filter that partially shears the zygotes – so carefully wash the filter after each use.

   b. **Trypsin method:** Incubate eggs with a tiny amount of very crude trypsin (less than a mg), added as a powder to the top of the beaker containing the eggs. Incubate for the time it takes for the eggs to settle to the bottom of a 250 ml beaker (about 5 min). Decant the trypsin-containing SW and wash 3x in ASW before fertilizing the eggs. Hints for the sloppy scientist: 1 mg of trypsin/250 ml of SW will do the trick. In fact just sprinkle a little trypsin that could be at least within striking distance of that amount will eliminate the fertilization membrane. Note: With the trypsin method there will be no fertilization membrane so you will not know until first cleavage how many eggs were fertilized. The trypsinized eggs are not as easily fertilized so fertilize with a dilute sperm suspension then fertilize again with a slightly more concentrated sperm suspension about a minute later. When the eggs settle wash them several times to remove excess sperm that otherwise die and attract bacteria.
c. **DTT:** (from Vacquier) Treat eggs with 10mM Dithiothreitol in ASW (Dilute eggs 1:1 into 20mM DTT, pH9.4). Treat 5-7 min. Wash 2-3x with ASW. The eggs can then be fertilized or activated with acetic acid parthenogenetically (Gradually bring eggs to 5.5mM Acetic acid, then dilute with ASW containing 10mM TRIS at pH 8.0; eggs should activate when they reach pH 5.5 or slightly above).

4. **Acid Arrest of the Fertilization membrane:** (from H.E. Lehman) One can stop the progression of the fertilization wave in acid. To do this make up a solution of ASW at pH 4.6-4.8. Adjust the pH using 1N H₃PO₄. Fertilize the eggs normally then simply pipet a sample of eggs into a tube containing the acid sea water at different intervals after sperm addition. This procedure will freeze the progression of the elevation of the hyalin membrane. Hint for the fastidious: If you want the timing to really be precise you can give the eggs a 10sec. pulse of sperm then bring the eggs + sperm to 0.01% SDS in SW. This will kill the ability of future sperm activity but the eggs will continue to develop in this concentration. This method will assure that the eggs could have been fertilized only during a 10sec window.

5. **Elimination of the hyaline layer:**
   a. **The Kane Method:** Incubate the embryos in a solution of 1M glycine, 2mM EDTA for about 1 min. Wash the embryos back into ASW. The hyaline is solubilized into the glycine. This method is useful if one wants to get hyaline but it is difficult to keep living cells through this harsh treatment. The next method is not as efficient in isolation of hyaline but it tends to keep the cells alive.
   b. **The McClay Method:** Suspend the embryos in HEM (see media formulary). Incubate the embryos in HEM (1ml embryos/40ml HEM) until a small sample of embryos can be easily pipetted into a single cell suspension (usually 5-15 min depending upon the species). Pellet the embryos in a hand centrifuge and the hyaline material plus single cells will be in suspension in the supernatant. Note for those who do adhesion experiments: This technique works for dissociation of many species and the cells are fairly healthy. It does not remove absolutely all the hyaline from all the cells, however (immunofluorescence shows some cells to still have some hyaline). See also: dissociation techniques.

6. **Centrifuged eggs:** (From Harvey, 1956; as modified by McClay). Make up 1M sucrose (she made it up in tap water). With the 1M sucrose stock make sucrose:SW (85:15) and (65:35). In a microfuge tube add 0.5ml of each to form a two layer step gradient. Add eggs drop-wise (so they mix the sucrose a bit). Spin at max on a microfuge for three min. You should have three bands, an upper nuclear merogone layer, a middle layer of elongated whole eggs with segregated cytoplasm, and a lower layer of merogones that are a nucleate and pigmented. Hints for the adventurous: By varying the speed and duration of the spin you can enrich for populations of merogones or of elongated 'shmoos'.
   
   An additional series of methods can be found in Maruyama, et al. (1986)(Maruyama,Yamamoto,Mita-Miyazawa,Kominami, & Nemoto, 1986). These methods utilize 1M sucrose that is diluted with different amounts of ASW to achieve step gradients. For example, 45:55, 50:50, 55:45 sucrose:SW, and a bottom of 0.65M sucrose has been used to separate different fragments and schmoos. In our experience there are differences between
species so fooling around with concentrations, centrifugal times, and with force will be necessary.

7. **Hybridization between species:** The trypsin protocol (3b) is used to prepare the eggs. This method destroys the vitelline envelope, presumably by punching holes in it so the fertilization envelope never forms. At the same time the trypsin destroys the species-specific barrier to cross-fertilization. Thus, the washed trypsin-treated eggs can then be fertilized with sperm of any species. One can achieve 95%+ levels of fertilized hybrids in this way. Hints to optimize successful hybridization: First, try to fertilize in alkaline SW (1ml 1N NaOH/liter of SW, pH ca. 9). Then if that fails, to increase the percentage try to fertilize by the progressive method - first fertilized with a normal dilute sperm suspension. Two minutes later fertilize again with a 5x concentrate of the normal sperm suspension. Then each two min fertilize with progressively more concentrated sperm suspensions. The two min. wait is essential since those eggs already fertilized will have an appropriate block to polyspermy in place in the two min. The hybrids can be raised by stirring culture, however, incubate the embryos through about 120 cell stage as a monolayer since early cleavages of hybrids are easily sheared to dissociate the embryo. The embryos can then be transferred to stirred cultures.

8. **Embryo dissociation:** See details of the following protocols: (McClay & Fink, 1982) (Fink & McClay, 1985) (McClay, 2004). Put simply, incubate embryos in HEM or in CF, depending on the species, then dissociate by trituration with a 10 ml syringe (no needle) with the embryos in a 50 ml plastic centrifuge tube. Each species is different, and the embryos vary widely with embryonic age so you must experiment first in order to get good cell suspensions. Once the cells are dissociated you can eliminate most of the whole-undissociated embryos (if there are any) by hand centrifuging the embryos to the bottom of the centrifuge. Further cleaning up of the cell suspension can be done by filtering through 12-15µm Nitex. Finally, pellet the cells in a clinical centrifuge by spinning at 3/4 speed for about 3 min. Keep the cells in CF until the last minute before starting an aggregation experiment. Note, if you are dissociating young cleavage stage embryos, you can simply soak the embryos (without fertilization membranes) in CF from fertilization onward until the dissociation.

