CHAPTER 33

Sea Urchin Gametes in the Teaching Laboratory: Good Experiments and Good Experiences

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Overview

Sea urchin gametes are exceptional for demonstrating cell and developmental phenomena and have long been classic material in college classrooms and, more recently, in high school laboratories. Here, we present salient information on using them in the teaching laboratory along with representative exercises which demonstrate many of the phenomena of early development. A major focus is using the microscope as a tool, allowing the student to obtain meaningful results without having to learn complex and time-consuming new techniques for each lab session. Emphasized are labs which can be completed in one to two days. Also emphasized is the ability to use this material for inquiry-based teaching, in which the student can ask questions and quickly obtain answers to these questions. The outcome is a highly satisfying experience for both student and instructor.

I. Introduction

Sea urchins have provided classical material for laboratory experimentation in the classroom, in part, because of their rich heritage and history and, in part, because of the ease with which urchin embryos can be fertilized and grown in the lab. Another advantage, which will be stressed in this chapter, is the use of this material for inquiry-based science. This approach allows the student to ask specific questions regarding embryonic development and obtain answers in the classroom setting. Inquiry-based teaching allows the student to experience the

scientific process and, hopefully, to use this knowledge to better appreciate science as encountered in the real world. In ideal cases, it can open up to the student the possibilities of science as a lifetime career.

Sea urchin material provides such inquiry-based science education because the answers to such inquiries can often be obtained simply and easily in the course of single laboratory exercises. Many experiments have endpoints that can be scored microscopically, such as successful/unsuccessful fertilization or as effects of various experimental procedures on cell division or normal development. The relative technical simplicity of working with embryos means that a student does not need to spend most of the laboratory time learning a new technique; rather, he or she can simply observe early development with the microscope, ask questions about what is observed, and quickly obtain answers. Based on these answers, students can then ask another question, obtain an answer, and ask a different question. This satisfying iterative process has made sea urchin labs a favorite in many universities and even high schools, where initial experiments can lead to student-directed research projects. Indeed, the best-liked introductory biology lab at Stanford is the sea urchin lab.

Good sources of information on using sea urchins for instruction can be found on several Internet sites. The most comprehensive is "Sea Urchin Embryology" developed and maintained by Chris Patton of the Epel lab at http://www.stanford.edu/group/Urchin/index.html. Almost 300 pages long, it provides information, diagrams, videos, animations, and overheads on how to obtain sea urchins for classroom use, suggested lesson plans, suggestions for advanced experiments, and numerous videos and animations useful in the classroom setting. Spanish versions of the major parts of the site are also provided. The site maintained by Eric Davidson's lab at CalTech (http://sugp.caltech.edu:7000/) also provides current research information on the sea urchin genome, which is expected to be complete in 2003/2004.

II. Obtaining Adult Urchins and Gametes for the Classroom

A. Maintaining Adult Urchins

A major problem in using sea urchins is obtaining and maintaining adult sea urchins. Also of concern are issues of availability of material on a year-round basis. These issues are covered in Chapter 1 of this volume. Short-term maintenance of the urchins can be easily achieved in saltwater aquaria (see chapter 1 and Web site). A single order of urchins often can be used to support labs over the course of several weeks. Long-term maintenance is more problematic unless one has extensive experience with these aquaria. Teachers should also be sensitive to ethical and environmental concerns surrounding the treatment of this biological material.

B. Storing Eggs Useful for Classroom Experiments; an Alternative to Maintaining Aquaria

A simple protocol we (D. E., C. P.) have developed which eliminates problems in maintaining urchins in saltwater aquaria for long periods of time is to shed and store the gametes for later classroom use upon delivery of the adult urchins. The principle is based on our finding that ''death'' of sea urchin eggs after spawning results from bacterial action once the eggs are shed. To avoid this problem, the eggs are stored in simple antibiotic solutions that maintain the gametes in a fertilizable condition for at least one week, allowing use for many different classroom exercises. Simple storage of sperm at low temperatures will similarly preserve these gametes for 4 to 5 days.

Urchins are spawned as described in Chapter 1 and in the following text. The sperm are not spawned into seawater but are collected as a ''dry'' paste and stored in closed small tubes (conical ''Eppendorf-type'' are ideal) at 0 °C on ice in the refrigerator. They are good for 4 to 7 days under these storage conditions.

Eggs are spawned into Millipore-filtered seawater (SW) or Millipore-filtered artificial SW (do not use autoclaved seawater). The eggs settle quickly in the seawater and the supernatant is removed and the eggs resuspended in fresh filtered seawater. This settling of eggs, removing supernatant seawater, and resuspension is repeated three times in this sterile seawater. It is imperative to remove as much of the supernatant seawater as possible, since this contains the original contaminating bacteria. In a laboratory setting, this can be accomplished most easily with a water aspirator; in a classroom setting, a turkey baster is handy for removal of the supernatants.

After the third wash, the eggs are then resuspended in seawater containing a mixture of two antibiotics, sulfamethoxazole (final concentration at 200 ug/ml and trimethoprim (final concentration of 10 ug/ml). These concentrations are almost at the limits of solubility in seawater and getting them into solution is time-consuming. Continuous stirring and periodic adjustment of the pH are required until the antibiotics are dissolved (~2 h). At the end of the dissolution procedure, the antibiotic solution is passed through a Nalgene bacterial filter. Using this mixture of sulfamethoxazole and trimethoprim will permit storage for at least a week with *Strongylocentrotus purpuratus* eggs and up to three weeks with *Lytechinus pictus* eggs. If difficult to obtain both antibiotics, sulfamethoxazole alone will work although the eggs will not last as long. Although other antibiotics are not as effective, ampicillin will suffice for several days. More detailed protocols are described at http://www.stanford.edu/group/Urchin/protocol.html and in a paper in preparation (Epel *et al.*, in preparation).

Optimal storage is obtained under dilute conditions, 0.1 to 0.2% v/v%, as a monolayer in Falcon-type tissue culture flasks. Alternatives are baking dishes or other glass or plastic containers that allow the eggs to be stored in a quasi-monolayer condition. Storage temperature for *L. pictus* and *S. purpuratus* is 10 to 15 °C; lower temperatures lead to lysis of the eggs. We have not tried these procedures on eggs from other species but assume that similar principles will

apply, albeit the storage temperatures should be adjusted to the ambient temperatures of the adult sea urchins.

III. Some Guidelines for Using Sea Urchin Gametes in the Classroom

Before describing specific experiments, we present several tips on handling gametes that will make the exercises more successful. These are based on our years of experience in the classroom and will allow you to avoid the most common pitfalls that students will experience.

A. Storage of Diluted Sperm during Classroom Exercises

You will need to use dilute sperm suspensions to fertilize eggs, but a problem in classroom use is that the diluted sperm quickly lose their ability to fertilize eggs when they are diluted in seawater. We have found that *S. purpuratus* sperm at a 1% concentration in seawater stored on ice will retain their fertilizability for at least 12 h. Similar procedures might work with sperm from other urchin species.

B. Temperature

Eggs of *S. purpuratus* develop in nature at temperatures of 12 to 15°C, so development of this species should be followed at 12 to 17°C. If this is not possible, one can fertilize them at room temperature; fertilization and cleavage stages will proceed, although later development will probably not be successful. Development is too slow in a refrigerator at 4°C, unless you can adjust the refrigerator to a higher temperature. An option used in our local high school is to keep the culture vessels in a Styrofoam chest in which the water is adjusted with ice to 15°C or to float the embryos in bottles in a cooled aquarium. Worst case: in the absence of below-ambient temperature equipment, try to keep the cultures in the coolest part of the room. Other species such as *Arbacia punctulata, Lytechinus pictus*, and *L. variegatus* develop well at room temperature, and have the added advantage of rapid development.

