Int. J. Dev. Biol. 47: 161-164 (2003)

The color purple: analyzing alkaline phosphatase expression in experimentally manipulated sea urchin embryos in an undergraduate developmental biology course

JULIE DRAWBRIDGE*

Department of Biology, Rider University, Lawrenceville, New Jersey, USA

ABSTRACT In these laboratory exercises, developed for a sophomore/junior-level undergraduate course in Developmental Biology, students explore the processes of differentiation and morphogenesis in sea urchin embryos by monitoring the spatio-temporal expression pattern of the endoderm marker, alkaline phosphatase. Once students have determined the normal alkaline phosphatase expression pattern, they are asked to treat sea urchin embryos in some way that perturbs normal morphogenesis. Their task is to discover whether the chosen treatment perturbs both morphogenesis and differentiation of the gut or only morphogenesis. The ease with which sea urchin embryos can be cultured and manipulated provide the Developmental Biology instructor with a powerful system for inviting students to explore questions regarding differentiation and morphogenesis.

KEY WORDS: *sea urchin, alkaline phosphatase, gut development*

Background Information

The author teaches at a small, liberal arts institution. Her teaching emphasizes learning biology by doing biology, both in the classroom and in her research laboratory. The author's research focuses on understanding the molecular basis of kidney morphogenesis in amphibian and fish embryos. Many of the co-authors on her scholarly papers and abstracts are undergraduates who have done research in her lab.

Representative Publications

DRAWBRIDGE, J., WOLFE, A.E., DELGADO, Y.L., and STEINBERG, M.S. (1995). The epidermis is a source of directional information for the migrating pronephric duct in *Ambystoma mexicanum* embryos. *Dev. Biol.* 172: 440-453.

DRAWBRIDGE, J., and STEINBERG, M.S. (1996). Morphogenesis of the axolotl pronephric duct: a model system for the study of cell migration *in vivo*. *Int. J. Dev. Biol.* 40: 709-713.

DRAWBRIDGE, J., and STEINBERG, M S. (2000). Elongation of axolotl tailbud embryos requires GPI-linked proteins and organizer-induced, active, ventral trunk endoderm cell rearrangements. *Dev. Biol.* 223: 27-37.

DRAWBRIDGE, J., MEIGHAN, C., and MITCHELL, E. (2000). GDNF and GFRα-1 are components of the axolotl pronephric duct guidance system. *Dev. Biol.* 228: 116-124.

Introduction

The embryos of the sea urchin have been used as a model experimental system for the study of cell and developmental biology for over a century. Much of what we know about the processes of fertilization, protein translation, signal transduction and early embryonic development comes from investigative work on these embryos. They are members of the phylum Echinodermata, which also includes star fish, brittle stars, sand dollars and sea cucumbers. Sea urchins are deuterostomes—animals in which the site of the blastopore marks the anal opening—and, as such, share some basic developmental mechanisms with vertebrates.

Sea urchin embryos provide excellent experimental material for the teaching laboratory for several reasons: Adults are easy to maintain and spawn; fertilization and embryonic development occur outside the female; eggs and embryos are large and clear, so it is possible to watch both the inside and outside of living, developing embryos. Sea urchin embryos are also easily manipulated, thus allowing one to do technically simple experiments which illustrate important aspects of cell and developmental biology. In this set of laboratories, developed for a sophomore/junior level undergraduate Developmental Biology class, students use a direct detection method to assay for tissue-specific expression of the gut enzyme alkaline phosphatase (AP). AP is a digestive enzyme expressed in the guts of most animals, as well as in tissues such as bone, liver and kidney. In sea urchin pluteus larvae, AP is expressed only in the gut epithelium and therefore serves as a good marker for differentiated endoderm. Since no antibodies are required to detect endogenous AP, this technique can be used on

***Address correspondence to:** Dr. Julie Drawbridge. Dept. of Biology, Rider University, 2083 Lawrenceville Rd., Lawrenceville, NJ 08648, USA. Fax: +1-609-895-5782. e-mail: drawbridge@rider.edu

0214-6282/2003/$25.00

Printed in Spain
www.ijdb.ehu.es

almost any species of sea urchin, is cheaper than antibody-based detection methods, and is quick and easy for students to do in a single laboratory period. Once students have mastered the AP staining technique, they can perturb development and ask whether gut differentiation can occur in the absence of normal development.