9. **Cell Aggregation:** There are many aggregation protocols. If one wants a highly quantitative protocol see (Fink & McClay, 1985) (McClay, 1986b; Lotz, Burdsal, Erickson, & McClay, 1989; McClay, 2004). If one wants a simple protocol for aggregates, one that is highly adaptable for sorting out of cells in aggregates is found in (Bernacki & McClay, 1989). Briefly, cells are first made purely as single cell suspensions, then centrifuge the cells into a pellet and gently add ASW. After about 5 min, gently flush the pellet and break it up into smaller cell aggregates and culture these chunks. Hints for the experimenter: One can mix a few fluorescently labeled cells with unlabeled cells so that one can follow the sorting or lack of sorting of one cell type from another. See cell labeling methods.

10. **Cell or embryo labeling methods.**

    b. **Dyes:** RITC covalently links to amine groups of proteins and labels cell surfaces. FDA is a membrane permeable dye that is cleaved by endogenous esterases to yield a fluorescent product that cannot come back out of the cell once cleaved. Both dyes are used when donor tissues are to be labeled. DiI and DiO are lipophilic dyes that label cell surfaces. They are
advantageous when single cells or small groups of cells are to be labeled. All dyes will eventually become internalized and become punctate as the embryo develops but are suitable for use from the cleavage stage up to the pluteus stage. Other dyes form Molecular probes work well also.

1) **Rhodamine**: (From (Ettensohn & McClay, 1988)). Rhodamine B isothiocyanate (RITC, Sigma #R-1755) is stored in a dessicated chamber at -20°C. Fresh staining solution is prepared immediately before use. Dissolve a couple of crystals of RITC in 50µl DMSO. Add 1 µl of this to about 2ml of ASW. The color should barely be pink. If it is deep pink or red it is too concentrated. Too much RITC and you risk toxic effects on the embryos. Incubate embryos to be labeled donors in freshly diluted RITC for a few min. Sample the embryos by fluorescence microscopy to determine when they have accumulated enough dye. Wash the embryos out of the dye and resuspend in ASW. Always culture a few rhodamine labeled embryos as controls to assure yourself that the rhodamine label has not poisoned the embryos or cells. Rhodamine dextran (17,000 mw) is injected as a cell lineage marker at 0.1% in water. Fluorescent dextran suitable for fixation (5% lysine-fixable fluorescent dextran, M_r=10,000; Invitrogen) is injected according to Ruffins and Ettensohn (1996).

2) **DiI (or DiO)** Several approaches are used. Since DiI is lipophilic there are several ways to introduce it to embryos.

a. **Wesson oil injections**: Dissolve DiI in ETOH (5mg/ml). Mix with Wesson oil. After the Wesson oil has taken up the dye it can be microinjected into embryos just beneath the plasma membrane where the dye will diffuse into the membrane compartment. You can also get DiI directly into Wesson oil and avoid the ethanol but this takes longer to dissolve. In either case it is wise to filter the dye before using on embryos.

b. **Wand method**: Take one drop of 5mg/ml DiI in ETOH and place on a glass slide. The drop will spread and dry. Repeat several times to build up a layer of DiI on the slide. When ready to coat needles, add one drop of DiI-ETOH to the slide and before it dries sweep prepared needles through the concentrated solution. Small crystals of DiI should accumulate and dry on the tip of the microneedle. This needle will serve as a labeling wand. Simply hold the needle against the cell of interest for several minutes (using the micromanipulator), and the cell will be labeled (Technique from Seth Ruffins and Chuck Ettensohn; with DiI12, 3-5 min is sufficient; DiI18 requires about 8 min of contact for the dye to transfer. Efficiency of dye transfer will also be stage dependent and sometimes can be improved by removal of the hyalin layer.)

c. **Salt and pepper method**. By adding the DiI-ETOH mixture directly to sea water and shaking vigorously you will get small microcrystals of DiI in suspension. If you now incubate embryos in SW in this mixture you will randomly label blastomeres. Add 10µl of the 5mg/ml DiI:ETOH stock to 1ml SW. Vortex the mixture to disperse the microcrystals. Add 50-100µl of this stock to 10ml SW. In a 50ml tube, gently shake the embryos in the staining solution for about 10-15 sec. Resuspend the embryos in SW. You can then select embryos carrying the appropriately labeled cells for further study under a fluorescent dissecting microscope. If you want to film the embryos by time lapse you can remove the cilia (see pg 7).
You may need to adjust the ratio of DiI stock to SW according to stage or to density of cells to be labeled.

d. Iontophoresis: (from Birgbauer and Fraser, 1994) Load a filament-containing electrode with dye (5mg/ml in EtOH) and place on the needle holder. Tape the grounding wire to the stage or kiehart chamber. Place the needle in the embryo holding chamber and then insert tip of the diI-containing electrode against desired cell. Pulse with minnow (9-10nA current) for 15-45 seconds, depending on the size of the cell. When dye is transferred, a small, sticky bolus will appear at the needle tip. Check for labeling success by examining the embryo under fluorescence. Note: the “minnow” is a small hand held iontophoresis device that operates on a nine volt battery. All parts can be purchased from Radio Shack for less than $30 and a wiring diagram can be obtained from the McClay or Fraser labs.

e. Rubber cement (from Scott Fraser) Allow diI-EtOH to soak into a small drop of rubber cement. Coat a glass shard or probe of desired shape/size in the rubber cement/dye mixture and place embryo against the dye for several minutes. This method allows one to label a patch of cells on one side of an embryo, a quartet of micromeres, etc. Remember to let the dye/rubber cement to dry completely before placing near embryos.

11. Micromere isolation: A number of techniques have been devised. We use one adapted by Rachel Fink (Fink & McClay, 1985), from a technique originally published by Okazaki (Okazaki, 1975). Briefly, eggs are fertilized in PABA and fertilization membranes removed. Zygotes then are placed into CF and grown to the 16 cell stage in CF. Some species can be put into CF at the 8-cell stage and kept there until the next cleavage is complete. They are then dissociated by gently pipetting. The blastomeres are then layered on a discontinuous sucrose gradient (2% sucrose/CF on 6% sucrose/CF {using a stock of 0.75M sucrose in CF as 100% sucrose/CF }). You can build the gradient in a 50 ml glass centrifuge tube: use about 10ml of 6%, topped by about 10 ml of 2% sucrose-CF. Add cells in about 2-5ml of cell suspension. Allowed the cells to settle at 4°C, through the upper sucrose layer. The separation takes about 15 min. The micromeres stop at the the 2-6% interface and the macromeres and mesomeres continue settling. Collect the micromeres by slurping the layer interface gently and adding the cells to a centrifuge tube. Pellet the cells in a clinical centrifuge at 3/4 speed, 3 min and Resuspend in ASW. Micromeres can then be cultured in vitro in ASW plus ca. 2% horse serum, or as recently shown, in nM concentrations of VEGF. For larger quantities of micromeres one can construct a rectangular chamber out of two old gel plates separated by plastic 1 cm thick. The layers of sucrose are added, then the dissociated cells, and after about 30 min. at 4° the separated micromeres can be skimmed off the length of the chamber at the 2-6% interface

