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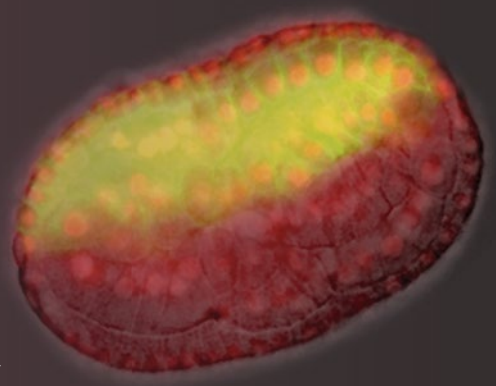

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Developmental Biology of the Sea Urchin and Other Marine Invertebrates

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Preface

Sea urchins and other marine invertebrates have contributed fundamentally to our knowledge of cell, molecular, and developmental biology. These contributions, ranging from the discovery of cyclins in the sea urchin, to cellular immunity in the starfish, to transmission of nerve cell signals in the squid giant axon, arise from the incredible diversity of these organisms, the ease with which samples can be prepared, and the availability of sufficient numbers of samples. With sea urchins, for example, you can relatively rapidly obtain large quantities of eggs all of which are arrested at the same stage of the cell cycle. This natural synchronization of these cells has proven useful for understanding the regulation and control of the cell cycle.

One of the most famous contributions of marine invertebrates to cell and molecular biology is the discovery of cyclins [1]. Over several summers at the Marine Biology Laboratory in Woods Hole, MA, Tim Hunt and colleagues demonstrated a protein band that disappeared in fertilized or activated sea urchin eggs, while the other proteins continued to be synthesized [2]. Further analysis showed that this protein band was continuously synthesized before being destroyed about 10 min before each cell division. Following this initial discovery, a similar behavior was discovered in cycling clam eggs and, as we now know, is universally responsible for regulating the cell cycle [3].

These interesting organisms have also contributed basic knowledge that leads directly to discoveries in human physiology with direct relevance to human health. For example, Ilya Mechnikoff and Paul Ehrlich shared the Nobel Prize in Physiology and Medicine in 1908 for their discoveries in immunity. In his Nobel lecture, Mechnikoff describes his work on comparative embryology as being crucial for the ultimate discovery of phagocytosis [4]. Enabling his discovery was the fact that many of the "lower animals" (his words) are transparent and thus allow visualization of cells within the living organism. He observed cells within the body cavity of bipinnaria larvae from the starfish and it occurred to him that these might be like blood cells from vertebrate organisms and that they may play a role in the individual's defense against microorganisms. He tested his idea by inserting a thorn (different sources say a rose thorn or a thorn from a tangerine tree) into the larva and observing that it was surrounded by mobile cells the following morning. In later studies, he observed similar behavior in "water fleas" (*Daphnia*) infected with microbes and then transferred his theories to humans and the infection-fighting role played by phagocytes [4, 5].

With the development of genome projects for many different phyla, the potential for these organisms to contribute more to our understanding of basic cell and molecular processes increases. This Method in Molecular Biology volume addresses the use of sea urchins and other marine invertebrates in the cell and molecular biology laboratory. It covers all aspects of the process beginning with caring for the animals, obtaining gametes, and producing embryos for a variety of organisms in the first chapters. We move on to describing

methods for imaging and other useful experimental tools for cell and developmental biology research. The final portion of the book will present a variety of molecular biological methods and strategies for utilizing the sea urchin genome.

Chapters 1–5 present methods for culturing and caring for a variety of marine invertebrate organisms. Hopefully, these chapters will inspire you to try a new model system in your own research program. In Chapter 1, Richard Strathmann describes techniques for the culture of marine invertebrates. These methods are streamlined and adaptable to the larvae of diverse animals. Many labs would like to give these fantastic animals a try and this chapter is just what you are looking for to get started. The provided methods are sufficient for taking your cultures from fertilization, through embryogenesis and up to metamorphosis. Chapter 2 is a very special chapter, written by Charles Lambert just before his passing in 2011. With great thanks to Gretchen Lambert, his chapter was completed and edited for inclusion in this volume. This chapter focuses on obtaining gametes, performing in vitro fertilization, and culturing embryos from the three different orders of ascidians. Because of their special place as basal chordates, understanding maturation, fertilization, and early development in this important group of animals has been very informative. The Drs. Lambert worked on these intriguing animals throughout their productive careers and have inspired many scientists along the way. Chapter 3 by Anthony Pires describes the culture of the widespread limpet *Crepidula fornicata* from spawning to metamorphosis. This mollusc has emerged as a very useful model system for the study of embryonic and larval life histories because of the development of useful tools including a well-resolved fate map, development of loss- and gain-of-function strategies, and an emerging genomics resource [6]. The culture of the mysterious placozoan *Trichoplax adhaerens* is described by Andreas Heyland and colleagues in Chapter 4. These basal metazoans have interested scientists since their discovery in 1883 because they were difficult to classify, appearing perhaps as cnidarians or sponges [7]. They have also generated recent interest since the publication of their 98 million base pair genome because they show great promise for understanding the evolution of the basic animal body plan [8]. A method for culturing bryozoan larvae and colonies is presented by Michael Temkin in Chapter 5. The Bryozoa are a phylum of diverse aquatic invertebrates that are found in fresh and salt water. There are over 4,000 living species known! Bryozoans are found in the fossil record, but their relationship to other organisms is not certain, making them an interesting group to study for evolutionary reasons.

The field of cell biology developed in response to advances in technology, particularly along with advances in microscopy. The light microscope allowed direct visualization of cells and some subcellular structure, which continued to advance following the introduction of electron microscopy that allowed for visualization of subcellular detail and even to the level of individual proteins. In Chapters 6–9, methods are described for acquiring and analyzing visual information in cell and molecular biology. George von Dassow details methods for processing of images from confocal microscopes in Chapter 6. This is a very practical and useful chapter written in an engaging style. Among many other things, readers will learn how to process 3D images, how to use the free ImageJ software, and how to deal with background noise. In Chapter 7, George Shinn details methods for imaging chaetognatha internal anatomy by light and transmission electron microscopy. The detailed methods described are applicable to most any marine invertebrate organism. There is a particularly extensive and useful **Note** section in this chapter, which will allow the novice to avoid the common pitfalls encountered when learning how to process embedded and sectioned specimens for light and electron microscopy. John Buckland-Nicks gives an excellent description of scanning electron microscopy in Chapter 8 with a focus on visualization of marine

invertebrate gametes. The chapter describes some background theory before giving detailed steps that could be replicated for any organism. Particular attention is paid to the delicate nature of gametes, particularly oocytes. Again, the extensive use of the **Notes** section provides the reader with sufficient advice for successfully adopting these methods in their own laboratory. In Chapter 9, Robert Burke and colleagues give a detailed description of several techniques useful for imaging neural developing in sea urchins. They consider many topics that will be of interest to anyone using light microscopy to study cell and molecular biology (which is probably all cell and molecular biologists). Some items covered include a comparison between the use of paraformaldehyde and methanol as fixatives. What is compatible with in situ hybridization techniques? Can this be combined with immunolabeling of a specific protein antigen. Even if you've done these methods before, you will learn something new in this chapter.

Chapter 10 by Anthony Morgan and Antony Galione describes the preparation of sea urchin egg homogenates that preserve biological function. These preparations have been invaluable for the study of signal transduction in these cells, particularly the regulation of calcium release in response to a variety of agonists. The homogenates allow for visualization of both calcium release from internal stores and the study of the uptake of calcium as the signal is completed. Using homogenates also removes the necessity of microinjection for introducing molecules or proteins into the system. However, microinjection remains a useful method for studying cell biology in these systems, as detailed in Chapter 11 by Alex McDougall, Karen Wing-man Lee, and Remi Dumollard. They describe methods for the microinjection of mRNA expressing fluorescently tagged proteins and for following the expression of these proteins from the egg to the tadpole in the ascidian. As they mention, this method could be combined with a variety of gene knockdown methods to give a very comprehensive picture of specific developmental processes. While described for the ascidian, these methods will also certainly be applicable and useful and most any system that allows for microinjection (basically every egg)! Methods for isolating specific cell types from the sea urchin are described by Celina Juliano, S. Zachary Swartz, and Gary Wessel in Chapter 12. The sea urchin model system has been extraordinarily useful for understanding gene expression in the early embryo and during cell specification. This chapter details methods for isolating these different types of cells, based upon gene expression patterns, which will allow for downstream analysis of specific cell lineages that would not otherwise be possible. This lab thinks outside the box, so you don't know what ideas may be generated by reading their protocol. Chapter 13 by Samantha Cihal and David Carroll describes a method for labeling cell surface proteins in living starfish oocytes and methods for the further analysis of these proteins and, perhaps, their interaction with intracellular signaling molecules. It is hoped that this procedure would be generally useful to most cell types.

The Hedgehog signaling pathway is an important regulator in many different developmental processes and in a wide variety of organisms. It represents a fundamental decision-making pathway. Methods to study the contribution of this pathway to development in the sea urchin by microinjection of morpholino oligos or with small molecule inhibitors are described in Chapter 14 by Jacob Warner and David McClay. Sea urchins have been useful model systems for the study of fertilization and also for the early developmental events that occur following fertilization. In Chapter 15, Jolanta Kisielewska and Michael Whitaker report a clever method for using fluorescence to monitor DNA replication in living cells, along with methods for introducing other fluorescently labeled proteins into these embryos.

Protein phosphorylation regulates the activity of many signaling molecules, in both a positive and negative fashion. As more is learned about developmental mechanisms, it is

becoming clear that the phosphorylation state of these signaling proteins is critical for their function. In Chapter 16, Jose Escalona and Stephen Stricker detail methods for an immunoblotting method to quantifiably analyze protein phosphorylation changes in nemertean oocytes during maturation. These methods can be easily adapted for use in other oocytes or cell types. One big advantage to the method described is that it is film based and, as such, will be useful in a wide variety of situations, including when working at marine laboratories.

In Chapter 17, Aditya Sethi, Robert Angerer, and Lynne Angerer detail methods for the analysis of gene regulatory networks during development in the sea urchin using multicolor fluorescent in situ hybridization (*FISH*) combined with immunohistochemistry. These techniques will allow spatial and temporal resolution of gene expression changes in whole embryos. Julio Harvey outlines a very practical application of a DNA hybridization assay in Chapter 18 to identify specific organisms from environmental water samples. This protocol, called the sandwich hybridization method, makes use of two different DNA probes—one to capture the target and the other for identification. This separation allows for an increased signal-to-noise ratio and it also allows for quantification in the described microarray format.

As mentioned above, the sea urchin has proven to be a perfect model system for the study of fertilization. In recent years, tremendous progress has been made in identifying components of the fertilization pathway and to understand the mechanism of the initial activation event. Chapter 19 by Michelle Roux and Kathy Foltz takes this analysis to the next level by describing methods for the analysis of either individual signaling molecules or global signaling pathways at fertilization in the sea urchin system. Because of the development of the sea urchin genome project (*see* Chapter 20 in this volume), modern genomic and proteomic methods can be applied to this fundamental biological problem. This chapter does a fantastic job introducing the promise and the potential problems that can be encountered when beginning such a research program. Chapter 20 details bioinformatic methods for efficient use of the sea urchin genome data. Andrew Cameron takes us through SpBase, the home of the Sea Urchin Genome Database. From this starting point, the proteins, genes, and genome data can be analyzed using a variety of publicly available software programs. The possibilities are tremendous and available for all to take advantage.

The super-versatile starfish oocyte system is utilized in Chapter 21 by Eiichi Okumura, Masatoshi Hara, and Takeo Kishimoto. They describe methods for the preparation of antibodies for use as specific inhibitors in the oocyte (and even in the nucleus). The protocol details each step of the way, from designing and producing the antigen, to immunizing the animals and purifying the antibodies by affinity chromatography to the microinjection process. This is a remarkably comprehensive chapter and will prove very useful for labs looking to design a project from start to finish to understand the function of a specific protein. In our final chapter, Tetsuo Kida, Shinjiro Matsuda, Atsushi Kuyama, and Tetsuo Toraya describe a method for identifying the 1-methyladenine receptor using photoaffinity labeling. The chapter also details their progress in the identification of a promising target for the long-sought-after 1-MA receptor.

Melbourne, FL, USA ***David J. Carroll***

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Chapter 1

Culturing Larvae of Marine Invertebrates

Richard R. Strathmann

Abstract

Larvae of marine invertebrates cultured in the laboratory experience conditions that they do not encounter in nature, but development and survival to metamorphic competence can be obtained in such cultures. This protocol emphasizes simple methods suitable for a wide variety of larvae. Culturing larvae requires seawater of adequate quality and temperature within the tolerated range. Beyond that, feeding larvae require appropriate food, but a few kinds of algae and animals are sufficient as food for diverse larvae. Nontoxic materials include glass, many plastics, hot-melt glue, and some solvents, once evaporated. Cleaners that do not leave toxic residues after rinsing include dilute hydrochloric or acetic acid, sodium hypochlorite (commercial bleach), and ethanol. Materials that can leave toxic residues, such as formaldehyde, glutaraldehyde, detergents, and hand lotions, should be avoided, especially with batch cultures that lack continuously renewed water. Reverse filtration can be used to change water gently at varying frequencies, depending on temperature and the kinds of food that are provided. Bacterial growth can be limited by antibiotics, but antibiotics are often unnecessary. Survival and growth are increased by low concentrations of larvae and stirring of large or dense cultures. One method of stirring large numbers of containers is a rack of motor-driven paddles. Most of the methods and materials are inexpensive and portable. If necessary, a room within a few hours of the sea could be temporarily equipped for larval culture.

Key words Algal medium, Antibiotics, Culture, Food, Larvae, Seawater, Stirring, Temperature, Toxic, Vital stain

1 Introduction

A great variety of marine animals can be reared from embryo to metamorphic competence if the seawater (SW) is of sufficient quality and the temperature within the tolerated range. Different foods are best for different larvae, but a few species of easily cultured algae suffice for larvae of many taxa. Much is learned from larvae reared in the laboratory, but conditions in laboratory cultures are unlike conditions in the sea. The embryos and larvae in laboratory culture are at much greater densities than in nature [1], without the usual complement of other plankton and with differing dissolved organic compounds, light, and motion of water. Food, sometimes interacting with temperature, is known to affect the

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form, size, and organic composition of larvae and the postlarval juveniles subsequently formed [2–6]. Absence of predators in cultures can also limit the range of forms because predators affect form or size of some larvae [7]. Natural and artificial seawaters differ in dissolved materials, which can affect larval development. Thus, larvae and postlarval juveniles reared in the laboratory can differ from those encountered in nature. Whether the differences are trivial or important for interpretation of results depends on the question being asked.

This protocol emphasizes simple methods suitable for a wide variety of marine invertebrate larvae that are heavier than SW and either eat phytoplankton or do not require food. The methods are adequate for rearing hundreds to a few thousands of individuals to metamorphic competence. Some variations for rearing one or a few larvae per container and for rearing larvae that are buoyant, hydrophobic, or eat larger prey are also described. Access to SW of good quality (from a location within a few hours of the sea) is assumed, though artificial seawaters have been adequate for some larvae. Materials and Methods are listed for several different purposes so that subsets can be chosen for particular needs.

Almost all described methods are for batch cultures with periodic water changes. Marine larvae and the reasons for culturing them are much too diverse for a single protocol to cover all useful methods. Additional information is in guides and protocols that focus on culture of diverse larvae [8], echinoids [9], and algae [10]. The web provides useful information on both methods and supplies. A literature search often uncovers methods best suited to a particular kind of larva. Although some general suggestions for inducing metamorphosis are offered, methods for starting cultures from gametes and fertilization [8] and for inducing metamorphosis of competent larvae differ greatly among taxa and are both beyond the scope of this protocol and described elsewhere.

2 Materials

Solutions should be made with deionized or glass distilled water. Water deionized by reverse osmosis (RO water) does not need to be at the highest level of purity. If these are lacking, other freshwater of high quality can be tried. Rinsing containers and stirrers with distilled or RO water avoids residues from tap water.

Some tools, such as saw, drill, and hot-melt glue gun, will be needed to construct a stirring frame or container for hatching *Artemia*. A glass scoring tool (stylus tipped with a substance harder than glass) is useful for breaking glass tubes. Some common laboratory supplies are not specified separately as materials.

2.1 Culture Containers

Choice among the following depends on total volume of SW, number of replicate containers, and whether there are numerous or single larvae per container (*see* **Notes 1–3**).

1. Glass jars of 3–4-liter (~1 gal) volume.
2. Glass dishes of a bit more than 100 ml volume with sloping sides (such as custard dishes from Corning or others).
3. Shot glasses.
4. Plastic multiwell culture plates (Falcon™) with 6, 12, or 24 wells, petri dishes, or (as a substitute) plastic ice cube trays.
5. 125- and 500-ml Erlenmeyer flasks for algal cultures (*see* **Note 4**).
6. Aluminum foil or (if using a microwave oven) glass beakers, paper, or heat-resistant plastic as caps for flasks.

2.2 Stirrers for Cultures

Although it is possible to change the water frequently and rear sparsely populated cultures without a stirrer, many cultures require stirring. Described below are three possible methods of stirring—paddle stirring, aeration, and shaking.

1. Paddle stirrer for up to 18 large jars: components for a paddle stirrer are listed in 1a–e; assembly instructions are given in 1f (a smaller stirrer is depicted in Fig. 1) (*see* **Note 5**).
   (a) Fixed frame: ~5.2 m (~17 ft) of pipe of ~25-mm (~1 in.) outer diameter (OD) for a frame of outer dimensions ~100 cm long, 60 cm wide, and 46 cm height (~40 × 23 × 18 in.) with a pipe ~15 cm (6 in.) long to hold the motor; 5 T-joints and 4 corner joints that fit the pipe; 4 screws longer than the pipe OD to prevent rotation of the T-joint and pipe at the end of the frame that supports the motor.
   (b) Swinging frame: ~3 m (~10 ft) of pipe of ≤22-mm (≤7/8 in.) OD for a frame of ~94 × 50 cm (37 × 19.75 in.), four corner joints that fit the pipe.
   (c) String: ~9 m (30 ft) (for six rows of string on the fixed frame to support paddles and six rows of string on the swinging frame to push paddles, with enough extra string to tie ends through holes in the frames); also string to hang the swinging frame below the fixed frame with distance of ~15 cm (~6 in.) at each corner.
   (d) A small electric stirring motor, 6–10 rpm, with holes in its casing for mounting; two bolts with nuts of the diameter and length needed to mount the motor; acrylic sheet (or any rigid durable light material that can be drilled and sawed) of ~6 × 10 cm and ~0.3 cm (~1/8 in.) thickness for mounting the motor; two bolts with nuts for attaching

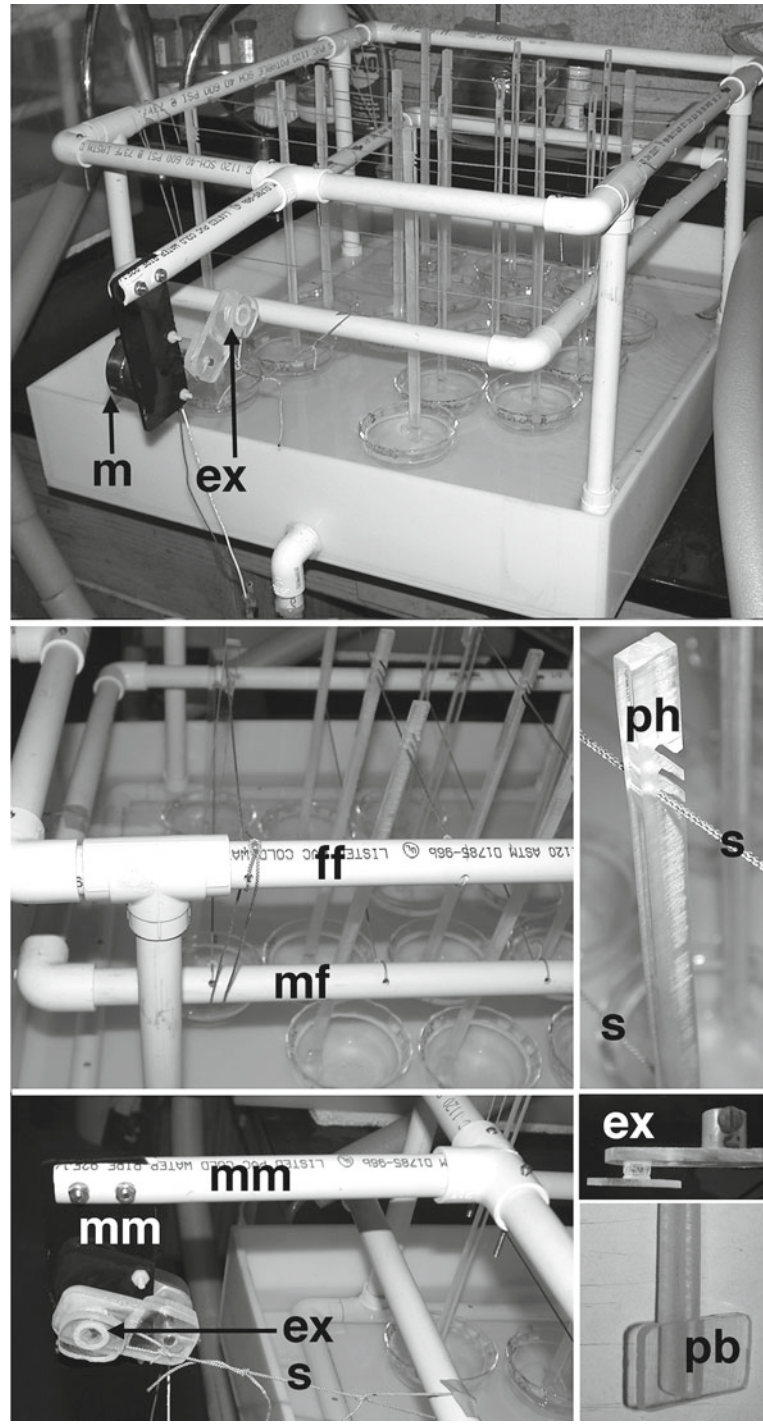

Fig. 1 Frame, motor, and paddles for stirring numerous cultures: *ec* arm for eccentric attachment for string that pulls the moving frame, *ff* fixed frame, *sf* swinging frame, *m* motor, *mm* motor mount, *pb* paddle blade, *ph* paddle handle, *s* string

acrylic sheet to PVC pipe; pieces of acrylic for constructing an arm of ~3×8 cm mounted to the motor's shaft for eccentric attachment of the string that pulls the swinging frame; one bolt as a set screw for attaching the eccentric arm to the motor shaft.

(e) Paddles: an acrylic sheet (Plexiglass) ~0.3 cm (~1/8 in.) thick with an area of about 1,000 cm^2 (0.12 yd^2) for 20 paddle blades, each ~8×6 cm (~3×2 in.), and an acrylic sheet ~0.7 cm (~0.25 in.) thick and ~1,700 cm^2 (~0.2 yd^2) for 20 paddle shafts, each ~48 cm (~19 in.) long and ~1.6×0.7 cm (~5/8×1/4 in.) in cross section.

(f) Assembly instructions for paddle stirrer: drill holes along the pipes for each side of the fixed frame for strings for hanging paddles. Spaces between holes (and strings) should allow room for rows of culture containers along the strings, though if jars are offset, the space between strings can be less than the diameter of the jars. Drill holes in the pipes for each side of the swinging frame for strings at the same interval. These strings push the paddles. Friction is sufficient for all joints of the assembled frames except for the end of the fixed frame that holds the motor mount. At the end of the fixed frame at which the motor is mounted, drill holes through each end of the T-connector and pipes at the middle and through the two connectors and the pipes at each corner for four screws that prevent rotation of the T-connector and end pipes under the weight of the motor. Tie strings taut across the fixed frame and the swinging frame. Tie four strings to hang the swinging frame from the fixed frame, near the ends on each side.

After cutting shafts and blades, fasten the blade to shaft with chloroform, which dissolves and fuses the acrylic and then evaporates. Make blades small enough to traverse most of the width of the culture vessel without hitting the sides but large enough for stirring. A large volume of water should move slowly near the bottom of the culture vessel. Cut a series of notches for suspension of each paddle from the string on the fixed frame. Several notches will allow adjustment of height of the paddle blade above the bottom of the vessel.

Drill holes for mounting the motor near one end of the small sheet of acrylic plastic or other rigid material. The drilled holes should match the holes in the motor housing and also include a hole for the shaft of the motor. Insert the other end of the sheet into a slot cut in the PVC pipe extending from the T-connector and fix it in place with bolts through two holes drilled through pipe and acrylic sheet. Fix the motor to the acrylic sheet by bolts

through the drilled holes. From pieces of acrylic, fashion a rotating arm that at one end is fixed to the motor's shaft by a setscrew (possibly reinforced with glue but not hot-melt glue) and that at the other end has a place for a loop of string. Form a loose loop at the end of the string at the rotating arm and tie the other end of the string to the end of the swinging frame. Tape the string at the swinging frame to prevent the loop from slipping along the pipe.

When the frame is assembled, adjust the distance that paddles swing by adjusting the length of the string connecting the rotating arm to the swinging frame and by adjusting the lengths of the strings connecting the swinging frame to the fixed frame.

2. Stirring with air bubbles: aquarium air pump, flexible tubing, gang valves to adjust airflow, cotton for a filter in the tube between pump and gang valves, and glass tubes or pipettes for ends in the cultures.
3. Stirring by shaking of multiwell plates: shaker table for rotation at 40–60 rpm [9]. If the shaker becomes warm, use a sheet of insulating polystyrene foam plastic between the cultures and shaker.

2.3 Seawater Solutions

1. If possible, obtain SW that is at least as clean as the water where the larvae are found in nature. SW from bays with ports, marinas, or city or industrial runoff may be contaminated with toxic materials (*see* **Note 6**).
2. Large plastic containers with plastic screw caps for collecting SW.
3. Thermometer, for checking adequacy of temperature control and for estimates of development rates (*see* **Note 7**).
4. Refractometer for approximate estimates of salinity with units for salinity (ppt). Needed if salinity of the source SW varies and is sometimes lower than that tolerated by the larvae.
5. Artificial seawaters have been used to rear larvae with varying success, usually less successfully than with natural SW. Commercially available artificial seawaters include Crystal Sea Marinemix, Instant Ocean, Coralife, and Tropic Marin, Petco. Alternatively MBL general purpose artificial SW with trace metals can be mixed as follows with distilled or RO water for all solutions ([11], *see* **Note 8**): 24.72 g NaCl, 9 ml of 1 M KCl, 9.27 ml of 1 M $CaCl_2{\cdot}2H_2O$, 22.94 ml of 1 M $MgCl_2{\cdot}6H_2O$, 25.5 ml of 1 M $MgSO_4{\cdot}7H_2O$, 2.15 ml of 1 M $NaHCO_3$ in a 1-l flask. Add the $NaHCO_3$ after the solution is almost to the liter volume and, if sterilizing, after autoclaving. Bring solution to final 1 l volume. For larval culture, include trace metals [9], as in the MBL trace solution SW formula [11]: use same recipe as above but with only 8.27 ml of the

1 M KCl and 0.089 g KBr per liter; 0.003 g NaF per liter; 0.037 g $SrCl_2 \cdot 6H_2O$ per liter; and 0.024 g H_3BO_3 per liter. The MBL artificial SW can be adjusted to pH 8. TRIS buffer is commonly used but may harm some larvae.

2.4 Seawater Filters

Choice of filters is a matter of need, cost, and convenience. Coarse filtration is often sufficient, but removal of small particles is useful when one must increase consistency in food supply by minimizing contaminating organisms or when one is culturing larvae removed from capsules at early stages.

1. Micropore membrane filters of cellulose nitrate/acetate, commonly 0.22- or 0.45-μm nominal pore size and 47 mm diameter (as from Corning, Gelman, Millipore).

   Use an in-line filter holder if there is sufficient upstream pressure from a SW system. If system pressure is low or if one is using stored SW, then use a suction filter apparatus: a vacuum pump, vacuum hose, and trap between filter apparatus and vacuum pump. The trap can be a flask with two-hole stopper with inlet and outlet tubes. Glycerin is a nontoxic lubricant for inserting glass tubes into rubber stoppers.
2. Bag filters: polyester felt filters with nonmetal rings are available in nominal 1- to 10-μm pore sizes, though particles larger than that can pass (from McMaster Carr or other suppliers).
3. Paper filters and funnel: many paper filters are adequate, including coffee filters.
4. Mesh filters: nylon screens (Nitex mesh, as used for plankton nets) with 30–200-μm mesh openings. Remove the bottoms from plastic beakers or other containers and attach mesh with hot-melt glue. A 30-μm mesh excludes most multicellular animals and transmits organisms of sizes eaten by many larvae. Larger meshes that are still substantially smaller than the larvae allow water changes by reverse filtration (*see* Subheading 2.10). Use a beaker of ≥400 ml for filtering SW for cultures. Use a beaker that fits within the culture vessel for reverse filtration: a beaker of ~50 ml fits into 100 ml in a custard dish, of ~150 ml can be lowered into a large jar.
5. Flexible tubing as a siphon for removing large volumes in reverse filtration.
6. A turkey baster for removing volumes of a few hundred ml (*see* **Note 9**).

2.5 Glassware Cleaners

1. Plastic fiber scrubbing pad (without detergent) for glass culture containers and paddles.
2. Bottle brush for Erlenmeyer flasks.
3. RO or distilled water for rinse following tap water and before air drying and storage (*see* **Notes 1**, **2**, and **10**).