Here, I present a method for performing AP staining in sea urchins, as well as some ways in which this assay can be used to engage students in an experimental investigation of differentiation.

Procedures, Supplies and Solutions

Procedures

Methods for spawning sea urchins vary from lab to lab; many commercially available developmental biology lab manuals provide good instructions for handling sea urchins. In addition, there are many sources of AP stains. Those based on BCIP/NBT should all work well on sea urchin embryos. The following handling procedures and AP staining procedures have worked well for me in the undergraduate teaching laboratory setting, and require no specialized equipment. Required supplies and solutions are itemized below.

Spawning Adults

One-half to one milliliter of 0.5 M KCl is injected into the adult with a 1-cc syringe inserted into the soft tissue around the mouth (Aristotle's lantern). This procedure is repeated two to four more times at different spots around the mouth. The animal is swirled gently, dorsal side down, then set down on a paper towel. Within 5 min, gametes should appear on the dorsal side of the animal as they are extruded from the five gonadopores: eggs usually look yellowish-orange and slightly translucent; sperm is white. If the animal is female, she is turned dorsal side down on top of a beaker of 10-20°C (temperature depends on the species of urchin) artificial sea water (ASW; see solution list below) so that the eggs are shed directly beneath the surface of the ASW. Males should be inverted on top of a dry beaker and put into the refrigerator while the eggs are being prepared. "Dry" sperm can be pipetted into microcentrifuge tubes and stored at 4°C for several days.

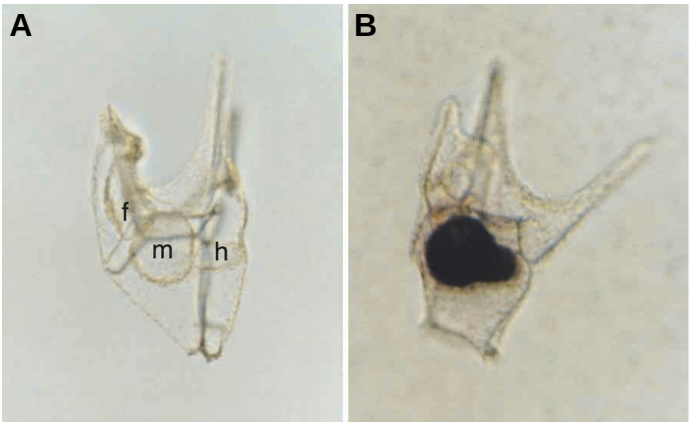

Fig. 1. Alkaline Phosphatase (AP) staining in pluteus larvae of *Lytechinus pictus*. Lytechinus pictus *plutei were fixed or fixed and stained as described. The larva in* **(A)** *has not been stained; the larva in* **(B)** *shows the purple AP stain localized to the gut. Abbreviations: f, foregut; m, midgut; h, hindgut.*

After the female has finished spawning and most of her eggs have settled to the bottom of the collection beaker, dejelly the eggs by pouring the egg suspension slowly and steadily through a 100-µm Nitex screen into another beaker. When the dejellied eggs have mostly settled to the bottom of the beaker, decant the ASW and refill the beaker with fresh ASW. Repeat several times.

Fertilization

Pipette a small volume of eggs into a full beaker of ASW so that no more than a single layer of eggs settles to the bottom. (If you are lucky enough to possess paddle mixers that keep the embryos suspended, you can culture the embryos at higher concentration.) Activate sperm by diluting a single drop from a Pasteur pipette into 50 ml ASW. Pipette or stir to disperse the sperm. The solution should be slightly turbid. Add 2-5 drops of diluted sperm per 100 ml of diluted eggs. Mix the sperm and eggs well by stirring. Check a sample of eggs for fertilization envelopes after 2 min; add a few more drops of sperm if fertilization is not 100%. After the fertilized eggs have settled to the bottom, decant as much ASW off the eggs as you can. Refill the beaker with fresh ASW, let eggs settle to the bottom and decant ASW again. Repeat. Embryos to be reared in sulfate-free sea water (SFSW) should then be transferred to a beaker of SFSW and rinsed in SFSW several times (at least 5). To rinse, the eggs should be allowed to settle to the bottom of the beaker of SFSW; the SFSW decanted and fresh SFSW added to the beaker. Culture embryos to the desired stage.