C. Need to Wash Eggs

During prolonged storage, substances are released from the eggs or the jelly layer of the eggs that can interfere with fertilization. If eggs have been shed more than an hour earlier, it is a good idea to wash the eggs before adding sperm. Since eggs are heavier than seawater, they will settle by gravity in the beaker and the supernatant can be removed and fresh seawater added.

Sometimes, settling is slowed by the presence of the jelly layer around the eggs; to speed up the washing, one can use low-speed centrifugation. The eggs are

delicate, however, so be gentle when adding fresh seawater to the eggs or when resuspending the eggs in seawater.

D. Inadvertent Fertilization

It is very easy for students to accidentally fertilize the entire batch of eggs that is to be used by the class through contaminating the eggs with sperm from the sperm pipet. To avoid this, let the students know of this problem and, most importantly, label the pipettes being used for sperm or eggs.

E. Polyspermy

Polyspermy will result in abnormal development and is to be avoided (unless you wish to have a lab around this···which can be very interesting). To avoid polyspermy, emphasize to the students the need to fertilize with a very dilute sperm suspension. Details about sperm dilution will be given.

F. Overcrowding

Overcrowding can affect development. Proper development requires good oxygenation, which can be achieved by gentle stirring, as with a rocker-type stirrer. Since these may not be available in a classroom setting, keeping the eggs as a monolayer on the bottom of a beaker or other type of glass vessel will work just fine.

G. Egg Transparency

Transparency varies with the different species. Ideal, transparent eggs, which allow easy visualization of the nucleus and mitotic apparatus, are those of the genus *Lytechinus*. The eggs of *Arbacia* and *Strongylocentrotus* are relatively opaque so such cytological detail will be more challenging to see in the classroom. However, *S. purpuratus* has many advantages for classroom use, such as a prolonged reproductive season and robustness in the laboratory. Later developmental stages are easily seen with the microscope in all species of sea urchins.

IV. Basic Introductory Labs

Here we present a series of laboratory exercises that we have used in our classrooms at Stanford, University of California at San Diego, Susquehanna University, high schools on the Monterey Peninsula, and also in various international courses in which we have been involved over the years. These exercises provide specific examples that can be used as described or as ideas or scaffolds for use in specific situations. They start out with simple microscopic observations with

light or phase microscopes that are essential introductory labs. We follow these with suggestions for a variety of experiments that could be used in the aforementioned inquiry-based science approach, some of which will require more specialized equipment as well as time beyond a normal laboratory period.

Most of the lab directions and experiments presented are the basis for handouts that we have used in our various classes. Instructors should feel free to use the text for their classes, as presented or modified for their particular settings. There are also excellent student exercises, handouts, and transparency texts available on the sea urchin embryology website (http://www.stanford.edu/group/Urchin/protocol.html).

A. Basic Protocol for *In Vitro* Fertilization and Observation of Early Development

This laboratory is a good introduction to events of fertilization and early embryonic development and works well with all sea urchin species we have used. The level of instruction written here is for introductory college labs and advanced high school instruction. It can also be adapted for upper division college labs.

1. Spawning Gametes

This part of the exercise is great if you have lots of sea urchins; it allows the student to see the adult urchin and understand how spawning takes place. The teacher should be sensitive to the ethical concerns of sacrificing the animals. This concern can be avoided if a seawater aquarium is available, since the animals can be placed back into the aquaria after spawning and will generally survive (but requires that the animals be kept moist while spawning and be placed back into the aquarium as soon as possible). Alternatively, the instructor can provide gametes to the students, either by inducing spawning just before the lab or using gametes obtained through the long-term egg and sperm storage procedure described earlier.

The sexes are separate and the gametes are spawned into seawater with fertilization and embryonic development taking place external to the adult body. Gravid animals spawn when injected with 0.5 M KCl [the K^+ ions depolarize a muscle which surrounds the outer surface of the ovary or testis and the eggs or sperm are forcibly extruded into the seawater through five gonopores on the aboral surface (the opposite surface from the mouth)]. To begin, the needle on the syringe of KCl is carefully inserted into the soft tissue around the mouth (Aristotle's lantern). When the needle comes to rest on the inner surface of the test, the syringe is depressed to release approximately 1.0 ml of the solution. This is repeated in two different areas, followed by gentle shaking of the animals. After injecting an animal, the hypodermic needle should be rinsed in tap water to kill any sperm that might have stuck to the needle so as to avoid unwanted fertilization of eggs (e.g., the next animal injected might be a female).

Within 1 to 5 min of injection, the gametes appear on the topside. The only way to distinguish males from females in most species is to see what comes out—eggs

are orange, red, or yellow, depending on species, and sperm is creamy white and thick. Students may have trouble distinguishing the two initially until they see both.

Females are placed upside down on a beaker of seawater and the eggs settle to the bottom. It may take up to 10 min to spawn completely. Once the eggs are spawned, they should be "washed" through several changes of seawater, allowing them to settle by gravity to the bottom of the beaker after each addition of seawater. Eggs should be fertilized within a few hours of spawning, unless prepared for long-term storage as has been described.

Sperm are stored "dry," so males are placed upside down on an empty beaker. After 10 min, the sperm should be removed from the urchin surface with a Pasteur pipet and transferred to a small Eppendorf-type tube for storage in the refrigerator or on ice. Stored on ice, the sperm cells remain capable of fertilizing eggs for 4 to 5 days.

2. Jelly Removal

Sometimes, one would like to remove the jelly coat to facilitate handling the eggs (the jelly coat causes the eggs to be buoyant and impedes settling). This can be done by straining through Nitex mesh or cheesecloth; if using Nitex, use a mesh size that is about the diameter of the eggs (75 micron for *S. purpuratus* and 120 micron for *Lytechinus*). To do this, a suspension of eggs is poured through several layers of cheesecloth or one layer of Nitex into a new beaker. Use additional seawater to rinse the cheesecloth to remove any remaining eggs. Then wash the eggs several times to get rid of any jelly and broken eggs.

B. Observation of the Gametes

1. Eggs

The eggs are between 80 and 120 μm in diameter, depending on the species and 2 to 4 million eggs are present in one ml of packed eggs. Sea urchin eggs (and eggs of all species of the Echinoderm class "*Echinodea*") are unusual in that, unlike all other animal eggs (except for coelenterates), the mature eggs (ova) are post-meiotic and are arrested in the interphase of the first mitotic division (G1). Each egg contains a single haploid pronucleus (female pronucleus) with an intact nuclear envelope. The eggs are metabolically dormant and synthesize protein at very low rates. When they are fertilized, their metabolism is markedly activated, with large increases in protein synthesis (particularly the synthesis of cyclins necessary for cell division), appearance of new transport systems, alterations in the cytoskeleton, and initiation of DNA synthesis (Epel, 1997). These explosive changes in sea urchin eggs at fertilization have been well studied as a model for similar changes after fertilization in all animal eggs. Additionally, fertilized sea urchin eggs provide natural synchronously dividing eukaryotic cells for the first

two divisions and present an exceptional model for understanding cell division in all eukaryotic cells (Sluder *et al.*, 1999).