2.6 Sterilization of Solutions

1. Autoclave.
2. If an autoclave is unavailable or inconvenient, then a hot plate, stove top, or microwave oven can be used (*see* **Note 11**).

2.7 Antibiotics

Antibiotics are usually unnecessary but are helpful when embryos or larvae are removed from egg capsules at early stages. Streptomycin and penicillin are often used in combination.

1. Streptomycin (dihydrostreptomycin) at 50 mg/l [9] or 50–200 mg/l [8].
2. Penicillin at 50 mg/l or ~100 U/ml [9] or 25–150 mg/l [8].
3. Gentamicin at 40 mg/l [12].

2.8 Food for Larvae That Eat Algae

1. Algal species commonly used for larvae that capture small particles include *Isochrysis galbana, Pavlova lutheri, Rhodomonas lens, Skeletonema costatum, Chaetoceros gracilis*, *Dunaliella tertiolecta, and Nannochloropsis oculata* (*see* **Note 12**).
2. f/2 enrichment medium for algal cultures (*see* **Note 13**): to make the f/2 enrichment from one's own stock solutions [10], add to 950 ml of filtered SW the following components using specified stock solutions dissolved in distilled or RO water: 1 ml of 75 g/l stock solution of $NaNO_3$, 1 ml of 5 g/l stock solution of $NaH_2PO_4{\cdot}H_2O$, 1 ml of 30 g/l stock solution of $Na_2SiO_3{\cdot}9H_2O$, 1 ml of trace metal solution [into 950 ml of distilled or RO water, dissolve the following components: 3.15 g $FeCl_3{\cdot}H_2O$, 4.36 g $Na_2EDTA{\cdot}2H_2O$, 1 ml of 180 g/l stock solution of $MnCl_2{\cdot}4H_2O$, 1 ml of 22 g/l stock solution of $ZnSO_4{\cdot}7H_2O$, 1 ml of 10 g/l stock solution of $CoCl_2{\cdot}7H_2O$, 1 ml of 9.8 g/l stock solution of $CuSO_4{\cdot}5H_2O$, 1 ml of 6.3 g/l stock solution of $Na_2MoO_4{\cdot}2H_2O$; bring total volume to 1 l], 0.5 ml of a vitamin solution [into 950 ml of distilled or RO water, dissolve the 200 mg thiamine·HCl, 1 ml of 1 g/l stock solution of biotin (vitamin H), 1 ml 1 g/l stock solution of cyanocobalamin (vitamin B_{12}); bring to 1 l volume with distilled or RO water and store frozen in quantities convenient for thawing and use]. Bring total volume of f/2 medium to 1 l with more filtered SW and sterilize. Omit the silica for algae that do not require it for their skeletons or when growing algae with SW rich in silica.
3. Centrifuge capable of up to ~3,000×*g*: for separating algal cells from their culture medium.
4. Hemacytometer with Neubauer rulings: for cell counting.
5. Lugol's fixative for fixing algal cells: dissolve 2 g potassium iodide and 1 g iodine in 100 ml distilled water. Other recipes maintain this ratio of solutes but with greater or less concentration in water. Some add acetic acid or sodium acetate. Presumably all are sufficient to fix algal cells for counting.

2.9 Food for Larvae That Eat Small Animals

1. *Artemia* cysts (from aquarium shop, mariculture supply, or biological supply). To hatch *Artemia* cysts, use the following: aquarium pump with tubing, container with tube entering bottom for air bubbling, and aquarium heater (or warm room). An inverted plastic jug with stopper with tube for air hose inlet at the narrow bottom (formerly the top) and its former bottom removed is cheap; a separatory funnel with air entering the bottom outlet is convenient; an air stone in any nontoxic container with narrow bottom is sufficient. To separate hatched *Artemia* nauplii from debris: a fiber-optic or other light that can be directed at a small area. Alternatively, an opaque container with hole drilled in its side, placed inside an outer transparent container. The opaque container can be constructed from any completely opaque nontoxic material that can be drilled. Because the *Artemia* nauplii are hardy, the inner container can also be made opaque by covering it with black tape. Algae (cultured as described above) can be used for feeding *Artemia* nauplii, if daily hatching is inconvenient.
2. The rotifer *Brachionus plicatilis* has been used to feed those zoea larvae of crustaceans and fish larvae that need smaller prey. Obtain from aquarium or mariculture supplier. *I. galbana*, *N. oculata*, or other algae rich in appropriate highly unsaturated fatty acids can be used to feed the rotifers.
3. Natural plankton: use a plastic bucket or large plastic bottle in conjunction with nylon (Nitex) mesh filters of mesh sizes that retain desired plankton and eliminate others. Cut away the bottom of ≥200-ml plastic beakers and replace it with Nitex attached with hot-melt glue.
4. Other foods: suppliers for mariculture and aquarists sell foods like Instant Algae® (killed algae of several species) that are appropriate for rotifers, *Artemia*, and possibly some marine larvae.

2.10 Changing Seawater in Larval Cultures

1. For changing seawater in 3 l jars by reverse filtration, construct Nitex filters on a beaker of ~400 ml as described in Subheading 2.4, **item 4**, and attach a cable tie or other plastic handle for lowering the filter into the jar; use in conjunction with Tygon® or other nontoxic tubing for siphoning.
2. For changing smaller volumes (e.g., custard dishes) by reverse filtration, use a Nitex filter on a beaker of ~50 ml in conjunction with a turkey baster.
3. For removing individual larvae: Pasteur pipettes with latex or other bulbs (*see* **Note 14**). A Bogorov tray can aid the search for larvae (*see* Fig. 2).

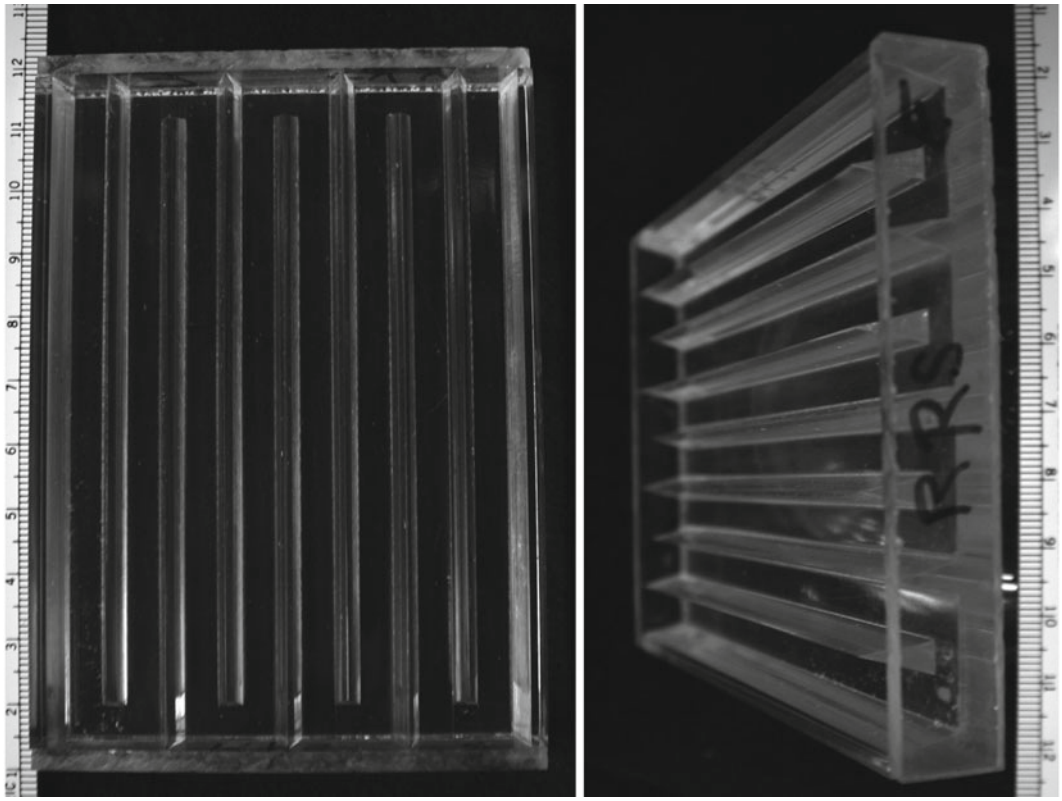

Fig. 2 Bogorov tray in two views. Ruler for scale is in cm and mm

2.11 Handling and Observing Individual Larvae

1. Microscope slides, cover glasses, and Plasticine (modeling clay).
2. If it is necessary to break microscope slides, pipettes, or glass tubing, use a tool for scoring the glass prior to breaking (*see* **Note 14**).

3 Methods

3.1 Stirring Cultures

Stir cultures by swinging paddles, rotating paddles, aeration, or placing cultures on a rotating platform (*see* **Note 5**). Low densities of larvae permit fast and normal development with fewer failures, with densities depending on the size of the larvae. For sea urchin larvae, densities of ~1 ml^{-1} are sufficiently low at early stages; densities of ~0.1 ml^{-1} are better and sufficiently low at advanced stages. Larval body volume, biomass, metabolic rate, and maximum clearance rate (volume of water cleared of food per time) are indications of suitable larval densities.

3.2 Cleaning Containers and Stirrers

1. With few exceptions (e.g., multiwell tissue culture plates and disposable pipette tips), rinse new containers, paddles, and filters with RO water or filtered SW.
2. After use, scrub culture vessels, paddles, or other materials with a plastic fiber scrubber (without detergent) in tap water. Then rinse twice with RO (reverse osmosis) or distilled water before air drying. Unless there is contamination from dust, materials rinsed and air dried in this way can be used directly without more rinsing (*see* **Note 10**). If distilled or RO water is unavailable, materials can be rinsed with filtered SW before use. Also, some tap water is an adequate rinse for some larvae.

3.3 Collecting Seawater

1. SW of high quality from a non-contaminated source (*see* **Note 6**) may change as it passes through the pipes of a SW system. Low pH of SW exiting from a system of pipes is expected, has not been reported as a problem for larval culture, and can be remedied by aeration or other stirring. A change in suspended particles is expected and can be remedied by filtration.
2. Water can be collected from the ocean and stored. If possible, collect away from shore. If possible, collect from below the sea surface to minimize material from surface slicks. Remove large particles with a bag or paper filter and store in a refrigerator.
3. If using a commercial artificial SW, mix the dry product thoroughly before mixing a subsample with water. Check that adequate trace metals are included in the artificial SW. Artificial SW lacking adequate trace metals can be supplemented with f/2 trace elements, which include the chelator EDTA [9] (*see* Subheading 2.8, **item 2** and **Note 13**). For the MBL artificial SW, use the version with trace metals (*see* Subheading 2.3, **item 5**).

3.4 Filtering Seawater

1. Remove unwanted particles by filtration. Coarse filters are often adequate. A 30- or 50-μm nylon mesh is sufficient to remove predators and most animal eggs, while passing a variety of edible particles, but such coarse filtration may also necessitate more labor in cleaning cultures. A bag filter removes most particles greater than 10 μm. A paper filter is slower than a bag filter but satisfactory.
2. Membrane filters help remove smaller particles and greatly reduce introduction of other organisms. Micropore filters with a 0.45-μm pore size are commonly used. Prefilter SW with a fiber prefilter if the membrane filter clogs rapidly. With a SW system of sufficient pressure, in-line filter holders for filters of 47-mm diameter are convenient. Otherwise suction filtration is required. The low pressure associated with suction removes dissolved gases from solution, which then re-equilibrates.

Because membrane filters may contain materials to enhance wetting, it is prudent to discard the first water through the filter, especially when filtering a small total volume. Filtration removes most organisms but does not provide sterile SW.

3. If culturing larvae that have been removed from capsules at early stages, filter SW with a 0.22-μm or 0.45-μm membrane filter and then sterilize or pasteurize. Sterilize in an autoclave at ~120 °C for ~15 min or pasteurize by heating SW to 60 °C for 20 min on a hot plate or stove top or heat in a microwave oven just to initiate boiling (*see* **Note 11**). Antibiotics may be needed.

For algal cultures, sterilize SW as above, after adding the enrichment medium. Autoclave flasks containing the enriched SW medium at ~120 °C for ~15 min or microwave just to the point of boiling (*see* **Notes 11** and **13**).

3.5 Adjusting Temperature, Salinity, and Light Regimes

1. Control temperature if room temperature is not within the tolerated range of the larva or if development rates are needed. If available, an aquarium with a continuous flow from a SW system can be used as a water bath, but comparisons of development rates require more accurate temperatures, as with a cold room or a temperature-controlled water bath (*see* **Note 7**).
2. If the source of SW for cultures is an estuary with varying salinity, use the refractometer to compare salinities to the salinities where the larvae occur (*see* **Note 6**).
3. Ordinary room light of a wide range of intensities, spectra, and light/dark durations is usually adequate for the larvae. Light affects the behavior of some larvae and affects the growth of contaminating organisms and algae introduced as food. Alas, observations of larval development seldom include data on light.

3.6 Changing Seawater in Cultures

1. Change water more frequently at higher temperatures and at greater concentrations of larvae. A water change every 2 days at high temperatures (>20 °C) and less often (~twice a week) at low temperatures (~10–15 °C) is usually sufficient. The balance between the desire for less variable culture conditions and aversion to repetitive work decides the frequency of water changes. If growth at particular concentrations or kinds of food is being compared, frequent changes with renewed food may be necessary even when larvae are at low density because algal concentrations change from causes other than larval grazing.
2. Reverse filtration is rapid and sufficiently gentle for most larvae. Use a filter screen with mesh size substantially smaller than the larva but large enough to pass much of the debris. Use a siphon for cultures of large volume and a turkey baster for small ones.

3. Many mollusk veligers and some other larvae are unharmed if the culture is poured through a filter that is submersed in a bowl.
4. Larvae that swim to light, swim upwards, or sink concentrate themselves, aiding transfers.
5. In cultures maintained for weeks, aggregates of material larger than the mesh size will accumulate. If larvae do not separate themselves from debris by their swimming, concentrate the larvae by reverse filtration and then transfer them to clean SW with a pipette with an orifice larger than a larva (*see* **Note 14**). To remove all larvae from a volume of water without repeated searches of a dish, pour water into a Bogorov tray (Fig. 2) and scan along the troughs, taking care not to miss larvae under the meniscus at the edges of troughs.
6. Change containers and paddles when they are visibly fouled or more often.

3.7 Providing Algae as Food for Larvae

1. A convenient medium for growing algae is f/2 in SW. It is easier to purchase the medium than to make it but useful to know what is in it (*see* Subheading 2.8, **item 2** and **Note 13**). Filter SW. Add the enrichment to the SW before sterilization. The listed algae will grow well even after vitamins in the enrichment are lost from heating. Mix well, divide into Erlenmeyer flasks, and cap each flask with a double layer of aluminum foil that extends a few cm down the neck of the flask. Autoclave enriched SW at ~120 °C for ~15 min. After capped flasks are autoclaved, they can be stored for months at room temperature. If an autoclave is unavailable, heat in a microwave oven (*see* **Note 11**). For microwave heating, cover each flask with a glass beaker, paper, or heat-resistant plastic cap instead of aluminum foil.
2. All of the algae listed in Materials grow at room temperatures (~17–22 °C). A window without intense sunlight is usually adequate. A fluorescent light tube suspended about 20 cm from the algal cultures is more consistent. For slower growth, maintain stock cultures with fewer daily hours of light, at lower temperatures, or both. Culturing the cells on agar, with transfers by a flame-sterilized metal inoculation loop, is also possible [10]. To promote algal growth, swirl flasks every 1 or 2 days. Grow more food than needed and discard excess algal cultures rather than being caught short of food. Feed larvae with algae that are still growing. Algae that have depleted nutrients and slowed or ceased growth change composition. More frequent transfers of smaller food cultures provide food of more uniform quality than when feeding larvae from an aging algal culture of large volume. Look at change in color of algal cultures to see if they are still growing, nearly constant, or in decline. When transferring algae to a new flask of medium, handle caps

so that fingers do not touch the cap where it touches the flask's opening or the flask near its opening. For faster growth, air bubble algal cultures with an aquarium air pump, a cotton plug as a filter in the air line, and a gang valve to distribute air to flasks through multiple tubes. Do not air bubble stock cultures because it increases risk of contamination.

3. Add enough algae that the larvae have some algal cells in their guts, but do not overfeed. The SW should have little or no discernible color from the algae (*see* **Note 15**).

   For a known ration, count algal cells per volume of suspension and then add a volume of the suspension that gives the desired number of cells ml^{-1} in the larval culture. To avoid contamination from pipetting from the culture flasks, pour some of the algal culture into a separate container. Mix by swirling, decant a subsample of a few ml into a vial, and add a drop of Lugol's fixative. (Killing is necessary before counting if the algae are motile.) Place the cover glass on the clean and dry hemacytometer, mix the vial with a Pasteur pipette, touch the tip of the pipette to the groove of the hemacytometer chamber, and release the liquid, immediately removing the pipette so that the chamber fills evenly with no overflow. Add fluid to the chamber only once and do not overfill. The thick cover glass supplied with a Neubauer hemacytometer maintains a constant depth of the chamber of 0.1 mm. Each chamber of a hemacytometer with Neubauer ruling is divided into nine squares, 1 mm on each side (Fig. 3). A count of one of the nine squares gives number of algal cells per 0.1 mm^3. (Each of the 1-mm^2 squares is subdivided in a different way.)

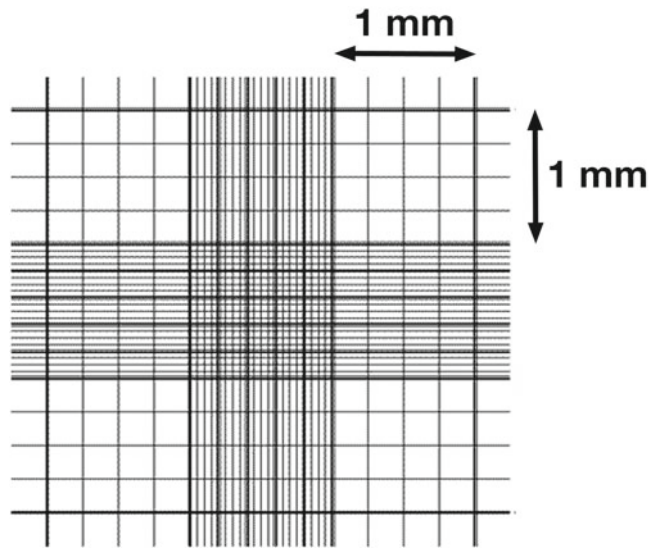

Fig. 3 Neubauer ruling on hemacytometer, with 1-mm × 1-mm square indicated

Variation in counts for different fillings of the chamber is high. Count from at least two separately filled chambers. A total of ≥200 cells gives an approximate estimate of concentration. Count enough of the 1-mm^2 squares for an adequate count. Clean the hemacytometer with alcohol and wipe dry.

4. Because organic content of small algal cells is nearly in proportion to volume, adjust numbers of cells added to cultures according to approximate volume. As examples, maximum ingestion for echinoid larvae with *R. lens* or *D. tertiolecta* occurs at about 5,000–10,000 cells/ml [13]. For feeding with *R. lens* alone, 5,000 cells/ml should provide near maximal growth if the larvae are at low concentrations. *D. tertiolecta* is smaller than *R. lens*, and *I. galbana* is about half the diameter of *D. tertiolecta*. About 5,000 cells of *D. tertiolecta* ml^{-1} and 40,000 cells of *I galbana* ml^{-1} should provide near maximal ingestion rates for many kinds of echinoderm larvae. Maximum ingestion for bivalve larvae with *I. galbana* occurs at about 100,000–150,000 cells/ml [14]. For feeding with *I. galbana* alone, that concentration is about right for many mollusk veligers, or it could be offered as a 1:1 mix of *I. galbana* and other small algae, such as *C. gracilis*, *N. oculata*, or *P. lutheri*. For approximate estimates of algal density, determine the relation between optical density and cell concentrations for suspensions of cells of each species used. The relationship does not give an accurate estimate of cell numbers because optical density changes with the state of the algae. When using two algal species as food, additions of similar total cell volumes from each species can be based on comparison of optical density by eye. It is often unnecessary to increase concentrations of food during larval growth and development (*see* **Note 16**).
5. Examples of successful feeding: different larvae grow best with different foods. The algae listed in Materials are in common use, but other algal species are also good foods. Check the literature for foods that have been successfully used for the larvae being reared. Here are some suggestions. Although the maximum sizes of algal cells captured commonly increase as larvae grow, the minimum size changes little for many echinoderm and mollusk larvae. Thus, one size, adequate for the earliest stages, can be used throughout their development [15, 16].

Numerous algae have been sufficient in combinations and several when added as single species, though reported diets may have had different supplements from unknown contaminant organisms. For echinoderm larvae, try *R. lens* alone [17] or in combination with another alga or try a mixture of *D. tertiolecta* and *I. galbana* (T-ISO) [3, 18, 19]. *C. gracilis* [20] and *D. tertiolecta* have each been used alone, although *D. tertiolecta* is a poor single species diet for some larvae. Development of sand dollar larvae was faster with

a mixed diet of *I. galbana* and *D. tertiolecta* than with either alga alone [3, 18, 19].

Molluscan veligers have been reared with *I. galbana* (usually T-ISO) alone or in combination with *C. gracilis*, *P. lutheri*, or *N. oculata*. Molluscan veligers capture small particles and small veligers do not ingest large particles. Large gastropod veligers have been reared with the larger cells of *Rhodomonas* sp. [21].

A nemertean pilidium has been reared through metamorphosis with a *Rhodomonas* sp. alone.

Barnacle nauplii have been reared with *S. costatum* alone [4].

3.8 Providing Animals and Natural Plankton as Food for Larvae

Larvae of some decapod crustaceans can be reared on *Artemia* nauplii as their sole food; others require small prey [22] or require smaller prey at early stages and grow better with larger prey at later stages [23, 24]. Many small animals can serve as food. Rotifers are a small prey and *Artemia* nauplii a larger prey that are widely available from aquarium shops and mariculture suppliers (*see* **Note 17**).

1. Store *Artemia* cysts cool and dry in a tightly capped container. Wash cysts in filtered SW before use. Hatch at a density of ~5 g of cysts per liter of water with illumination with a temperature of ~20–30 °C (best at ~28 °C) in water heavily aerated from below. The water can be SW of full salinity or SW somewhat reduced in salinity. Aerated SW should have a pH near the desired pH 8. Hatching is in ~2 days at 20 °C.

   Attract hatched nauplii to light in a small part of the hatching chamber (after stopping aeration) to harvest them separately from unhatched cysts and hulls. If nested opaque and clear chambers are available for harvesting, shine the light at the hole in the inner opaque container so that the nauplii swim out into the surrounding container to be harvested as food. Because hatched nauplii rapidly deplete their nutrient reserves, they should be used within the day of hatching or be fed (*see* **Note 17**).
2. The rotifer *Brachionus plicatilis* has been used as a smaller prey for some zoea larvae of decapod crustaceans and fish larvae [8, 25]. Obtain from an aquarium store or mariculture supplier. Feed rotifers with algae such as *I. galbana* and *N. oculata*, and transfer a portion of the culture to new water and containers at intervals (*see* **Note 17**). The rotifers can be reared in dense cultures. For harvesting, separate rotifers from algae and debris and concentrate them by filtration, or they can be settled by adding distilled or RO water for 5–15 min and then resuspended in filtered SW before use as food.
3. Natural plankton as food: for algal cells, collect SW in a large bottle, held beneath the water's surface. Pour the water through a mesh of ~30 μm and use it as the SW for larval culture. Collect zooplankton with a plankton net. After collection, resuspend

the concentrated plankton in a larger volume of SW to reduce mortality. Large plankton can be eliminated by pouring the sample through a mesh that passes desired prey. If necessary, smaller plankton can be eliminated by reverse filtration.

3.9 Culturing Buoyant or Hydrophobic Larvae

1. Larvae that float at the surface are often fragile. Ruptured embryos or larvae produce a slick of decaying material, and the floating larvae must be provided with clean SW. Although culture in still water has succeeded, improvements are mesh-bottomed beakers suspended in a SW aquarium with continuously renewed water [26, 27] or in similar bottomless beakers and aquarium with a drip of filtered SW from above [28].
2. Hydrophobic larvae, such as opisthobranch veligers and rhizocephalan nauplii, become caught in the surface film. One remedy is sprinkling flakes of cetyl alcohol on the surface of the culture [8, 29]. Another remedy is a drip to the surface of a culture [27], which can be achieved with air bubbled to one arm of a Y-tube that lifts water from an outer beaker and drips it onto the surface of a culture in an inner mesh-bottomed beaker [8]. Yet another remedy is to eliminate the air-water interface by sealing larvae and their food within bottles that are then rotated on a roller; continuous light was provided and oxygen did not decline [30]. A plankton wheel, to which capped containers are strapped and then rotated at ~1–2 rpm around the wheel's axis, might also suffice to keep larvae and algae suspended in containers without an air-water interface.

3.10 Observing Larval Condition During Rearing

1. Keep track of development by removing a sample of larvae. Observing larvae in a dish under a dissecting microscope minimizes stress but precludes close observation (*see* **Note 18**). For observations with a compound microscope, avoid crushing larvae under the cover glass by supporting the cover glasses with a small quantity of Plasticine (modeling clay) at each corner. Some larvae can be entrapped by compression of the Plasticine supports until the larvae are touched by cover glass and slide.
2. To aid observations of living larvae, the following anesthetics can be used for reversibly reducing larval motility [8]: isosmotic $MgCl_2$ (0.33 M) mixed ~1:1 with SW relaxes muscles of many kinds of larvae without permanent damage but does not prevent swimming of some ciliated larvae; chloretone, a few drops of 1 part saturated aqueous solution and 2 parts SW added to the water; MS-222, final concentration of ~0.5 mg/ml in SW (store the stock solution in the dark; ineffective if it turns yellow); propylene phenoxytol, stock solution 1.5 % in distilled or RO water, refrigerated, >100× dilution in SW to anesthetize larvae.

3. If sampling is to be done without returning larvae to the culture, larvae can be slowed with polyethylene oxide of ~5×10^6 molecular weight, added as a powder to the microscope slide, or killed by ethanol or a fixative, but replacement of the larvae following observation is often necessary when checking sparse cultures of larvae. Some larvae can be immobilized by very slight compression from the supported cover glass or entrapped in a cul-de-sac formed by slivers of glass or other materials under the cover glass.
4. Vital stains can be used to distinguish between larvae subjected to different initial treatments and then reared in the same container [8, 31]. For echinoderm larvae and some others, incubate 5–20 min in SW with 5 ppm of neutral red; Nile blue sulfate can be used similarly if two stains are needed [32]. Extent of adverse effects has varied. To control for staining effects, reverse staining between treatments, with replicates for each stain-treatment combination. Alternatively, calcium carbonate shells and skeletons can be marked with fluorescent stains (tetracycline or calcein) that also mark a growth line at the time of staining. Bivalve larvae were stained with calcein at 50 and 100 ppm for 2 and 3 days [33].

3.11 Stimuli That Induce Metamorphosis

Stimuli differ among species and include materials from conspecifics, prey, and substrata [34].

1. Because bacterial exopolymers stimulate settlement of many larvae [35], a dish left in an aquarium can acquire a biofilm that may induce settlement.
2. Water flow and surface topography also affect settlement [36].
3. An elevated concentration of potassium ions induces metamorphosis of larvae of several phyla, but the effective concentrations (between ~10 and 30 mM excess K^+) differ among species [37].

4 Notes

1. Many materials are generally nontoxic when used in larval culture**:** polyethylene and Tygon® tubing, polyethylene containers (and many other plastic containers), acrylic plastic (including acrylic plastic joined by chloroform as solvent, after the chloroform evaporates), PVC (as in SW systems, after continuous flow has removed solvents or catalysts), hot-melt glue, clear silicone glue (after the acetic acid evaporates), nylon plankton netting, bag filters, membrane filters, glass filters, most paper filters (including coffee filters), membrane filters, parafilm, and plastic wrap as covers.

Advantages of glass containers include weight-related stability, durability with repeated scrubbing, and high melting point (if heat sterilization is necessary). Advantages of plastic are low cost and light weight for transport.

Rinse most new containers, tubing, and filters before use because of the possibility of toxic residues from the factory or storage. Exceptions are materials like disposable pipette tips and multiwell culture plates.

Glassware and other materials for cultures can be specially labeled (as with an "E" for embryological). If glassware is contaminated, special cleaning is required, or the "E" should be removed and it should placed with non-"E" stock.

2. Many materials are often toxic, especially if residing in batch cultures: latex tubing, black rubber tubing, black rubber stoppers, some heavy metals in solution, and some metal objects. Also, potentially toxic are containers with a residue of formalin, glutaraldehyde, osmium tetroxide, or detergents from previous uses.

   Where most materials are for culture of embryos, glassware that may have toxic residues because of exposure to toxic materials can be specially marked (as with an "X"). Avoidance of other potential sources of toxicity:

   When in doubt and especially when filtering a small volume of water, discard the first water through a filter.

   Clothespins with a piece of labeled tape attached are convenient for labeling cultures, but check that metal parts are not corroded. Corroded metal could be shed into the culture.

   Avoid hand lotions.

   An air stream used for stirring can introduce noxious materials with dust. A cotton plug should be inserted in air lines between the aquarium air pump and the cultures. A strong air stream from room ventilation may blow noxious materials into open cultures. If that appears to be a problem, cover cultures with a sheet of clean plastic.