AP Detection in Whole-Mount Sea Urchin Embryos

Fill 15-ml tubes with suspended embryos from each developmental stage and/or experimental treatment being used. Concentrate the embryos by pulse spinning in a centrifuge and pouring off as much SW as possible. Be careful. Hard spins can destroy the embryos; as a rule of thumb, we never spin embryos at more than 1000 rpm or for longer than 30 s in a tabletop centrifuge.

Fill each tube with ice-cold methanol; make sure the embryos are well suspended. Set the tubes on their sides in an ice bucket, resuspending the embryos every minute or so. At exactly 15 min after adding methanol, stand the tubes upright in the ice and let the fixed embryos settle to the bottom of the tube for up to 5 min. Aspirate or decant off the methanol and resuspend embryos in ice-cold ASW. Let the embryos settle to the bottom of the tube by gravity. Once the embryos have settled aspirate or decant off the ASW.

Let the embryos settle by gravity in ice-cold ASW. Decant ASW and resuspend in ice-cold Developing Buffer. Let the embryos settle by gravity in Developing Buffer, then aspirate off as much Developing Buffer as possible. Add AP Substrate to each tube to just cover the embryos. Put the embryo suspension from each tube into the wells in a depression slide and observe samples in a compound scope. The presence of AP is indicated by a purplish blue color. To stop the staining reaction, pipette off substrate solution and refill the depression well with phosphate-buffered saline (PBS). Photograph your results immediately; the morphology of stained sea urchins will deteriorate over time, so fixed and stained embryos should not be stored.

Supplies Needed

- Adult sea urchins for spawning: There are many suppliers of adult sea urchins on both the east and west coasts of the United

States. The species used should be picked based on breeding season and egg pigmentation. Species such as *Arbacia punctulata*, with darkly pigmented eggs, should be avoided.
- 1 ml tuberculin syringes with 26 gauge needles
- beakers (50, 100, 250 and 500 ml) for collecting eggs and sperm and culturing embryos
- 100 μm mesh size nylon screen such as Nitex
- low-temperature incubator or cooling water bath for embryo culture: The temperature at which sea urchin embryos are cultured depends on the species. For example, *Lytechinus variegatus* can be cultured at room temperature, but *Lytechinus pictus* and *Strongylocentrotus pupuratus* embryos need to be cultured at cooler temperatures, approximately 15°C. If incubators and/or cooling water baths are not available, a cool-water bath set up in an insulated container will maintain a constant enough temperature for embryo rearing.
- 50 ml and/or 15 ml polypropylene test tubes; 1.5 ml microcentrifuge tubes
- tabletop centrifuge
- microscopes
- depression slides

Solutions Needed

- The formulae for making Artificial Sea Water (ASW) and Sulfate-Free Sea Water (SFSW) are given in Table 1.
- 0.5 M KCl
- PBS
- ice-cold methanol
- BCIP (5-bromo—4-chloro -3'-indolylphosphate *p*-toluidine salt) - 50 mg/ml in dimethylformamide. This stock solution of BCIP should be stored at –20°C.
- NBT (Nitro-blue tetrazolium chloride) - 50 mg/ml in dimethylformamide. NBT stock should also be stored at –20°C.
- Developing Buffer: 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$
- AP Substrate: Add 6 μl/ml NBT stock and 3.5 μl/ml BCIP stock to 1 ml Developing Buffer. Make immediately before use; keep cold, and protect from light.

A Sample Set of Teaching Laboratories Exploiting the AP Staining Technique

While redesigning my Developmental Biology course to incorporate more experimentation into the laboratory, I found that it is essential to "introduce" students to the embryos that they will be working with before they are asked to design any experiments. Therefore, most of my teaching laboratory exercises run for at least 2 weeks; the first week students learn how to handle their experimental organisms and become familiar with staging embryos, the second (or later) week students design and perform an experiment on those same embryos.