To observe, drops of egg suspension are placed on a slide and viewed using the low power (10×) objective. The eggs may be dark in color due to yellow, purple, or red pigment granules (species-dependent). Students should focus up and down to see the round egg pronucleus (about 1/20th of an egg's diameter), which is most apparent in transparent eggs, such as those of *Lytechinus*. Students should note the egg pronucleus in an otherwise homogeneous, but granular, cytoplasm and observe that the nucleus is not in the center of the egg but is eccentrically placed on one side (as some eggs will be viewed from the ends or poles, this eccentricity will not be observed in all eggs; the instructor can use this as a means of demonstrating how the microscope image varies, depending on orientation of the cells). Eggs can also be viewed under higher (40×) power. Before going to higher power, students should add a "footed" coverslip by placing several small pieces of broken coverslip around the drop of eggs, then placing an intact coverslip on this support. This prevents the coverslip from crushing the eggs while protecting the objective lens from corrosive saltwater.

Occasionally, immature oocytes are present in the egg solution. These are easily identified because they contain a large germinal vesicle (pre-meiotic oocyte nucleus) inside of which is a round, extremely dark nucleolus. (If a large percentage of the ooctyes are immature, a new female should be spawned.)

The eggs are surrounded by transparent jelly layers, about one-half an egg diameter in thickness. This jelly layer cannot be seen because of its transparency but can be inferred since the cytoplasmic surfaces of the eggs will not touch each other and the eggs will be evenly spaced on the slide. The jelly coat can be directly visualized by adding a few drops of calligraphy ink (such as Sumi ink, which can be purchased at art stores) suspended in seawater to the slide. The ink particles cannot penetrate the viscous jelly coat, which then appears as an inkfree clear zone around each egg (see Fig. 1). The sperm must swim through this jelly coat, and there are a plethora of substances that affect sperm motility, cause the acrosome reaction, and induce chemotaxis (Mengerink and Vacquier, 2001). A procedure to isolate the egg jelly to study these sperm-altering substances will be described, after the fertilization protocol.

2. Sperm

One ml of undiluted semen contains 4×10^{10} sperm cells and the haploid amount of DNA in a single sperm cell is 0.85 picograms (*S. purpuratus*). The sperm cell (spermatozoan) is composed of a tail (flagellum) about 50 μm in length and a head 3 μm in length (approximate dimensions vary with species). Between the sperm flagellum and the head, and connected to the sperm head, is a midpiece made of a single mitochondrion.

A phase contrast microscope with a 100× oil immersion objective is needed to see details of the sperm morphology. The stored semen should be diluted 1:1000 in

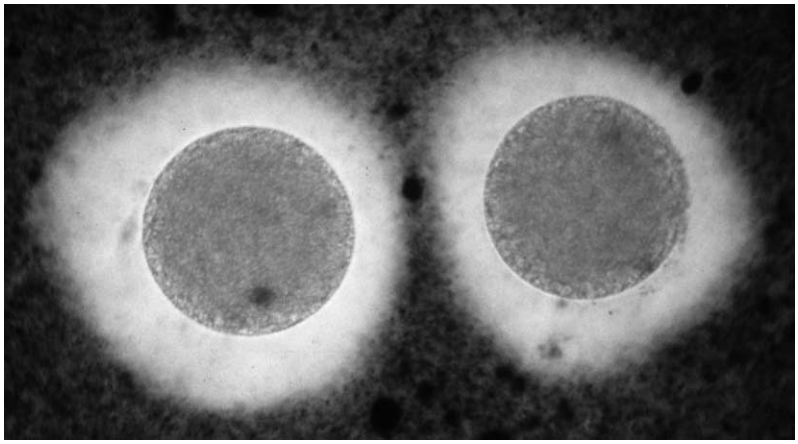

Fig. 1 A jelly layer surrounds the unfertilized sea urchin egg. The jelly layer is normally not visible because it is transparent. Here it is made visible by suspending the eggs in a thin suspension of Sumi ink (Japanese calligraphy ink). The jelly layer excludes the particles of ink and the layer then stands out as a clear structure against the black background of the ink suspension.

seawater. Then, a drop of the sperm suspension is placed on a slide and covered by a coverslip. Excess seawater from the slide can be drained by turning the slide on edge on a tissue, then flipping the slide over, coverslip down, on filter paper or Kimwipes. A slight pressure is applied by thumb for 1 s. The slide can then be turned over and viewed under high power with oil. Many of the sperm will have undergone a spontaneous acrosome reaction and are now stuck to the glass by their acrosomal process (this acrosome reaction is necessary for fertilization). Structures that should be discerniable are the flagellum (50×0.2 μm) and the head (3×1 μm). The head contains a single, giant, donut-shaped mitochondrion which comprises the sperm midpiece. The conical nucleus makes up most of the volume of the sperm head. At the most anterior region of the nucleus is the acrosome granule, with a clear space, the nuclear fossa, separating the granule from the nucleus. This will be visible only in non-acrosome reacted sperm.

If a phase contrast microscope is not available, sperm can be observed under bright field. At 40×, details of sperm structure are not readily discernible, but with a fresh sperm dilution, the beating of the flagella is easily seen.

C. Fertilization

To fertilize eggs in small batches, one medicine dropper/pipette full of eggs from the beaker into which the female was spawned is added to a clean beaker with approximately 30 ml of seawater. The eggs will have settled so the students must

be careful to pipette from the bottom of the beaker. The more eggs that are transferred, the more easily the fertilized eggs for observation will be found.

A fresh sperm dilution (1:1000) is made as has been described. Generally, 1 ml of this diluted sperm added to the 30 mls of eggs will be sufficient for fertilization without inducing polyspermy. The sperm are added and the beaker is swirled to mix the gametes. After 2 min, during which time the cortical granule reaction occurs and the fertilization envelope is elevated and hardened, a drop of eggs from the bottom of the beaker is placed on a slide and observed with the 10× objective. Students should count the number of fertilized and unfertilized eggs on the slide to determine a percentage of fertilization. It is readily possible to count 100 eggs or more, provided sufficient eggs were transferred to the beaker initially, allowing students to obtain quantitative data. In most cases, a percentage of 90% or better represents successful fertilization. If the percentage is less than 90%, a fresh sperm dilution can be made and the eggs can be refertilized, as has been described. Eggs fertilized in the first batch will be protected from polyspermy because of the presence of the fertilization envelope.

Once students are confident that they can distinguish fertilized eggs from unfertilized eggs, they can attempt to observe the elevation of the fertilization envelope directly. In order to do this, a drop of unfertilized eggs is placed on a slide and the student focuses on a small cluster of eggs at 10× magnification with no coverslip (emphasize not to use higher power without a coverslip!). A drop of diluted sperm is added and students watch for several minutes to directly observe fertilization (an increased dilution factor should be used here; if there are too many sperm, it will be difficult to see the elevation). Students can time the elevation in several eggs by repeating the procedure with fresh eggs and sperm.

It should be possible to see sperm attachment to the egg surface. In 30 to 60 s (depending on the species), the elevation of the vitelline layer (VL) begins, followed by its transformation into the fertilization envelope (FE). The elevation of the VL initiates at the site of sperm–egg fusion, looks like a blister as it begins, and sweeps around the egg as a circular wave (Fig. 2). The flagellum of the fertilizing sperm may be visible, appearing straight and motionless, sticking out of the blister.

The VL to FE transformation is a result of the cortical reaction which involves the exocytosis of thousands of membrane-enclosed cortical granules which are attached to the inside surface of the egg plasma membrane (see Fig. 2). After the cortical reaction completes, a clear gelatinous layer, the hyaline layer, begins to become apparent on the surface of the egg. The hyalin protein that makes up the hyaline layer also originates from the cortical granules and forms a calcium-dependent gel that is necessary for adhesion of the blastomeres during development. The layer can be easily seen in *S. purpuratus* embryos in 5 to 10 min after fertilization. It forms more slowly in *L. pictus* embryos and should be apparent by 20 min after fertilization (Matese *et al.*, 1997).