12. Primary mesenchyme cell isolation: (Ettensohn & McClay, 1987). Dissociate mesenchyme blastulae by washing the embryos several times over a period of about 5 min. in CF, or by other dissociation protocols (see cell isolation techniques). Add the cells to panning dishes containing Wheat germ agglutinin (WGA, coat 100x20 polystyrene tissue culture dishes (Falcon No. 3003) with WGA {Sigma #L-9640}, 1mg/ml, 20 min at RT. Save the WGA solution since it can be used to coat about 10 dishes. Rinse the dishes 5x with dist. H2O. Block the plate with 100% FBS for 20 min (this can be reused also), then wash 4x with dist. H2O.). These WGA plates can then be used for several rounds of panning for primary mesenchyme
cells. Add 16ml of cell suspension (5-6 x 10^6 cells/ml) to the plates and let the suspension sit undisturbed for 10 min. Pour the suspension off and wash the plate gently but thoroughly 2x with CF by swirling the CF around the plate and decanting. Finally, squirt the plate with CF using a pasteur pipet to remove the primary mesenchyme cells. Confirmation of the purity of PMC’s can be performed with FITC WGA (Sigma #L8131)(stock 1mg/ml in SW, used at 1:100 for 1min), or with a monoclonal antibody that recognizes PMC's.

12b. **Skeleton isolation** (From Armstrong and McClay, 1994) Wash embryos into CaCO_3-saturated dH2O. Add equal volume of chlorox to dissolve soft tissue. After a few min. gently triturate to remove the soft tissue and wash the skeletons 3X in CaCO_3-saturated H2O.

13. **Ectoderm isolation:** (From (McClay & Marchase, 1979)). Ectoderm can be isolated easily from mid-gastrula onward. Simply incubate embryos in HEM or in CF until a brief trituration dissociates the ectoderm and leaves the mesoderm and endoderm associated with the basal laminar bags. In order to get fairly pure ectoderm incubate in the dissociation medium for a period short of the optimal period for total embryo dissociation. The goal is to remove some of the ectoderm but to error on the side of not removing all of the ectoderm (and thereby gain contamination of mesoderm and endoderm). Hand centrifuge the larger clumps, then filter the supernatant containing ectodermal cells through 15 mesh Nitex. The cells can then be washed and collected in CF.

14. **Endoderm isolation:** This tissue can be isolated in quantity toward the end of gastrulation onward. Incubate the embryos in 1M glycine, 2mM EDTA for 3-5 min. Pellet the embryos and resuspend them in CF. Triturate carefully and take a sample frequently to inspect by microscopy. You should see the ectoderm peel away easily and the endoderm + mesoderm will stay behind with the basal lamina. This can be purified by washing on a piece of 28 mesh Nitex. The endoderm will be retained and the cells will be washed through the mesh. The endoderm retentate can then be further dissociated if one wants a single cell preparation. An alternative method that is gentler is to incubate briefly in 1M glycine followed by incubation in HEM until the ectoderm easily dissociates from the endodermal bags plus mesenchyme.

15. **Deciliate the embryo:** Cilia can be stripped easily by a 10 sec wash in double strength SW (or for that matter in 1/2 strength SW; it’s the osmotic shock that does the trick). To 1L of ASW add 39g NaCl. Place 10 ml of this solution in a centrifuge tube. Add 1ml of concentrated embryos and immediately stir then centrifuge on a hand centrifuge. Immediately withdraw the 2x ASW and replace with ASW. The embryos will survive and will regenerate their cilia.

16. **Release cortical granules:** (From (Hylander & Summers, 1981)). Cortical granules normally are bound to the inside of the plasma membrane. They can be released in 3% urethane. Make up a 6% solution of urethane and dilute 1:1 with an egg suspension. Incubate 5-10 min. The eggs are washed and then can then be pelleted using the centrifugation method to pellet the cortical granules in the cytoplasm. Reports on fertilization of these eggs are inconsistent, however in at least some cases the eggs will fertilize.

17. **Immunofluorescence.** (Whole Mounts) McClay lab protocols.
Protocol 1

1. As little as 1-20 min fixation in methanol (RT) works for most antigens. Alternatively one can fix in ice cold 100% methanol (-20°C) for 20 min. (20 minutes is maximum). For different antibodies this fixation may vary. We find some antigens are killed by extended methanol fixation. Alternatively, fix with formaldehyde or paraformaldehyde (see below).

2. Rinse into ASW + 5% normal goat serum (ASW-NGS), store in ASW-NGS. Embryos can be stored for several days in the refrigerator at this point, - with a small amount of azide in the ASW-NGS (0.005%). When ready to stain add no more than 10 embryos per well to the first well of a Terasaki plate column, in 8 µl of ASW-NGS.

3. Transfer embryos to antibody in well # 2 of the Terasaki plate column, and incubate at 4°C for overnight. Generally the incubations can be done in 5µl of diluted antibody with no more than 10 embryos per well. Dilute the antibody in ASW-NGS – dilutions will vary depending on the strength of the antibody. If there time is limiting, generally one can get away with an incubation in primary antibody for 2 hrs at room temperature.

4. Rinse 4x in ASW-NGS (8 µl per well- in wells # 3-6 of the Terasaki column).

5. Incubate in 5µl secondary antibody at a 1:200 dilution in ASW-NGS (column well # 7 of the Terasaki plate). The secondary antibody will carry a fluorescent tag. If the primary antibodies are made in two different animals (eg. Rabbit and guinea pig), or are two different immunoglobulin classes (eg. IgM and IgG), the double staining can be done simultaneously both with the two primary antibodies, and with the two secondary antibodies)

6. Rinse 4x in ASW-NGS (column wells # 8-11).

7. Transfer embryos to 35% glycerol-ASW-NGS (8µl in column well #12). Initially embryos will float on top of the glycerol-ASW-NGS. Allow them about 5-10 min to sink – this allows the glycerol to gently penetrate the embryos to maintain their shape. At this point embryos can be stored at 4°C for several days before examining – the fluorescence gradually fades if longer than a few days.