3. Large jars permit automated stirring of ~2 l of water by a paddle, with 100 or more larvae per jar. Searching for larvae cultured as single individuals is aided by rearing them in dishes with sloping sides that fit under a dissecting microscope. Custard dishes containing ≥100 ml of SW are large enough for automated stirring by a paddle. Shot glasses are useful for individual larvae but not large enough for paddle stirring. Multiwell culture plates (Falcon™) are convenient for single larvae. They are usually used without stirring, though multiwell plates can be rotated on a shaker at 40–60 rpm [9].

4. A 125-ml Erlenmeyer flask is convenient for continuing stock cultures in ~50 ml of water. A 500-ml flask is convenient for growing food for larvae in ~200 ml of water. The low water

level gives a greater surface for gas exchange. Flasks for food can contain a greater volume of water if aerated.

Aluminum foil is a convenient cap when sterilization is by autoclave or by pasteurization with a hot plate but cannot be used with a microwave oven.

5. The protocol gives dimensions of a stirring frame and paddles of dimensions for large jars, but dimensions can be altered to fit the space and containers used for culture. In contrast, the smaller stirrer in Fig. 1 (with PVC pipe of smaller diameter) was made for custard dishes holding 100 ml of water and one or few larvae per dish.

   PVC plumbing pipe and joints make a frame that can be disassembled for transport. Cut legs of the fixed frame long enough and hang the swinging frame low enough that the swinging frame pushes each paddle across the width of a culture container. The swinging frame must clear the legs of the fixed frame and clear the top of culture jars.

   The motor can be quite lightweight because little force is required. Supporting the motor on an extension from the stirring rack makes positioning of the motor relative to the stirring frame easier. String is the lightest and most convenient way to support and push paddles, support the swinging frame, and join the eccentric attachment on the motor to the stirring frame. The motor pulls the frame forward and gravity moves the frame and paddles back.

   Acrylic plastic sheets (Plexiglass) are a good material for paddles because they can be cut with a power saw and joined with chloroform, which dissolves the plastic, then evaporates without a residue. Hot-melt glue might substitute for chloroform but has not been tried. Other materials may provide easier construction of paddles, but I know of no reports on successful alternatives.

   Stirring distributes larvae and food more evenly. An advantage of the frame and paddles is that a large volume of water is moved with low shear. Another is that a rack with one motor can stir at least 20 jars for a total of 40 l of SW. That permits culture of 4,000 larvae at a low density of 1 larva per 10 ml. With stirring, many kinds of small larvae, such as those at early stages, can be reared at higher densities, ~1 ml^{-1}, if necessary. Multiple jars also provide replication. Each jar provides one replicate for a statistical test without pseudoreplication; numerous jars provide more replication per treatment (e.g., 20 jars allow 5 replicates of 4 treatments). When a paddle stirrer is scaled down for 100 ml volumes in custard dishes, individual larvae develop in a large volume of moving water.

   A disadvantage of paddle stirrers is that cultures are open to the air, which can introduce other organisms from SW spray

if an aquarium is used as a water bath. In rooms with a strong airflow, dust may contaminate cultures, necessitating a dust cover. Rotating paddles, with a motor on a plastic cover for each jar, is one alternative, but many motors are required. Magnets mounted on the shaft from a motor can drive rotating paddles in numerous jars [17], but the water volume is more limited than with the stirring frame.

6. Source of SW is important, whether it is piped to the lab or collected in a bucket. SW from bays with ports, marinas, or urban or industrial runoff is often contaminated with toxic materials. SW in recirculating systems that serve several aquaria is usually of poor quality for larval culture.

   Common salinities along open coasts are 32–35.5 ppt. Many larvae, though from open coasts, tolerate salinities down to 30 ppt or lower.

7. An indication of tolerated temperatures is the range where the larvae develop in nature. Within a species, temperature tolerances for development can change with season or differ between regions [38, 39]. Temperature control for cultures is often needed in laboratories heated or cooled for human comfort. A heating-cooling circulating bath (e.g., Thermo Scientific [formerly Haake] or Lauda-Brinkmann) can pump freshwater to a higher external bath for cultures, with the water returned by gravity. Prevent corrosion of the circulating heater-cooler by using RO or distilled water and avoiding SW spills into the bath. Water level in unstirred containers should be near the level of water in a cold bath to avoid heating above the bath's waterline. If relying on a temperature-controlled room, decrease short-term variation in temperature by placing containers in a large water bath.

8. SW is nearly 1 osM. To maintain osmolarity when substituting ions, one can add salts in a solution of ~1 mol of ions, as in 0.5 M KCl or 0.33 M $MgCl_2$, although solutions isosmotic with SW of 35 ppt would be 0.53 M KCl and 0.37 M $MgCl_2$ [11].

9. Alternatively a filter can be connected to a siphon. One method is to cut off the flat part of the screw cap for a 50-ml polypropylene centrifuge tube (Falcon™), screw the cap over Nitex mesh, trim off excess mesh material, cut off the pointed end, and seal a tube into the hole in the pointed end with hot-melt glue [9]. A smaller filter can be made by attaching the Nitex over the large end of the disposable plastic tip for a 1,000-μl automatic pipette; fasten the Nitex mesh with a short band cut from Tygon® tubing that fits snuggly; then insert the small end of the pipette into the flexible tubing.

10. "E" containers for cultures can be exposed to SW, tap water, distilled water, or RO water. Plastic fiber scrubbers

(without detergent, sold for household use) and a bottlebrush can be used to remove organic material from glassware without solvents. If additional cleaning is considered necessary, dilute sodium hypochlorite (household bleach), dilute acetic acid, and dilute HCl can be used without compromising "E" ware by a toxic residue if rinsing is thorough. Also, substances that are metabolized, such as ethanol and acetic acid, are adequately removed by rinsing.

11. With usual culture methods, neither larval nor algal cultures are free of bacteria. Although contamination with bacteria harmful to larvae has occurred in aquaculture [40], cultures with bacteria but without other eukaryotes are usually sufficient for algae used to feed larvae. Nevertheless, sterilization is needed to minimize contamination of algal cultures and to protect larvae that have been removed early from egg capsules.

    The duration of microwave sterilization depends on the volume of fluid and the power of the microwave. With a 700-W microwave and 1.5 l of SW, bacteria were eliminated in 5–8 min [41].

12. Obtain starter algal cultures from UTEX the Culture Collections of Algae at the University of Texas at Austin, the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Carolina Biological Supply, or other suppliers of algal cultures. Reports of value of algae added as foods do not preclude presence of other material in the diet. Algal cultures designated as the same species but from different isolates may differ in quality. Cultures of algae are designated by codes, such as the T-ISO strain of *I. galbana*. Noting the culture code is good practice, though as algal cultures are passed around, this information is often lost and thus unreported. Algae with the same culture number may diverge in quality and cell size during repeated growth and transfers. Without extraordinary precautions, algae maintained as food are rarely if ever free of bacteria. Larval cultures become contaminated with other organisms introduced with the starting embryos or larvae or later. Dissolved and particulate organic material accompanies the SW. Of the listed algae, *Rhodomonas* spp. grows more slowly in culture and more commonly fails as a culture, but it is convenient as an adequate sole food for many echinoderm larvae.

13. Buying Guillard's medium f/2 for addition to SW is more convenient than making it from reagent grade ingredients. It can be obtained from Fritz Pet Products (500 Sam Houston Rd. Building F, Mesquite, Texas, 75149-2789). Component A, with the vitamins, should be refrigerated for storage and both components (A and B) can be stored refrigerated and used for years.

14. To adjust a pipette to larval sizes, enlarge a Pasteur pipette by scoring and breaking it or pull the pipette to a finer tip after heating it in a gas flame.

    If finer control is needed, construct a braking pipette by pulling a capillary tube until there is a narrow constriction at one end, then cementing it into a glass tube so that the constriction is at the inner end, restricting airflow. Attach a flexible tube to the pipette instead of a bulb. Suck or blow on the tube to control airflow and thus water flow. Consistent constrictions in the capillary tube can be achieved with an automatic pipette puller, as used for microelectrodes [42]. For a braking pipette with larger capacity, pull a portion of a Pasteur pipette to the desired tip diameter for the outer end and a very small diameter for the inner, braking end.

15. For many kinds of larvae, algal medium need not be removed from algal cells used as food, but some larvae are sensitive to materials dissolved in algal cultures. Also, comparisons of effects of different kinds or concentrations of algae are best made without confounding effects of different quantities of enrichment solution and algal exudates. Medium can be removed from the algal cells by centrifugation and resuspension of algal cells in seawater. A few trials will indicate the time and speed necessary to pellet the algae; 1 min at ~2,300 rpm (~3,000 × *g*) is a recommended centrifugation from a protocol that lists the same algae as this one [9]. Discard the supernatant and resuspend the pelleted cells in a volume of filtered SW that gives a cell concentration convenient for counting. After the first trial centrifugation, inspect algal cells for damage.

    If undesired effects from algal medium are suspected, algae used for feeding (as distinct from stock cultures of algae) can be grown in simplified nutrient enrichment with inorganic nitrate and phosphate added to SW at the same concentration as in the usual enrichment medium.

16. Some protocols recommend increased concentrations of algae as larvae grow. Larger larvae can clear a larger volume of water of food per time, but for many ciliary-feeding larvae, the satiating concentration of food changes little during their growth and development [13, 14]. If larvae are not so concentrated that they clear much of the SW of food between water changes, then the same concentration of algae can be used throughout their development.

17. The quality of *Artemia* nauplii as food varies among populations, from maternal effects, and from their diet. The quality of rotifers (*B. plicatilis*) as food varies similarly. *B. plicatilis* supported the development of crab zoeae to the megalops stage when the rotifers were fed with *I. galbana* but not when they were fed with *D. tertiolecta* [25]. There is an extensive literature

on how to improve *Artemia* and *B. plicatilis* as food and on their genetics inspired by their use in aquaculture [43, 44]. The web and instructions from suppliers are sources for methods beyond this protocol.

18. No single measure fully represents the stage of development because larvae develop with different body proportions or sizes at stage under different conditions [2–6]. Some echinoderm larvae divide or bud to produce dwarfed larvae [7]. For measurements, use a microscope equipped with an ocular micrometer that is calibrated with a stage micrometer.

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Chapter 2

Obtaining Gametes and Embryos of Ascidians

Charles C. Lambert

Abstract

Ascidians are basal chordates that have become increasingly important for understanding chordate evolution. They comprise three orders. In the orders Phlebobranchia and Stolidobranchia, most species freely spawn eggs and sperm, whereas members of the order Aplousobranchia form colonies that brood their eggs and broadcast sperm. In the two free spawning orders, eggs and sperm are easily obtained for in vitro fertilizations. In the third order, slices of colonies yield gametes and embryos of all stages. Methods are described for obtaining gametes, performing fertilizations, and culturing embryos. Also included are methods for removing follicle cells and vitelline coats from oocytes.

Key words Ascidian, Tunicate, Aplousobranchia, Phlebobranchia, Stolidobranchia, Demembranation, Defolliculation

1 Introduction

The phylum Chordata includes the subphyla Vertebrata, Tunicata, and Cephalochordata. Tunicates are currently considered to be the sister group of the vertebrates [1]; they include the class Ascidiacea which are all sessile as adults and the pelagic Appendicularia and Thaliacea. Ascidians include both solitary and colonial forms. All tunicates are marine and require a salinity of at least 20 parts per thousand for development. All ascidians are hermaphroditic; some are protandric, but most are simultaneous hermaphrodites. Self-fertilization is possible in some species, but many are self-sterile [2]. The ascidians include three orders: Aplousobranchia, Phlebobranchia, and Stolidobranchia [3]. Aplousobranchs are all colonial with small zooids only a few mm in length embedded in a common tunic matrix. Each zooid in the colony is cloned from buds. Because of the small size of the zooids, only a few eggs are produced per zooid. The eggs are generally large and may take

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weeks to develop fully. Aplousobranchs spawn large complex sperm which fertilize brooded eggs. This type of reproduction has been termed spermcast reproduction [4]. Aplousobranch embryos are difficult to culture outside the colony, so most studies of their development come from embryos dissected from colonies. The location of embryos within the colony varies from family to family. Embryos may be within the zooid atrium, in brood pouches, or in the basal tunic of the colony.

Phlebobranch and stolidobranch ascidians are mostly solitary free spawners with eggs from 100 to 300 μm. Thousands of eggs are released at each spawning with fertilization occurring in the sea. These eggs are small compared to those of aplousobranchs, and the sperm is also much smaller and less complex than that of aplousobranchs. In the Stolidobranchia, the family Styelidae includes a number of colonial species. *Botrylloides violaceus* produces small eggs with a true placenta forming very large and complex larvae which may take several zooid generations to develop [5]. Only a few phlebobranchs are colonial. *Ciona intestinalis* is a large solitary ascidian that has been classified as either aplousobranch or phlebobranch in various publications. It has some properties of both groups. Based upon its small and simple sperm, its egg and tadpole larva, as well as its large size and solitary nature, it is considered a phlebobranch here.

2 Materials

Traces of detergents, soap, fixatives, and metal salts are toxic to gametes and embryos. Thus, it is best to use new glassware and plasticware whenever possible. Glassware can be washed with Micro cleaner and rinsed several times with very hot tap water followed by distilled water rinses; all glassware and instruments must be free of contaminants. If in doubt, wash.

2.1 Buffers and Seawater Solutions

1. Buffer A: 0.56 M NaCl 1 % EDTA (tetrasodium) pH 4.2.
2. pH 6 SW: with a pH meter measure pH and add 1 N HCl dropwise to clean SW to pH 6.
3. pH 9 Tris SW: with a pH meter add dropwise 1 N Tris to pH 9.

2.2 Solutions for Removing Vitelline Envelopes

1. Treat eggs with 0.1 % trypsin solution in SW for 2 h. Pipette to remove membranes. Wash thoroughly.
2. Buffer B: to 20 ml seawater (SW), add 0.15 g dithiothreitol, 0.72 g CHES, and 0.02 g pronase E = Sigma P6911 or P5147 pH 9.2 (make fresh).

3 Methods

3.1 Obtaining Gametes and Embryos from Aplousobranchs

Colonies are invariably asynchronous with regard to blastogenic and embryonic stages, making it necessary to collect and dissect several colonies to be assured of obtaining the required stages. Since aplousobranch eggs are brooded for extended periods and difficult to culture in the laboratory, it is usual to dissect colonies and isolate the required stages [6, 7]. Place the colony in a 60 mm Petri dish and slice it into 1–3 mm slices. Under a dissecting microscope, remove the required embryos. In *Distaplia* (family Polycitoridae), the embryos are embedded in the colony within a brood sac that may be independent of the zooids. In *Aplidium* [7] and other species from the family Polyclinidae, as well as other polycitorids, zooids brood their embryos in enlarged thoracic atria. Didemnid embryos are brooded in the tunic at the base of the colony below the zooids. Thus, it is important that the basal tunic is included when colonies are collected. This is also true for the colonial botryllid stolidobranch *B. violaceus*, though in other botryllids, the embryos are brooded in the zooids. Swimming tadpoles can be collected with a tadpole collector in response to light following darkness [8].

3.2 Obtaining Gametes and Embryos from Phlebobranchs

Store adults under continuous light to prevent light-induced spawning [9]. *Ciona* and *Corella* spawn when exposed to light for about 30 min after a 1 h dark period [9–11]. *Ciona* is self-sterile, so be sure to spawn or dissect at least two if you want fertilization [12]. Dissection is necessary for other phlebobranchs.

1. In the family Ascidiidae which includes *Ascidia*, *Ascidiella*, and *Phallusia*, remove the tunic to expose the gonoducts which are easy to see when full of gametes (*see* **Note 1**).
2. Dry the animal thoroughly with a Kimwipe, being especially thorough in the region of the gonoducts.
3. The oviduct overlies the sperm duct. Using a needle held parallel to the oviduct, carefully make an opening, and the eggs will stream out. The eggs are held together by a viscous mucus coating. Pick up the eggs on the *outside* of a dry Pasteur pipette. The mucus will stick the eggs to the outside of the dry pipette. Keeping the operating area dry prevents the eggs from streaming away (*see* **Note 2**).
4. Remove the tunic from *Ciona*, and open the atrium with fine scissors to expose the gonoducts. Dry the animal and remove eggs as for other phlebobranchs. After the eggs are removed, the sperm can be collected "dry" (i.e., in a concentrated mass) with a dry Pasteur pipette or a positive displacement pipette if sperm are scarce. Store sperm in an iced Eppendorf tube. Dry sperm may be stored up to a week in the refrigerator.

3.3 Obtaining Gametes and Embryos from Stolidobranchs

Store animals under continuous light to prevent unscheduled spawning [13] and to accumulate gametes. As is the case for *Ciona* and *Corella*, *Molgula* will spawn in response to a short light cycle [11]. Many species of *Styela* and sometimes other stolidobranchs will spawn if stored in the dark for 12 h or so and then exposed to light for 12 h. Many stolidobranchs are self-sterile, so it is necessary to use two or more animals to start a culture. If you wish to experimentally fertilize a culture, it is necessary to use a self-sterile species so that fertilization does not occur while dissecting eggs (*see* **Note 3**).

Many but not all species of the stolidobranchs including *Boltenia*, *Herdmania*, *Pyura*, *Styela,* and *Halocynthia* are self-sterile. *Molgula* spp. are generally self-fertile [2]. A few solitary stolidobranch and phlebobranch species are ovoviviparous. In this case, opening the atrium will release the brood.

1. To obtain gametes, clean epibionts from the tunic and bisect with a razor blade through the siphons. Remove from tunic and lay out the isolated half animals in a 60 mm Petri dish covered with SW. With forceps, remove and discard the branchial basket to expose the ovary and testis in the body wall. Oocytes with intact germinal vesicles are stored in the ovary. Germinal vesicle breakdown occurs within 30 min of dissection in pH 8 SW. If you want oocytes with intact germinal vesicles, dissect in low pH SW [14].
2. Remove the gonads and place in 35 mm Petri dish, chop with fine scissors, then pipette in and out of a Pasteur pipette. Be sure the scissors are clean and not contaminated with formaldehyde or detergents.
3. Pour through coarse (300 μm) Nytex. Squirt SW through tissue remaining on filter to remove more eggs and sperm. Pour egg/sperm suspension into 100 ml beaker with SW. Use a larger beaker if necessary.
4. Wash oocytes in a beaker by placing a 100 μm filter which is attached to the bottom end of a 38 mm Plexiglas tube 70 mm long into the beaker. Withdraw supernatant SW from the Plexiglas tube with a large-volume syringe or pipette to remove the supernatant. The filter allows removal of supernatant without loss of mature oocytes. Wash until supernatant is clear of sperm, adding more SW to the beaker as needed. This method also removes small immature oocytes that pass through the Nytex mesh. The Nytex screen prevents eggs from entering the interior of the tube where the supernatant is collected (*see* **Note 4**).
5. Eggs from colonial styelids are obtained by dissection as described for aplousobranchs. Embryos of *B. violaceus* are in the base of the colony. Thus, be sure to collect the whole

colony and not just the surface layers. The embryos of *Botryllus* spp., other *Botrylloides* spp., and other colonial styelids are brooded inside the atrial cavity of the individual zooids.

4 Fertilizations and Culture Methods

1. To insure synchronous fertilization, soak eggs for an hour in pH 6 SW. Wash 4× with normal pH 8 SW.
2. Dilute dry sperm 1:1,000 with clean normal SW or pH 9 Tris SW (*see* **Note 5**).
3. Add 10 ml diluted sperm to 100 ml of egg suspension. Most ascidians do not have a fertilization membrane. Thus, it can be difficult to know if fertilization has occurred before cleavage. However, if you examine the eggs with a 100× microscope 5 min and 15 min after fertilization, you will see that fertilized eggs are now quite elongate or pear shape [15]. About 10 min after fertilization wash off exogenous sperm with clean SW (*see* **Note 6**).
4. For short-term culture, you can leave the embryos in a monolayer in the bottom of the beaker where they will develop well to hatching without any aeration or agitation. Swimming tadpoles can be collected from the upper levels of the beaker; they are photopositive when first hatched but become photonegative when ready to settle.
5. For large cultures, place the embryo suspension in a 1 gallon jar and agitate with a plastic propeller at 30–60 RPM. Large quantities of tadpoles and newly metamorphosed tadpoles may be cultured in this manner.

4.1 Follicle Cell Removal and Isolation

1. To remove follicle cells of phlebobranch eggs, place eggs in Ca^{2+}-free SW containing EGTA and agitate vigorously [16]. Alternatively, rapidly squirt eggs in and out of a syringe with a 26 gauge needle (*see* **Note 7**).
2. For stolidobranchs like *Boltenia* and *Halocynthia*, removal of follicle cells is accomplished by a modification of a method by Fuke [17]. Oocytes are placed in a 15 ml centrifuge tube of Buffer A and shaken periodically for 30 min followed by 20 passages through a 163 μm Nytex filter. Follicle cells can then be separated from oocytes by passage through a 100 μm Nytex filter and pelleted in a clinical centrifuge at Ravg 1600 (Rmax 2683; 4,000 RPM) for 10 min and then re-suspended in SW [18].

4.2 Removal of Vitelline Coats

Originally vitelline coats were removed with needles, and this method is still in use when it is desirable to avoid the use of enzymes

[19]. To remove the vitelline coat, follicle cells, and test cells of *Phallusia*, agitate eggs in trypsin [20] (*see* **Note 8**).

The following method works on *Ascidia*, *Ciona*, and *Boltenia* eggs.

1. Add oocytes to freshly made Buffer B.
2. Rock gently 1.5–2 h (Nutator or other device that gently agitates gametes).
3. Place in tube, and shake fairly vigorously.
4. Wash by settling in clean SW.

5 Notes

1. Wear gloves when dissecting ascidiid tunicates as blood will leave a green stain on your fingers.
2. Do not suck up the eggs as they will stick to the inside of the pipette and are difficult to recover.
3. To avoid self-fertilization in self-fertile species, dissect eggs into pH 6 SW and wash the eggs with pH 6 SW until free of sperm. Return the eggs to pH 8 SW and observe at expected time of cleavage to be sure that no self-fertilization occurred [10].
4. Be sure to aspirate supernatant slowly enough that eggs are not forced through Nytex. Check with microscope to be certain that no eggs are being lost.
5. Check motility of diluted sperm without a coverslip using dark-field illumination and 40–100× magnification. To obtain dark-field illumination, shift the phase rings of a phase contrast microscope slightly off center. If sperm do not show vigorous translocational swimming, use pH 9.2 (Tris) SW to make another sperm preparation.
6. Left over sperm will rot and bacteria tend to kill embryos.
7. Be careful to examine *Ciona* eggs with a compound microscope as many treatments break the elongated *Ciona* follicle cells leaving the base of the cells still attached to the vitelline coat.
8. Another demembranation method utilizes higher pH and thioglycolic acid [21]. Demembranated eggs and embryos stick and lyse when they contact glass or plastic. To prevent this, coat glassware, plasticware, and cover slips with 1–2 % agar or as follows: soak briefly all glassware, plasticware, etc., in 0.1 % gelatin, 0.1 % formaldehyde in distilled H_2O. Rinse in tap then distilled water, drain thoroughly, air dry, and store until needed [22].

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Chapter 3

Artificial Seawater Culture of the Gastropod *Crepidula fornicata* for Studies of Larval Settlement and Metamorphosis

Anthony Pires

Abstract

The slipper limpet, *Crepidula fornicata*, is a gastropod mollusc of growing importance as a research model in developmental biology and as an invasive organism. The large (>1 mm) veliger larvae of this species are well suited for neuroethological investigations of settlement and metamorphosis. In this chapter, methods are described for conditioning adult broodstock, growing microalgal food for larvae, and culturing larvae to metamorphic competence in artificial seawater. A protocol is also presented for obtaining electrophysiological recordings of ciliary arrest spikes from intact, behaving larvae, as putative neural correlates of larval settlement.

Key words Gastropod, Veliger, Larva, Settlement, Metamorphosis, Neuroethology, Ciliary arrest

1 Introduction

The calyptraeid gastropod, *Crepidula fornicata*, is a cosmopolitan species of unusual importance. Although historically significant in classical studies of embryogenesis [1], it is only in the last decade that *C. fornicata* has re-emerged as a valuable model system for lophotrochozoan development (comprehensively reviewed in ref. [2]). In addition to its new utility for cellular, molecular, and genomic studies of early development, this species has been the subject of numerous studies of growth, energetics, and life history [3–9]. Regulation of larval metamorphosis, a key developmental and ecological transition, has been studied with respect to environmental cues and constraints [10–12] as well as internal signaling mechanisms [13–16].

Native to eastern North America, *C. fornicata* ("slipper limpet") is an invasive species that has become established in the American Pacific Northwest, Europe, and Japan [17, 18]. Its superabundance in some European locales has raised concerns about ecological

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impacts (reviewed in ref. 19). This range expansion has increased its availability as an object of research and added to the value of understanding all aspects of its biology. However, despite the substantial volume of literature dealing with post-hatching larval biology of *C. fornicata*, a complete protocol for rearing larvae to metamorphic competence has not been published.

The goal of the present contribution is to facilitate the use of *C. fornicata* in mechanistic studies of larval settlement and metamorphosis, by providing simple culture protocols that are adapted for inland laboratories without ready access to natural seawater. As reproductive adults are prerequisite for larval culture, the first protocol given is for conditioning adult broodstock. Adults of *C. fornicata* are suspension-feeding protandric hermaphrodites that live in "stacks" of 2–15 individuals; females brood eggs that hatch as veliger larvae [3, 7]. Few rigorous studies exist of the seasonal timing of gametogenesis, oviposition, and larval release by *C. fornicata* in nature [19, 20]. In the Bay of Brest, France, adults produce three or four broods per season with peak hatching periods occurring between March and August [19]. Adults collected from populations near Woods Hole, MA, and in the southern Puget Sound, WA, USA, yield larvae within a few days of collection during June and July; Woods Hole adults appear to be mostly spawned out in August ([21], personal observation). The protocol given here for conditioning broodstock out of season reliably produces high-quality naturally hatching larvae when used with adults obtained from Woods Hole between December and May. This protocol uses small volumes of artificial seawater and a commercially prepared concentrated food source and does not require permanently established aquaria. Alternative methods for obtaining embryos out of season have recently been published [22].

The larval rearing protocol presented here is derived from methods described by Pechenik [4], modified with respect to larval density, food ration, and use of an artificial seawater medium. Under the rearing conditions described below, brooded embryos hatch as planktotrophic veligers at about 450 μm and grow 35–40 μm/day, usually becoming competent for metamorphosis at 10–11 days at 800–900 μm. If cues for metamorphosis [23] are withheld, competent larvae of 1,200–1,500 μm may be easily obtained before spontaneous metamorphosis occurs between 3 and 4 weeks post-hatch. I also present a simple protocol for culturing the larval food source, the flagellate *Isochrysis galbana*, using artificial seawater and a minimal amount of natural seawater (no more than 1 L/week for a typical small-scale laboratory culture operation).

The large size of larvae of *C. fornicata* is a distinct advantage for neuroethological studies of settlement and metamorphosis. As an example of the types of manipulations that are possible with large veligers, I present a protocol for electrophysiological recording of ciliary arrest spikes from intact larvae. The recording

electrode is a tether of fine platinum wire [24, 25] that allows larvae to swim in the water column or interact with a substrate. The ciliary arrest spike is an action potential in the preoral ciliated cells that provide the propulsive force for swimming [26–29] and may be a neural correlate of larval settlement behavior [30–32].

2 Materials

Adult broodstock conditioning, algal food culture for larvae, and larval culture utilize Instant Ocean artificial seawater (ASW) (United Pet Group, Cincinnati, OH, USA). Dissolve 800 g ASW salts in 20 L deionized, distilled, or reverse osmosis-purified water. Adjust final salinity to 32 ppt with a refractometer or a hygrometer. If the water source does not incorporate a purification stage that removes organics, it is advisable to pass the water through a bed of activated carbon media (e.g., using a canister-type aquarium filter) before dissolving the salts. Use this preparation of ASW without further filtration for adult conditioning and larval cultures. Filtration for algal medium is described below in Subheading 2.3.

2.1 Conditioning Adult Broodstock for Spawning Out of Season

1. Adults of *C. fornicata* may be field collected or purchased from the Marine Resources Center at the Marine Biological Laboratory, Woods Hole, MA, USA. Keep adults cool (≤10 °C) and damp during transport from collecting site (*see* **Note 1**).
2. Shellfish Diet 1800 (Reed Mariculture, Campbell, CA, USA): This is a mixed-species microalgal concentrate. Store at 4 °C according to the supplier's instructions. Use within 12 weeks of purchase.

2.2 Culturing Veliger Larvae to Metamorphic Competence

1. Sieves: Larvae are collected and transferred on sieves made from Nitex mesh (Sefar AG, Heiden, Switzerland). Cut the bottom off of a 400 mL tri-corner disposable plastic beaker, and use hot-melt glue to apply a panel of mesh to form the bottom of the sieve. 150 and 236 μm sizes are most useful.

2.3 Culturing the Flagellate Isochrysis galbana (T-ISO) as a Larval Food Source

1. Starter cultures of *Isochrysis galbana* (strain T-ISO) are available from UTEX (The Culture Collection of Algae, University of Texas, Austin, TX, USA), CCMP (The Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, Boothbay Harbor, ME, USA), and Mariculture Technologies International (Oak Hill, FL, USA).
2. Guillard's f/2 medium: This is a half-strength formulation of the "f medium" originally given by Guillard and Rhyther [33]. F/2 medium is available as a two-part concentrate from Kent

Marine (Franklin, WI, USA). Add 130 μL each of Part A and Part B to each liter of a 1:1 mixture of ASW and natural seawater and sterile-filter at 0.45 μm. Transfer to sterile 1 L screw-top stock bottles. Store at 4 °C (*see* **Note 2**).