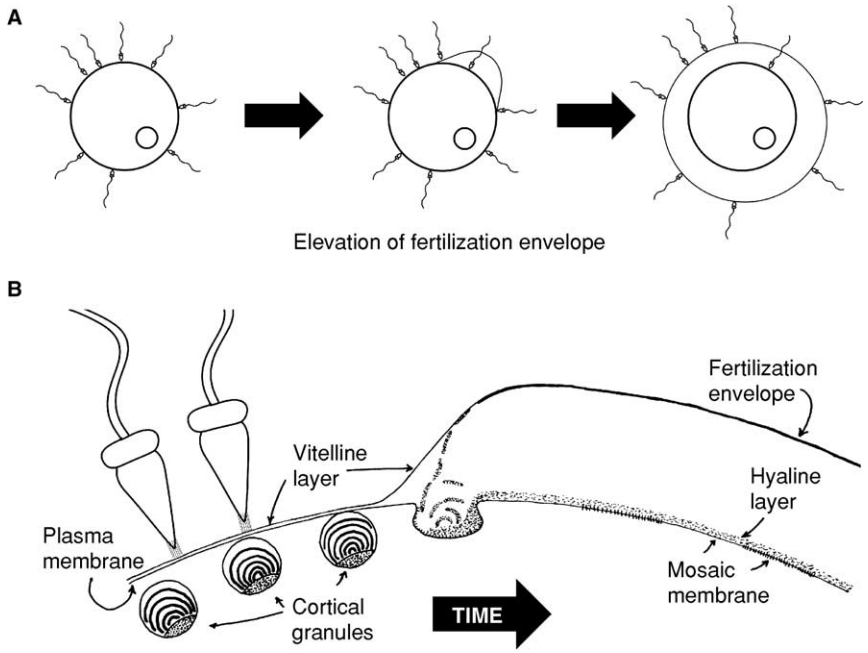

Fig. 2 A cartoon illustrating the elevation of the fertilization envelope. Part A depicts the elevation of the envelope as seen in an intact egg. As seen, the elevation begins as a blister on the surface, at the site of sperm-egg fusion. This then travels or propagates around the egg over the next 30 s, resulting in the elevation of the envelope. Part B depicts the mechanism of elevation, which involves the fusion or exocytosis of cortical granules with the plasma membrane of the egg. This fusion releases the granule content, which provides the structural materials for the membrane and its hardening over the next few minutes.

D. Observing Post-Fertilization Events

The transparent qualities of sea urchin eggs allow easy observation of the movement of the pronuclei, cell division, and later stages of development. To do this, a larger batch of eggs should be fertilized and resuspended in a dish with a large surface area-to-volume ratio so that the eggs will receive sufficient oxygenation. For class purposes, this could be in baking dishes, custard dishes, or other dishes. Embryos should be added to the dishes until a sparse monolayer forms. If available, one can also increase oxygenation by agitating the eggs in the dishes on a rocking platform.

1. Visualizing Nuclear and Mitotic Events—Light Microscope

Pronuclear fusion and mitosis are most easily seen with transparent eggs, such as those of *Lytechinus*. With these eggs, you will be able to observe with low-power microscopic magnification that the originally eccentric pronucleus moves to the

center of the egg at about 20 to 30 min after insemination and that the nuclear envelope disappears as the egg enters late prophase. One can see these stages with much more detail using higher magnification (40×). Before going to higher power, a "footed" coverslip should be added, as described previously.

In transparent eggs, one can usually see directly the nuclear changes and formation of the sperm pronucleus and formation of mitotic apparatus. In the more opaque eggs, one will have to compress the eggs slightly. This can be done by withdrawing water from under the coverslip. With the footed coverslip, this is easily done by wicking off excess seawater by placing a paper towel or piece of filter paper next to one edge of the coverslip. The broken piece of coverslip will prevent the eggs from being crushed and lysed. With this compression, one should be able to see the nuclear movements and the fibrous mitotic apparatus. Timing-wise, the mitotic events will begin at approximately 50 to 90 min after insemination, depending on the temperature and species. Cytokinesis will begin about 90 to 120 min after insemination, and the successive divisions will then occur about every 30 to 60 min (again, times will vary with species and temperature).

2. Visualizing Nuclear Events—Fluorescence Microscope

If a fluorescent microscope is available, the dramatic events taking place in the nucleus during sperm–egg fusion and mitosis can easily be visualized.

Eggs are incubated in seawater containing 1 uM Hoechst 33342 for 10 min (Hinkley *et al.*, 1986). The eggs are then washed 2 to 3 times in seawater. This stain labels the eggs, so that if the eggs are used for subsequent fertilization, one should be able to visualize the fusing sperm, the movements of sperm and egg through the cytoplasm, the fusion of the two nuclei in the egg center, and the formation of the chromosomes during metaphase and their separation during anaphase of the first mitotic division. This will require high-power magnification (40×) and some compression of the eggs, so the footed coverslip technique will be necessary.

E. Later Developmental Stages

Depending on time, students should be able to observe the initial cleavage stages of development during the lab period. They may then be asked to return periodically to the lab during the next few days to observe later important developmental stages of embryonic development. Alternatively, batches of embryos fertilized by the instructor prior to the lab period can be made available for observation. Once the embryos hatch and begin to swim, it becomes more difficult for students to find and follow them under the microscope. Drops of embryos can be placed on slides that have been coated with protamine sulfate or poly(L)lysine (Mazia *et al.*, 1975), which provides a charged surface on the slide to which the embryos will adhere and become immobilized for easier visualization.

V. Inquiry-Based Experiments Using the Basic Fertilization Protocol

Fertilization and cleavage stage cell division are convenient systems for inquiry-based education since they occur in the timeframes of the typical classroom laboratory. Next, we describe some possible experiments that students can do which can lead to questions about the molecules and mechanisms involved in fertilization and early development. Many of these depend primarily on microscopic observation and the students should realize that to gain the imagination needed to be a good experimental cell biologist, a scientist must observe the behavior of living cells with his or her own eyes. Students will often look and then stop observing. It is important for them to realize that one cannot spend too much time observing living cells with a microscope. As Daniel Mazia stated about microscopy, "important discoveries continue to travel to us on beams of light." These beams provide a wonderful vehicle to learn and we hope these exercises will achieve this goal.

A. Chemotaxis

There are many examples in cell biology and development where cells sense a chemical gradient and move toward it or away from it. Slime mold amoebae move up gradients of cAMP to aggregate into the "cellular slug" stage. In mammals, white blood cells move to wounds and infections. Neural crest cells migrate in the vertebrate body to form many parts of the body. This directed movement is often a form of chemotaxis, or chemoattraction; few examples of the phenomenon are known in molecular detail in eukaryotic cells.

When treated with soluble egg jelly, sea urchin sperm cells show a change in swimming behavior that is some kind of an unexplained chemotaxis (Eisenbach, 2004; Ward *et al.*, 1985). It is easy to demonstrate this phenomenon using isolated egg jelly and sperm. To isolate egg jelly, the coat is solubilized by exposing the eggs to seawater at pH 5.0 for 2 min. This is done by putting a pH electrode in the egg suspension and gently stirring the eggs by hand while slowly dripping in 0.1 N HCl until the pH reaches 5.0. After 2 min, the pH is quickly readjusted to pH 7.8 by adding 1:100 1 M HEPES buffer, pH 8.0. The eggs are allowed to settle and the supernatant seawater, which contains the dissolved egg jelly, is removed and centrifuged to remove egg fragments. The isolated egg jelly solution then can be stored on ice or frozen for later use.