8. Mount embryos on a slide by adding embryos to a 2µl drop of 35% glycerol-ASW-NGS. Use two narrow strips of double-stick scotch tape as spacers to prevent the coverslip from crushing the embryos. The 2µl drop is used so that addition of a coverslip creates a circle of fluid that does not reach the edge of the coverslip. That way one avoids mixing oil (from oil immersion lenses) with the glycerol – which blows up the embryos. Sometimes it is advisable to place the droplet next to the edge of the coverslip so the orientation of the embryos can be manipulated. In that event, be careful if you use oil for the microscopy.

Protocol 2.

1. Incubate embryos in 3.7% formaldehyde, 10mM TRIS, pH 8 in ASW on ice for 10-15 min. Be careful to use disposable plastic or glassware for this and all subsequent steps.
2. Incubate embryos in ice cold acetone (-20o) for 10 min.
3. Wash with acetone-ASW (1:1).

Protocol 3
1. Fix embryos in 2% paraformaldehyde at pH8 in 1.33X ASW to prevent embryos from swelling. Then wash embryos in ASW and stain as usual. To make up the paraformaldehyde, dissolve the powdered reagent in ASW using a microwave oven to quickly heat the ASW until the paraformaldehyde dissolves. Alternatively, dilute 16% paraformaldehyde to 2%. Fix 5-20 min., wash into cold MeOH for 1-2 min, then transfer to ASW-NGS and proceed as in protocol 1.

18. Basal laminar bag isolation: (From McClay & Marchase, 1979; Harkey & Whiteley, 1980, with modifications by Jeff Hardin)
1. Start with embryos at the prism to early pluteus stage of Lytechinus. If S. purpuratus is used the Harkey Whiteley preparation is the method of choice. Lytechinus bags are harder to isolate so the following protocol can be used.

Reagents:
1. 10mM Sodium Bicarbonate; .01% Triton X-100; 10-20mM EDTA made in deionized water
2. 10mM Sodium Bicarbonate made in deionized water

Protocol: (all on ice)
1. Concentrate and pellet embryos by spinning at 500 rpm for one minute.
2. Lyse embryos in Bicarb/Tx-100/EDTA solution (5ml packed embryos/45ml solution). Let incubate for 5-10 minutes on ice with occasional gentle trituration.
3. Spin at 1000 rpm for 2 minutes.
4. Pour off supe and repeat steps 2 and 3.
5. Resuspend pellet in Bicarb and spin at 1500 rpm until pellet is formed. This takes a little more time for each wash. Wash three times. Maximum spin time should be no more than 15 minutes.
6. Pour off Bicarb on last spin and freeze pellet at -20o C.

19. Oralize or aboralize embryos: To oralize embryos can be treated with nickel to eliminate most, if not all, aboral-specific genes. The means by which this is accomplished is not yet understood though recent data suggest nickel interferes with a crucial calcium transporter necessary for some aspect of aboral specification. (Method after Hardin, et al., Development 1992).

1. Fertilize embryos in ASW. After 10 min transfer embryos to 0.5-1.0mM NiCl in SW (use a range for your first experiment to find appropriate concentration.
2. Wash embryos out of the nickel solution at the early mesenchyme blastula stage and development will continue beyond that stage. If left in NiCl the embryos will tend to arrest.
To aboralize it is necessary to eliminate the Nodal pathway. To do this at the top of the pathway, eliminate p38 activity. This MAPK activates a transcription factor that activates transcription of Nodal in the oral ectoderm.

1. Fertilize embryos. Add SB203580 (Calbiochem) at 20 µM.
2. Wash embryos out of SB at mesenchyme blastula stage.

20. **Animalization**: (From many sources) A number of agents will cause the phenomenon known as animalization. The following reagents and their concentrations have been shown to work.
   a. NaSCN - 1% (0.124M). Wash eggs 2x with CF. Add eggs to 1% NaSCN. Incubate for about 20 min. Wash and return eggs to ASW. Fertilize and incubate embryos normally.

   b. In the vital dyes below, incubate the embryos in the dye beginning at the 8-16 cell stage. Monitor development microscopically.
   Evans Blue -0.0025% or Congo Red 0.0025% - Evans blue and congo red are sulfonic (HSO3+) vital dyes.

21. **Vegetalization**: Make up a stock of 0.1M LiCl in ASW.
   1. Fertilize eggs normally and raise to the 8-16 cell stage.
   2. Begin treatment in Li (30 and 50 mM LiCl) – again the range gives one working margins.
   3. Remove from Li at the flattened vegetal plate stage just before the primary mesenchyme cells ingress.
   4. Wash the embryos into ASW. At gastrulation the vegetalized embryos will begin to show abnormal morphology.

22. **Exogastrula**: Embryos can be induced to exogastrulate by growing the embryos in 0.001% SDS-SW (From (Coffman & McClay, 1990)).

23. **Conversion of unequal cleavage to equal cleavage**: (From Langelan & Whiteley, 1985). Fertilize eggs normally and raise to the 4-cell stage. Incubate the embryos in SDS (20-25 µg/ml in SW) for 30 to 60 min. (shorter times for embryos that develop faster). Observe 4th cleavage. It should be equal rather than unequal.

24. **Embryo cut and paste methods**:

   a. **Micromanipulation of blastomeres**: (From Ettenson & McClay, 1986; Ettenson & McClay, 1988; McClay and Logan, 1996, McClay, 2004). These approaches enable you to transfer cut and paste any combination of cells until the 60 cell stage.
   1. Obtain Pyrex glass capillary tubes (O.D 0.86mm, I.D. 0.51mm, Drummond Scientific).
   2. Pull capillaries to make long needles. The glass needles should be flexible when gently pressed against the bottom of a chamber.
   3. If joystick micromanipulators are available set them up so the needles approach each other at 90°, with each almost parallel to the stage (about a 10° angle approaching the focal point), Prepare a Kiehart chamber with two coverslips (McClay, 2004). For cut and paste experiments with Lytechinus or Paracentrotus fill the chamber with CF. For experiments with
Strongylocentrotus fill the chamber with HEM + 5% goat serum (to prevent everything from becoming too sticky).