3. Cotton plugs sized to fit mouths of 500 mL Erlenmeyer culture flasks. Fallout of fibers into media will be minimized by wrapping cotton plugs in cheesecloth. Flasks are plugged, capped with aluminum foil, and autoclaved empty (*see* **Note 3**).
4. 22 cm lengths of 5 mm (O.D.) glass tubing, fire polished to reduce sharp edges. These provide aeration for algal culture flasks.
5. A hemacytometer for counting algal cell density.

2.4 Electrophysiological Recording of Velar Ciliary Arrest Spikes

Ciliary arrest spikes may be recorded with standard instrumentation for extracellular electrophysiology, i.e., a differential AC amplifier of 1,000×–10,000× gain, interfaced with appropriate data acquisition software. Standard input cables are terminated with fine-wire electrodes that require the following:

1. Electrodes are prepared from 25 μm coated platinum wire (7690 from A-M Systems, Carlsborg, WA, USA). Cut a 10 cm length of wire, and remove insulation from 1 cm at the proximal (cable terminal) end by passing through a fold of 600-grit aluminum oxide sandpaper (*see* **Note 4**).
2. Terminals to connect electrodes to input cables: Copper flat-tip micro alligator clips (BU-34C from Mueller Electric, Akron, OH, USA).

3 Methods

3.1 Conditioning Adults for Spawning Out of Season

1. Set a lighted incubator to the ambient seawater temperature of collecting site, or 5 °C (whichever is higher), and 14-h:10-h light:dark cycle. Chill ASW to this temperature (*see* **Note 5**).
2. Open shipping container of adult *C. fornicata*, remove any cold packs, and equilibrate temperature 1 h in an incubator set as in **step 1**.
3. Transfer each "stack" of adults to 2 L chilled ASW in a 1-gal glass jar. If stacks are small (4–5 adults), 2 stacks may be housed together in a single jar. Provide moderate aeration through a piece of 5 mm (O.D.) glass tubing.
4. Let animals acclimate overnight without feeding.
5. Raise the incubator temperature by 1 °C/day. Change the water every other day.
6. Begin feeding on the first day when the temperature is equal to or greater than 10 °C. On the first feeding day, add a single

feeding of 300 μL Shellfish Diet to each jar. Animals should completely clear the water within 3–4 h. On subsequent days, feed 300 μL Shellfish Diet twice daily. Do not add second daily feeding until the first has cleared.

7. Continue to raise the temperature by 1 °C/day, until temperature in the incubator reaches 20 °C. Continue to feed twice daily, changing water every other day. Maintain incubator at 20 °C.
8. First releases of larvae should occur within 2–3 weeks after the temperature passes 15 °C. Most releases occur in the late afternoon. A second peak of releases should occur about 2 weeks after the first. Subsequent releases are smaller and less reliable; it is advisable to terminate adults after the second peak of releases and replace with freshly conditioned broodstock.

3.2 Culturing Veliger Larvae to Metamorphic Competence

1. Remove aeration from an adult jar in which a release of larvae has occurred. Let stand for 30 min for debris to settle. Larvae are strongly geonegative immediately after release and will accumulate near the top of the jar.
2. Collect larvae by siphoning into a 150 μm sieve suspended in a beaker of ASW. Avoid siphoning near the bottom of the jar.
3. Lift the sieve to drain and momentarily strand the larvae on the mesh surface, and then gently rinse larvae into a finger bowl of ASW using a squirt bottle of ASW.
4. Collect larvae with a Pasteur pipet (*see* **Note 6**). Count 500 larvae (*see* **Note 7**) into a glass jar containing 2 L ASW at 20 °C (*see* **Note 8**).
5. Feed with T-ISO, adding enough to achieve 10×10^4 cells/mL final density in the 2 L culture volume (*see* **Note 9**). Cultures are static—do not aerate or stir.
6. Change water every other day. Pour the contents of the culture jar into a 236 μm sieve suspended in a beaker, such that waste water overflows the beaker while larvae are retained within the sieve. Transfer larvae to a clean culture jar containing 2 L ASW, as in **step 3** above, and feed with T-ISO as in **step 5** (*see* **Note 10**).

3.3 Culturing the Flagellate Isochrysis galbana (T-ISO) as a Larval Food Source

Comprehensive methods for culturing marine algae are given in ref. [34].

1. Set incubator to 27 °C, with continuous light cycle (*see* **Note 11**).
2. Pour 330 mL f/2 medium into a sterile, cotton-plugged 500 mL Erlenmeyer flask.
3. Insert a sterile glass aeration tube between the plug and the neck of the flask. Connect to air pump, and bubble gently in incubator for 30 min to warm the cold medium.

4. Inoculate culture flask with 8–10 ml of a dense culture of T-ISO, using a sterile pipet (*see* **Note 12**). Continue gentle aeration (2–3 bubbles/s) while in growth period.
5. Use T-ISO for feeding while in log-phase growth (typically at a cell density of $4–7 \times 10^6$ cells/ml, after 4–6 days).

3.4 Electrophysiological Recording of Velar Ciliary Arrest Spikes

1. Pipet a larva in a minimum volume of ASW onto a sheet of weighing paper on a dissecting microscope stage.
2. Blot or pipet away excess ASW, leaving larva immobilized in an amount of ASW no larger than its own body volume.
3. Gently blot larval shell with a twisted bit of laboratory tissue.
4. Apply a minimal amount of cyanoacrylate glue to apical area of larval shell, using a sharpened toothpick.
5. Lay electrode obliquely across shell, so that the tip of electrode extends beyond the margin of shell by about 1 mm (*see* **Note 13**).
6. Lift the electrode, with larva tethered at the end, and quickly transfer larva to recording chamber filled with ASW.
7. Attach proximal end of electrode to micro alligator clip terminal on amplifier input cable. It is convenient to clamp the terminal end of the cable in an XYZ manipulator to facilitate positioning of the larva within the recording chamber.
8. Form distal end of recording electrode into a hook using fine forceps (Dumont #5 Biologie or equivalent) so that the distal end of the electrode is near the base of one velar lobe, without interfering with retraction/protrusion of the velum (Fig. 1).
9. Position larva in water column, in a perfusion stream, and/or to enable crawling on a substrate, using the manipulator that secures the end of the input cable. Bend the distal portion of the electrode as needed to bring the larva into an appropriate posture with respect to perfusion flow or orientation of the substrate.
10. A typical recording is shown in Fig. 1. As in many electrophysiological applications, the quality of the recording depends on proper shielding, grounding, and noise reduction techniques (*see* **Note 14**).

4 Notes

1. There is no need to ship animals in seawater; they remain in good condition in damp packing material in a foam cooler for 24–36 h after collection.
2. Sterile filtration is easily accomplished with an autoclaved vacuum filtration flask and funnel/filter support that uses 47 mm × 0.45 μm membrane filters (09-719-2E from Fisher

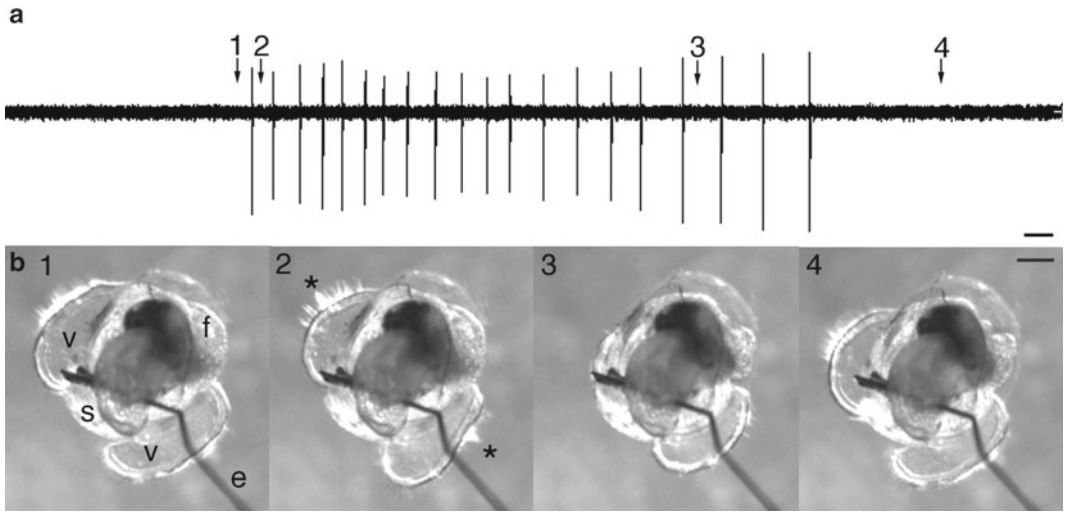

Fig. 1 Correlated electrophysiological (**a**) and video (**b**) recording of spontaneous ciliary arrests in a 14-day (post-hatch) competent veliger of *Crepidula fornicata. Numerals* in (**a**) correspond to *numbered video frames* in (**b**). 1. Several minutes of swimming without ciliary arrests preceded the first ciliary arrest spike. E, electrode; f, foot; s, shell; v, velar lobes. 2. A momentary ciliary arrest followed the first spike. *Asterisks* indicate interruption of metachronal wave of ciliary beating. 3. Complete ciliary arrest and partial collapse of the velar lobes followed a sustained burst of arrest spiking. 4. Normal ciliary beating gradually recovered over a period of 5 s after the last spike. Scale bars, 1 s (**a**) and 200 μm (**b**)

Scientific, Pittsburgh, PA, USA, or similar). Filtration is much more efficient if the 0.45 μm membrane filter is topped with a 2.7 μm glass-fiber prefilter (Whatman GF/D or similar, Whatman International, Maidstone, England). Prepared f/2 medium may be stored at 4 °C for several months.

3. I have followed the common marine laboratory practice of avoiding the use of fixatives, soaps, and detergents in glassware to be used for cultures. Glass jars used to culture adults and larvae *C. fornicata* are cleaned by rinsing with hot tap water and scouring with an aqueous paste of food-grade baking soda on a nylon scrub pad, rinsing in tap and deionized water, and air-drying. Erlenmeyer flasks used to culture T-ISO are similarly cleaned with a brush and baking soda and are occasionally acid-washed in 1 N HCl as needed to remove mineral deposits.
4. It is not necessary or desirable to remove insulation from the distal (larval) end of the electrode; the cut cross section of exposed platinum provides sufficient electrical contact.
5. This protocol has been used to condition adults of *C. fornicata* that have been collected at Woods Hole, MA, USA, between December and May. It should be applicable to winter and spring spawning of adults from other populations as well. Its purpose is to achieve an appropriate temperature for predictable oviposition and rapid development of brooded

embryos while avoiding temperature shocks that can cause premature oviposition or mortality of adults. Real-time and historic surface seawater temperatures at Woods Hole are available [35] and may range between 1 and 15 °C during the indicated months.

6. It is easy to damage larvae with the shearing forces and sharp glass apertures of Pasteur pipets. Pipet gently. For handling larger larvae Pasteur pipets should be broken back and fire-polished to relieve sharp edges, so that apertures are at least 1.5× the larval shell diameter.
7. It is essential to count larvae into cultures, rather than attempt to estimate numbers in pipetfuls of larvae. "Eyeball" estimates nearly always undercount numbers of larvae and lead to density-dependent problems of poor growth, shell fouling, and mortality.
8. Larvae and conditioned adults may be kept in an incubator at 20 °C on a 14:10 light:dark cycle but also do well on a laboratory benchtop, away from direct sunlight, at 18–24 °C room temperatures and ambient room lighting. Temperature effects on growth rates and larval duration have been described [5].
9. Higher larval growth rates have been reported by feeding with T-ISO at cell densities of $15–18 \times 10^4$ cells/ml [5]. My experience has been that the lower food cell density given in this protocol minimizes problems of shell fouling, clumping, and mortality while still supporting vigorous growth at the larval density indicated.
10. Larvae should begin to become competent for metamorphosis at 10–11 days post-hatch. Metamorphosis consists of loss of the larval velum and may be assayed by exposing larvae to a conspecific adult, ASW conditioned by a conspecific adult, or ASW in which the concentration of K^+ has been elevated by 15 mM by the addition of KCl [13, 23].
11. Good results will usually be achieved in typical lighted incubators designed to support plant growth. If growth lights are not permanently installed in the incubator, use a pair of 457 mm, 15 W full-spectrum "plant and aquarium" fluorescent tubes (F15 from General Electric, Cleveland, OH, USA, or similar), positioned 10–15 cm from culture flasks. These tubes fit standard household 18″ fluorescent fixtures.
12. In order to avoid contamination it is good practice to subculture only from cultures that have not yet been opened and used for feeding.
13. Attaching the electrode to the shell and forming the hook at the end of the electrode are critical steps, because the quality of the recording depends on the proximity of the electrode tip to the velar lobe. Practice is required. It is best to err on the side of allowing excess length of electrode wire to protrude

past the margin of the shell in **step 5**; any excess can be trimmed with fine scissors in **step 8**.

14. The recording chamber should be set up on a grounded steel plate within a Faraday cage. The monopolar recording electrode occupies one terminal of a differential amplifier's input cable; for best common-mode noise rejection the second terminal should be connected to a silver wire immersed in the recording bath near the tethered larva. The recording bath should be grounded.

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Chapter 4

Trichoplax adhaerens, an Enigmatic Basal Metazoan with Potential

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Abstract

Trichoplax adhaerens is an enigmatic basal animal with an extraordinarily simple morphological organization and surprisingly complex behaviors. Basic morphological, molecular and behavioral work is essential to better understand the unique and curious life style of these organisms. We provide basic instructions on how *Trichoplax* can be cultured and studied in the laboratory emphasizing behavioral and cellular aspects.

Key words Placozoa, Behavior, Immunohistochemistry, In situ hybridizations, Cloning, Chemotaxis

1 Introduction

Trichoplax adhaerens is a species from the phylum Placozoa that has been the subject of considerable investigation in recent years. The newly emerged interest in this enigmatic creature arose from its basal position within the animal kingdom as well as its extraordinarily simple morphological organization (*for recent reviews see* refs. [1, 2]). Superficially one might believe that *Trichoplax* is an amoeba (*for general overview of Trichoplax morphology see* Fig. 1 and ref. [3]). Instead *Trichoplax* is reported to have 4–5 cell types and several thousand cells. More importantly, it shows a remarkable array of behavioral responses to its environment, reproduces both sexually [4, 5] and asexually [4] and defends itself chemically against predators [6]. It also relies on a broad array of developmental and physiological signaling pathways for essential cellular and molecular functions [7].

"It is a riddle, wrapped in a mystery, inside an enigma"
Winston Churchill, *cited in* Miller and Ball 2008

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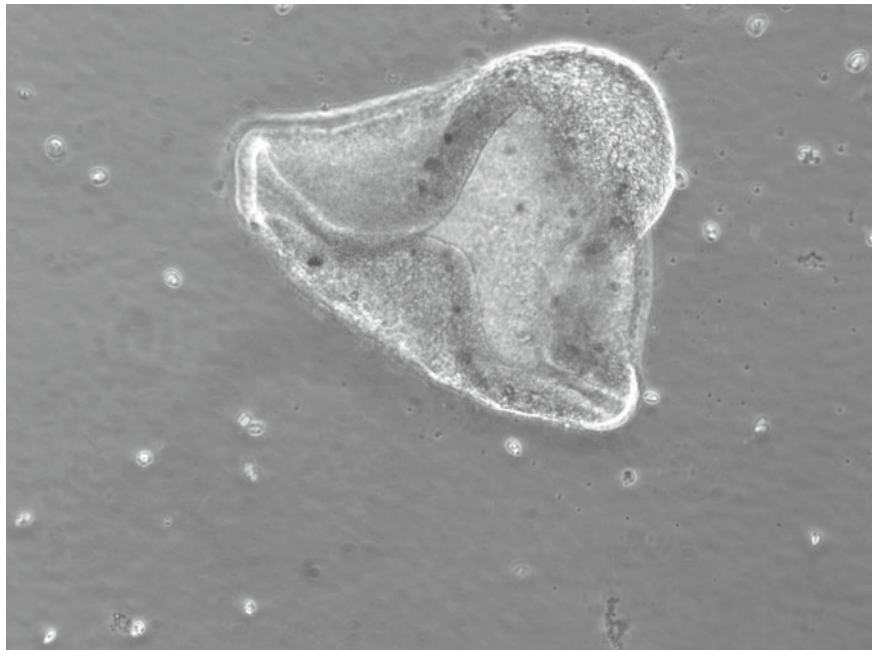

Fig. 1 Photograph of *Trichoplax* under DIC illumination. Notice the uneven folding pattern of the outer rim of the animal (F: folds). Dark spots seen along the rim as well as in the center are shiny spheres (SS). Recent research suggests that these cells contain toxins that are used as predator defense mechanisms [6]. The isolated spots visible in the culture dish are unicellular algae (*Cryptomonas* sp.) that are added to the *Trichoplax* cultures. After dying, they help build a biofilm on which *Trichoplax* can feed. Scale bar: 200 μm

Trichoplax occurs abundantly in tropical and subtropical marine habitats around the globe, including coral reefs and mangrove habitats [8]. Based on this wide distribution and the lack of any meaningful morphological characters that could be used to distinguish putative species, it seems unlikely that placozoans are a phylum of a single species (*T. adhaerens)*. Indeed, several newer molecular studies document a significant divergence of individuals between geographically separated populations [7, 9].

The phylogenetic relationship of placozoans within the animal kingdom and especially in relation to other basal groups such as the cnidarians, ctenophores, and sponges remains controversial, despite increasing genomic information from a diversity of organisms (*for discussion see* refs. [7, 10, 11]). Still, this issue is highly relevant for the evolutionary interpretation of future morphological and molecular questions. For example, if *Trichoplax* species are more basal than sponges, the last common ancestor of all metazoans could have been comparable to *Trichoplax*, i.e., morphologically simple with a small genome and a large mitochondrial genome. However, if placozoans are more derived than sponges the simple morphology of *Trichoplax* could be a secondary simplification.

Intriguingly, many genes with essential biological functions related to development, the nervous system and metabolism can be identified in the *Trichoplax* genome [7]. This is remarkable as no research has so far produced evidence for any *bona fide* neurons or developmental processes such as gastrulation and neurulation.

The cellular organization of *Trichoplax* is simple but it provides all essential functions for a free-living lifestyle, thus almost certainly excluding the possibility that *Trichoplax* is a parasitic form. Individuals are characterized by a dorsal and ventral ciliated epithelium. They incorporate food through specialized digestive cells in the ventral epithelium which secret digestive enzymes and can phagocytose digested food particles. The cell arrangement between the two ciliated epithelia is relatively loose; *Trichoplax* does not have coeloms and the only specialized cell type in this region is the fiber cell which may be used for a diversity of functions, including locomotion and cell communication. Studies suggest that these fiber cells can produce cytoplasmic extensions in isolation [12] and it is possible that these differentiated cells fulfill functions analogous to those of neurons and/or muscles. *Trichoplax* also has regular cell–cell junctions between epithelial cells. However, the basal lamina appears to be missing and so far nobody has been able to identify a true extracellular matrix (ECM), although the genome reveals genes that are coding for proteins that are known to interact with the ECM [3, 7]. Finally a function for an additional structure was recently identified. The shiny spheres (Fig. 1) have been shown to be effective in deterring predators by inducing paralysis [6].

The methods outlined in this chapter provide a basis for morphological, molecular, and behavioral work on *Trichoplax*. Linking these three aspects will contribute to our understanding of reproduction, growth, and metabolism. Therefore, this chapter is intended for researchers who are starting work on *Trichoplax* and who require information on how to culture and experimentally manipulate these organisms.

2 Materials

1. *Cryptomonas* sp. (from UTEX at the University of Texas at Austin, stock number LB 2423—http://web.biosci.utexas.edu/utex/).
2. f/2 growth medium (commercially available or http://www.sbs.utexas.edu/utex/media.aspx for protocol).
3. Pyrex® 90 mm × 50 mm evaporating dishes (part # 3140).
4. Low-melting point agarose (Fisher Scientific # BP165-25 or comparable product).
5. Disposable borosilicate glass Pasteur pipets.
6. 6-Well culture plates (Corning Costar part # 3527).

7. Phosphate buffer with Triton (PBT): 0.1 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl, 0.2 % Triton X-100.
8. DABCO mounting medium: 1 % DABCO in 90 % glycerol and 10 % PBS (pH: 8.0–9.0).

3 Methods

3.1 Collection and Maintenance

Several colonies of *Trichoplax* can be maintained with approximately 1 h of time investment per week (*see* **Note 1**). Seawater can be natural or artificial, ideally with salinity between 30 and 35 ppt (*see* **Note 2**); specimens generally appear to tolerate higher salinities (>35 ppt) better than lower salinities (<30 ppt).

There are two components to maintaining a *Trichoplax* colony: first, establishing the algal foodstock (unicellular pyrenomonas algae), and second, setting up and maintaining the *Trichoplax* dishes. The long-term maintenance of colonies requires healthy algae cultures which should be prepared at least 7–10 days before the first *Tricholplax* culture is established (*see* **Note 3**). It is also essential that embryologically clean glassware is used for all processes related to *Trichoplax* cultures (*see* **Note 4**).

Trichoplax can be collected in tropical and subtropical regions around the globe (*see* ref. [8] and **Note 5**).

3.2 Algal Cultures

Culturing *Trichoplax* requires the maintenance of axenic algae cultures as well as a working stock.

1. A commonly used unicellular algal species for *Trichoplax* cultures is *Cryptomonas* sp. (*see* **Note 6**) which can be cultured at room temperature with a 12:12 light:dark cycle. If faster growth is required they can be kept under continuous light and supplemented with CO_2 (*see* **Note 7**).
2. Axenic algal cultures are maintained separately from the working stocks. In order to keep the algae culture axenic, sterile techniques should be used at all times (*see* **Note 8**).
3. Working stock solutions are inoculated with 5 ml of axenic stock solution. Depending on the conditions (i.e., light, CO_2 temperature), these cultures will take 1–2 weeks to grow to a usable density (*see* **Note 9**).
4. Working stock cultures are maintained in sterile 1 l bottles filled with 500 ml of f/2 (250 µl of f/2 component A and 250 µl component B in 500 ml of FSW). Use a small aquarium pump connected to a 5 ml plastic transfer pipette to aerate (air and CO_2; *see* **Note 7**) the cultures. Also make sure that the cultures are covered with aluminum foil or Parafilm™ to avoid contamination.

3.3 Trichoplax Cultures

Maintaining *Trichoplax* cultures requires very little space, water, and time. The instructions below outline a simple protocol for the successful maintenance of a healthy *Trichoplax* population in the lab.

1. *Trichoplax* cultures are maintained in covered Pyrex 90 mm × 50 mm evaporating dishes filled approximately 90 % with filtered seawater (FSW: 0.2–0.45 μm; *see* **Note 10**), allowing sufficient air exchange between the culture and the environment.
2. Water should be changed every 2 weeks, but the culture will survive up to several more weeks without a water change. When changing the water of the cultures carefully pour off 70 % of the water in the container without exposing the *Trichoplax* to air. Gently add autoclaved FSW with the proper salinity until the dish is 90 % full and add 2 ml of algal feeding solution (densities can vary but the algae culture should be in a healthy part of its growth and not overly dense; *see* **Note 9** on algae culture for details).
3. When starting a new culture of *Trichoplax*, begin with an autoclaved dish and add the water. After adding the water place 10–15 *Trichoplax* into the dish then add 2 ml of the algal feeding solution and place a lid loosely on the new culture (*see* **Note 11**).
4. Start two new cultures per month and discard cultures after 6–12 months.

3.4 Behavioral Analysis

While *Trichoplax* has a distinct upper and lower ciliated epithelium, it lacks an anterior–posterior polarity. When *Trichoplax* is observed under a microscope, their net directional movements are so slow that they are almost imperceptible. Slightly more rapid indications of activity include changes in folding and invagination patterns of the organism (Fig. 1, Online Supplement 1). These folds can occur towards the center or the perimeter of the organism and are usually visible as subtle color changes under the microscope.

Time lapse photography, however, reveals a richer array of behavioral patterns in *Trichoplax*. Specimens can be observed under a microscope with a capture frequency of one frame per 5–10 s. This will result in continuous movements in the final video and allow tracking as well as qualitative and quantitative analyses of these behaviors.

3.5 Recording Behaviors

In order to setup simple time lapse recordings, specimen are transferred into small petri-dishes (35 mm diameter) with glass inserts and placed on a dissecting microscope with transmitted light or an inverted microscope (*see* **Note 12**). Videos can be captured for up to 4 h without any major water evaporation. This is based on

a total water volume of 4 ml/dish (*see* **Note 13**). Following is a summary of behaviors that can be observed using time lapse recordings. Note that various folding patterns, invaginations and flipping can be observed under real time conditions, while all other patterns require time lapse recording.

3.6 Identifying Specific Behavioral Elements

1. Folding: *Trichoplax* shows distinct folding patterns of the dorsal and ventral epithelium. A part of the dorsal and ventral epithelium is elevated away from the substrate while the sides of the fold move closer towards each other. These folds generally occur along the outer edge of the organism and are critical components of flipping behavior (*see* below).
2. Invagination: Invaginations are similar to folding (*see* above) in that the perimeter of the organism is changing shape. In contrast to folding they do not involve a significant elevation of the epithelium away from the substrate.
3. Rippling: Animals create folds (*see* **step 1**) along the outer edge but there are more and smaller folds in comparison to the folding pattern described above.
4. Rotating: The animal rotates without any major displacement in a specific direction.
5. Flattening: The outer edge of an individual moves outwards. Distinct movements in the center can occur (*see* Online Supplement 2). This generally co-occurs with reduced displacement per time (*see* also description of feeding behavior below).
6. Flipping/Turning: Since *Trichoplax* can distinguish between up and down (or as others have referred to as dorsal and ventral) they show distinct behavioral patterns when they are placed upside-down. The sequence begins with the even folding of the outer edge into a cup like shape (*see* also **step 1**). The raised borders of the epithelium then move in opposite directions until the down-facing epithelium faces upwards and the animal unfolds.
7. Feeding: As noted by others (i.e., [13]) feeding is accompanied by a specific set of behavioral patterns that can be clearly and repeatedly seen in time lapse recordings (Online Supplement 2). To further characterize feeding behavior, we compared the activity patterns of the same individual on dishes with and without naturally grown biofilm. During exposure to biofilm, *Trichoplax* displayed specific and recognizable behavioral patterns. This behavior consisted of noticeable flattening, resulting in increased surface area, sometimes to almost double their original surface area (*see* Fig. 2 and **Note 14**).

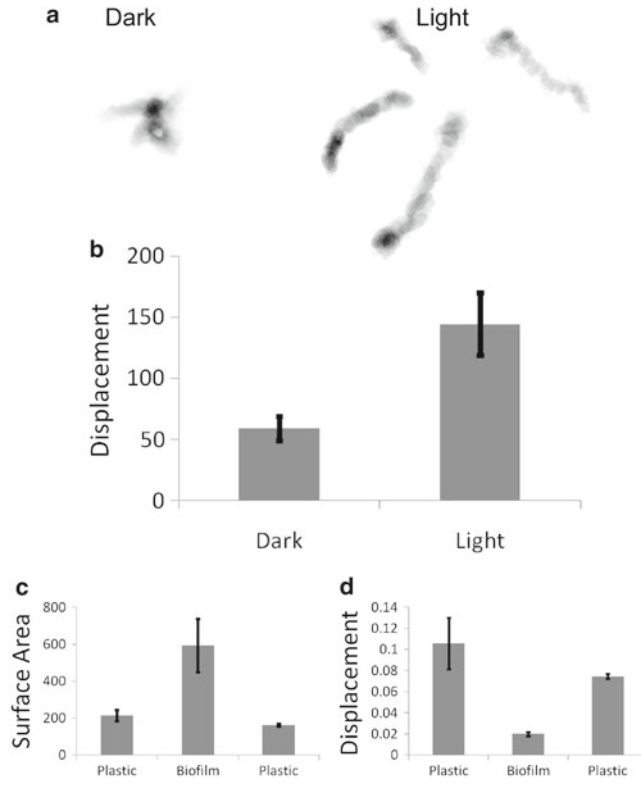

Fig. 2 *Trichoplax* response to light and biofilm. Under dark conditions, *Trichoplax* moves significantly less then under light conditions (**a**). This difference can be quantified using video analysis (**b**)—*y*-axis is in pixels/frame. We also compared the surface area and displacement of *Trichoplax* and response to biofilm and found that animals move significantly slower on biofilm while increasing their surface area (**c** and **d**)—*y* axis in (**c**) is in pixels in (**d**)—*y*-axis is in pixels/frame. For more details on feeding behavior and video analysis *see* text and Fig. 4. Scale bars: 4 mm

3.7 Identifying Social Behaviors

1. Touching: Two individuals are making contact with each other without any folding occurring.
2. Brushing: Folds are created along the outer edge of animals in response to direct contact between two individuals. In an extreme case, this can result in an extensive contact zone between the individuals on their ventral epithelia.
3. Aggregation: Multiple individuals in a dish form large aggregates that are all connected with each other, potentially forming a syncytium. We observed such aggregates in apparently healthy cultures with abundant biofilm growth. Online Supplement 3 shows a time lapse video analysis over 12–24 h of such aggregates. Individuals are budded off or fuse together with the aggregate on a regular basis.

3.8 Pharmacological Trials: Dissolved Chemicals

Experiments can be used to analyze behavioral patterns in response to neurotransmitters, putative chemoattractants, biological extracts, or other chemicals. Compounds can be diluted in seawater or presented as a local source within the experimental arena (Fig. 3). We discuss two complementary types of assays that can provide novel insights into the behavior of *Trichoplax*.