TABLE 1

ARTIFICIAL SEA WATER (ASW) AND SULFATE-FREE SEA WATER (SFSW) FOR CULTURING SEA URCHIN EMBRYOS

salt	ASW (g/l)	SFSW (g/l)
NaCl	24.72	27.72
KCl	0.67	0.67
$CaCl_2 \cdot 2H_2O$	1.36	1.36
$MgCl_2$	-	9.32
$MgCl_2 \cdot 6H_2O$	4.66	-
$MgSO_4 \cdot 7H_2O$	6.29	-
$NaHCO_3$(*)	0.18	0.18

(*)$NaHCO_3$ is added after all other salts have dissolved. Adjust final pH to 8.1-8.3 with NaOH.

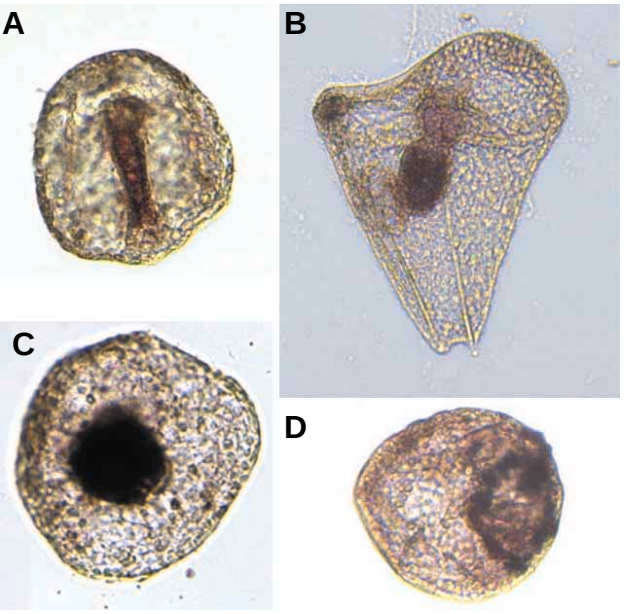

Fig. 2. Alkaline Phosphatase (AP) positive cells in embryos reared in Sulfate-Free Sea Water (SFSW) compared with Artificial Sea Water (ASW)-raised controls. Strongylocentrotus purpuratus *embryos were reared in either ASW (A, B) or SFSW (C, D) and stained for AP.* **(A)** *shows gut staining at the late gastrula/early prism stage;* **(B)** *shows staining in the early pluteus larva.* **(C,D)** *show AP staining in embryos cultured in SFSW until sibling controls reached the early pluteus stage. Embryos reared in SFSW show no signs of gastrulation. AP-positive cells differentiate on the surface; the "spot"* **(C)** *and "ring"* **(D)** *distribution of AP-positive cells at the vegetal pole are typical staining patterns. The significance of these different patterns is not understood. Note that unstained tissues in* Strongylocentrotus purpuratus *embryos are intrinsically more pigmented than the* Lytechinus pictus *embryos shown in Fig. 1.*

Therefore, in the first sea urchin lab, students practice handling and spawning adult sea urchins, stage embryos, stain embryos for AP, and, by so doing, learn when AP can first be detected (late gastrula; early prism), and where AP is made (gut epithelium) in normal embryos. Figure 1 shows AP staining in pluteus larvae of *Lytechinus pictus*. Note that staining works especially well in *Lytechinus* embryos because their eggs have very little pigment. Species like *Arbacia punctulata* would probably not work well with this assay because of the dark pigmentation of *Arbacia* eggs.

In the next week (or two) of the sea urchin labs, I have students pick one way of disrupting morphogenesis, formulate a hypothesis about whether or not they think gut AP will be expressed in such embryos, and design an experiment to test their hypothesis. There are many simple ways to intervene in development of sea urchin embryos by simply adding or removing something from the sea water culture medium. To name a few: Addition of LiCl vegetalizes the embryo and causes exogastrulation (Hörstadius, 1973; Nocente-McGrath *et al.*, 1991); zinc animalizes the embryo (Czihak, 1975);

16-cell embryos can be dissociated in Ca++-free sea water and micromere, mesomere and/or macromere aggregates can be cultured and analyzed (Hynes and Gross, 1970); and, as shown here, gut formation can be prevented by rearing embryos in SFSW (Anstrom *et al.*, 1987; Lane *et al.*, 1993).