4. Add embryos to the chamber and the assembly is placed on a microscope at a 45° angle to the converging microneedles. One of the microneedles is broken prior to the experiment using a razor blade – carefully cut off the tip, place on the microscope then slam the needle tip on the face edge of the coverslip until it breaks to give you a square hollow tip with the desired opening diameter (I try to obtain an opening of the same diameter of the cells I am about to transfer). Attach that needle to a mouth pipet tube and it will serve as your suction device. The other needle, controlled with the other joystick, serves as the “thumb” for the suction pipet. With practice one can do many manipulations of embryos and cells.

b. Removing PMCs: The suction needle is broken to the diameter of micromeres. For PMC removal mount embryos at very early mesenchyme blastula stage into Kiehart chambers in which the coverslip has been treated with 1% protamine sulfate. Penetrate the blastula wall with the needle. I do this by denting the embryo with the pipet, then I gently strike the table below the microscope – this pops the needle into the blastocoel and dislodges the PMCs. Additional blow will cause the PMCs to exit from the blastocoel, or you can vacuum the PMCs into the pipet.

c. Tissue deletion or transfer:
   1. Prepare a kiehart chamber with embryos at the desired stage
   2. Blunt a needle to about 10 µM (smaller if you want to transfer micromeres, larger if you wish to transfer macromeres or mesomeres. Ideally the bore of the pipet should be slightly smaller than the diameter of the cell to be transferred). Practice will be necessary to dictate the size relative to the operation you wish to perform.
   3. Transfer a bunch of embryos into a Kiehart chamber, insert the two needles at 90° to one another, then begin your experiment.

1. Remove micromeres: Simply manipulate an embryo so you can see the micromeres, approach them from the side with the suction pipet, suck off two in a row, adjust needle focus to suck off the second two.

2. Mosaic analysis. Transfer a few red “donor embryos” into a chamber along with a few host embryos (we dye the hosts green generally). At the 60 cell stage get used to the orientation of the embryo. Suck out two host cells at the desired site of insertion. Then pick up the equivalent two donor cells and carefully place them in the host hole. This manipulation is time-dependent because the host immediately begins to close the hole once its cells are removed so it is advisable to first remove the donor cells an place them near the future host before attacking the host.

3. Recombinations: Any embryo recombination can be made. Generally I cut the embryos using the needle as a saw and the suction pipet to hold the embryo in the correct position. After cutting the pieces as desired, recombine the two or more parts to reconstitute the embryo of the recombinant pieces. Hold the needle and suction needle as a “+” over the recombinant, gently pressing down for about 10 – 15 seconds to get the two pieces to stick to one another.

d. Cutting eggs: Eggs can be cut easily with a simple very thin pulled glass needle.
   1. Cutting can be accomplished simply by pushing down on the egg or early embryo. The wound quickly heals and the egg or embryo is subdivided into two.
   5. The embryo can now be transferred via mouth pipet to a small chamber for culture.
e. **Embryo fusion**: Embryos can be parabiosed rather easily. Simply incubate the embryos at the 16 cell stage in CF for about 30 min. Return the embryos to ASW and place them in close contact until gastrulation. Contact can be enhanced by pelleting the CF-treated embryos.

25. **Lineage markers available**: The following list is not exhaustive but lists some of the markers that can be obtained from workers in the field. These are available for your use in the course.

**Oral Ectoderm**: Ecto V (Coffman & McClay, 1990). This monoclonal antibody stains the oral ectoderm and can be used for western and immunoprecipitations.

Nodal – (Duboc, et al., 2005). In situ probe.

Aboral ectoderm: Spec 1 (Lynn, Angerer, Bruskin, Klein, & Angerer, 1983); CyIIIa (Schott, Lee, Britten, & Davidson, 1984). These genes are differentially expressed in the aboral ectoderm and can be detected by in situ hybridization on S. purpuratus. LvS1 is a Digoxigenin riboprobe that can be used on Lytechinus Tbx2/3 (Gross and McClay, 2003; Croce and Gache, 2003) In situ probe.

**Primary mesenchyme**: 1D5 (Mouse monoclonal IgM) (McClay, Cannon, Wessel, Fink, & Marchase, 1983). This monoclonal antibody and a number of others that have been produced in other laboratories will stain primary mesenchyme cells. The MSP130 DIG-riboprobe identifies gene for the molecule recognized by 1D5.

**Secondary mesenchyme**: SMC1 and SMC2 (both are mouse monoclonal IgMs) Sp-12 (Tamboline & Burke, 1989), is an antibody that can differentially stain secondary mesenchyme if one plays with the titer of the antibody (it also stains primary mesenchyme). A myosin DIG riboprobe can identify the secondary mesenchyme cells very easily.

**Endoderm**: Endo 1 (McClay et al., 1983)(Wessel & McClay, 1985). This antibody stains the midgut and hindgut of the embryo beginning at gastrulation. DIG- Endo 16 recognizes S. purp. endoderm and DIG LvEn recognizes Lytechinus endoderm.

**Endoderm – Alkaline phosphatase**. (Sigma Kit#85) Add several drops of 0.25% Naphthol-MX phosphate buffered to pH 8.6 to embryos or cell suspension. Add 1-2 grains of fast blue RR salt to the suspension. Alkaline phosphatase will develop, if present in about 5 min.

**Hyalin**: 183 (Adelson & Humphreys, 1988) is a monoclonal antibody that selectively binds to the cell adhesion site of hyalin (Adelson & Humphreys, 1988). The antibody can be used for immunofluorescence, westerns or immunoprecipitations. DIG-hyalin is the corresponding riboprobe.

**Basal Lamina**: Ib10 (Wessel & McClay, 1987). This antibody stains several collagens and is diagnostic for the basal laminar vesicles of the eggs.

**Aboral ectoderm**: Ic10 (Nelson & McClay, 1988), and (Alliegro & McClay, 1988). These antibodies both identify an oligosaccharide on several apical proteins all of which

**Ciliated band**: Monoclonal 295 recognizes just the ciliated band.

**Anti β-catenin**

**Anti vasa**

**Anti Ng-CAM**

26. **Injection of eggs to introduce RNA, morpholinos, or DNA constructs - general.**

**Set Up:**

1. Prepare protamine sulfate-coated p60 dish lids:
Make 0.25% protamine sulfate: make ~50 ml, combine dH₂O and powder and vortex until dissolved. This may take 10 minutes. Store at 4°C. Use the lids of P60 dishes. Draw a line on the outside of the dish using a straight edge. Set up 10 lids and apply a small volume of PS solution along the line with a P1000, using the tip to spread the PS along the line, leaving a stripe of PS on the center of the dish. After the 10th dish, start timing for 2min. During this time, use a pasteur pipet to make a scratch on the edge of the PS stripe opposite from the line, within the PS. Thus, you have a stripe of PS with a scratch inside the edge along one side, and the line drawn on the other side. When 2min are up, use a squirt bottle to rinse the dishes with dH₂O, and leave them upside down to dry. I typically coat 20 or 40 in groups of 10, depending on how much injecting I am doing. The dishes may be used immediately after rinsing, or may be used after drying for more than a week.