The goal of these trials is to test the physiological activity of a chemical. The basic design consists of four phases. Standard conditions for behavioral recording include 30–60 min trials using time lapse with 1 frame every 5–10 s.

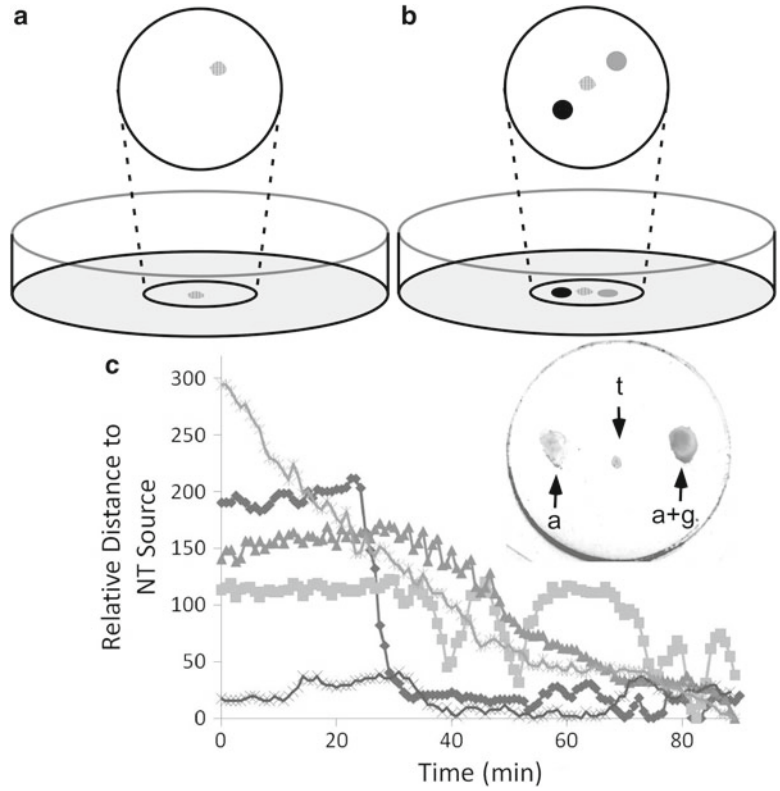

Fig. 3 Summary of experimental designs for behavioral analysis in response to chemicals. (**a**) *Trichoplax* is placed in the experimental arena and the petri dish is filled with 4 ml of filtered seawater. Washes can be done without having to remove *Trichoplax* from the arena. Point-source experiments (**b**) can be done with chemicals mixed into small agarose blocks which are then placed into the experimental arena. The relative distance of *Trichoplax* to any of these point sources can be plotted as a function of time (**c**). In this experiment one agarose block contained a 10^{-3} M Glycine (a + g), while the other block only contained agarose and solvent vehicle. Four out of five trials showed a clear tendency to move towards the glycine source relative to the control source. Note that the measurement shown on the *y*-axis is relative to the control and therefore does not have any units

1. One or multiple *Trichoplax* are placed in the experimental arena (Fig. 3) and allowed to settle for 30 min. It is important that individuals are only exposed to ambient light during this time and that vibration and other disturbances are avoided (*see* **Note 15**).
2. Recording can then be started for 30–60 min using standard conditions (*see* above).
3. The water is carefully removed from the dish with a glass or plastic pipette, ensuring that *Trichoplax* remains submerged in a small pool of water inside the experimental arena (*see* **Note 12**). The dish is then refilled with experimental solution. Recording should be started immediately after the addition of solution in order to capture fast behavioral responses.
4. The chemical solution is removed and the dish is refilled with filtered seawater. It is important to rinse the dish at least three times before starting to record in order to dilute chemicals (*see* **Note 16**).

3.9 Pharmacological Trials: Point-Source Trials

The goal of these trials is to test the response of *Trichoplax* to a local chemical source. In contrast to the dissolved chemical trials outline above, point-source trials can be used to analyze chemoattractants or repellent responses.

1. In preparation for a trial, one needs to prepare low-melting point agarose with and without the inclusion of a known concentration of a specific chemical or extract (controls will contain water or the specific solvent that was used to dissolve chemical). If the compound is relatively heat stable, regular agarose can be used. Mix 0.1 g of agarose with 10 ml of FSW and warm up in a water bath or microwave oven until agarose dissolves. Next, aliquot liquid agarose into 1.5 ml Eppendorf™ tubes and add the dissolved chemical. Mix well before gelling occurs. Tubes can be kept in the refrigerator or freezer until they are used for the experiment (*see* **Note 17**).
2. Before an experimental trial, bring the agar to room temperature and stab it with a disposable borosilicate glass Pasteur pipette to remove a small cylinder from the tube that can be easily pushed out of the pipette into a plastic dish. Use a fine scalpel to cut a small slice of this cone under a dissecting scope. Transfer experimental and control slices carefully to opposite sides of the experimental arena filled with FSW using forceps. In addition to these replicated experimental trials, two types of replicated control trials have to be performed where chemical versus chemical and control versus control blocks are presented (*see* **Notes 18** and **19**).

3.10 Video Analyses

Time lapse videos can be imported into ImageJ [14] using the Bioformats LOCI plugin (http://www.loci.wisc.edu/software/bio-formats) and be transformed into .avi videos using JPEG compression as well as variable frame rates to keep file size small.

For both qualitative and quantitative analyses of videos, ImageJ [14] can be used to create summary videograms [15], track either single or multiple individuals, and acquire morphological measures. Below are point by point instructions on how to setup and perform a video analysis (Fig. 4).

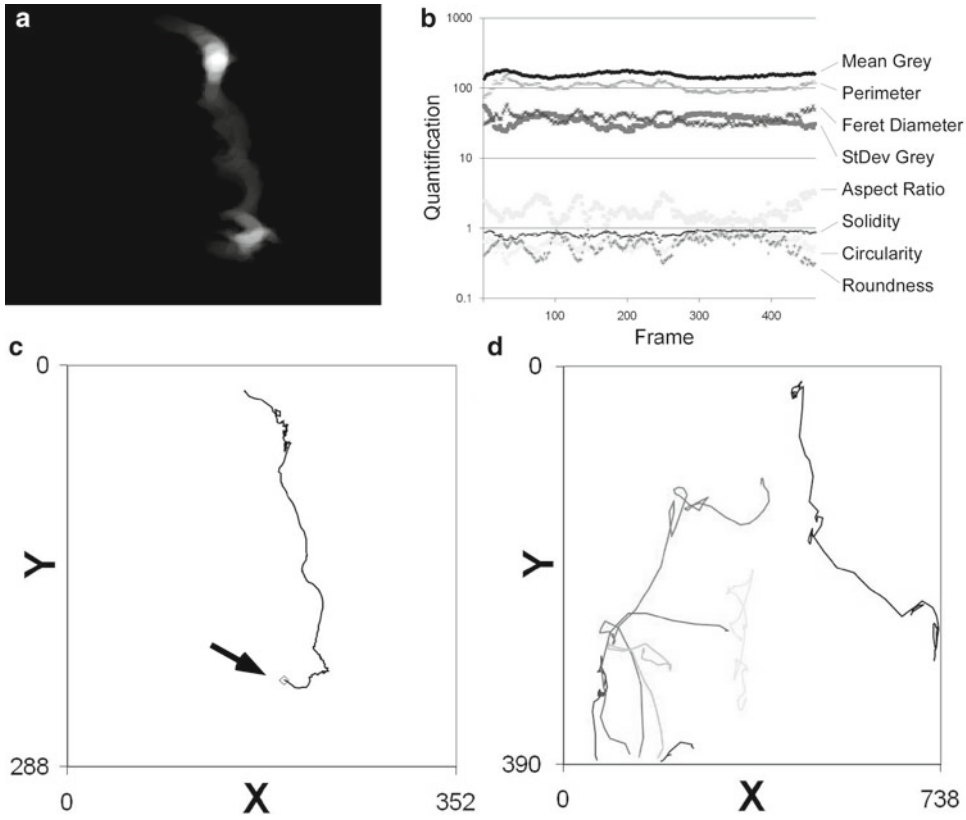

Fig. 4 Video analyses of *Trichoplax* behaviors include tracking either single or multiple individuals as well as quantification of morphological characteristics associated with different behaviors. (**a**) Videogram [15] showing the activity pattern over a 76 min video by a single *Trichoplax* crawling, pausing, and then continuing to crawl; Scale bar: 800 μm. (**b**) Several particle analysis measurements (ImageJ) show changes that correlate with the change in behavior. Mean gray value decreases as the animal becomes slightly darker, while the standard deviation (StDev) of the gray value increases as rippling occurs during the pause. In addition, the more circular morphology is apparent in a number of measures, including increased circularity, roundness, and solidity, and decreased aspect ratio, feret diameter, and perimeter. (**c**) Locomotory track of the *Trichoplax* shown in (**a**) and (**b**), based on coordinates acquired using the Analyze Particles command in ImageJ (*arrow* indicates start location). (**d**) Locomotory paths of five *Trichoplax* monitored simultaneously in a different video (duration: 3 h). Seven tracks are visible since two *Trichoplax* crawled out and then back into frame (start/return points indicated by the different symbols). Tracks were generated automatically using the MTrack2 plugin in ImageJ

1. Contrast: The critical step in automating behavioral analyses is ensuring adequate contrast between the *Trichoplax* and the background. This can be achieved either by: (1) ensuring all portions of every *Trichoplax* in the video are consistently darker (or lighter) than the background, or (2) using background image subtraction to enhance contrast of moving objects [15, 16]. Briefly, either a blank frame without any *Trichoplax* in view or a mean frame, averaged across the entire video (which blurs the moving *Trichoplax* into the background), can be subtracted from every frame in the video, generating a video with just the moving *Trichoplax* in view. Once adequate contrast is achieved, selection of an appropriate threshold will isolate the *Trichoplax* in a stack of binary images corresponding to each frame of the source video (*see* **Note 20**).
2. Videograms: Videograms provide a quantitative activity summary of a video clip [15]. To generate these, the binary image stack is simply summed, generating a single image with pixel values proportionate to the number of frames a *Trichoplax* was active in that pixel location. These can be used for both qualitative comparisons of different treatments or quantitative analyses of activity levels (based on the pixel gray values).
3. Tracking: To track individual *Trichoplax* in the binary image stack, the "Analyze Particles" command in ImageJ [14] generates x and y coordinates that indicate the centroid of an ellipse fit to the *Trichoplax* shape. When all frames are processed, these then provide a two-dimensional locomotory track for the *Trichoplax* in the video. Semiautomated tracking of multiple individuals can also be achieved using the MTrack2 plugin (downloadable from http://valelab.ucsf.edu/~nico/IJplugins/MTrack2.html) (*see* **Note 21**).
4. Morphological Measurements: In addition to tracking object locations, the "Analyze Particles" command in ImageJ [14] provides access to a range of morphological measurements (both pixel values and shapes) that can aid quantitative analyses of *Trichoplax* videos. Any of these can be gathered at the same time as tracking by using the "Set Measurements" command, selecting the appropriate measures and choosing "Redirect To" the original source video stack (this allows calculations based on the video pixel values).
5. Example Analysis: As an example of the outlined method we present a behavioral analysis of *Trichoplax* to light. For these analyses, source videos of single *Trichoplax* at 1 frame per 30 s were used. To enhance contrast, a mean frame was calculated, and this was then subtracted from every frame in the source video. The video was then cropped to exclude areas outside the area where *Trichoplax* behavior occurred. The resulting cropped and motion enhanced video was converted to a binary

image stack, with the threshold set to ensure the entire *Trichoplax* shape was present in the binary image. The particles were analyzed to find the *Trichoplax* locations, with three important settings: (1) Since the chosen threshold introduced some spurious smaller noise particles due to contrast fluctuations, the minimum particle area was set to filter out the noise, leaving just the *Trichoplax* as part of the analysis. (2) The measurements were redirected to the original video to allow pixel gray values to be part of the measurements. (3) A noise-less binary image stack including just the *Trichoplax* was generated using the Show Masks option. Videograms were generated by summing the resulting binary image stacks (after removing the inverted LUT that is applied by default and interferes with the summation). The measurements table including the *x* and *y* coordinates was saved, and Excel (Microsoft Corp.) was used to calculate frame-by-frame displacements which we then summed over each trial and compared between treatments (Fig. 2; *see* **Note 22**).

3.11 Immunohistochemistry Methods

In recent years more research on *Trichoplax* has began to focus on molecular and cellular processes. Although still rudimentary, some useful protocols exist for basic methodologies such as immunohistochemistry (IHC), in situ hybridizations (ISH) and even some transient knockdown techniques have been employed.

Standard IHC can be used for *Trichoplax*. We tested the IHC method outlined below using antibodies raised against serotonin, alpha- and beta-tubulin and histone H3. Other authors [17] also presented IHC data based on an RFamide antibody using this method. Figure 5a shows ciliary stain of *Trichoplax* using alpha-tubulin antibody (monoclonal anti-α-tubulin antibody, clone DM1A; Sigma Chem. Co.). Note that live and fixed *Trichoplax* have a broad range of autoflourescence. Figure 5b–d shows images of live *Trichoplax* at three commonly used wavelengths (used for the visualization of FITC, TRITC, and Cy5). Fixation generally quenches autoflourescence slightly.

1. Fixation overnight at 4 °C. 4 % paraformaldehyde in seawater can be used for the fixation, but specimens have often been observed to disintegrate during subsequent processing (*see* **Note 23**). The addition of 0.25 % of glutaraldehyde enhances tissue preservation (*see* **Note 24**).
2. Five washes in PBT 10 min each at room temperature (RT).
3. Two washes in blocking solution (PBT, 10 % goat serum), first wash 5 min, second wash for 1 h at RT.
4. 1° Antibody incubation (Blocking solution, 1° antibody 1:200–1:5,000) overnight at 4 °C.
5. Five washes in PBT, 10 min each at RT.

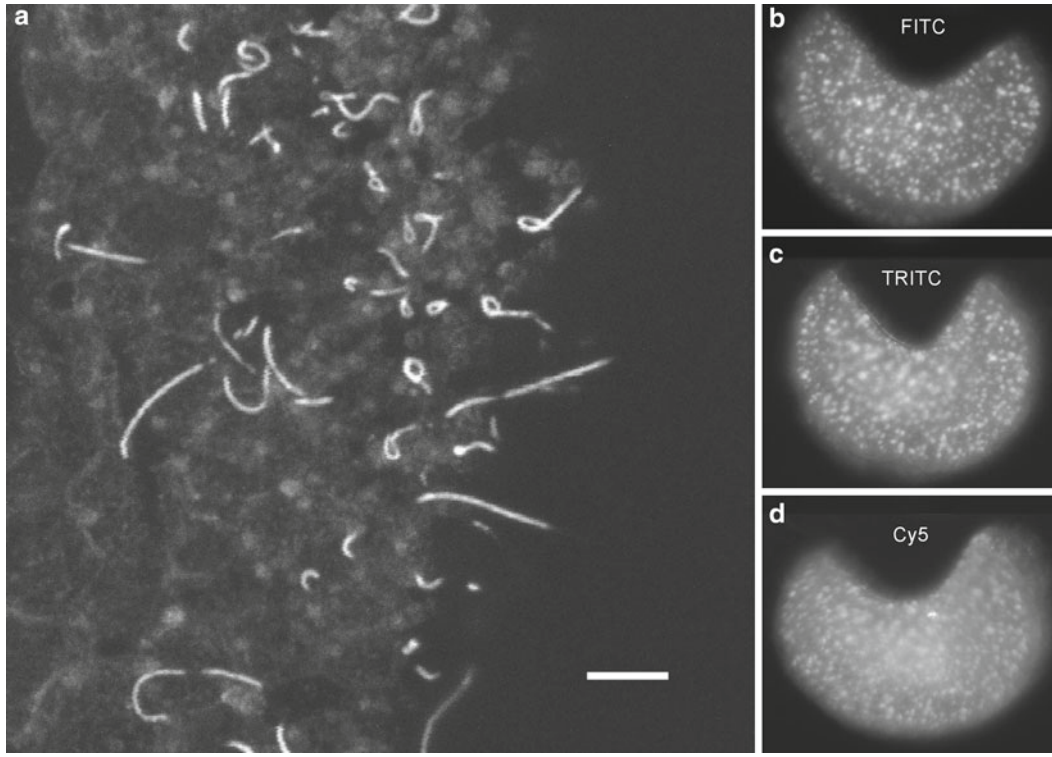

Fig. 5 Alpha-tubulin staining of *Trichoplax* using immunohistochemistry protocol further explained in the text (**a**). Scale bar equals 150 μm. *Trichoplax* produces significant amounts of autofluorescence in all three commonly used channels: FITC, TRITC, and Cy5 (**b**–**d**). Scale bars on *panels* (**b**–**d**) are 90 μm

6. 2° Antibody incubation (PBT, 2° antibody 1:400–1:1,000), 3 h at RT or overnight at 4 °C.
7. Five washes in PBT, 10 min each.
8. Mount on poly-L-lysine coated slides with DABCO mounting medium (*see* **Note 25**).

3.12 Molecular Methods

Over the past years, several molecular methods have been successfully applied to *Trichoplax*. For example, several groups have successfully employed ISH with *Trichoplax* [18–23]. While the protocols differ slightly from group to group, the most important steps are comparable. In our experience, *Trichoplax* can be fixed in 4 % paraformaldehyde and post-fixed in ice cold 100 % methanol for 10 min. We also noted that proteinase K treatment is generally not necessary. Finally, hybridizations in heating ovens give the best results.

Two types of transient knockdown techniques have been applied to *Trichoplax*: double-stranded RNA and morpholino antisense oligonucleotides. Both techniques have resulted in successful knockdown effects as documented by [19].

DNA and RNA extraction methods have been successfully applied to *Trichoplax* and do not require special techniques. Protocols for DNA extractions can be found in Pearse [8]. In our experience, one requires at least 30 specimen to successfully isolate >20 ng/μl high-quality RNA using RNAqueous®-Micro Kit (Ambion).

4 Notes

1. The amount of time needed to maintain *Trichoplax* largely depends on whether filtered seawater is readily available and whether the cultures are fed with live algae or rice (*see* Subheading 3.1).
2. The salinity can be measured with a handheld salinity meter or refractometer.
3. *Trichoplax* cultures can be easily maintained for several months using uncooked rice. Add 2–3 rice grains to the culture at each waterchange and remove the old ones. If cultures should be maintained longer however it is recommended to use live algae.
4. All glassware that is used for *Trichoplax* or algae cultures need to be embryologically clean. This means that it must be autoclaved and not exposed to any soap, detergent, or bleach at any time. It is generally recommended that a separate area with dedicated tools is set aside in the lab for cleaning.
5. *Trichoplax* can be shipped in 50 ml Falcon tubes. Rinse the tube with deionized water then fill it with FSW. Place the desired number of *Trichoplax* inside the tube and wait 10 min for the *Trichoplax* to attach themselves. Gently add more water until the tube starts to overflow, at this point stretch Parafilm™ over the top of the tube and tighten the lid on the tube.
6. We tested different algae species and the best results were achieved with *Cryptomonas* sp. (stock number LB 2423 from UTEX): The Culture Collection of Algae; The University of Texas at Austin; 1 University Station A6700, Austin, TX 78712-0183, USA; (ph) 512-471-4109; (fax) 512-471-0354.
7. If cultures are supplemented with CO_2 it is recommended to keep it at a concentration of approximately 1 % which can be achieved by gently bubbling CO_2 into the algae cultures. This cannot be done with the axenic stocks.
8. Recommended protocol for maintaining axenic algae cultures: First, open the sterile algae culture immediately after arrival and transfer 1 ml of culture into two autoclaved tall disposable borosilicate glass test tubes with either a breathable plastic lid or a sterile cotton plug. To the same tubes add 4 ml of sterile

FSW containing 0.05 % f/2 components A and B (i.e., 2 μl component A and 2 μl component B; note that this stock can also be prepared in larger quantities and kept at 4 °C). Care should be taken not to cross-contaminate components A and B (use different pipette tips). This and all subsequent transfers should be done under a laminar flow hood using sterile pipette tips and glassware. The axenic culture needs to be changed once every 2 weeks by transferring 1 ml of the original culture into a new tube with 4 ml f/2. One such culture (5 ml total volume) can also be used to setup a new working stock culture (*see* below) after at least 1 week of growth.

9. To estimate algal density take a small sample from these cultures and count the number of cells every 2 days using a hemocytometer. Do not use the culture to feed *Trichoplax* once the growth curve reaches a plateau (the maximum cell density ranges from 1,000 cells/μl under low light conditions to 3,000 cell/μl under high light conditions). Once the working stock culture has reached this point, transfer 100 ml into 500 ml growth medium (250 μl of f/2 component A and 250 μl component B in 500 ml of FSW) to setup a new culture. Note that this transfer should be done maximally four times before a new axenic culture sample is used to inoculate the working stock culture.
10. While cultures can be maintained in plastic dishes we had better success with glass dishes.
11. Alternatively, cultures can be inoculated with algae up to a week before adding *Trichoplax* so that a biofilm can grow.
12. Differential Interference Contrast (DIC) illumination is preferred for time lapse recordings but regular brightfield illumination can be used as well. We have also had success using agarose plates (prepared with seawater) as a suitable substrate for behavioral experiments.
13. In a 4 h trial the salinity will likely increase. One strategy to avoid excessive evaporation and perform longer trials is to cover the dishes. With this setup, water condensation on the lid can obscure observations if the room temperature is higher than the initial water temperature. This problem can be partially solved by using an inverted microscope.
14. During feeding the animal crawled noticeably more slowly across the substrate compared to when it was in the dish without biofilm. *Trichoplax* also appeared to thicken around the periphery while flattened. The inner flattened portion became thicker in some parts, moving outward towards the periphery in a wave-like motion, almost as if the *Trichoplax* was "kneeding" the surface of the arena. While on the biofilm, *Trichoplax* seemed to exhibit this behavior several times, and each time

was followed by a short bout of movement to a slightly different position before flattening again. After the *Trichoplax* moved to a new location, it was apparent that biofilm had been removed from the bottom of the arena.

15. The conditions during this adjustment phase should be identical to those used during the experimental trial.
16. Removing too much water can lead to immediate disassociation of *Trichoplax* due to surface tension.
17. Make sure to always use the same agarose stock for the experimental and control trials.
18. Agarose will become almost transparent under water and it is therefore advisable to mark the position of the experimental and control blocks. Furthermore the relative position of these blocks should be randomized between trials (*see* Fig. 3 for details). Behavioral patterns as well as directional data can be extracted from these assays (Fig. 3 and Subheading 3.4).
19. Point-source trials can be performed with multiple individuals with and without video recording. For example, multiple replicates can be run simultaneously without recording in 6-well culture plates. At the end of each trial the positions of individuals can be recorded and travel distance relative to the chemical source calculated.
20. Contrast fluctuations that result in spurious objects in the binary images can be removed, provided they are found in consistent locations, are consistently smaller than *Trichoplax*, or have consistently higher aspect ratios than *Trichoplax*.
21. This method can automatically provide tracks for *Trichoplax* that do not touch each other; however manual intervention is necessary to separately identify two or more individuals that contact each other, and thus will be counted as one particle by the software.
22. Note that the light environment needs to be kept constant when performing behavioral trials.
23. Because *Trichoplax* specimens tend to disintegrate during histological processing, they can also be pre-mounted on poly-lysine coated slides and all steps can be performed on slides. See also additional notes on fixations above.
24. Addition of 0.5 % glutaraldehyde noticeably increases background fluorescence, while specimens fixed in 4 % paraformaldehyde with the addition of only 0.1 % glutaraldehyde often fell apart.
25. DABCO mounting medium can be prepared using a 10× PBS or TBS buffer solution that has the correct amount of DABCO dissolved in it. Add 1 ml of that solution to 9 ml of glycerol (check for autoflourescence) and prepare 1 ml aliquot that can be frozen at −20 °C.

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Chapter 5

Rearing Gymnolaemate Bryozoan Larvae for Cellular and Molecular Analyses

Michael H. Temkin

Abstract

Gymnolaemates represent the largest group of extant bryozoans, having more than 3,000 described species. Gymnolaemates display a diverse array of reproductive and developmental patterns including planktotrophy, lecithotrophy, and matrotrophy. The larvae of gymnolaemates have been broadly grouped into three types, cyphonautes (shelled, feeding), pseudocyphonautes (shelled, nonfeeding), and coronate (unshelled, nonfeeding), although each group is heterogeneous and probably includes various morphologies that are largely undescribed. Here, methods for rearing bryozoan colonies and larvae are presented.

Key words Bryozoan, Larvae, Coronate, Cyphonautes

1 Introduction

Gymnolaemate bryozoans have either a feeding (cyphonautes) or a nonfeeding (pseudocyphonautes or coronate) larval form [1–3]. In the majority of gymnolaemates, fertilized eggs are retained by maternal individuals and may be brooded in one of several different ways for about 2–3 weeks until a fully developed nonfeeding larva is released [1, 2, 4–9]. The bulk of the nutrients to support the development of nonfeeding larvae is supplied by the maternal individual either to the egg during vitellogenesis before ovulation or to the embryo via a placental-like system after spawning [1, 2, 5]. In some species of the genera *Alcyonidium*, *Biflustra*, *Conopeum*, *Electra*, *Hislopia*, and *Membranipora*, fertilized eggs are not retained by maternal individuals and embryos develop into planktotrophic, cyphonautes larvae [1–3, 10–12].

Gymnolaemate larvae usually spend some amount of time swimming in the water column before settling and undergoing metamorphosis to form an "ancestrula" (i.e., the first member of a new bryozoan colony) [1, 2, 13, 14]. Nonfeeding larvae swim from several hours up to 2 days after release, although shorter times are more typical if a suitable substrate is present [13, 15, 16].

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Extending the swimming period by preventing coronate larvae from undergoing metamorphosis can significantly increase the time it takes to complete metamorphosis, reduce the overall size of the ancestrula including the lophophore, and decrease the survivorship of ancestrulae [16–18]. In comparison to nonfeeding larvae, cyphonautes larvae may swim for a month or more in the water column before becoming competent to undergo metamorphosis depending on the supply of food. For example, cyphonautes of *Membranipora* sp. have been estimated to spend at least 4 weeks in the water column before metamorphosis [19, 20]. Cyphonautes can extend their developmental period when food is scarce by resorbing portions of the juvenile rudiment, which regrow and become functional when food becomes available again [21].

As larvae approach metamorphosis, their responses to environmental cues change. In particular larvae become negatively or neutrally phototaxic and positively geotaxic (*see* refs. 1, 2, 13, 22) which causes them to swim toward benthic substrates. Gymnolaemate larvae explore potential settlement sites with the pyriform organ, a composite sensory and glandular structure [1–3, 13]. Gymnolaemate larvae have been demonstrated to choose settlement sites based on the presence of biofilms (*see* refs. 1, 2), the presence of congeners or conspecifics (e.g., [23, 24]), or the age and species of kelp (e.g., [25, 26]). Gymnolaemate larvae may temporarily attach to a substrate using secretions of the pyriform organ, but permanent attachment occurs during metamorphosis through the eversion of the internal sac which becomes cemented to the substrate by mucopolysaccharide adhesive secretions [1, 2]. The reorganization of the larva into the ancestrula during metamorphosis is extensive and may take from one to several days to complete depending on the species [1, 2, 14, 16].

The method used for rearing larvae of any particular gymnolaemate bryozoan depends on the reproductive pattern of the species. For example, rearing cyphonautes larvae involves collecting and caring for embryos and larval stages [21]. In contrast, rearing nonfeeding larvae involves the care and maintenance of adult colonies until larvae are released [27–35]. Here, methods for the general rearing and maintenance of gymnolaemate bryozoans, obtaining nonfeeding and cyphonautes larvae, and obtaining ancestrulae and rearing young colonies are presented.

2 Materials

2.1 General Care and Maintenance of Bryozoan Colonies

Cultures of *Rhodomonas* sp. (catalog number CCMP757, CCMP, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA) prepared accordingly as described elsewhere [36].

Containers to hold colonies (*see* **Note 1**).

100 and 1,000 mL Tri-Pour® polypropylene, disposable beakers.

10 and 20 μm Tri-Pour® filter baskets. These filter baskets are made by modifying polypropylene Tri-Pour® polypropylene beakers (*see* **Note 1**).

Small paintbrushes.

2.2 Obtaining Nonfeeding Coronate Larvae from Gymnolaemate Bryozoans

1. Black or opaque plastic box.
2. Fiber-optic light source or gooseneck lamp.

2.3 Rearing Cyphonautes Larvae of Membranipora membranacea

500 mM Ethylenediaminetetraacetic acid (EDTA), pH 8: Add 350 mL of deionized water to a 1-L beaker containing a stir bar. Weigh 93.06 g disodium EDTA and 9.5 g NaOH (*see* **Note 2**). While monitoring with a pH meter, slowly add the disodium EDTA to water in beaker with stirring. In order for the EDTA to completely dissolve, adjust the pH to near 8 with NaOH. Once all the EDTA is dissolved adjust the final pH to 8 with NaOH and bring the volume up to 500 mL with deionized water. Alternatively, 500 mM disodium EDTA can be purchased from many suppliers, including Promega, Madison, WI, USA (catalog numbers V4231 and V4233).

0.1 mM EDTA in 10 μm filtered seawater: Filter seawater through a 10 μm filter basket and add 200 μL of 500 mM EDTA per liter of seawater. Make sure that the solution is mixed well.

20 mL plastic syringes.