The SFSW culture technique allows students to ask whether AP-positive gut cells can differentiate when normal gut morphogenesis fails. Gastrulation in sea urchin embryos consists of three morphogenetic events: (1) ingression of primary mesenchyme cells (PMCs) into the blastocoel; (2) invagination of the vegetal plate epithelium; and (3) elongation of the invaginated tissue to form the archenteron (gut). Invagination begins when cells of the vegetal plate secrete chondroitin-sulfate proteoglycan (CSPG) into the inner lamina of the hyaline layer. Absorption of water by CSPG causes the vegetal epithelium to buckle into the blastocoel (Anstrom *et al.*, 1987; Lane *et al.*, 1993). When sea urchin embryos are cultured in SFSW, CSPG cannot be made, thus primary invagination and subsequent formation of the archenteron cannot occur (Anstrom *et al.*, 1987; Lane *et al*., 1993). I like the SFSW treatment because students will typically hypothesize that, since no gut tube forms, no AP expression will be seen in treated embryos. As shown in Fig. 2, such embryos DO contain AP-expressing cells at the vegetal pole. Thus, the students are forced to ask themselves why differentiation of gut cells can occur in the absence of a gut, and grapple with the idea of specification of cells in the absence of proper morphogenetic movements.

Although I've been using sea urchin AP staining to demonstrate tissue-specific gene expression since 1995, I've only added the second experimental laboratory the last three times I've taught Developmental Biology. Therefore, my students have not yet tried all the treatments described above. So far, two lab groups have chosen SFSW treatment and one has chosen lithium. Of those two, the SFSW treatment gave more interpretable results, i.e., more of the treated embryos lived. I would suggest to anyone thinking of incorporating these exercises into a laboratory course to either be available for lots of hands-on trouble-shooting when initiating a new experiment, or to try a new treatment before the students do. Finally, be creative. The list of treatments I've suggested is not exhaustive. The sea urchin AP assay can be used to detect the teratological properties of anything one can dissolve in sea water.

Summary

The techniques presented here for detecting gut AP in developing sea urchin embryos provide the Developmental Biology instructor with a powerful tool for investigating tissue-specific gene expression during normal and disrupted embryonic development. The exercises are technically simple and, therefore, work well in the hands of a novice; the fixation and staining procedures can be done during one laboratory period; and the staining should work for most species of sea urchin. The first laboratory in this unit provides the students with both the facts and techniques required to initiate subsequent experimental investigations. The second laboratory is designed to provide the instructor with the opportunity to engage the students in experimental design and scientific inquiry. Students have a variety of options for choosing how to disrupt development, and, therefore, a variety of ways to approach questions regarding gut cell differentiation.

Acknowledgements

I would like to thank the Rider University Department of Biology and the Rider Science Education and Literacy Center (SELECT) for supporting my efforts to improve science teaching. I would also like to thank Rebecca Lumpkins, Anthony Pennello and Brooke Smith—three of the participants in the Spring 2002 Developmental Biology course—for providing the photographs in Fig. 2.

References

ANSTROM, J.A., CHIN, J.E., LEAF, D.S., PARKS, A.L. and Raff, R.A. (1987). Localization and expression of msp130, a primary mesenchyme lineage-specific cell surface protein of the sea urchin embryo. *Development* 101: 255-265.

CZIHAK, G. (1975). The sea urchin embryos. In *Biochemistry and Morphogenesis*. Springer, New York, pp.473-508.

HÖRSTADIUS, S. (1973). *Experimental Embryology of the Echinoderm*. Clarendon, Oxford, UK.

HYNES R.O. and GROSS P.R. (1970). A method for separating cells from early sea urchin embryos. *Dev. Biol.* 21: 383-402.

LANE, M.C., KOEHL, M.A.R., WILT, F. and KELLER, R. (1993). A role for the regulated secretion of apical extracellular matrix during epithelial invagination in the sea urchin. *Development* 117, 1049-1060.

NOCENTE-MCGRATH, C., MCISAAC, C. and ERNST, S. (1991). Altered cell-fate in LiCl-treated sea urchin embryos. *Dev. Biol.* 147: 445-450.