2. Prepare mRNA:
Clone cDNA or fragment into pCS2, which should give maximum expression. Linearize ~10 ug (check for completeness on a gel) and phenol extract plasmid DNA, ppt and resuspend to 0.5 µg/µL. Transcribe & cap RNA using mMessage mMachine kit (Ambion), as per instructions, using SP6. DNase (kit) treat 15m. Dilute reaction to ~100 ul, add 1/10 vol. 5M NH₄OAc (kit), extract and isopropanol ppt (1 vol). Wash in 70% EtOH, and resuspend to 10 µL in depc dH₂O (kit). Aliquot 0.5 ul and store –80°C. {* Or, do not aliquot, but save diluted RNA at –80 and reuse. I haven’t really tried this yet, but Jeni does it for everything.} Quantitate with the spec (0.5 ul to 50 is 1:100, wash cuvette and blank well before reading RNA). Run 0.5 ul on a formaldehyde gel, a single prominent band should be visible.

3. Prepare rolling mouth pipets. These pipets should be pulled so that a long thin end is produced, with an inner diameter just larger than an egg, to make rolling/rowing as easy as possible. Wash the pipet well after using to prolong its life. To pull these pipets, begin with a long pasteur and heat the neck of the pipet, where the thin part changes to the wide part, in the flame until soft. Remove it from the flame and the quickly pull the thin part. Test new pipets with eggs to see that the pipet isn’t too narrow, deforming the eggs, or too wide, allowing them to pass double file.

Injection of eggs:
1. Pull needles using needle puller. Do NOT disturb the omega-shaped foil. Have someone show you how to use the needle puller. Do not contaminate needles with RNases.

2. Prep RNA and load needles. Dilute RNA using 20% glycerol, with or without dye. Spin down for ~3m to remove particulates (that can clog needles). Load needles by pipeting a small drop (~0.75 ul) into the end of the needle and allowing capillary action to pull it in to the tip.
Load needles with injection sample using one of the long loading tips in the back of the main embryology room. Slide the end of the loading tip inside the needle as far as it can go and expel a small amount of the injection sample.

• It is easier to see the position of the loading tip in the needle against a dark surface.

• You can load all the needles you will need if you inject fast enough, and use them before they are dry or use one of the wet chambers with needle holder. OR you can load one solution at a time only when you are ready to inject.
An alternative method can be used when the needle capillary tube has a filament: build your own needle holder using clay, place the needles with tip down and pipet 0.5 µl of solution on the opening at the top of the needle. By gravity the solution will go to the tip of the needle in about a minute.

3. Spawn female urchin by injection with 0.5M KCl. Collect eggs in sea water (ASW), allow them to settle, and replace ASW. Dejelly eggs: pour through 102µ nitex 3X, and allow them to settle. Wash with ASW 3X. Dejellying and washing can be done while other steps are being carried out, or afterwards, but dejellied eggs should not wait around unnecessarily, since they will quickly degenerate. Transfer dejellied eggs to a small beaker in SW and keep covered.

4. Set up: Add ASW to protamine sulfate dishes and put into humid chamber (optional: include PABA in ASW to ease removal of embryos prior to hatching. Dissolve PABA at 0.15 g/100 ml in ASW). Sperm dilution tubes should also be made ready: partly fill 1.5ml eppendorfs with ASW (or PABA), 1 per dish. Bring filled needles, dishes, sperm tubes, rolling pipets, 200 ul pipetman, and sharpie to injection area.

5. Turn on the picospitzer: The microinjection station is comprised of a picospitzer apparatus, micromanipulators and the microscope. Turn on the power to the picospitzer; open the valve for air pressure. The standard pressure is 40 psi and the usual pulse duration is 10 to100 msec. However in practice the pulse time, as well as the pressure, may be adjusted as necessary depending on the size of the needle opening. Be sure that the time is set as msec and not min. Turn on the light of the microscope. Alternatively the picospitzer can use continuous flow for injection.

6. Roll eggs and fertilize. Rinse rolling pipet a few times to lubricate inner surface, then draw up a few centimeters of eggs and roll them onto PS dish alongside scratch. Immediately fertilize by pipeting diluted sperm along the row of eggs, then label dish with sharpie.

Eggs don't like to be in contact with the protamine sulfate for too long, so once they are rowed proceed immediately with fertilization. Pipet 0.2- 0.5 µl of sperm and dilute in ASW. Use a pipette (Pasteur pipette or a 200µl pipette) to transfer some of the liquid to the egg plate. You may observe a slightly white and opaque quality to the dilution. Swirl the plate to equally distribute the sperm. Check for fertilization. To verify fertilization, observe the formation of the fertilization membrane within a minute of fertilization. As you are observing note the presence of the sperm easily detectable under 20X magnification. If you see too much sperm swimming around, wash the eggs by gently removing as much water as possible and replace it by fresh water. This can be done several times until you get rid of the excess of sperm.
7. **Align eggs under microscope and position needle.** Open the nitrogen tank. Push back stage (‘north-ward’) to its limit, then position dish so that lowest eggs are still north of the field of view. Scroll upward to the top of the line of eggs, observing fertilization, then move to center the scratch in view. Insert needle into holder and tighten, then insert holder into manipulator clamp and tighten. Use coarse adjustments to lower needle tip into ASW, then move it into the light path. Turn on the picospritzer, and adjust pressure to 7 or 8. Focus on the tip of the needle, centering it in the lower left of the field (to compensate for joystick resting point). Refocus on the scratch, then carefully lower the tip to the plane of focus.

8. **Adjust needle flow.** Use joystick to gently break the tip of the needle on the scratch. Flow is best observed with needle just out of focus. Use pressure settings to adjust flow rate.

9. **Inject.** Raise needle and return to eggs. Focus on egg equater, then adjust needle height until tip is in focus. Touch needle to egg, raising or lowering so that contact is equatorial. Inject by denting egg with needle, then tapping scope base if needed. The injected bolus should be visible, and shouldn’t exceed ¼ diameter of the egg, and shouldn’t flow in too vigorously. Pull needle back out smoothly. If flow diminishes or stops, try to clear needle by pressing clear button on spritzer. If this does not help, re-break needle until flow is restored. In the first couple of eggs adjust the pulse duration and psi in order to inject 2-5 pl. Injection volume relative to the egg size:
12. **finish**: As soon as you finish injecting the plate, wash the eggs to remove the excess of sperm (if you haven't done so already), or to dilute the PABA-SW. Finish by adding roughly 1 volume of antibiotic sea water. Put the plates in a large Petri dish or box with some paper towel soaked with dH$_2$O (optional); labeled the plate with name and date and incubate at 18-23 deg. For Lytechinus, 12-15° for Strongylocentrotus, or about 18° for Paracentrotus.