Sterile 0.22 μm Fisherbrand® syringe filter disks (Fisher Scientific, Dallas, TX, USA).

35 × 10 mm Fisherbrand® Media-Miser Disposable Petri Dishes (catalog number 08-757-11YZ, Fisher Scientific, Dallas, TX, USA).

Swinging paddles on a rack stirring mechanism constructed according to Strathmann (*see* Chapter 1).

Cultures of *Rhodomonas* sp. and *Isochrysis galbana* (catalog number CCMP1323, CCMP, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA) prepared according to [36].

2.4 Obtaining Ancestrulae and Growing Colonies

1. Glass or plastic containers.
2. Depending on the species, either artificial substrates including glass, plastic, acetate, and ACLAR® (Ted Pella Inc., Redding, CA, USA) or natural substrates such as shell, wood, macroalgae, or chicken egg inner shell membrane can be used.
3. Seawater with 10 mM excess KCl: Dissolve 0.745 g of KCl per liter of 0.22 μm filtered seawater.

3 Methods

3.1 General Care and Maintenance of Bryozoan Colonies

1. Gently clean bryozoans of sediment and organisms that are adhering or growing on the surface of colonies (*see* **Note 3**). Try to remove as much sediment and as many organisms as you can, recognizing that colonies will likely not be pristine after you have finished cleaning them. Depending on the growth form (e.g., encrusting sheets, creeping or erect stolons, or arborescent), colonies may be cleaned using a small paintbrush, a forceps, and/or your fingers. Rinsing colonies in flowing seawater is also helpful.
2. Place cleaned colonies into containers filled with seawater at the appropriate temperature and salinity. Since gymnolaemate colonies have many different shapes or sizes, and can grow on a wide variety of substrates (e.g., rock, wood, shell, algal fronds, sea grass, barnacles, ascidians, and other bryozoans), the choice of container will depend on the species, the presence and type of substrate, how much material was collected, and whether colonies are being maintained in a flowing seawater system or static containers (e.g., bowls or jars). Bryozoans can be sensitive to anoxia, and overcrowding should be avoided, especially when colonies are kept in static containers (*see* **Note 4**). Colonies may be hung or floated upside down to reduce the accumulation of sediment and fecal material on the colony surface.
3. Gymnolaemates can be fed *Rhodomonas* sp. (10,000 cells/mL) as a supplement or as a replacement for food in natural seawater [29–31, 33–35, 37] (*see* **Note 5**).
4. Reproductive colonies in many gymnolaemates can be distinguished by the presence of embryos developing in specialized brood structures such as ovicells or embryo sacs (*see* **Note 6**). Embryos are often yellow, orange, purple, or red in color. In some cases, brooding embryos can be easily observed by using dark-field microscopy (Fig. 1). In species that do not brood embryos, such as *Membranipora membranacea*, reproductive individuals have pale pink or tan disk-shaped eggs located within maternal coeloms and intertentacular organs (ITO) (egg-spawning organs) formed between the basal portions of the distomedial tentacles (Fig. 2).

3.2 Obtaining Nonfeeding Coronate Larvae from Gymnolaemate Bryozoans

1. Place colonies that are brooding larvae in the dark (*see* **Note 7**).
2. After at least 5–8 h, expose the colonies to light.
3. If possible dim the room lights, and place a light at the side of the container. A fiber-optic light source works well here.
4. Gymnolaemate coronate larvae from many species are positively phototactic after release and swim toward the light source [2, 13, 22, 28, 38]. After a short time, larvae accumulate on

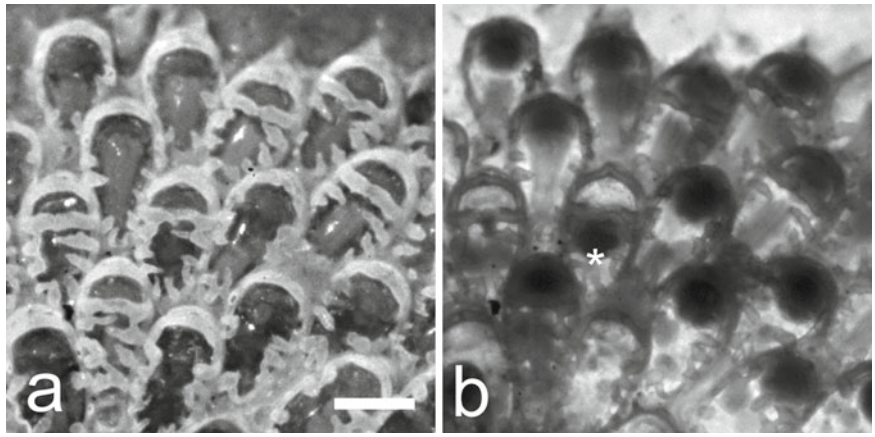

Fig. 1 Individuals of a colony of *Cribrilina* sp. viewed through a dissecting microscopy using epi-illumination with a fiber-optic light source (**a**) or dark-field microscopy (**b**). In the dark-field image (**b**), dark spheres are embryos in ovicells except for the one marked with an *asterisk* which represents a primary oocyte within an ovary. Scare bar = 250 μm

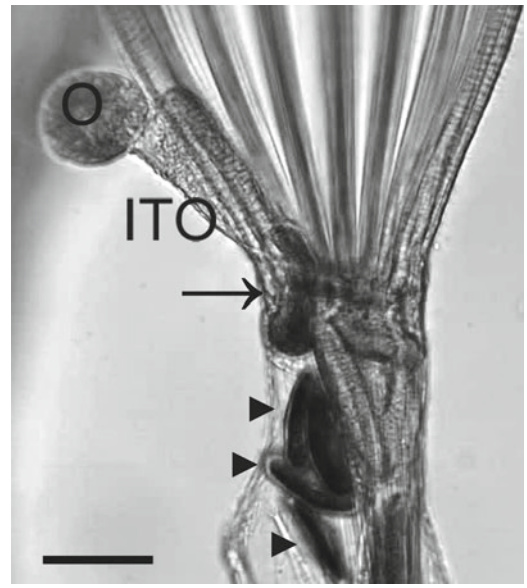

Fig. 2 The lophophore of a reproductive individual from a colony of *Membranipora membranacea* possessing an intertentacular organ (ITO) and coelomic oocytes (*arrowheads*). One disk-shaped primary oocyte (O) has just been spawned through the ITO while a second primary oocyte (*arrow*) is entering the ITO from the maternal coelom. Scare bar = 100 μm

the side of the container near the light source. If the species you are working with releases larvae that are photoneutral, then larvae can be made more apparent by illuminating the container from the side. Larvae will appear as small refractile, spherical objects swimming in spiraling trajectories.

5. Larvae can be transferred to tubes or other containers using glass or plastic pipettes.
6. Larvae will typically swim for several hours before beginning to settle and undergo metamorphosis (*see* **Note 8**).

3.3 Rearing Feeding Cyphonautes Larvae of Membranipora membranacea

1. Place colonies of *M. membranipora* [39] on algal substrate in a glass dish containing 0.1 mM EDTA in 10 μm filtered seawater, cooled to ambient seawater temperature (*see* **Note 9**).
2. Allow *M. membranacea* colonies to spawn fertilized eggs for about 2 h (Fig. 2) (*see* **Notes 10** and **11**). Colonies may be kept at room temperature during this time. A slight increase in water temperature may increase spawning. Fertilized eggs are disk shaped but will become spherical after egg activation (*see* **Note 6**).
3. After about 2 h, transfer colonies to a new bowl of 0.1 mM EDTA in 10 μm filtered seawater or return to normal seawater.
4. Fill a 35 × 10 mm Petri dish approximately halfway with 0.22 μm filtered seawater using a 20 mL syringe fitted with a filter disk.
5. Use either a mouth pipettor fitted with a glass pipette pulled from a glass tube (*see* **Note 12**) or a 200 μl micropipettor fitted with a plastic tip to transfer embryos (*see* Fig. 3) from the bottom of the dish to the small Petri dish containing 0.2 μm filtered seawater (*see* **Notes 13** and **14**). Do not transfer more than between 100 and 200 embryos per dish to avoid overcrowding.
6. Once embryos begin to swim (usually between 24 and 30 h depending on temperature), provide them with *Rhodomonas* sp. (5,000 cells/mL) and *Isochrysis galbana* (40,000 cells/mL) [21] (*see* **Note 15**).

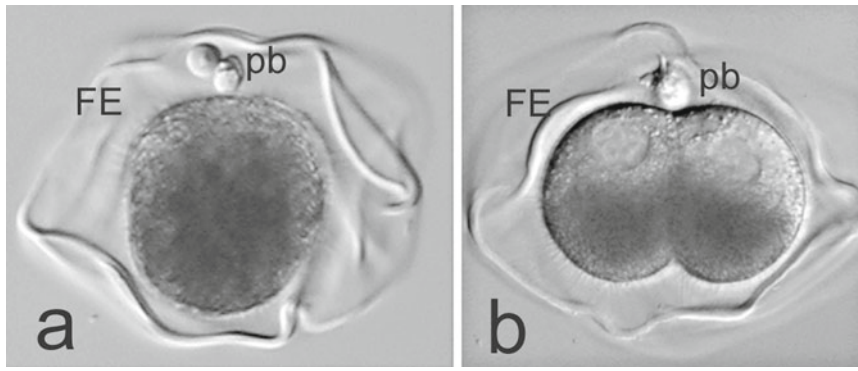

Fig. 3 Differential interference contrast microscopy images of (**a**) zygote and (**b**) two-cell stage of *Membranipora membranacea*. Polar bodies (pb) can be seen at the animal pole of each stage contained within the wrinkled fertilization envelope (FE)

7. For long-term rearing, transfer larvae to larger containers that are stirred at about 8 rpm with a swinging paddle stirring apparatus (*see* Chapter 1).
8. Change water and food at least weekly.
9. At about 12 °C, larvae may be competent to undergo metamorphosis in about 4–5 weeks [20].

3.4 Obtaining Ancestrulae and Growing Colonies

1. Rinse substrates in seawater overnight. Many gymnolaemate species will settle on plastic, acetate, and *ACLAR*® in addition to natural substrates such as macroalgae, wood, shell, and chicken egg shell membranes (*see* **Notes 16** and **17**).
2. Fill bowls, dishes, or multiwell plates with 0.22 μm filtered seawater. To reduce variability in when larvae settle, metamorphosis can be induced using 0.22 μm filtered seawater containing a 10 mM excess of KCl [40, 41].
3. Place appropriate substrates into the containers. Polystyrene weighing dishes can serve as both a container and substrate [35].
4. Transfer larvae competent to undergo settlement and metamorphosis into containers. An individual larva or a small number of larvae can be added to a well of a multiwell plate (*see* **Notes 18 and 19**).
5. Following the initial phases of metamorphosis, transfer substrates with ancestrulae into containers with fresh seawater.
6. Once ancestrulae evert lophophores, feed them *Rhodomonas* sp. at a concentration of 10,000 cells/mL.
7. Colonies can be grown in the lab or moved to the field (*see* **Note 20**).
8. As encrusting colonies increase in size, initial substrates can be glued to larger substrates using a cyanoacrylate adhesive to provide for more surface area for growth [34].

4 Notes

1. Depending on the species, we often use a variety of clear Pyrex glass bakeware to hold colonies. We find 10 oz Pyrex custard cups, 4.8 quart oblong baking dishes, and 8-in. square baking dishes to be readily available and the most convenient for use with a dissecting microscope. A system that works well in running sea tables can be constructed out of modified Tri-Pour® polypropylene beakers and PVC pipe. We cut the bottoms from the Tri-Pour® beakers with a band saw and cover the open bottoms with Nitex mesh (150–500 μm), using either hot glue or silicon glue to hold the Nitex in place. The more open the mesh the easier it is to transfer water between the basket and the sea table. Modified Tri-Pour® beakers are

suspended in the sea table by placing them in a rack built from 2.5 cm rings of PVC pipe that are glued together along their sides at points of contact. The diameter of rings depends on the size of the Tri-Pour® beakers. For example, 1 L beaker baskets can be used for holding large colonies or many colonies, whereas individual small colonies or young colonies can be kept in 100 mL Tri-Pour® beaker baskets. The height of the rack can be set by using nylon screws and nuts to mount the rack on to a set of legs consisting of ¾ in. PVC pipe. In this system water can be changed in the Tri-Pour® baskets by raising and lowering the basket in the rack. Also *see* refs. 27, 28.

2. NaOH is hydroscopic and will quickly pick up water from the air if left exposed in an uncovered weigh boat. We keep some NaOH in a capped 15 mL plastic centrifuge tube and briefly open tube when we are adjusting the pH of the EDTA solution.
3. Bryozoan colonies are a habitat for many organisms, including nudibranchs, ascidians, pycnogonids, turbellarians, polychaetes, small arthropods, and nematodes. Many of these organisms eat either adult tissue or larvae [42]. Removing as many of these organisms from colonies will reduce the risk of adult and larval mortality due to predation.
4. It is important to consider the oxygen consumption of living substrates, such as algal fronds or ascidians, when choosing containers or transporting colonies. In closed systems large algal biomass can cause anoxic conditions very quickly in the dark.
5. Many types of food have been used to feed bryozoans [37]. I have tried to use commercial preparations of algal mixtures for bivalve aquaculture to replace *Rhodomonas* sp.; however, these algal mixtures alone did not support the long-term growth of colonies in the lab.
6. Gymnolaemates spawn sperm (or sperm aggregates) through the tips of the tentacles. Spawned sperm enter maternal coeloms and fertilize either ovarian or coelomic primary oocytes. Although sperm–egg fusion occurs internally, eggs typically do not begin to develop until they are spawned from the maternal coelom (*see* refs. 4, 10).
7. Depending on facilities, it is often convenient to place colonies in a dark box. A dark box can be constructed out of a black or an opaque plastic box or tote. The size of the box will be determined by the containers holding your colonies. Boxes can be adapted with ports to attach hoses for a flow-through system in a sea table.
8. Bright fluorescent illumination (130–170 $\mu E/m^2$ s) has been used to inhibit larvae of four species of *Bugula* (*B. neritina*, *B. simplex*, *B. stolonifera*, and *B. turrita*) from initiating metamorphosis and thus prolonging the length of the larval phase of development for more than 2 days [16–18].

9. The reason why 0.1 mM EDTA enhances developmental success of *M. membranacea* is uncertain. EDTA chelates divalent cations. However, the low concentration of EDTA used to increase developmental success of *M. membranacea* embryos would not alter the concentrations of Ca^{2+} or Mg^{2+} in seawater. The addition of disodium EDTA to seawater does decrease the pH of the seawater by 0.4 U, but this pH change alone does not stimulate *M. membranacea* oocytes to undergo egg activation [10].
10. In *M. membranacea* sperm aggregates of 32 or 64 sperm called spermatozeugmata are spawned through the two distomedial tentacles [10, 43]. Spawned spermatozeugmata are collected by the feeding currents of zooids and can enter maternal individuals through the ITO. Sperm aggregates disassociate and sperm–egg fusion occurs at ovulation, but egg activation does not occur until eggs are spawned through the ITO [10].
11. This protocol will generate a batch of embryos that all begin to develop within the same 2-h window. Consequently, there will be a range of cleavage stages present among the embryos during the first few hours after development has been initiated. Once the early larval stage is achieved, individuals in a batch are indistinguishable from one another. An alternative to natural spawning to achieve greater synchrony within a batch of embryos is to cut open reproductive individuals, releasing eggs into 0.1 mM EDTA in 0.2 μm filtered seawater, cooled to ambient seawater temperature. Even though eggs have not been spawned through the ITO, eggs will undergo activation and develop normally [10].
12. We make mouth pipettors from borosilicate glass tubing (3–5 mm outer diameter), latex rubber tubing (inner diameter should match outer diameter of glass tubing—1/8 in. will often work), and a tip for a 1 mL pipette. Cut glass tubing to lengths of approximately 3.5 in. Make glass pipettes by heating the center of the glass tubing over the flame of a Bunsen burner until it begins to soften. Pull the tubing until the diameter of the center of the tube is about 0.5 mm but the space inside the tube is not closed. After the tubing has cooled, break the tubing to separate the two glass pipettes. If you pull until the lumen of the tubing has closed, you can sometimes still obtain a useful pipette by breaking the glass back to where the tubing is open. Fit the wide end of glass pipette into one end of the latex tubing and the narrow end of 1 mL pipette tip into the other end to use as a mouthpiece. Finer control of the pipette can be obtained by inserting a 1 in. piece of capillary tubing (5 mm outer diameter, 0.25–1 mm inner diameter) into the latex tubing between the glass tip and the mouthpiece.
13. Embryos should be transferred into 0.22 μm filtered seawater as early cleavage stages. Embryos grown in seawater containing

0.1 mM EDTA through late cleavage stages usually do not develop correctly.

14. *M. membranacea* eggs and embryos are very sticky and will adhere very readily to the sides of pipette tips and dish bottoms. Try to gently expel embryos from pipettes into dishes. Embryos that tightly adhere to surfaces may not develop correctly or may remain stuck to the surface as larvae.
15. Larvae seem to do better if food is available as soon as they become competent to feed. If you are only interested in early larval stages, then larvae can be fed a diet of *Rhodomonas* sp. or natural seawater filtered through a 20 μm Nitex mesh filter basket.
16. Observations of metamorphic events and colony growth are often easier to make with transparent substrates such as clear or translucent plastic, acetate, and ACLAR®.
17. Cyphonautes of *M. membranacea* settle preferentially on kelp (e.g., *Laminaria*) (e.g., [25, 26, 40]). Punches of kelp fronds can be prepared using a cork borer. In response to being cut, macroalgae like *Laminaria* produce extensive amount of mucus and should be rinsed overnight in seawater before being used as a substrate.
18. Conspecific larvae may settle gregariously [23, 24].
19. A multiwell plate with 24 or 48 wells works well. The construction of 96-well plates makes observations of events inside the well difficult with a dissecting microscopy. In most instances, you will probably want to use untreated multiwell plates as containers for larval settlement. There are a variety of multiwell plates used for tissue culture that are coated with substances such as poly-L-lysine, laminin, gelatin, and collagen.
20. Mating colonies has been most often achieved by growing two or more colonies in the same container or in close proximity to one another (e.g., [31–33, 35]) rather than by collecting and distributing sperm between different colonies [34]. Fertilization events are not easy to manipulate in gymnolaemates for a variety of reasons. First, sperm spawned into the external seawater enter maternal individuals and sperm–egg fusion occurs internally. Second, sperm fuse with primary oocytes while they are still at a very early vitellogenic stage in many cheilostome species that brood their embryos. In these species, a primary oocyte containing a sperm nucleus may not begin developing for weeks, depending on how many primary oocytes are present within a particular ovary. Consequently, it is difficult to determine when an egg was fertilized. Third, colonies usually contain both functional male and female individuals, so some level of self-fertilization is almost always a possibility. Lastly, males do not spawn dense aggregates of

sperm. Thus, sperm are not easy to collect in large numbers, although *C. hyalina* may be an exception [34]. Recent studies of self-fertility in *C. hyalina* [33] and *B. stolonifera* [35] indicate that selfing may produce high levels of sterile offspring. These results suggest the potential importance of sperm behavior and sperm–egg interactions in the fertilization process to avoid selfing [43, 44].

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Chapter 6

An Invertebrate Embryologist's Guide to Routine Processing of Confocal Images

George von Dassow

Abstract

It is almost impossible to use a confocal microscope without encountering the need to transform the raw data through image processing. Adherence to a set of straightforward guidelines will help ensure that image manipulations are both credible and repeatable. Meanwhile, attention to optimal data collection parameters will greatly simplify image processing, not only for convenience but for quality and credibility as well. Here I describe how to conduct routine confocal image processing tasks, including creating 3D animations or stereo images, false coloring or merging channels, background suppression, and compressing movie files for display.

Key words Confocal microscope, ImageJ, Stereo images, False coloring, Kymographs, Image processing

1 Introduction

This chapter describes strategies to display and present data generated by a confocal microscope—image series collected over focal height or time or both—using primarily the free image processing program ImageJ (http://rsbweb.nih.gov/ij/). This is neither the fastest nor the most sophisticated image processing program, but it runs on essentially any computer platform; it is readily extended using macros or easy-to-program plug-ins; and, because the code is freely available, a user modestly fluent in Java can determine exactly what the program is doing to images. Therefore many of us are content with ImageJ for all of our image processing and analysis. Virtually all commercial image processing software offer similar functions at some level. When such a package does not seem to do what you want, ImageJ is a helpful adjunct. In this chapter I also make use of PhotoShop and QuickTime, which are both widely available and by no means unique in their functions.

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I omit discussion of sophisticated methods for dealing with challenging or extensive datasets, such as deconvolution, automated segmentation, or volume rendering and 3D measurement. Available software for such techniques changes rapidly. Furthermore, at present relatively few embryological applications truly require advanced image processing: careful sample preparation and data collection will usually obviate the need for image manipulation beyond what is outlined here.

Before discussing *how* to manipulate images it is worth considering *whether* to do so. The short answer is that there is simply no avoiding it. However, the complete displacement of film photography by digital imaging, in science as well as everywhere else, and the widespread availability of image manipulation tools, naturally makes people suspicious about the credibility of published figures.

This is not quite so new a problem as it seems: the journals of a century ago were filled not with photos but with pen-and-ink drawings. These are no more nor less credible than the confocal micrographs of today, even though clearly the interpretation of a stained tissue section into a drawing on the page involves a deep act of manipulation. Compared to drawing, however, digital image processing is both easier to do and harder to detect. The issue is not so much outright fakery or deception but rather that we humans find recognizable color images so compelling and therefore intrinsically credible. Poor choices of enhancements, color combination, or rendering can deceive unintentionally.

Few journals yet provide detailed guidelines for image manipulation. The most extensive and well-thought-out set, to my knowledge, has been prepared by the Journal of Cell Biology, which now also makes it easy for authors to deposit original raw data alongside figures. Hopefully other journals will follow the JCB's lead. Their advice, drafted by Mike Rossner and Kenneth Yamada [1], boils down to this: a few straightforward steps are always acceptable and need not be mentioned; a few obvious deceits are universally unacceptable; everything else needs to be disclosed to the reader. This is simple, commonsensical, and costs nothing whatsoever.

For the specific purpose of this chapter, I have adapted their advice, with a few additions, to a list of Ten Commandments:

1. "*God made crop and the contrast stretch; all else is the work of man*," paraphrasing the mathematician Leopold Kronecker, who actually said this about the counting integers. He did not mean that one should not do computations involving irrational numbers. By analogy, in scientific image processing, it is always appropriate to crop to a region of interest. It is also universally desirable to set, judiciously, the white and black point, as long as nothing is thereby obliterated. Everything else—from gamma adjustment to convolution filtering—deviates from obviousness and should be disclosed upon publication.

2. *Honor thy original data, and keep track of what you did to it.* Hard disks are so cheap that no one should hesitate to keep a copy of the originally collected files. All image processing should be judged according to whether the chain of manipulations preserves the essence of these original data.
3. *Thou shalt not blot out specific parts of images*, i.e., to remove dirt or extraneous structures or "nonspecific staining." Image quality intuitively implies something about the depth of investigation, and touch-up undermines this relationship. To quote Rossner and Yamada [1], "If you would have redone an experiment to generate a presentation-quality image in the days before the digital age, you should probably redo it now." This does not proscribe masking to systematically extract specific features, but obviously the use of masks is among the "all else" that should be explained.
4. *Thou shalt not montage together pieces of different images*, unless the boundaries remain obvious to any viewer. Otherwise, to montage different specimens together risks misleading the viewer about the number, context, or independence of observations.
5. *All color is false color*; therefore, use it only when it is required, i.e., to show two labels together, or for highlighting zones of intensity within an image with a single label. When color is required, color tables should be selected to optimize either for the final medium of reproduction (i.e., print or screen) or for human visual perception, hopefully both.
6. *Rotate images by 90° or 180° if at all possible*; this preserves original pixel values. If it is necessary to rotate images by intermediate angles—e.g., so that all of a set have the anterior of the animal to the left—then do so as few times as possible, because this operation involves resampling of the original intensity values in which new pixel values are interpolated between original ones.
7. *Scale images by integer multiples* to avoid interpolation as much as possible. This is for a similar reason to #6: if an image must be enlarged, e.g., for print purposes alongside others, or to show a blown-up inset, then stretching it by integer multiples *without interpolation* amounts to the least alteration of original data. Likewise for image reduction. However, I disagree with Rossner and Yamada [1] that nearest-neighbor "interpolation" should be preferred for arbitrary scaling. For non-integer multiples, this method's only virtue is that it so obviously suffers for its sins: the new pixel values are just as invented as in better interpolation methods, but they are *less accurate*. If images must be scaled by non-integer values, use good interpolation *precisely because* it better matches the original data.

8. *Save compression for last*. Useful data is easily ruined by blithe use of "lossy" image compression before measurement, rendering, or construction of figures. The approximations made by the compression algorithms used for JPEGs or QuickTime and AVI movies can ruin subsequent efforts to create detailed renderings or extract believable measurements.
9. *Whatever you do, do it to the whole image*. To change the intensity in one portion but not the other is potentially misleading. I can think of only one exception, which itself proves the rule: background subtraction, either by subtraction of a reference image or by Fourier filtering, changes intensities (and other features) more or less in various parts of the image, but in either method the entire image participates. Of course, both methods of background subtraction should be disclosed.
10. *Always present your best image*. Say what? Is it not a problem that people show only the singularly remarkable, most compelling pictures? Not if you have reason to believe that they are realistic. As Rossner and Yamada [1] point out, "the quality of an image has implications about the care with which it was obtained." All kinds of factors make it difficult to achieve the perfect image: the specimen was not oriented quite conveniently, the labeling was weak this time, the laser was flickering, etc. Anyone who has any experience with microscopy (or even ordinary photography) knows that to have one great picture required taking many mediocre ones. No amount of (honest) PhotoShoppery will change this. Yes, one can get lucky; yes, the best picture might not always be representative; and yes, someone might choose exceptional data to display because of preconceptions about the ideal results. But on average, the best picture will also be the most representative one. That is usually why it is the best.

2 Materials

Most embryological experiments yield specimens or image sets that do not quite meet the ideals described in standard handbooks of confocal microscopy. With fixed material there are two principle reasons. The first encompasses the problems of labeling a sufficiently clear embryo or larva with sufficient brightness to enable collection of enough sections at high enough signal-to-noise to capture the desired structures.

The second reason is an optical constraint: most invertebrate embryos used in research today are small enough that high magnification objectives with a high numerical aperture (NA) are essential; yet these objectives suffer from a shorter working distance, and the fall-off in signal intensity and resolution as one travels from near the coverslip deeper into the specimen can be severe.

Live imaging over time adds a third major deviation from ideal conditions. The feature of interest is often likely to remain in a single focal plane only by luck, therefore mandating collection of at least a short focal series at each time point. But embryos change rapidly, necessitating frequent time points. Even for the nearly unbleachable sea urchin embryo expressing a GFP fusion protein, serious trade-offs against image quality and sampling density are often unavoidable.

Thus, embryologists simply cannot always achieve theoretically optimal resolution or signal-to-noise. Even so, it is useful to keep certain targets in mind:

2.1 Optical Issues

1. *At 40× and above, or beyond 0.75 NA, one should usually choose any immersion lens over even the best dry lens.* Even with a good instrument and the best lens, ideal coverslips, etc., the dry lenses suffer the greatest loss of intensity and resolution with depth. Even at 20×, an immersion lens, if available, and even at the same NA as a dry lens, can dramatically improve results.
2. *Minimize the differences in refractive index between the immersion medium and the mounting medium and the coverslip.* Commonly used mounting media such as VectaShield or other buffered glycerol preparations have refractive indices significantly different from glass, immersion oil, or water. They are convenient and clear some specimens (like early sea urchin embryos) quite well, but even apparently clear specimens will suffer from spherical aberration due to the refractive index difference to the extent that the far side of the embryo may be severalfold darker than the near side and far less detailed both laterally and axially.

   For exceptionally clear specimens, if a water-immersion objective is available, it may be preferable to mount specimens in aqueous buffer. This has the additional virtue that the specimen is likely to suffer little distortion if it remains in water. A water-immersion lens might be the best choice for live embryos as well.

   The best resolution and brightness, however, will usually be obtained using oil-immersion objectives and a mounting medium with a refractive index close to that of glass. Despite its many drawbacks, Murray Clear (2:1 mix of benzyl benzoate and benzyl alcohol) remains the most rewarding mounting medium for even those invertebrate embryos that are relatively clear (*see* ref. [2], section IV, for method).
3. *Oversample about twofold* … Most modern confocal microscopes will happily estimate *Z*-axis section thickness as well as report lateral resolution. Such estimates assume somewhat more favorable conditions than pertain at the far side of a yolk-rich embryo. Even so, it usually behooves one to sample more densely than the estimated resolution. The estimated section

thickness is based on the ability to clearly resolve objects along the *Z*-axis, not the ability to reconstruct a continuous edge without striations. If one samples too sparsely, intensity gaps will make any reconstruction look like a pile of tortillas.

4. … *but match Z-axis sampling to XY pixel size* if practical. If it is *not* practical to match *Z*-sampling to *XY* pixel size—sectioning an entire embryo at 0.1 μm steps is both tedious and pointless—then choose a *Z*-step that is an integer multiple of the pixel width. This can reduce the need for interpolation in later processing.

2.2 Labeling Issues

1. *Choose good modern fluorophores.* This is the only general advice on offer. Most dyes in the Alexa, BODIPY, or Cy families are both bright and highly photostable, and for all but very scarce labels these should tolerate collection of a well-sampled focal series of well over a 100 sections. Shun fluorescein but not rhodamine; the latter is especially useful if you are stuck with only a 543 HeNe laser.