To observe chemotaxis, sperm are diluted about 1:100 with seawater. Then, 0.5 ml of diluted sperm is placed in a 12 × 75 mm glass tube and 0.5 ml of the egg jelly solution is added. Within 10 s, sperm begin to cluster, and they form clotlike aggregates which can be seen with the eye at the bottom of the tube. (The tube should be held up to the light to observe this.) People used to think the cells were sticking to each other in a true agglutination reaction. However, careful

observation under the microscope demonstrates that the sperm are freely moving very rapidly, as bees do in a swarm (Collins, 1976). It is as if there is something in the center of each ball of swarming sperm that is attracting the cells. In 2 to 5 min, the clusters of sperm dissociate and the sperm swim freely in a once-again homogeneous suspension.

The swarming behavior of sperm cells by egg jelly can also be observed microscopically on a slide. Four drops of egg jelly solution are placed on a slide and two drops of freshly diluted sperm (1:100) are added. The clusters of sperm are irregular in shape for a few seconds before they become perfectly spherical swarms. In 2 to 5 min, the attraction is lost, the swarms spontaneously disperse, and the cells are evenly distributed in the field of view. Students can time these events of clustering and dispersion.

There are many investigative questions students can then begin to ask about this system; for example, will the same sperm swarm again if fresh egg jelly is added? Is the reaction species-specific? What is the effect of temperature on the reversal of swarms? (The lipids in the sperm membrane are frozen at about 7 °C.) How do proteases, which would digest sperm membrane receptors, affect the ability of the sperm to cluster? A solution of 0.2 mg/ml pronase in seawater makes a good test solution for this question—two drops of this solution are placed on a slide, and two drops of sperm suspension are added and mixed in with a pipette tip. After 10 min, four drops of egg jelly solution are added and the ability of the sperm to swarm is determined by comparing the response to a no-protease control.

B. Formation of the Fertilization Envelope

There are numerous examples of cells secreting glycoproteins that "harden" (intermolecular covalent crosslinking) to form tough structures that protect the cell and the organism. The epidermal cells of invertebrates such as insects and crustacea secrete an exoskeleton that hardens. Mussels secrete attachment threads of protein that are transformed to extremely resistant filaments. Reproductive stages of many animals are encased in hardened coats, such as insect egg chorions or the resistant cysts of protozoa and flatworms. One mechanism of hardening of such secreted proteins is by the formation of intermolecular crosslinks between adjacent proteins, which then are rendered extremely resistant to enzymatic and chemical attack. The hardening of the fertilization envelope of the sea urchin embryo occurs within a few minutes of sperm addition, and makes an excellent model system for asking questions about this hardening process (Kay and Shapiro, 1985).

1. Background on Transformation of the Fertilization Envelope

The egg is covered by a thin extracellular matrix called the vitelline layer or VL [it also contains the sperm binding (bindin) receptors]. The VL is about 30 nm

thick and is made of a loose meshwork of glycoproteinaceous fibers that are intimately bonded to the cell membrane. In fact, you can't isolate the VL without destroying the egg.

Directly under the cell membrane are about 15,000 cortical granules (CG) that contain 6% of the egg protein. (Many other species, including mammalian eggs, also have CGs). At the point of sperm fusion the CGs begin to fuse with the egg membrane and release their contents extracellularly under the VL. The exocytosis of the CG sweeps around the egg as a circular wave, taking 30 s to complete. This is evidenced as the elevation of the VL and its transformation into the FE (see Fig. 2).

The mechanism of VL elevation and transformation to the hardened FE results from the interaction of the cortical granule components with the VL. First, a trypsinlike-protease, released from CGs, detaches the VL from the cell membrane. Simultaneously, two other proteins, one structural and called proteoliaisin (PLN), and the other an enzyme called ovoperoxidase, come out of the CG bound to each other in a 1:1 stoichiometry. This dimeric complex assembles in paracrystalline order on both the inner and outer surface of the elevating VL. The VL thus acts as a template for these proteins, which is part of FE assembly (Correa and Carrol, 1997; Weideman *et al.*, 1987).

The secreted proteins, aligned in an orderly array (paracrystalline) on the VL template, are now crosslinked together by action of the ovoperoxidase. This protein functions as both a structural component and an enzyme in FE formation and hardening. The ovoperoxidase uses H_2O_2 (hydrogen peroxide) to catalyze the formation of di- and tri-tyrosine crosslinks among adjacent molecules. These crosslinks are resistant to hydrolysis in boiling 6N HCl; if you hydrolyze purified FEs in HCl to break all peptide bonds, and then separate the amino acids by HPLC, you get a large peak of di-tyrosine and a smaller peak of tri-tyrosine (Shapiro, 1991).

But, where does the H_2O_2 come from that is the substrate used by the ovoperoxidase in the crosslinking reaction? If seawater had that much H_2O_2 in it, we ocean swimmers would all be blondes. The answer is that the egg has a cytoplasmic enzyme called ovo-oxidase that uses O_2 and nicotinamide adenine dinucleotide phosphate (NADPH) to catalyze the reaction: $NADPH + H^+ + O_2 \rightarrow NADP + H_2O_2$. A protein kinase C is physically associated with the oxidase and appears to activate it at fertilization. (A fascinating question is why the H_2O_2 is not toxic. The answer is that the eggs possess novel compounds that neutralize the effects of the peroxide and associated free radicals produced by the H_2O_2. These compounds—1-methyl-4-mercaptohistidines—are found in most marine invertebrate eggs at concentrations of 3 to 5 mM and are called ovothiols [Shapiro, 1991].)

The FE protects the embryo from physical damage and microbial attack and persists as a tough protective micro-incubation chamber until about the 600-cell blastula stage. At that time, the blastula secretes a hatching enzyme, a metalloendoprotease, that dissolves the FE. The embryo at this time has also synthesized and assembled flagella on the outer cells and can now swim in the plankton as an

independent planktonic organism. In two days, it becomes a feeding larva and then spends 30 days in the plankton growing and continuing its development until metamorphosis.

2. A Simple Demonstration of the Assembly and Hardening of the FE

A drop of unfertilized eggs is placed on a slide, then one drop of 5% sodium dodecyl sulfate (SDS) is added. What should happen is complete solubilization of the cells; nothing is left but a yellow liquid. This is then repeated with fertilized eggs which have had elevated FEs for at least 5 min. What the student should see is that everything dissolves except the FE, leaving beautiful clean FEs on the slide that are resistant to SDS. In fact, one can boil the FEs in 5% SDS and they still do not dissolve.

3. Experiments Demonstrating the Mechanism of Assembly and Hardening of the Fertilization Envelope (FE)

a. Use of Inhibitors to Examine the Role of the Trypsinlike Cortical Granule Protease that Detaches the Vitelline Layer from the Egg Cell Membrane

Drops of concentrated unfertilized sea urchin eggs are placed in a series of tubes containing 1 ml seawater plus soybean trypsin inhibitor (SBTI) at concentrations of 2.0, 1.0, 0.5, 0.25, 0.12, and 0.06 mg/ml seawater. This protein binds tightly to trypsinlike enzymes with a 1:1 stoichiometry and completely blocks proteolytic activity. A seawater control (no SBTI) should also be used.