13. Turn off microscope lights, picospritzer (remove the needle, close the pressure valve, use the manual firing and set up in min to drain the pressure back to zero, turn off the power), wipe down microscope stage, cover the microscopes. The sea water is highly corrosive to metal surface. **Clean up all the mess especially any sea water spills! Are you sure you turned off everything?**

Comments:

1. Initially, RNA should be injected in a dilution series to determine an optimum injection volume. Dilute RNA at 1:10, 1:20, and 1:40 is usually a good beginning.

2. **Always** include a control (glycerol) injection. This should be the last dish injected, to also control for egg aging.

3. For the beginner, inject only dye into 2 or 3 dishes and assess morphology compared to a fertilized control. When 90% of injected eggs develop normally, begin experiments. Keep initial experiments to 6 dishes or so, although this number can be increased as proficiency is gained. Critical is the overall length of time from dejellying to fertilization of the last dish. If the eggs are robust, you have ~4 hours. If they are more delicate (the usual fare), there is less time until they lose it.

4. When dishes are too sticky, fertilization envelopes may spontaneously appear, blocking actual fertilization. First, coat dishes as recommended above. Second, fertilize immediately after rolling/rowing eggs.

5. When sea water is bad, fertilization envelopes will be close to eggs and non-concentric with eggs. Eggs may also look odd. When this is observed, it is very likely that the eggs will die after undergoing some cleavages. Don’t use carboys of SW that are almost empty.

6. It is probably better to remove embryos from the PS dishes and allow them to develop in fresh ASW. This is definitely true for eggs injected in PABA. Removal is least disruptive once a blastocoel has formed. 24 well plates are a good choice for incubation.
Sea urchin (*S. purpuratus*) Microinjection protocol

**Preparation**

1. **Gametes:** Eggs, sperm
2. **Solutions:** PABA, acidic sea water
3. **Needles**
4. **Injection samples**

1) **Gametes**

**Eggs:** Spawn first just by shaking. If nothing happens inject 0.5-1ml of 0.55M KCl. The color of eggs is yellow/orange. Once identified a female put her upside-down on top of a beaker filled with sea water (SW). The eggs will be released from the gonopores and will settle at the bottom of the beaker. If the female gave eggs just with a shaking, she can be reused. However keep her in a small container with fresh SW for at least an hour to be sure that she is no longer releasing eggs, and then put her back in the tank. Females injected with KCl will die, so DO NOT put back in the tanks.

**Sperm:** Undiluted (“dry”) sperm can be kept at 4°C or on ice for about a week. Sperm (white) is collected dry directly from the male using a Pasteur pipette; do not put a male upside-down on a beaker with sea water!

To dispose of used sea urchins, dump them in the water table room “dead animals” trash.

2) **Solutions**

**PABA sea water (PABA-SW):** PABA is Para-aminobenzoic acid sodium salt (SIGMA A6928, 100gr). PABA is used to prevent hardening of the fertilized membrane. Use PABA-SW at fertilization at a final concentration of 2mM: 150mg of PABA in 500ml filtered sea water. We have made up PABA-SW for you.

**Acidic sea water (ASW):** for de-jellying the eggs so that they can stick to protamine coated plates. The final pH of the sea water should be 4-5: use 30µl 0.5M citric acid in 15ml filtered sea water. The SW starting pH can vary from 7.5 to 8.2, for this reason some people prefer to adjust the pH exactly to 4.75 using the pHmeter.

We have made ASW for you.

3) **Needles**

**Needles:** we have made up needles for you. They are by the centrifuge in the back of the main room.

4) **Injection samples**

**Injection solution:** spin down at least 5 min to ensure any debris in the solutions is pellet, then use upper portion of the solution to load the needle. This step prevents clogging of needle during injection. *Very important!!*
See the reagents section for exact volumes for the Embryology Course; here’s our rough set of guidelines.

**DNA**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid/BAC DNA</td>
<td>X µl</td>
<td>1000-5000 molecules/2pl For BAC 25ng/µl</td>
</tr>
<tr>
<td>Carrier DNA</td>
<td>X µl</td>
<td>Ratio 1:5 molecules relative to the plasmid, No carrier DNA in BAC injection</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>0.6 µl</td>
<td>120 mM</td>
</tr>
<tr>
<td>Rhodamin Dextran 5 mg/ml</td>
<td>0.5 µl</td>
<td>Optional 0.5 mg/ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>Up to 5 µl</td>
<td></td>
</tr>
</tbody>
</table>

All the solutions for injection of DNA are sterilized by filtration throughout a 0.2 µm filter and are not autoclaved.

**RNA**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>X µl</td>
<td># of molecules depends on the prevalence of the transcript during sea urchin development</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>0.6 µl</td>
<td>120 mM</td>
</tr>
<tr>
<td>Rhodamin Dextran 5 mg/ml</td>
<td>0.5 µl</td>
<td>Optional 0.5 mg/ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>Up to 5 µl</td>
<td></td>
</tr>
</tbody>
</table>

All the solutions for RNA injection are made with DEPC water with sterile plastic-ware and then filtered with 0.2 µm filter.

**Morpholino Antisense (MASO)**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MASO</td>
<td>X µl</td>
<td>The concentration varies from 50µM to 300µM</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>0.6 µl</td>
<td>120 mM</td>
</tr>
<tr>
<td>Rhodamin Dextran 5 mg/ml</td>
<td>0.5 µl</td>
<td>Optional 0.5 mg/ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>Up to 5 µl</td>
<td></td>
</tr>
</tbody>
</table>

MASO is incubate at 55°C for 10 min before preparing the injection solution and the solution is kept at RT until injection time.

**Injection**

1. Prepare eggs: A) De-jelly the eggs; B) Row the eggs
2. Prepare for microinjection: A) Turn on the microinjection station; B) Load the needle; C) Mount the needle; D) Adjust and break the needle
3. Injection: A) Fertilization; B) Injection
4. Done
5. Turn off
6. Collect the embryos
1) Prepare Eggs

**De-jelly**: Dissolve the jelly coat so the eggs will stick to the protamine-coated plates. Use three regular dishes (no protamine coated plates). #1 is acidic sea water, keep the eggs here for few seconds to a max of 2 minutes; #2 and #3 are filtered sea water to wash. Swirl the plates to concentrate the eggs at the center of the plate and transfer between dishes with a Pasteur pipette.