2.3 Considerations for Time-Lapse Sequences

1. *Prefer many noisy time points over fewer, cleaner images.* This only applies when one finds oneself pushing the constraints of the instrument. If something moves between frames, too bad. If things turn out not to move much from frame to frame, then one can, if necessary, average successive frames to reduce noise. After all, this is approximately what one would do to collect a cleaner frame initially.
2. *Sacrifice axial resolution before lateral resolution, and lateral resolution before time resolution.* If pressed, that is, either by instrument constraints or by tolerance of the embryo or the label. Since the fate of most "4D" series is to be maximum projected and played back as a 2D movie, smooth motion is usually more important than absolute detail.
3. *Extensive photobleaching is harder to deal with than noisy images.* This is especially true if the goal is to measure intensity changes. One can "correct" photobleaching by measuring the average intensity fall-off and multiplying successive time points accordingly, but this can be hard to untangle from other reasons that brightness might change. Besides, however well one can estimate bleaching, brightening the bleached images at the end of the series inevitably yields a far noisier image than those at the beginning of the series.

Because ImageJ has excellent documentation, the procedures here assume that users have installed the standard distribution of ImageJ and familiarized themselves with basic operations. Certain plug-ins that can be downloaded from the ImageJ website (http://rsbweb.nih.gov/ij/plugins/index.html) are mentioned as needed. The most important of these is to install whichever one allows

ImageJ to read your instrument's native file format. For some commonly available confocals, like the BioRads, ImageJ may be able to open the native files without additional plug-ins, but for others the LOCI BioFormats package (http://www.loci.wisc.edu/software/bio-formats) or some more specific equivalent is required. Note that although many confocal operating programs will export data in a generic format (i.e., TIFF), this usually abandons essential metadata (e.g., spatial resolution and interval between time points) which the native files would provide.

3 Methods

3.1 Creating a "Rocking" 3D Animation from a Focal Series

A suitable focal series will consist of a stack of at least a few dozen sections.

1. Set white and black points before rendering, and reduce the data to 8-bit (choose the menu command **Image → Type → 8-bit**) or RGB (*see* **Note 1**).
2. Choose **Image → Stacks → 3D Project…**, select "Brightest Point" for the projection method, and choose Y-axis rotation. If ImageJ accurately reads the metadata, or you filled in spatial calibration information (using the menu command **Image → Properties…**), the slice spacing should be set already. Otherwise, fill in the appropriate number. Use an initial angle of −15°, a total rotation of 30°, and 1° increments. Ignore the transparency bounds (but *see* **Note 2**), leave the opacity at 0 %, and set both depth-cuing aspects to 0. Interpolation may improve results with stacks whose Z-steps are far apart.
3. This creates a new stack of 31 frames; the 16th frame is the straight-on view. Immediately rename it (**Image → Rename…**) to something which identifies it as the Y-axis rotation, such as "Y forward" (*see* **Note 3**).
4. Click in the window corresponding to the original stack to make it active, and again choose **Image → Stacks → 3D Project…**; leave all other settings the same, but choose X-axis rotation. Immediately rename the resulting stack accordingly.
5. For each of these new stacks, use **Image → Duplicate…** to create a copy of the entire stack minus the first and last frame: click the "Duplicate stack" checkbox, and type "2–30," taking the opportunity to assign a helpful name (e.g., "Y reverse"). Then use the menu command **Image → Transform → Flip Z** on each duplicate. Next, choose **Image → Stacks → Concatenate…** to join each series to its reversed copy, making sure that the reversed and trimmed version of each comes second. Again, take the opportunity to assign names ("Y swing"). There is no reason to retain the source stacks.

6. Now we wish to join the Y-axis stack to the X-axis stack. But to do so they must each start playing at the straight-on view, which is presently the 16th frame. To do this, for each rendered stack, use **Image → Duplicate…** to create a copy of frames 1–15, giving it a helpful name ("Y head"). Then delete those same 15 frames from each of "Y swing" and "X swing." Use **Image → Stacks → Concatenate…** to join "Y swing" to "Y head" in that order, likewise for the X-axis stacks; there is no reason to retain the source stacks.
7. The reordered "Y swing" and "X swing" stacks cannot be immediately concatenated because they are of different sizes. This is because the ImageJ projection function pads the window to make room for edges as the stack turns. If these are even numbers one can simply trim the edges (if they are, in fact, blank) or expand the canvas using **Image → Adjust → Canvas Size…** (choose "Center" positioning) so that both stacks are of the same size and can be concatenated directly. If, however, the numbers were not even, then the two stacks might end up misaligned by a single pixel after the canvas size is adjusted. To check this, set the Y and X swing stacks to the first frame—the straight-on view—and choose Process → Image Calculator…; instruct it to compute a difference between "Y swing" and "X swing," creating a new window, and click "No" when asked whether to process the whole stack. If the stacks are aligned, the resulting image will be black. The quickest way to figure out who needs to be nudged in which direction is to create a new window with two slices and the same dimensions as the swing stacks and then paste the first frame from each into each of the two new slices. Flip between to assess the shift, then execute the appropriate nudge on one of the swing stacks using **Image → Transform → Translate…**, and then concatenate them.
8. Choose **Image → Stacks → Animation Options…**, and select a frame rate between 15 and 30. Pressing the backslash key with the resulting stack active should play a smooth movie without jumps or pauses in which, starting from the straight-on view, the object swings first toward the left, then toward the right, then up, then down, and repeat. To add a pirouette after the rocking tour, create another Y-axis rotation from 0° to 360°, with 4° or 5° steps, delete the first frame of this new stack, and concatenate it to the end of the rocking stack.

3.2 Creating and Viewing Stereo Images

One cannot print the pseudo-3D animation created by the previous procedure. Only stereo images allow one to put a 3D view on a page. They work almost as well on screen, although they are limited by the number of pixels available. Most people with two not-too-different eyes can learn one or the other trick of seeing

stereo images; for those who cannot, they are no worse off than if you were to present a single projected rendering. If you can learn the trick, the view is far more compellingly three dimensional than any animation.

First I explain how to create a stereo triplet using ImageJ; afterwards I explain how to learn to see them and why a triplet. Suitable starting material is the same as for creating a 3D rocking animation (Subheading 3.1 above).

1. Choose **Image → Stacks → 3D Project…**, and select "Brightest Point" projection and Y-axis rotation. Make sure that the slice spacing is accurate. Use an initial angle of −3°, a total rotation of 6°, and 6° increments (*see* **Note 4**). Again, ignore the transparency bounds and opacity, and set both depth-cuing aspects to 0.
2. This creates a new stack of just two frames; one is destined for your left eye, and the other for your right. Move to the second frame, and choose **Image → Stacks → Add Slice** to create a blank frame at the end.
3. Move to the first frame, select the entire area (**Edit → Selection → Select All**, or press the A key), copy it (**Edit → Copy** or C), move to the new blank frame, and paste (**Edit → Paste** or V).
4. Choose **Image → Stacks → Make Montage…**, and specify 3 columns, 1 row, scale factor 1.0, and no borders.

   The resulting image is a stereo triplet, and it is used like this: by either of two tricks, one can "fuse" two adjacent images, each of which corresponds to the view angles appropriate for each eye, into a simulated 3D view. One pair of the triplet gives the view from the front, and the other pair gives the view from the back. By presenting triplets, viewers acquainted with either of the two viewing tricks will be equally able to see your data in 3D. *See* examples in Fig. 1 and **Note 5**.

   The two stereo viewing tricks are nearly the same: one has to make one eye look at one of the images, while the other eye looks at the other image (*see* **Note 6**). This means either letting your eyes diverge, or crossing them. This sounds easy, but the next step involves defeating a reflex that everyone has been honing since birth: the eyes must focus at a different distance than they converge. Most people can learn to do one or the other. Since I am naturally inclined toward the divergent method, I offer the following steps:
5. Display the triplet on screen, reducing to fit if necessary, and orient the screen as close as possible to be perpendicular to your line of sight. Keep your head level with the screen. For beginners it helps to place the screen somewhat further away or to make the images to be fused small enough that their

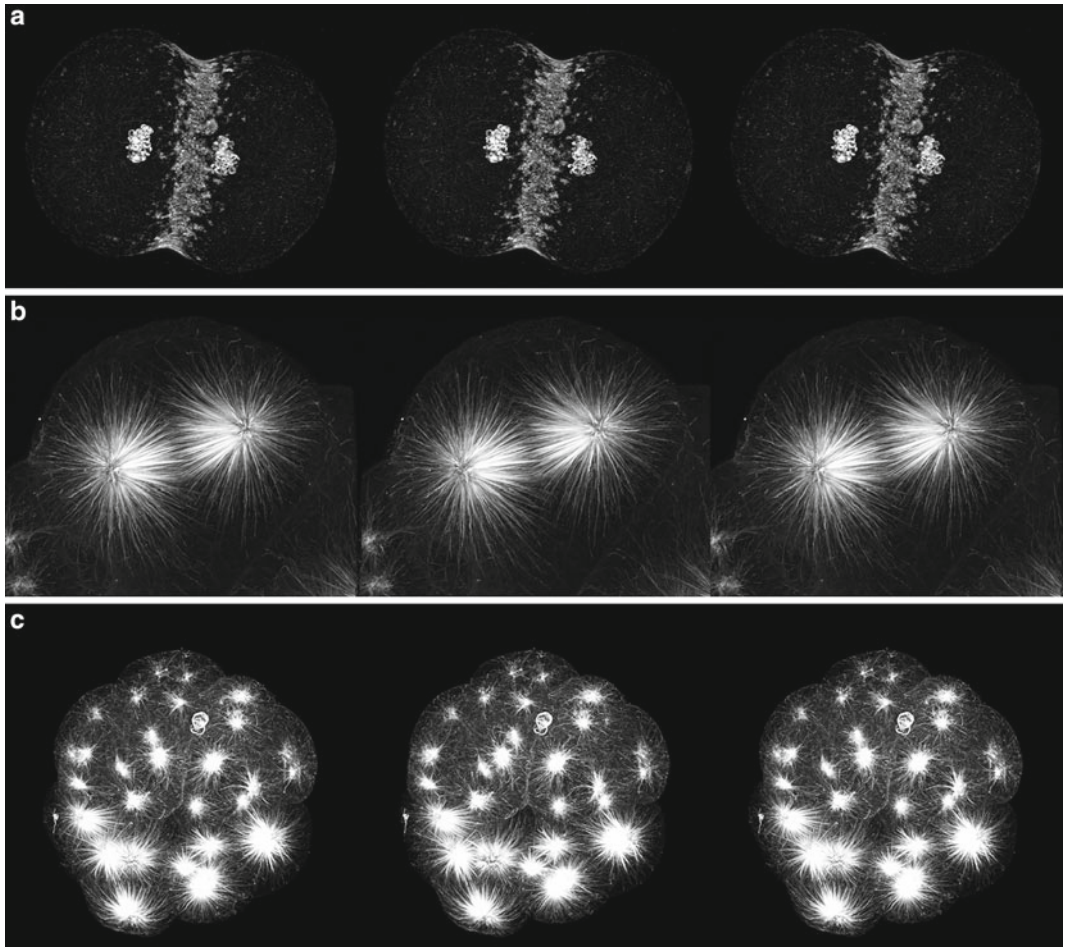

Fig. 1 Stereo triplets. (**a**) Cleaving sand dollar zygote, labeled with Hoechst to stain DNA and antibody to Ser-19-phosphorylated myosin II regulatory light chain. (**b**) Metaphase spindle in a single blastomere of an early embryo of the clam *Acila*. (**c**) Projection through 16-cell embryo of the clam *Acila*

centers are not much further apart than the viewer's eyes. (With practice, one can fuse larger and larger images, but eventually it becomes difficult to swing one's eyes far enough apart.) Try with simple geometric shapes first, like the cubes in Fig. 2a (first row).

6. Pay attention, at first, to either the left and middle images or the middle and right images. Fusing these will also fuse the others. Gaze at a point between the two images, and simply … space out.
7. As you space out (**step 7**), your eyes will wander. Correspondingly, the two images will wander as you lose focus on them (Fig. 2a, middle row). The key is *never to try to focus on them*. The moment you try to focus on the wandering images, your reflexes will take over and snap your eyes back to

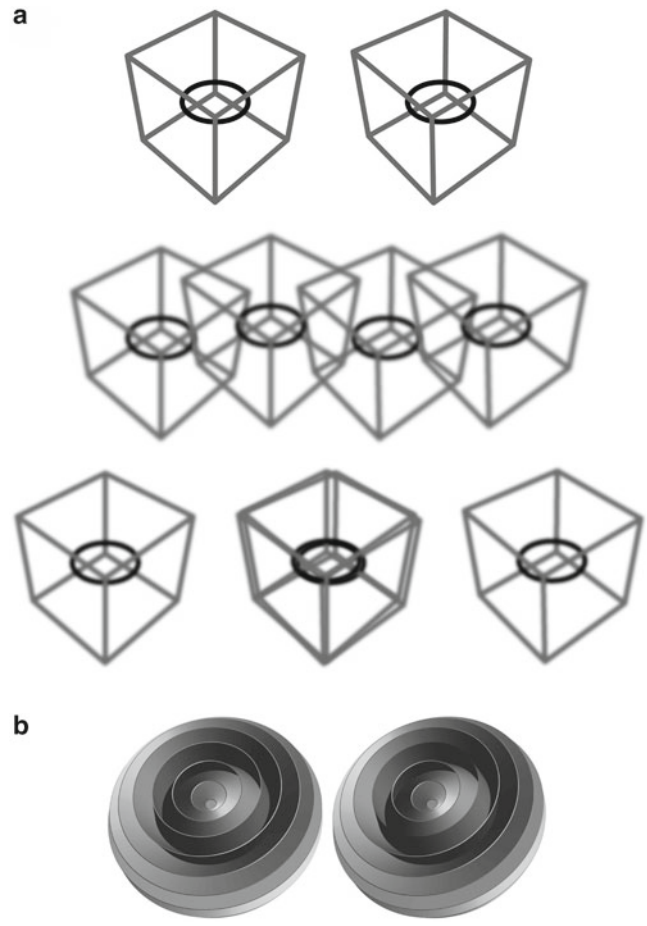

Fig. 2 Stereo viewing. (**a**) Simple geometric shapes, and the appearance at intermediate steps of fusion. (**b**) A somewhat more complex geometric shape

the "right" convergence distance. As the images wander farther apart, still out of focus, they will overlap. The goal is to get them to overlap completely (Fig. 2a, third row). If they start to separate, relax. If they are at the same longitude but different latitudes, tilt your head a little.

8. Once the out-of-focus images are right on top of each other, *do not do anything*. By some miracle, your eyes will discover that there is something to focus on there, and eventually it will just happen. The effect is usually transient at first, but gets better quickly with practice.
9. The cross-eyed viewing method is more or less the same, but instead of "spacing out," use a pencil or a fingertip in the foreground to make your eyes cross far in front of the screen or the page. Keep focusing on the fingertip, and move it closer or further from you, until you can perceive, fuzzy in the background, that the out-of-focus images have aligned on top of

one another. Again, do not *try* to do anything. Just gradually lose interest in your fingertip, and your brain might discover something else of interest in the background.

10. It sometimes happens that the forward and reverse images, once fused, do not seem to be at the same focal level. Nudging the center image one way or the other (select it, cut it, and paste it, and then use the arrow keys to nudge) while viewing "in stereo" will fix it.

3.3 Animating a Projected Slab

Simply projecting an entire focal series often obscures numerous features, especially those in the interior or on the far (that is, the inevitably dimmer) side. To retain the impression of sectioning while making use of three-dimensional data, one can animate a projected slab flying through the stack by repeated calls to **Image → Stacks → Z Project…**, which is exactly what is done by Nico Stuurman's "Running Z Projector" plug-in (available at http://valelab.ucsf.edu/~nico/IJplugins/; a slightly improved version of Stuurman's plug-in is available from my website, www.gvondassow.com/Research_Site/Methods.html). For many specimens, 5–10 sections give significantly greater feel for the whole compared to a single section. Usually the "Max Intensity" option is preferable.

To create an animation in which a single section or slab flies through against a background image of the projected data:

1. Create a single straight-on projection of the entire stack using **Image → Stacks → Z Project…** with the Max Intensity option. It is not necessary in this case to reduce the dynamic range to 8-bit.
2. This projection will be the background for each frame in the animation, so one needs as many copies of it as slices in the original stack. First choose **Image → Stacks → Add Slice** to create a second blank frame. Then select the entire first frame, copy, and paste it into the new blank frame. Finally, choose **Image → Stacks → Scale…**; leaving X and Y dimensions unchanged, choose "None" for the interpolation method, and for "Depth" enter the number of slices in the original stack.
3. The result is a stack of identical images. To serve as a background image, they must be dimmed substantially. Choose **Process → Math → Multiply…**; a factor of 0.3 is often effective.

4a. To overlay the original slices, use **Process → Image Calculator…** and request the maximum of the original stack and the stack of background images.

4b. To overlay a projected slab instead of a single slice, use my version of the Running ZProjector plug-in to create a stack with as many frames as the original stack and then do as in 4a.

5. To make the slice or the slab move in both directions through the stack, use **Image → Duplicate…**, then delete the first and last frame from the copy, run the menu command **Image → Transform → Flip Z**, and concatenate the forward and reverse frames.

Instead of using a dimmed, monochrome background, the projection can be shown in one color and the moving slice in another color. To do this, either skip **step 3** above, or use a milder dimming factor, and **step 4** should be to make a color merge between the stack of repeated projections and the series of slices or slabs.

3.4 Highlight a Horizontal or a Vertical Slab Within a Rocking Projection

First I describe how to highlight a subset of sections (a horizontal slab); alternate steps below explain how to highlight a vertical slab instead.

1. Since this recipe uses the 3D projection function, one must set the white and black point and reduce the stack to 8-bit before starting.
2. Decide which slice, or contiguous set of slices, you wish highlighted, and use **Image → Duplicate…** to extract them from the original stack.
3. The duplicated subset needs to be padded with blank frames on either side. For example, if you chose to highlight frame 35 out of a 100-frame stack, you will need to add 34 blank frames in front of it and 65 behind it. Use **File → New → Image…** to create stacks of blank frames of the appropriate size, and then use **Image → Stacks → Concatenate…** to assemble them with the slice or the slices to be highlighted.
4. The original stack needs to be dimmed out somewhat: make a duplicate of it, and then use **Process → Math → Multiply…** with a factor of 0.2–0.6.
5. Combine the result of **step 4** with the result of **step 3** using **Process → Image Calculator…** to compute the maximum of these two stacks.
6. With the resulting stack, proceed with Subheading 3.1 (or Subheading 3.2) using **Image → Stacks → 3D Project…** to create the desired rendering.

As in Subheading 3.3, one can use color, rather than brightness, for highlighting. Instead of **steps 4** and **5** above, do this:

4. Use **Process → Image Calculator…** to subtract the result of **step 3** from the original stack. This deletes the chosen slices from the original so that they have a more distinct color.
5. Combine the result of **step 4** with the result of **step 3** using **Image → Color → Merge Channels…**; choose colors such

that the highlighted slices are stronger than the background, check "Keep Source Images" so you can try again, and uncheck "Make Composite" so that an RGB stack (which can be used by the 3D projector) results. A suggestion, for a stack with fairly bold but sparse labeling (e.g., muscles), is to put the highlighted frames in the red channel, the original stack in the green channel, and the original minus the highlighted in the blue channel. This results in a yellow highlight on a cyan background. It may still be desirable to dim slightly the non-highlight frames.

To create a rocking projection with a vertical slab highlighted, the method is the same except that instead of blotting out selected entire frames for the highlight stack, one must blot out portions of all frames. Replace **steps 2** and **3** above:

2. Duplicate the original stack.
3. Decide which latitude or longitude to highlight: use the rectangular selection tool to pick out an entire band (from edge to edge), and then choose **Edit → Clear Outside** to delete the margins.

Proceed with **step 4**. The same series of steps can be applied with arbitrary combination of selected regions; the common theme is to blend the entire dataset, either dimmed or in a different color, with a masked version.

3.5 Color Merges and Color Coding

The main use of color for confocal microscopy is to blend two or more distinct labels for display. In this process of *color combination* or *merging* there is no meaningful notion of "true" color: although the detected light had a wavelength, and we distinguish different labels from each other by separating wavelength ranges, the collected data is monochromatic. Applying color is thus an instance of what is inauspiciously called "false coloring." Accordingly, one should feel no compulsion to, say, show rhodamine in red or fluorescein in green. The original wavelength does not matter at all. Instead, one should choose colors according to the nature of the data and human visual psychology [3].

If one has only a single channel to display, it is usually best to present it in grayscale. Even when one has several channels, displaying side-by-side grayscale images provides the maximum detail, the minimum obscurance of one channel by another, and makes the best use of the typical viewer's retina. In some applications, *color coding*—replacing the grayscale with a color table in which hue varies with intensity—can make images more informative, for example to co-display both low- and high-intensity portions of the image in the same panel or to highlight particular intensity bands. Color coding will be dealt with below.

For color combination, it is most convenient to work with images consisting of red, green, and blue channels. It makes

merging two or three channels trivial: in ImageJ, choose **Image → Color → Merge Channels…** and select appropriately; in Photoshop, create a new RGB-mode image of appropriate size; use the Channels palette to select red, green, and blue layers; and paste into them as needed.

This is rarely an ideal use of color, however. Pure red, pure green, and pure blue do not always print well, depending on the device. Moreover, for most people these primaries do not make optimal use of their color vision. This is not just about the color-blind, although this consideration too mitigates against a simple red–green merge.

It may seem like an odd claim: If our photoreceptors respond maximally to these primary colors, should not these be the best display channels? No: first, many people see very little dynamic range in pure blue, considerably more in pure green, and somewhat intermediate in pure red. This is because our blue light receptor is less sensitive than the others [3]. Second, any pure primary strongly excites only one-third of the available sensors, whereas mixed colors (like yellow) excite two-thirds, and therefore we can see mixed colors with higher resolution. The truth is a little more subtle than that, because the response spectra overlap substantially—and that is why we see the most detail in green—but nevertheless these rules are true enough that they have been extensively used to short-cut image encoding in video (both analog and digital) and in image sensors themselves (e.g., through the Bayer mask).

For these reasons, the ideal situation would be to present both the separate channels as monochrome images and a color merge that uses intermediate colors. If pure colors must be used for some reason, use green for the channel in which detail is most important.

The simplest two-channel color merge which satisfies all requirements—resolution, dynamic range, and accessibility for the commonest type of color blindness—is green and magenta. To make such a merge in ImageJ, use one raw image for green and put the other in both the red and blue channels. This combination requires no alteration of the original data, but if your neighbors were to paint their house green and magenta, you would likely consider moving. The next best combination is cyan and red, with the less detail-intensive channel red. The only other two-channel merge which leaves the data unaltered is yellow on pure blue. This is color-blind friendly but is usually suboptimal because humans differentiate intensities poorly in blue (also, blue prints worst of all).

Every other color merge requires that at least one of the red, green, or blue display channels contain information from multiple data channels. There are three ways to do this. First, one channel could be overlaid upon another, blotting out the underlying image except where the overlay is transparent. This requires some artistic control and does not often seem like it adequately preserves the

positive relationship between original data and displayed rendering. Second, the two original channels could be added or averaged, or third, the resulting channel could be computed from the maximum of the two originals at each point. For instance, to make a yellow/cyan merge, the blue and red channels consist of unaltered original data, whereas green is a blend. In ImageJ, with two data channels:

1. Optimize brightness and contrast, and any other adjustments, on the grayscale images alone, and then reduce them to 8-bit before merging. ImageJ (and PhotoShop) handles 16-bit/channel color just fine, but some surprises may occur.
2. Choose **Process → Image Calculator…**, and select the two original data channels; either add them or compute the maximum. Because it is not always obvious which of these is most useful, create and compare both candidates. If the two labels do not overlap extensively, the results are very similar. The Image Calculator will give the same name to each successive combination, so rename its products immediately to keep track.
3. Choose **Image → Color → Merge Channels…**; use the blend for green, one original for red, and the other for blue. Check "Keep Source Images" so that you may …
4. … try the opposite combination. Yellow and cyan are similarly perceived as both bright and detailed, but I often find that large areas of cyan make an image seem dark. This is likely because the eye can tell the difference between true cyan, i.e., 490 nm light, and the mixture created by a blend of blue and green light in a computer screen; most people, however, cannot tell true yellow light (580 nm) from a blend of red and green [2].

In ImageJ this works for stacks just like single images. For three channels the recipe is the same, except that *every* RGB channel gets a blend of two original data channels. There is one potentially significant difficulty: with the steps given above for two channels, the original grayscale data can be recovered from the red and blue channels of the resulting image. This is not possible if three channels are blended according to these instructions; only by using ImageJ's composite image format (or equivalent tools in PhotoShop) is it possible to apply intermediate colors without losing the independence of the original channels.

It is much easier to control colors and proportions using PhotoShop, although one cannot always figure out what, exactly, has been done, and PhotoShop does not handle stacks as naturally as ImageJ. To combine three data channels in PhotoShop:

1. Create a new RGB image of the appropriate size, and, by selecting the appropriate icon in the "Channels" palette, paste original grayscale images into each channel.

2. PhotoShop uses adjustment layers to accomplish certain image manipulations nondestructively. Choose **Layer → New Adjustment Layer → Channel Mixer…**; the controls are initially counterintuitive, but they ask, *for each final* display red, green, and blue, *how much the original* red, green, and blue will contribute. Start by turning everything monochrome: click the "Monochrome" button at the bottom, and then unclick it immediately. Everything turns dimly gray.
3. Now that all those distracting "original" colors are gone, choose "Red," for example, from the Output Channel menu. Using the sliders or the boxes in the Source Channels list, specify a positive contribution (try 50–80 % for each) from whichever original label should end up yellow, and from whichever one should end up magenta, and 0 % for whichever one is supposed to end up cyan. Do likewise for green and blue output channels; for exact intermediate colors (like yellow or cyan), assign the red and green output channels the same percentage from the yellow-to-be, and so on, but obviously to produce other colors (orange, say) one can make whatever mix one wants. To make one of the original channels appear in grayscale, assign the same percentage to all output channels.
4. Once finished, this channel mixer appears in the list of layers above the original RGB image. Double-click it to revise.
5. To alter the relative brightness of the original data channels, create another adjustment layer between the original image and the channel mixer. Click on the layer holding the original image, and choose **Layer → New Adjustment Layer → Levels…**; the Levels panel shows a histogram along which one can drag white and black points for each RGB channel.

If you have to blend more than three original data channels, you can in principle combine up to six by computing blends in ImageJ, but it is cumbersome: one must arrange that the green image contain all three of the intended green, cyan, and yellow components, likewise for the red and blue image … six calls to the Image Calculator and much trouble keeping track of components, but if you really need to do it, you can.

Up to now each channel remains monochromatic. This is virtuously simple, but a chronic problem is that the lower end of the dynamic range is almost inevitably obscured in color merges like these. What is the point of avoiding image distortion—sticking to linear adjustments, avoiding careless background subtraction, etc.—only to end up blotting things out through the back door?

Color coding can ameliorate this problem by exploiting our sensitivity to hue. So-called pseudocolor tables are widely used in certain applications (FRET or calcium imaging, for example) to bring out subtle variations in intensity. The color table (or LUT, for "look-up table" in ImageJ) specifies which RGB color

corresponds to each of 256 intensity levels in an 8-bit image. The color table may be arbitrarily specified, but for this purpose the most useful recipes are continuous transitions from one hue to a neighboring hue while increasing in intensity.

How, in color coding, to preserve the essential relationship between original intensity values and final perceptual intensity? With some effort and considerable theoretical ambiguity, one can calculate color tables which, according to various models of relative luminosity, best preserve this trait. Or one can tuck a calibration bar—a band that smoothly varies from black to white—into the original grayscale image. Whatever happens to the original intensity range will also happen to the calibration bar. In ImageJ choose **Analyze → Tools → Calibration Bar…**, and choose options appropriately. Problem solved.

I find most useful a particular color coding scheme which skews low intensities red and higher intensities progressively more yellow. This is easy to create and comes very close to preserving relative luminosity. To do so in ImageJ:

1. Starting with 8- or 16-bit grayscale image, apply white and black point adjustment, carefully trimming off any background peak; even a slight non-zero background will become visible. If working with 16-bit data, do not convert to 8-bit at this step (unless a calibration bar is needed—**step 2**—which will force this conversion).
2. If a calibration bar is desired choose **Analyze → Tools → Calibration Bar…** and apply desired options. This creates an RGB copy of the image; convert it to 8-bit by choosing **Image → Type → 8-bit**.
3. Duplicate the image twice by choosing **Image → Duplicate…**, giving the copies distinct names, e.g., "red" and "green." (Keep the original to revise gamma adjustments in **step 4**.)
4. For the "red" image, choose **Process → Math → Gamma…** and try a value of 0.6; for the "green" image, use the same command with a value of 1.5. (For the meaning of gamma, *see* **Note 7**.) Once you have applied the gamma adjustment, do not further adjust black and white points.
5. To combine these as red and green channels in a color image, choose **Image → Color → Merge Channels…** and choose the "red" image for the red channel, "green" for the green channel, and nothing for blue and gray; un-check "Create Composite," but check "Keep Source Images."
6. If the result is not pleasing, go back to **step 3** and try different numbers in **step 4**.

One must experiment with different pairs of gamma values for each dataset. The range 0.5–0.75 works well for red, and a complementary range 1.7–1.3 for the green. In the resultant color image,

for all pixels, the average of the red value and the green value should be as close as possible to the original grayscale value.