A few drops of the SBTI egg suspension are then placed on a clean slide. One drop of dilute sperm suspension is added and the eggs are observed to determine what happens to the elevation of the VL and its transformation into the FE. Students can ask questions regarding the effect of the different concentrations of SBTI as well as observe differences in sperm-binding behavior as compared to the no-SBTI control (see Vacquier *et al.*, 1972).

b. Preventing Di-tyrosine Crosslinking of the FE by Blocking the Egg Ovoperoxidase

Para-aminobenzoate (PABA) is an effective nontoxic inhibitor of the ovoperoxidase (the enzyme that forms the intermolecular di-tyrosine crosslinks). One ml of PABA dissolved in seawater at concentrations of 100, 50, 25, 12, and 6, 3, and 1.5 mM is placed in test tubes. Then 1 drop of concentrated unfertilized eggs is added to each tube. Three drops of each egg suspension are then placed on a slide and fertilized with a drop of diluted sperm. A PABA concentration-dependent effect is clearly seen on the elevation of the FE. When ovoperoxidase-mediated crosslinking is inhibited, then the FE becomes vulnerable to chemical agents which disrupt its molecular structure.

This can be demonstrated by repeating the above with a concentration of PABA that gives clear visual evidence that the FE is being affected. At 3 min after fertilization, 2 to 3 drops of 0.01 M DTT (dithiothreitol) in seawater (pH 9.1)

are added. This reagent will reduce disulfide (S-S) bonds that help stabilize the proteins of the FE. Additionally, 2 drops of 0.1 mg/ml pronase in seawater can be added to degrade the peptide bonds of the FE proteins. Since the ovoperoxidase was blocked by PABA, no hardening of the FE occurred and the DTT or pronase should destroy the soft FE, leaving a "naked" egg. [A seawater (no PABA) control should be run in parallel.]

c. Demonstrating that Disulfide Bonds Stabilize the Elevated FE after Tyrosine Crosslinking Has Hardened It

As has been noted, the FE is hardened by intermolecular di-tyrosine crosslinks. However, disulfide (S-S) bonds between cysteine residues are extremely important to the stability of the VL and also the completely elevated, hardened FE (Epel *et al.*, 1970). This can be demonstrated as follows. Two drops of egg suspension on a slide are fertilized with dilute sperm and allowed to sit undisturbed at least 5 min to allow complete crosslinking of the FE to occur (do not use a coverslip). Two drops of 0.01 M dithiothreitol (DTT pH 9.0) in seawater is then added. What you should see is that addition of DTT causes a dramatic increase in diameter of the FE due to breakage of S-S bonds and relaxation of the protein structure. Blowing air across the slide for several seconds will cause the oxidation of the DTT, the reformation of S-S bonds, and the shrinkage of the FE to its normal size. The cycle of expansion and contraction of the FE by breakage and reformation of S-S bonds should be repeatable on the same eggs by adding more DTT, followed by blowing.

Students can then ask a variety of questions about this process, such as, is the hardened FE susceptible to proteolytic destruction when the S-S bonds are reduced to SH? To test, 2 drops of normal fertilized eggs with hardened FEs are mixed on a slide with 2 drops of a 0.1 mg/ml solution of pronase in seawater. This control is then compared to fertilized eggs pretreated with DTT to expand the FEs. Protease effects will be time dependent, so the control and DTT-treated FEs must be observed for at least 5 to 10 min.

d. Demonstrating that Transglutaminase Activity is Also Involved in the Hardening of the FE

Transglutaminases are enzymes that crosslink proteins together by linking the epsilon amino group of lysine in one protein to the carboxyl group of aspartic or glutamic acids in another protein. If transglutaminase activity is involved in FE hardening, fertilization in the presence of transglutaminase inhibitors will yield soft FEs which should be destroyed by pronase and DTT (see, e.g., Battaglia and Shapiro, 1988).

Primary amine inhibitors of transglutaminase activity are cadaverine, putrescine, and glycine ethyl ester (GEE). This experiment to test their effects is set up just like the PABA experiment already described. Seawater buffered with 10 mM HEPES, pH 7.8, is used to prepare GEE at the following concentrations: 100, 50, 25, 12, 6, 3, and 0 mg/ml. One ml of each concentration of GEE is placed into a series of tubes, then 1 drop of a 10% egg suspension is added. After 2 min, 3 drops

of egg suspension are placed on a slide and fertilized by mixing in less than one drop of diluted sperm suspension using a pipette tip. Observe the formation of the FE. Does it look larger and thinner than the control? Additional questions can be asked, such as whether the FE formed in GEE is resistant to DTT and pronase.

C. The Role of Calcium Ions in the Fertilization Process

Calcium is required both for the acrosome reaction of sperm and for the activation of the egg, including triggering exocytosis of the cortical granules. There are a number of questions that students can ask about the role of calcium in the fertilization process, such as confirming that calcium is required for fertilization, determining where in the process calcium is required, and determining minimum levels of calcium required (see, e.g., Darszon *et al.*, 2001).

1. Is Calcium Required for Fertilization?

Five ml of calcium-free seawater (CaFSW) is placed in a tube and one drop of a 10% egg suspension is added. The eggs will settle through the CaFSW (or can be pelleted with very gentle centrifugation). The supernatant is removed with an aspirator and the eggs are resuspended in a fresh one ml of CaFSW. This procedure is repeated two more times to ensure that all the calcium is removed from the suspension. Then two drops of the suspension is placed on a slide and fertilized with small drop of a 1:100 dilution of sperm in CaFSW. In normal seawater, the FE elevates and hardens by 3 min after adding sperm. Note that, in this experiment, even 5 min after adding sperm, the FE is not present. This is because, normally, when sperm contact egg jelly, two calcium channels open in the sperm membrane, allowing calcium ions to come into the cell and trigger the acrosome reaction. Without having undergone the acrosome reaction, the sperm cannot bind to or fuse with the egg; thus, fertilization is inhibited. To demonstrate that calcium is the limiting factor, a small drop of 0.34 M $CaCl_2$ buffered with 10 mM HEPES at pH 7.8 can be added. This should result in the elevation of FE within about 2 min.

Students then can ask questions about how much calcium is required for acrosome activation by mixing CaFSW with normal seawater to create a range of calcium concentrations. Drops of eggs washed into CaFSW can then be added to these varying concentrations and fertilized by sperm diluted in the corresponding concentration to determine the level of calcium required for the acrosome reaction to occur and permit sperm binding and fertilization to occur (see also Collins and Epel, 1977).

2. The Role of Calcium Ions in Cortical Granule Exocytosis

A unifying feature of exocytosis, including the cortical granule reaction of sea urchin fertilization, is that it is mediated by a transient increase in free calcium ions. For example, in the unfertilized egg, the free calcium ion concentration (not

the total calcium) is about 100 nM. Fusion with a sperm causes the release of calcium from membranous channels that ramify throughout the cytoplasm and the free calcium ion concentration goes up to between 1 and 3 μM and this increase then triggers exocytosis of the CG, most likely by a conserved pathway involving SNARE proteins. This exocytosis and its calcium requirement can be easily studied with sea urchin egg cortical granule "lawns," which provide a model system for studying the mechanism of exocytosis (see review by Wessel *et al.*, 2001).

a. Isolation of Cortical Granule Lawns

The basis of the isolation is that eggs are adhered to a charged plastic dish and then sheared away, leaving a layer of plasma membrane with intact CG on the surface of the dish (see Vacquier, 1975).

A solution of one mg/ml protamine sulfate is used to coat a small circular area (2 cm) on the bottom of 5-cm-diameter tissue culture dish. It is best to circle the specific area with a marking pen on the bottoms and lids. After 1 min, the protamine sulfate is washed away with a jet of tap water, and the dish is then air dried. The dish can also be positively charged by applying a 2-cm-diameter circle of 1% alcian blue in water and then washing the dish with water and allowing it to air dry. The protamine remains stuck to the dish surface as a thin film of positively charged protein; as the surface of the egg is negative in charge, the eggs will electrostatically bind the dish surface and flatten out as hemispheres. Alcian blue works by binding to negatively charged polysaccharides on the cell surface.