Inject as in the protocol above.
Extras:
**Protamine coated plates:** Protamine solution 1% (W/V) of Protamine Sulphate (Sigma Cat# P4380, 25gr). I prefer to make the protamine solution fresh every time. Use the lid of the 60X15 mm Petri dishes. Some people use the bottom too. Organize the dishes into columns; fill each Petri dish of the first column with the solution. Incubate for 1 minute at RT; transfer the solution to the dishes of the second column repeat for all the plates. In the mean time rinse the plates of the column #1, then #2 and so on with continuous flow of dH₂O for 15 min. Air dry the plates Upside-down O/N. You can store the plates indefinitely in a dust free container.
In Situ Hybridization In 96 well plates  
Jenifer Croce/Villefranche Method Revised: 6.7.04

A. Rehydration of the Embryos
   1. Use a P-1000 to transfer 100-150 ul of fixed embryos to a well in the 96 well plate
   2. Add 100 ul of 50% MeOH/50% 1X TBST to the embryos and allow them to settle for 5 min at RT
   3. Wash the embryos with 150 ul of 50% MeOH/50% 1X TBST for 5 min at RT
   4. Wash the embryos with 150 ul of 1X TBST for 5 min at RT

B. Prehybridization and Hybridization
   1. Prehybridize the embryos for 45 min to 1 hr at 65°C in 150 ul of hybridization mix
   2. Hybridize the embryos overnight at 65°C in 150 ul of hybridization mix with 0.5 ng/ul of probe.
   3. Wash: 15 min in 150 ul of hybridization mix at 65°C
      15 min in 150 ul of hybridization mix at 65°C
      15 min in 150 ul of 50% hybridization mix/50% 2X SSCT at 65°C
      15 min in 150 ul of 2X SSCT at 65°C
      20 min in 150 ul 0.2X SSCT at 65°C
      20 min in 150 ul 0.1X SSCT at 65°C
      5 min in 150 ul of TBST at RT

C. Detection of the Probe
   1. Block the embryos in 150 ul of blocking solution (0.5% BSA and 2% heat-inactivated goat serum in 1X TBST) for 45 min to 1 hour at RT
   2. Incubate the embryos in 150 ul of Anti-Dig antibody diluted 1:2000 in blocking solution for 1 1/2 to 2 hours at RT
   3. Wash 2X 15 min then 4X 10’ with 150 ul of 1X TBST at RT
   4. Wash 2X 5 min with 150 ul of phosphate buffer I at RT
   5. Incubate the embryos in 150 ul of phosphate buffer II in the dark. Watch the color reaction—it can take 30 minutes to several hours.
   6. To stop the color reaction, wash the embryos with 150 ul of 50 mM EDTA in 1X PBST once for 5 min and a second time for 10 min at RT.
   7. Wash 3X 5 min in 150 ul of 1X PBST at RT. The embryos can be stored in the plates at 4°C for several days
8. Replace the PBST with 60 ul of 50% glycerol/50% 1X PBST and mount the embryos on slides with coverslips (~25 ul)

Solutions:

10X TBS
500 mM Tris-Hcl pH 8.0
1.5 M NaCl

1X TBST
1X TBS
0.05% Tween

20X SSC
175.3 g NaCl
88.2 g Na₃Citrate-2H₂O
Add water to 800 ml
Adjust pH to 7.0
Add water to 1 L

10X PBS
80g NaCl
2g KCl
26.8g Na₂HPO₄·7H₂O
2.4g KH₂PO₄
Add water to 800 ml
Adjust pH to 7.4
Add water to 1 L

Blocking Solution
For 10 ml in 1X TBST
500 ul of 100 mg/ml BSA
200 ul of Heat inactivated Sheep serum

Heat Inactivated Sheep Serum
Incubate the sheep serum at 60°C for 30 minutes, then store at –80°C

Hybridization Solution
25 ml ultrapure formamide
12.5 ml 20X SSC at pH 5.0
50 ul of 50 mg/ml heparin
50 ul of 50 mg/ml yeast RNA or tRNA
100 ul of 50% tween 20
12.3 ml of DEPC water

Phosphate Buffer I
100 mM Tris-Hcl pH 9.5
50 mM MgCl₂
100 mM NaCl
**Just before using, add 0.05% tween20 and 5 mM Levamisole

Phosphate buffer II
Phosphate buffer I with 4.5 ul/ml of NBT
(75 mg/ml diluted in 70% dimethylformamide)
And 3.5 ul/ml of BCIP (50mg/ml diluted in 100% dimethylformamide).
Double Fluorescent In Situ Hybridization for Sea Urchin embryos (Jeni Croce, May, 2008)

Everything should be done under a stereomicroscope to make sure you are not loosing embryos.

I. Fixation

Using a p-200 or a mouth pipette collect embryos to be fixed (50 to 100 embryos – 20 to 50 embryos will be used per probe or probes combination) into a 3-well or 9-well depression plate. At that point the volume of sea water you are pipetting doesn't matter.

In a separate well, put 150ul of Fix solution (8%PFA-20mMEPPS). Then, with a new, clean tip, collect ALL your embryos in a FINAL volume of 150μl of sea water and add them to the Fix solution all at once. Mix by up and down twice and fix for 1 hour at room temperature.

Wash the embryos in artificial sea water (ASW) once for 1 to 2 min at Room temperature (RT). Transfer your embryos using a p-20 or a p-200 from the Fix solution into 1ml of ASW present in new well. Try to pipette your embryos in the smallest volume of Fix solution you can (50 to 80ul). If you can’t repeat this wash one more time.

Wash twice in cold MeOH (4 degrees or -20 degrees MeOH). Again using a p-20 or p-200 transfer your embryos from ASW into 1ml of cold MeOH. During these steps the embryos should turn white (if your stereomicroscope has a black background and a top light) or dark brown (if your stereomicroscope has a bottom light). Note that the sea water will precipitate into MeOH. To reduce this precipitation and not lose your embryos transfer as little sea water as you can. If after the second wash you still have a lot of precipitate repeat the washes until most of it is gone.

After the MeOH washes transfer your embryos into a 96-well plate (one well per probe or probes combination). Put 20 to 50 embryos per well containing around 200ul of MeOH. Sealed the plate with parafilm and incubate at -20 degrees for at least 30min. You can leave the plate at -20 degrees for hours or days if you need to, just check for MeOH evaporation.

II. Rehydration

Take the plate out of the freezer, and add in each well 100ul of RNase free 1xTBST.

**Warning:** the probes you will use are RNA probes. RNA is sensitive to degradation by RNAses. So during the entire process of rehydration and hybridization make sure to keep...