The jelly coat of the eggs must first be removed by the low pH method previously described. The eggs are then washed three times in fresh seawater by settling or by gentle centrifugation. A few drops of concentrated egg suspension (10%) are placed in the circle and allowed to settle for 2 min. This results in the eggs sticking to the dish surface.

To make the CG lawns, the dishes are filled with cortical granule isolation medium (CGIM), which is calcium-free artificial seawater (CaFSW) with 10 mM EGTA to chelate the calcium ions, and adjusted to pH 8.0. After swirling for about 2 min to chelate all the calcium ions, the dish is tipped on its side and the eggs are squirted with a jet of CGIM from a squirt bottle. Sufficient force is used so that the egg cytoplasm is sheared away, leaving a perfectly clean preparation of CG bound to the exposed inner surface of the egg plasma membrane. All the excess liquid should be removed by flicking. The cortical granules can be observed by immediately dropping on a coverslip and observing with 40$\times$ objective of an inverted phase contrast microscope. (If the coverslip is floating, the excess liquid can be removed with a pipette.) The CGs are visible as a population of very regular granules about 1 μm in diameter.

b. Calcium and Exocytosis

To determine the effects of calcium, one student can observe the CG lawn through the microscope and have a another student add one drop of seawater to the edge of the coverslip. The CG will explode when the calcium ions hit them. The

wave of breakdown of the CGs can be followed by moving along with the seawater flow, using the stage micrometer and the low power objective. The explosion of the CG takes less than a second. This is the only available preparation of exocytotic vesicles that can be isolated in seconds in an intact and highly pure form that responds to μM concentrations of free calcium ions.

3. Examining the Role of Calcium in Egg Activation with the Use of Calcium Ionophores

Ionophores are low molecular weight natural products which dissolve in the plasma membrane or intracellular membranes of cells and make the membrane permeable to specific ions. For example, A23187 and ionomycin allow the movement of calcium and hydrogen ions across membranes from a region of higher concentration to one of lower concentration.

Ionophores can be used to ask questions about the role of calcium in egg activation without the use of sperm to fertilize the eggs. The principle of these experiments is to raise the calcium concentration in cells by adding ionophore to eggs. A stock solution of 2 mg/ml A23187, dissolved in 100% DMSO, can be used. Ten ul of this solution is added to 0.5 ml of seawater in a tube and mixed well. Then 0.5 ml of 2% vol/vol egg suspension is added, and a drop is immediately put on a slide and observed. Diluting the ionophore stock in this manner results in a 1% final concentration of DMSO and 38 μM A23187. Eggs should be observed quickly as the elevation of the FE occurs rapidly (see Steinhardt and Epel, 1974).

Students can then ask questions regarding differences between fertilization and ionophore activation. For example, they should be able to observe that, in sperm-fertilized eggs, the CG exocytosis sweeps around the egg cell as an expanding wave, beginning at the point of successful sperm interaction with the egg. In A23187-induced CG exocytosis, all the CG appear to fuse at the same instant and there is no wave of FE elevation; the FE elevates from the entire egg surface all in one instant.

Students can also use this system to confirm that the calcium responsible for the CG reaction is released from internal cytoplasmic stores. To test this, the eggs can be activated with the ionophore in calcium-free seawater. The ionophore will permeabilize the ER membranes that surround the internal calcium stores in addition to permeabilizing the plasma membrane. Note that a problem in seeing whether the eggs are activated is that the fertilization envelope will be very thin in calcium-free seawater, so careful observation, perhaps with phase microscopy, may be necessary. There will also be a time delay relative to the ability of the ionophore to stimulate CG exocyotosis in eggs in normal seawater, where there is a vast excess of calcium (10 mM).

D. Consequences of Parthenogenesis

Activating eggs, as has been described with ionophore, is a form of artificial parthenogenesis or initiating development without sperm. Students can ask questions about the ability of development to proceed in the absence of sperm by

observing what happens to the eggs activated with ionophore. Questions that can be asked include does the egg pronucleus center as it does in normal fertilization? Does the nuclear envelope break down? Does a mitotic apparatus form? Does the egg divide? These experiments will enable students to focus on the role of sperm in fertilization, such as the contribution of the centrosome used to assemble the mitotic spindle (see Schatten, 1994).

E. Consequences of Polyspermy

The role of the sperm can also be addressed by intentionally inducing polyspermy. This can be accomplished by addition of an excess of sperm or by adding excess sperm in the presence of soybean trypsin inhibitor (1 mg/ml). The consequences of the extra centrosomes for cell division are the most obvious, with the embryos going from one cell to multiple cell numbers at the first division. Students can also look at the nature of the mitotic apparatus under polyspermic conditions, such as observing multiple mitotic spindles instead of the usual bipolar spindle. This experiment thus demonstrates that the sperm brings in the centrosome and also that multiple centrosomes results in multiple divisions. Students can also observe the consequences on later development; the embryos will be abnormal since the chromosomes are distributed unequally to the daughter cells (see Schatten, 1994). Figure 3 depicts a polyspermic egg containing three sperm nuclei as observed under the phase contrast microscope. The nuclei were made visible by fixing the eggs in a 3:1 alcohol:glacial acetic acid mixture and then resuspending the eggs in 45% glacial acetic acid.

F. The Role of Protein Synthesis in Cell Division

A fundamental discovery made with sea urchins was that the synthesis of a specific protein after fertilization is required for cell division. This was first demonstrated by examining the consequences of inhibiting protein synthesis at various times after insemination. It was observed that applying these inhibitors shortly after fertilization prevented the later cell division (Wilt *et al.*, 1967). Most puzzling, however, was the finding that application of these inhibitors just before prophase had no effect on the cell division. This suggested that a critical protein was necessary for the cell division, but was made early in the cycle. This protein either acted early in the cell cycle or was already all synthesized by prophase, since inhibiting protein synthesis at prophase or later had no effect on that cell division. The answer to this question came from experiments by Tim Hunt and his colleagues, who showed that sea urchin eggs made a protein after fertilization, that this protein began to be degraded as the cell entered mitosis and it was resynthesized for the next cycle. This synthesis and degradation was cyclic and related to the cell division and Hunt named this protein "cyclin" (Evans *et al.*, 1983). Subsequent work revealed that this periodic synthesis and degradation was a normal part of the cell cycle in all organisms (and led to Tim Hunt's receiving a Nobel Prize for this important discovery).

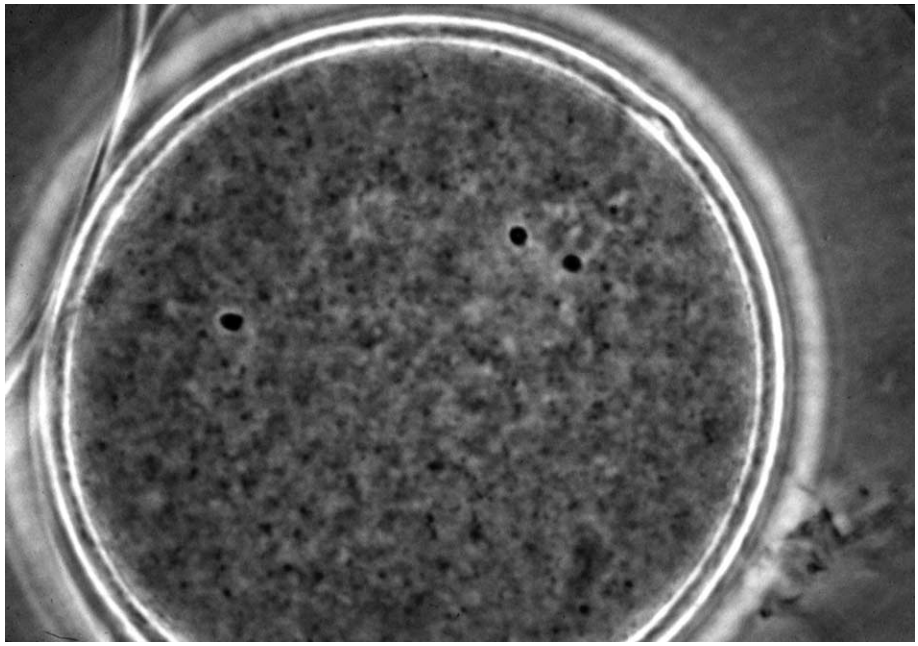

Fig. 3 Phase contrast micrograph of a polyspermic egg. Note the 3 sperm pronuclei, which are visible as dark spots within the egg. These eggs will not develop normally since each sperm brought in a centrosome, which will now direct the egg to form several mitotic apparatuses. These will compete for the chromosomes and there will be an unequal segregation of the chromosomes, which will upset the normal genetic regulation. The centrosomes will also each form a pole of a mitotic apparatus and the consequence will be that the cell will divide from one cell to 4 cells, corresponding to the number of centrosomes brought in by the sperm.

Students can demonstrate this essential protein synthesis in class. The experiment involves fertilizing eggs and then adding 100 uM emetine (a potent protein synthesis inhibitor) to the fertilized embryos at 10 to 15 min intervals after fertilization. The result will be that the cells will be prevented from entering mitosis until a specific time (in *S. pupuratus*, this is about 40 to 50 min after fertilization). After this time, the embryos will divide at the same time as the controls, but the next cell cycle will be inhibited. This concentration completely knocks out protein synthesis and indicates that synthesis of a protein is essential for cell division, but that this protein is completely synthesized long before the actual division takes place.

VI. Inquiry-Based Experiments in Later Development: Experiments on Differential Gene Expression

Sea urchin embryos are also excellent model systems for demonstrating differential gene expression during development. The expression of a number of cell-type

or stage-specific genes can be followed by immunolocalization of the expressed protein using antibodies.

A. Immunolocalization of Cell Type Specific Proteins

This type of experiment utilizes antibodies generated against proteins that are known to be expressed in a cell-type and stage-specific manner to determine when and where during embryonic development expression of the protein occurs. Students can either work outside of the scheduled lab time to grow and fix embryos of the appropriate stage, or the instructor can provide fixed embryos and have the students do only the staining. (For upper-level students, these experiments work well as independent study units, with groups of students starting cultures of embryos, monitoring developmental progress, and fixing batches of embryos as they reach the appropriate developmental stage.)

1. Basic Protocol

Additional details regarding immunolocalization and a list of available antibodies can be found in Chapter 15 (Venuti *et al.*) of this volume. This method is a basic protocol that can easily be performed by undergraduates.

One of the easiest fixation methods that works well for staining embryos with many different antibodies is cold methanol. Embryos of the appropriate stages are collected by gentle centrifugation. At least 0.1 ml of gently packed embryos should be obtained in order to prevent excessive loss during the washing steps. The seawater supernatant is removed by aspiration and replaced by 10 mls of ice-cold methanol. The embryos are gently dispersed into the methanol by inverting the tube several times. The tube is placed on ice for 20 min, during which time the embryos settle into a loose pellet. At this point, they become relatively brittle and sticky and must be handled carefully—washing must be done by allowing them to settle by gravity rather than by centrifugation. The methanol is replaced by seawater, and this wash is repeated two more times to ensure that all the methanol is removed. Following fixation, embryos can be stored at 4 °C for a week or more; the addition of a few drops of sodium azide solution will slow their degradation. An alternative fixation is to use 3.7% formaldehyde in seawater for 10 min on ice, followed by three washes in seawater.

To stain the embryos, an aliquot of 100 ul from the pellet of embryos at each of the various stages to be tested is placed in a 1.5 ml microcentrifuge tube. As much of the seawater supernatant as possible is removed, and replaced by 100 ul of antibody solution. Monoclonal supernatants are generally used undiluted for this procedure. The tube is gently mixed and the embryos are incubated in the antibody solution for 1 h at room temperature. The antibody solution is carefully removed with a micropipette and the tube is filled with seawater. The embryos are allowed to settle (both the incubations and washes involve a lot of dead time, so you may wish to have additional activities for these long waiting periods). An additional seawater wash is done to remove any unbound antibody.

The second seawater wash is removed and 100 ul of the secondary antibody, conjugated to a fluorochrome, such as fluoroscein or Texas red, is then added at the appropriate dilution as directed. The tube is incubated for 30 to 60 min, then the embryos are washed with seawater as has been described. (If a fluorescence microscope is not available, an alternative method for localization is to use a secondary antibody coupled to an enzyme such as alkaline phosphate. A substrate is then added to form a colored precipitate at the site of antibody binding, which can then be visualized with a normal light microscope.)

After the last wash, embryos are allowed to settle to the bottom of the tube and then removed with a pipette tip and mounted on a slide in a small drop of mounting medium containing an antibleaching compound. A coverslip is added, and the edges are sealed with fingernail polish. Once dry, the slide can be viewed immediately or stored. We often have students complete the staining in one laboratory session and then come in at a scheduled time during the following week to do the microscopic analysis of their data, including image capture and analysis.

2. Experimental Questions

A number of inquiry-based labs can be designed based on this basic protocol. Antibodies to known germ layer-specific proteins can be used to ask questions about differentiation of cells and the regulation of gene expression both temporally and spatially. For example, monoclonal antibody 6a9 stains primary mesenchyme cells (PMCs) following their ingression and works well on most species of urchin. Students can fix embryos at key developmental stages, for example, unfertilized eggs, early cleavage, blastula stage, mesenchyme blastula, and various timepoints during gastrulation and later larval development. Students can then stain the stages with 6a9 to determine at what point in development the PMCs begin to express the protein recognized by 6a9. A complete list of useful antibodies and their species specificity is found in the Venuti chapter [Chapter 15 (see also Ettensohn, 1990)].

This tool can also be used as a way of determining the effect of various treatments known to perturb development (lithium chloride, nickel chloride, actinomycin D, etc.) on gene expression. It allows students to monitor specific molecular changes along with observing changes in morphological phenotype. Students can also try agents of their own choosing in similar types of experiments.

VII. Experiments Using Morphology as an Endpoint

In terms of inquiry-based education, the later phases of development lend themselves to asking many questions about how development is affected by environmental factors. Some examples could be the effects of UV irradiation; effects of common pollutants, such as detergents; personal care products, which

have recently been shown to be present in the environment; hormone analogs; chemical pollutants, such as insecticides and herbicides. The student can pick a putative environmental agent and then observe the effects of this agent on later development. These experiments make excellent independent study projects, as different students can choose different agents.

VIII. Epilogue

The previously described exercises represent the tip of the iceberg of experiments that can be done with sea urchin embryos. Not covered here are experiments directly demonstrating new gene action during later development. This is an area of great research activity and intellectual ferment, but it has been omitted from this chapter since the laboratory study of such changes requires sophisticated equipment not available in most teaching laboratories. However, some experiments on the genetic changes in development can be done via computer analysis. As the sea urchin genome becomes available, many studies can be done with computer analysis of genes in the sea urchin genome and, for example, comparison with similar genes in other organisms or with similar genes in different species of sea urchins. The reader is referred to the sea urchin genome site http://sugp.caltech.edu:7000/.

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