This makes it quick and easy to color-code a single monochrome image, but it becomes cumbersome to composite several color-coded images within ImageJ (*see* **Note 8**). The same color code is trivial to apply in PhotoShop:

1. Open the grayscale image in PhotoShop, whether in 8- or 16-bit mode, and choose **Image → Mode → RGB Color**. This copies the grayscale information into each of three color channels which can be independently altered.
2. Choose **Layer → New Adjustment Layer → Levels…**, and check the box for "Use Previous Layer to Create Clipping Mask." This makes the adjustment layer apply only to the currently selected layer.
3. Suppress the blue channel: choose "Blue" from the "Channel" menu in the Levels dialogue box, and on the slider labeled "Output Levels," drag the white point arrowhead all the way to black.
4. Now adjust the gamma for red and green channels: under the "Input Levels" histogram, pull the midpoint arrowhead to the left for the red channel and to the right for the green channel, until you achieve the desired effect. Better, type a number in the middle box, noting that PhotoShop's gamma is the reciprocal of what you would use in ImageJ.
5. To revise the adjustment layer, double-click its icon in the Layers palette.

Any other layers to be merged can be similarly treated. For a single channel, the blue-to-cyan equivalent of the red-to-yellow gradient is far less effective, but it is the best choice for a complementary color scheme to apply to a second channel. The channel for which detail is most important should get the yellow. Blending a third channel imposes new complexities, and there is no good rule of thumb. Whatever color scheme is chosen, layer blending in PhotoShop goes like so:

1. Open individual grayscale images containing distinct *data* channels.
2. Create a new RGB image, and paste the first image as an entire layer (that is, not into a particular *display* channel of a layer); create and apply whatever adjustment layer is desired, if any, with a clipping mask as above.
3. Paste additional grayscale channels as entire layers above the first one, and create adjustment layers as needed.
4. In the "Layers" palette, the topmost item is a menu which allows one to specify the blending mode for the selected layer. For the bottom layer and all adjustment layers this should be

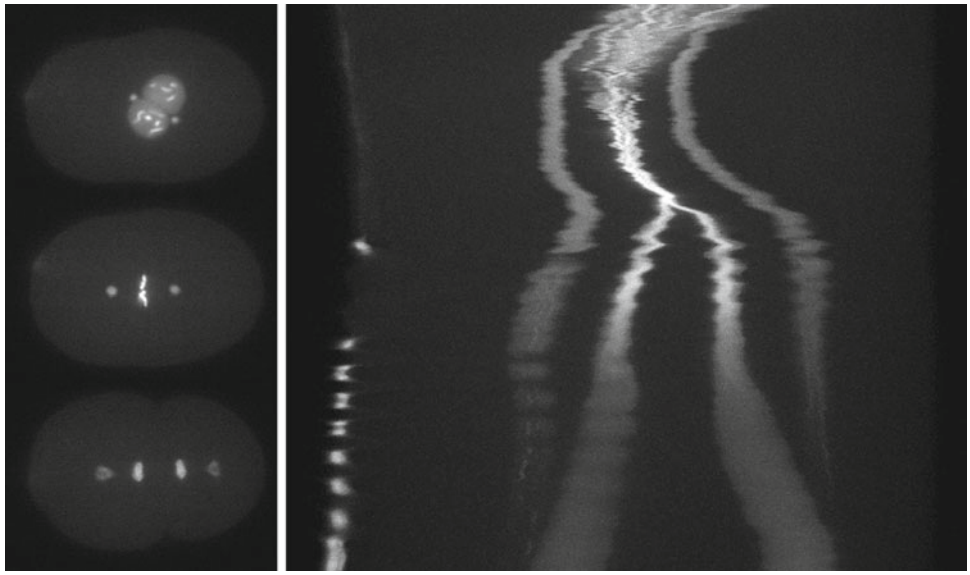

Fig. 3 Kymographs. The *left-hand side* of the figure shows three frames from a time-lapse sequence of a *C. elegans* zygote expressing both GFP–histone and GFP–gamma-tubulin; anterior is to the left. The *right-hand side* shows a kymograph with the anterior–posterior axis horizontal and time vertical. This kymograph was made by reslicing the image stack and maximum-projecting all the frames containing signal from centrosomes or chromatin

"Normal." For each data channel to be overlaid, choose either "Linear Dodge" to add intensities or "Screen" to simulate transparent overlay.

3.6 Kymographs

To depict directed motion in time-lapse sequences, the methods used for three-dimensional *spatial* data are often useful. Where moving objects are bright and relatively sparse, a single maximum projection can be surprisingly effective. When moving objects are densely packed, or when the entire medium flows, the most useful way to display and quantify motion is to create a kymograph—as in Fig. 3—a two-dimensional image in which one spatial axis is plotted against time.

If the time-lapse sequence has many frames (more than 100), and if the time resolution is good enough that each object moves less than its own diameter per frame, then the best way to make a kymograph is to reslice the image stack vertically along the dominant axis of motion and then project a subset of the resliced stack which contains the objects of interest (Fig. 4a):

1. Use the angle measurement tool in the ImageJ toolbar to determine the angle along which the predominant motion of the objects of interest takes place (or the angle of the axis along which motion is to be measured, e.g., the spindle or the anterior–posterior axis of an embryo).

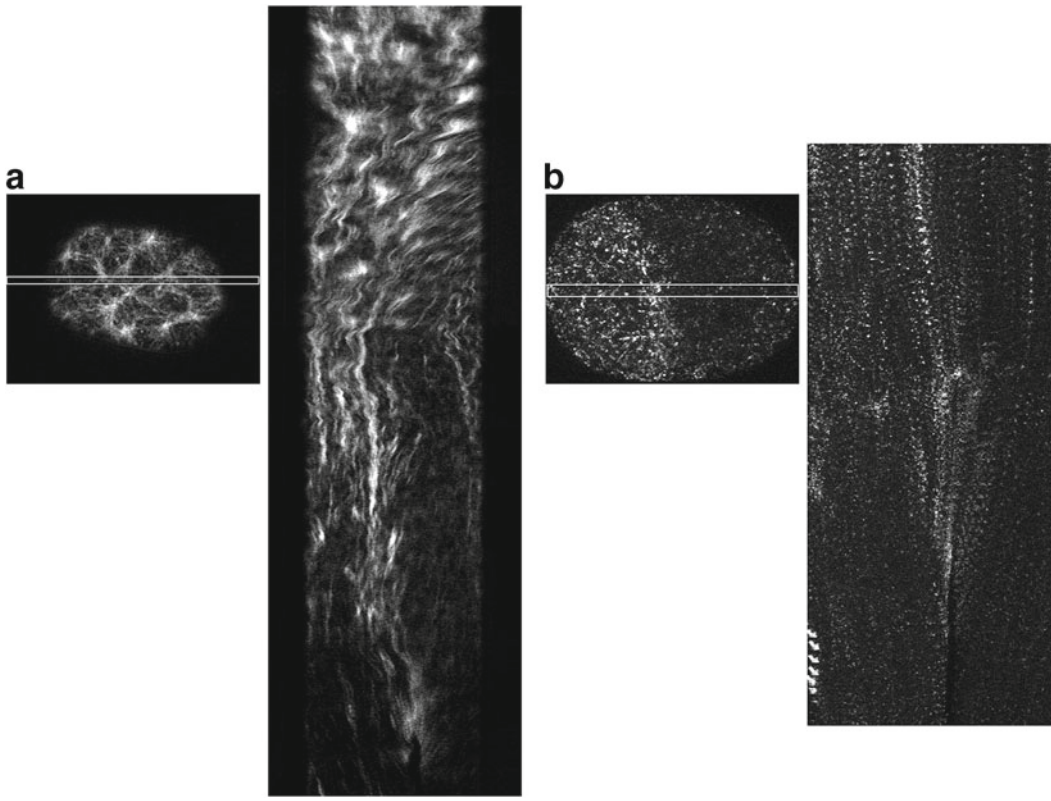

Fig. 4 Fine and coarse kymographs. (**a**) Kymograph produced by the first method described, from a time-lapse sequence of a *C. elegans* zygote expressing GFP–moesin to reveal actin filaments. The frame to the left shows the region covered by the kymograph. The top portion of the kymograph, which starts shortly after meiosis, illustrates active ruffling and anterior-directed flow; the middle portion exhibits little or no flow but static accumulations of actin in the anterior half of the cell; the bottom portion shows actin filaments flowing into the cleavage furrow. (**b**) Kymograph produced by the second method described, from a time-lapse sequence of a *C. elegans* zygote expressing GFP–NMY2 (non-muscle myosin II). The frame to the left shows the region used to make the kymograph, which covers the period immediately before and during cleavage furrow ingression (corresponds to approximately the bottom fourth of *panel* (**a**))

2. Select the entire rectangular region of interest from which a kymograph might possibly be useful. Use the menu command **Image → Transform → Rotate…**, and choose bicubic interpolation. A new rotated and cropped substack results.
3. Select a rectangular region of interest again, and choose **Image → Stacks → Reslice…**; specify that the reslicing should start from the top or from the left side (depending on whether the axis of motion is horizontal or vertical), and check "Avoid Interpolation (use 1.0 for spacings)" (*see* **Note 9**).
4. The stack resulting from **step 3** should have exactly the same dimensions as the region of interest within the input stack but with either width or height swapped with depth. For example,

if you started **step 3** with a 360 × 180 pixel region in a stack of 240 time points, and the reslicing went from the top, you should end **step 3** with a 360 × 240 stack with 180 frames. In each of those frames, time runs vertically and the original horizontal axis remains the same. Each is a kymograph along the horizontal axis, and you can choose the one you need. However …

5. … in most cases, a single-pixel-wide slice does not capture clear trails because small objects stray from frame to frame if they deviate even slightly from straight paths. Therefore, on the resliced stack run the "Running ZProjector" plug-in, with either average or maximum projection and an experimentally determined group size (try 5–20 frames). From the resulting stack, choose the one which best captures the motion of interest.
6. Velocities can be measured directly from kymographs. First, choose **Image → Properties…** and set the pixel width and height in the kymograph to correspond to the original pixel width (in microns) and frame spacing (in seconds; ignore the units here). Next, draw a rectangular selection box around the trail of interest. The toolbar will report the run (distance) per rise (time).
7. This is useful if objects move primarily along one axis. If they take complicated paths or move in diverse directions, the trails may be misleading, but the stack created in **step 3** can still be useful: following instructions in Subheading 3.1 or 3.2, one can render the resliced stack either as a 3D animation or as a stereo pair, which may help to visualize complicated trails (*see* Fig. 5). This helps with visualization but not quantitation, for which, in such cases, one must usually resort to particle tracking (but *see* **Note 10**).

For time sequences of fewer than 100 frames, and in which the individual objects move more than their own diameter from frame to frame, the method above will not work well. Instead, as long as the individual objects are more or less recognizable, one can make a kymograph by cutting out strips and laying them side by side (Fig. 4b):

1. Again, determine the predominant axis of motion using the angle tool, and rotate the stack appropriately.
2. Select a thin rectangular region of interest which contains the objects to be followed. On one axis, this strip should span more than the range over which motion might take place; on the other axis, the strip should be about the width of an individual object.
3. Use **Image → Duplicate…**, clicking the checkbox to copy the entire stack, to excise the strip at all time points.

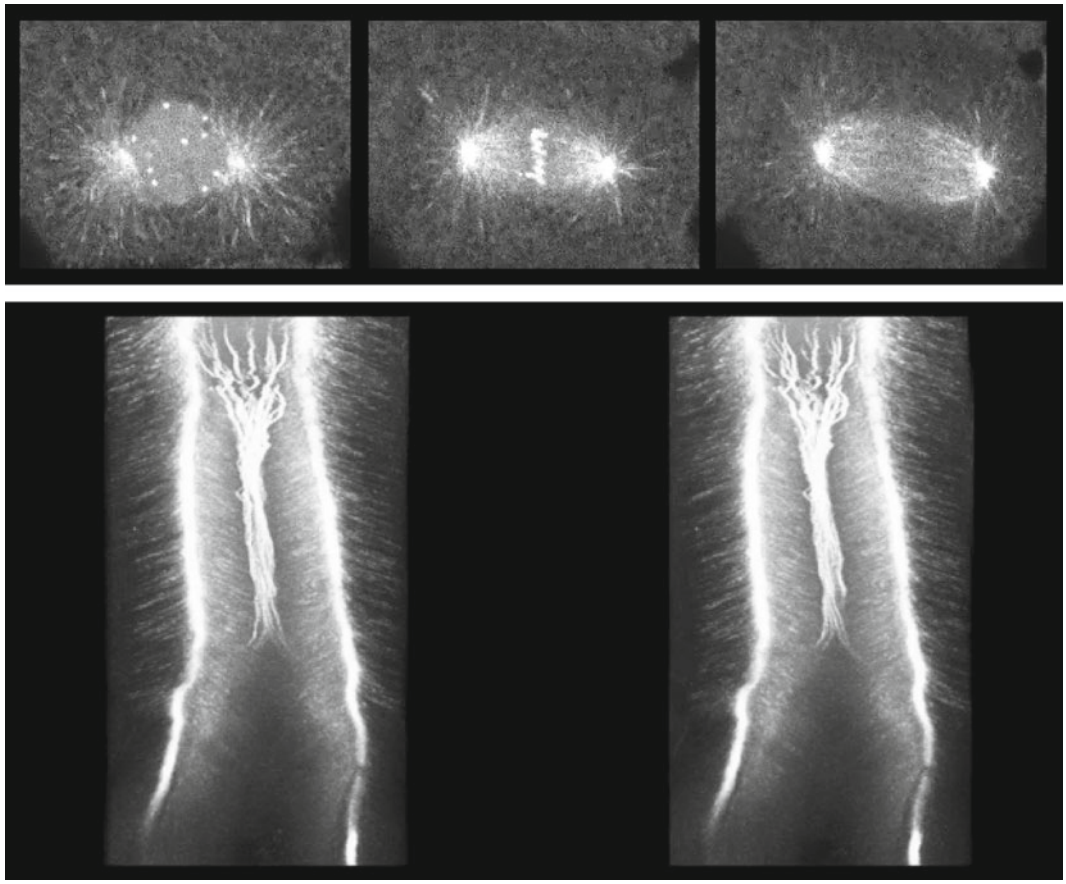

Fig. 5 Kymocube, stereo pair. The *top row* shows three frames from a movie, cropped to a single cell within a 32-cell sand dollar embryo injected with GFP–MCAK, which tracks microtubule tips and binds to kinetochores. In the stereo pair, in which time runs down the page, one can visualize the trails of kinetochores congregating to the metaphase plate and then disappearing as they separate in anaphase

4. Next use **Image → Stacks → Make Montage…** to lay out each strip in either a single row (vertical strips) or a single column. Specify 1.0 for the Scale Factor.

3.7 Suppressing Background with a Reference Image

Many embryos exhibit some autofluorescence, and certain preparation methods also suffer from nonspecific binding by probes, especially when targets are rare. It is sometimes possible to eliminate this kind of background signal by subtracting a clean, scaled image of the autofluorescence or the nonspecific binding (*see* **Note 11**). There are a couple of simple possibilities:

– If autofluorescence has a different spectrum than the label, one may be able to collect a reference image by activating a detection channel in which there is no specific label. For example, many batches of purple urchin embryos contain subcortical granules that are bright enough to pollute low-level GFP fluorescence.

These granules emit almost as much orange as green, so one can collect a second channel along with the GFP signal, scale the image so that it is slightly dimmer than the GFP channel, and subtract.

– Nonspecific binding by antibodies is often shared by variants of the same type; therefore, adding a different color of secondary antibody to the labeling mix may allow one to collect a reference channel (for example, if labeling with goat-anti-mouse alone gives diffuse nonspecific binding, goat-anti-rabbit from the same supplier is likely to do so also).

Both depend on having an empty channel to spare. For background subtraction to work well, both the image and reference should be as close to noise-free as possible. To subtract the reference image:

1. Both signal and reference images (or image stacks) should be open as separate windows. The black points must be set comparably, and any significant detector background (non-zero values outside the specimen) should be trimmed off. For 16-bit images, adjusting the displayed brightness and contrast do not alter the numerical values of pixels. Instead, for each image choose **Analyze → Histogram**; locate the lowest intensity peak, and determine its center by hovering the pointer over that part of the histogram. Then for each image use **Process → Math → Subtract…** to set the black point to equivalent levels.
2. Select a small region within the signal image where you believe there is only autofluorescence or nonspecific binding, and no specific signal. Select the same region in the reference image by choosing **Edit → Selection → Restore Selection**. Measure the average intensity in both selected areas using **Analyze → Measure** (if necessary, call **Analyze → Set Measurements…** to specify that the mean intensity is among the desired measurements).
3. The intensities in the reference image must be scaled by these measurements using **Process → Math → Multiply…**. The appropriate factor is ~0.8 times the ratio of the measurements made on signal and reference images; the factor of 0.8 means that, in the next step, only 80 % of the estimated background will be subtracted; adjust as needed.
4. Compute the subtracted image by choosing **Process → Image Calculator…** and making the obvious choices.

If autofluorescence is shared by two channels containing specific labels, it may be possible to compute an approximate background image from them. This only works if the two channels co-localize very little, but this is not as unusual as it seems: for example, if Hoechst or DAPI is used as a counterstain, and if the

other label is not present in the nucleus, then true co-localization should be virtually nonexistent. In such a case, the background is, approximately, everything that the two images have in common. If the images are relatively low-noise, then the common background can be computed and subtracted like so:

1. Open both images (or image stacks) in separate windows, and, as above, make sure that the black points are set equivalently.
2. Multiply the two images together using **Process → Image Calculator…**; make sure to check the box "32-bit (float) result." This creates an image which is dominated by features that the two images share.
3. Now use **Process → Math → Square Root** to return the common image to the same range as the originals.
4. As in the previous recipe, the background image needs to be scaled before applying it to the originals, but it has to be scaled differently for each channel, so use **Image → Duplicate…** to make a second copy of the common image with a name that associates it to a target channel.
5. For each signal image and one of the copies of the common image, select the same small region of interest that you believe to contain no specific signal, only background; as before, choose an area in one image, and copy the selection to the other window by activating and choosing **Edit → Selection → Restore Selection**. Use **Analyze → Measure** to measure mean intensity in each one.
6. One of the copies of the common image needs to be multiplied, using **Process → Math → Multiply…**, by 0.8 times the ratio of the measured value in channel #1 to the common image and the other copy by the analogous formula involving channel #2.
7. Subtract each background image from the corresponding signal image using **Process → Image Calculator…**.

See the example in Fig. 6. This is not foolproof and genuinely risks losing significant specific signal. It is probably only appropriate when no quantitative information will be extracted from intensity values.

3.8 Noise Reduction Using "Averaging" and Blurring

Since noise is the highest frequency—most rapidly varying—component in the image, one can suppress it (along with the finest details) by replacing each pixel value by some function in which values of the pixels within some radius contribute positively (*see* **Note 11**). Some suggestions include the following:

1. *Median instead of mean*: Because detector noise usually creates pixels with outlier values, the standard simplest possible function is not to compute the arithmetic *mean* of pixel values in the neighborhood, but rather the *median*. In ImageJ the

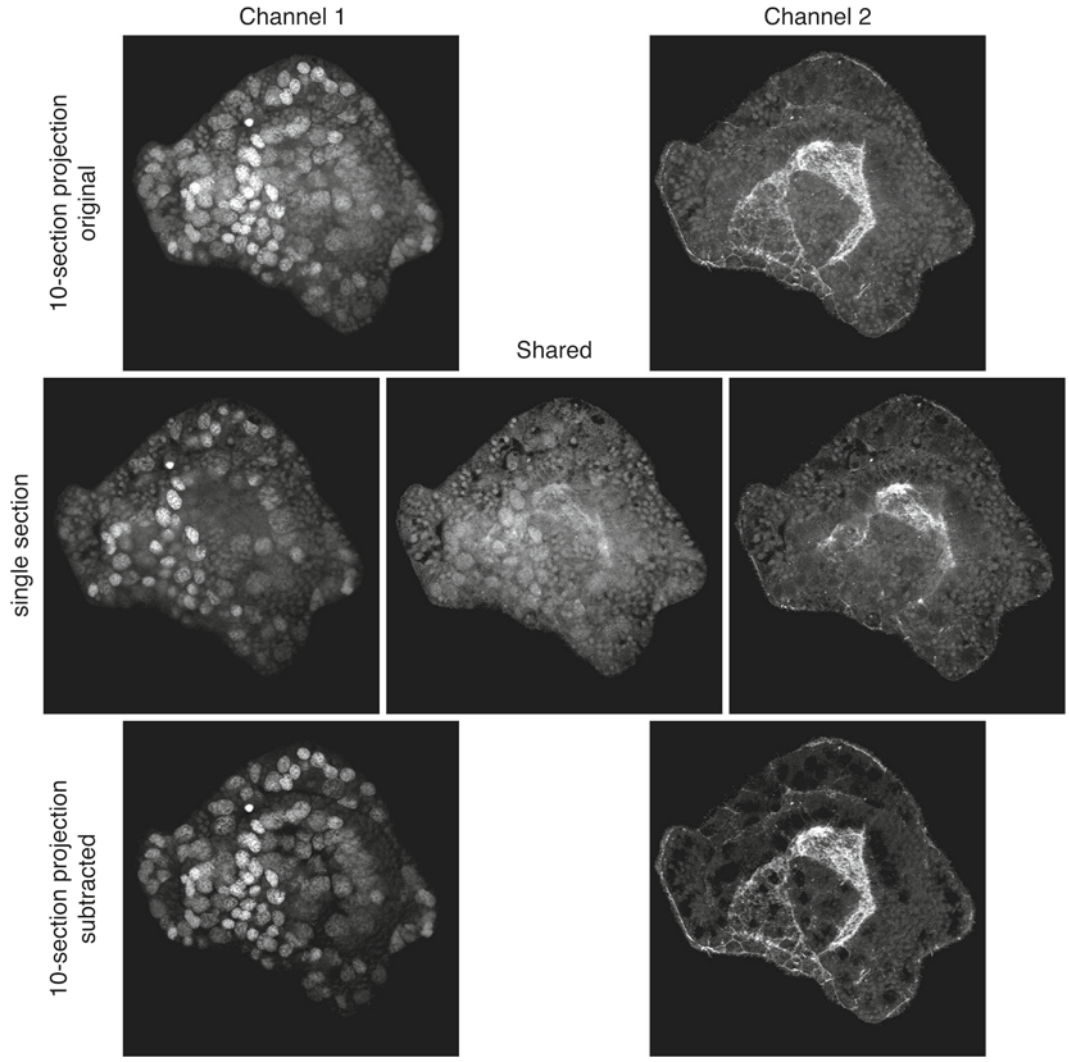

Fig. 6 Background subtraction. A shared background image is computed by multiplying channels together, taking the square root, and then scaling the result before subtracting from each channel. The images used in this demonstration come from a hatchling larva of the polychaete *Owenia collaris* stained with Hoechst (DNA) and phalloidin (actin filaments)

menu command **Process → Filters → Median…** has a single parameter, somewhat euphemistically called the radius; a value of 1.0 means that it uses a 3×3 block of pixels, 2.0 means a 5×5 block, and a value less than one means that it uses the center pixel and its four neighbors. **Process → Noise → Despeckle** is a median filter using radius 1; **Process → Noise → Remove Outliers…** is a median filter which allows one to set a threshold so that only things which deviate greatly are replaced.

2. *Gaussian blur*: The mean and median filters are pretty harsh because each pixel within the specified neighborhood contributes equally. For subtler smoothing use **Process → Filters → Gaussian Blur…**; the sigma value is the standard deviation of a Gaussian curve which is applied to each pixel—fractional values between 0.5 and 1.0 are a good alternative to the 3 × 3 mean.
3. *Convolution kernels*: For even better control, **Process → Filters → Convolve…** allows one to specify a custom filter. ImageJ's online documentation gives thorough explanations of how to use this feature, but note that entering the matrix ((1,1,1),(1,10,1),(1,1,1)) amounts to applying the mean filter with radius 1 and then averaging the resulting blurred image with the original; the matrix ((1,1,1,1,1),(1,4,4,4,1),(1,4,54,4,1),(1,4,4,4,1),(1,1,1,1,1)) blends one part of the 5 × 5 mean, one part 3 × 3 mean, and two parts original image. One cannot do the equivalent operation with the median filter in a single step, but one can create median-filtered versions with successively larger radii, and use the Image Calculator to blend them with the original to create a partially median-filtered image.
4. *Over depth* …: The sole fate of many focal series is to be projected straight-on. If noise within each frame is problematic (it adds up in the brightest point projection) then one can compute a grouped Z projection or a running Z projection (e.g., using the "Running ZProjector" plug-in) of two or three successive frames *averaged* together prior to maximum projection of the whole stack. If the series has been sampled sufficiently finely (that is, more frequently than the optical *Z*-axis resolution), this trick rarely alters any real image features.
5. … *or time*: When collecting time-lapse sequences one often prefers a faster frame rate at the cost of noisier individual images, meaning that each frame follows immediately after its predecessor, in which case computing the running average of two or three frames is equivalent to going back to collection time and deciding to average a few times after all. One can use the median (over time instead of XY space) with the "Running ZProjector" plug-in, but this is only meaningful with subsets of three or more frames; with two frames only the average makes sense.

3.9 Fourier Filtering to Remove Periodic Contaminants

Operating on the frequency-domain (Fourier-transformed) representation of an image is a powerful processing and analysis technique which is beyond the scope of this chapter to explore fully; the ImageJ online documentation includes several explanations with examples, and Wolf [4] provides a gentle introduction to the theory. Here I describe only one specific application, which is to remove periodic patterns that can appear in confocal images due to variations in laser intensity (Fig. 7a). These artifacts usually mean

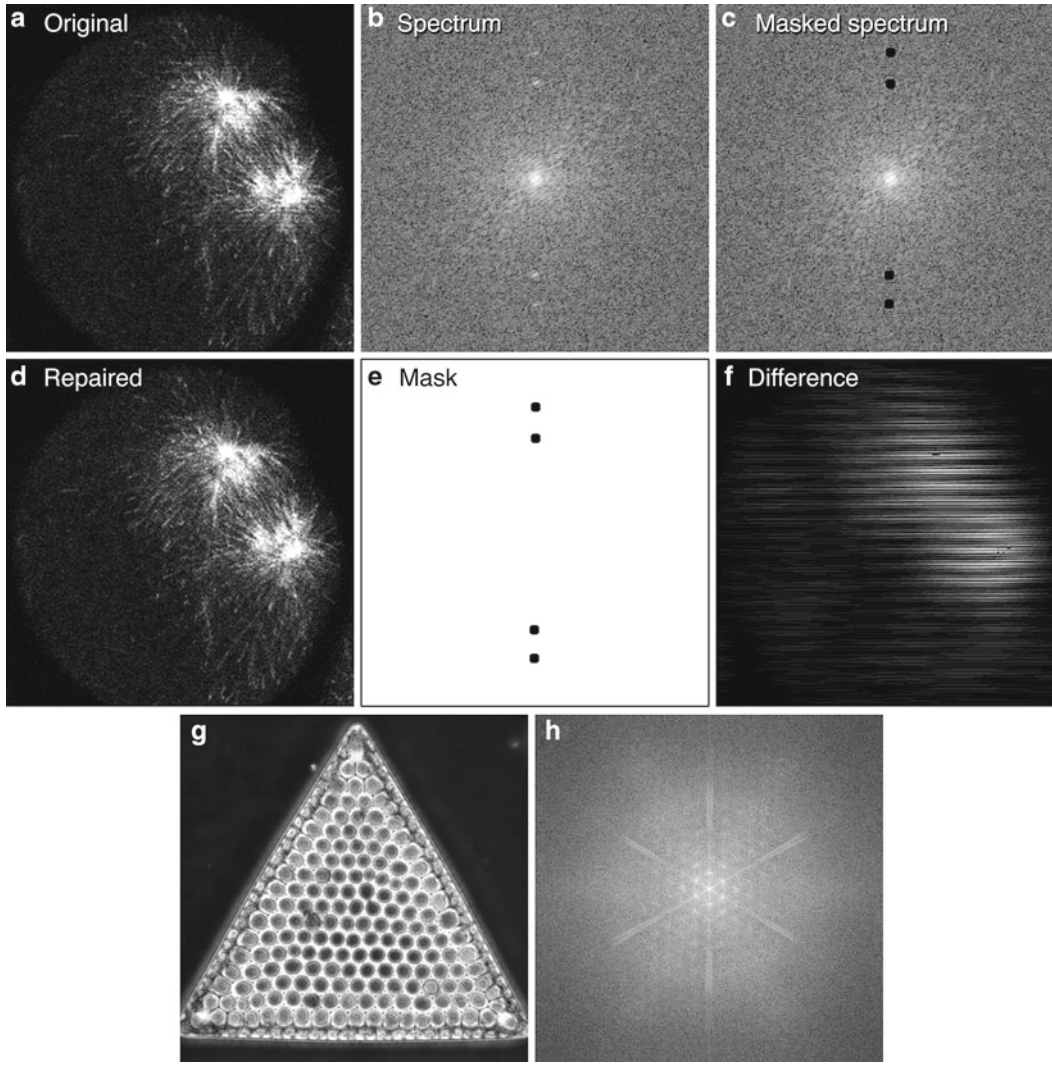

Fig. 7 Eliminating periodic noise. (**a**) Original image; single section from a time-lapse sequence of a sand dollar embryo expressing GFP–EB3, which binds growing microtubule tips. (**b**) Power spectrum of (**a**); FFT. (**c**) Power spectrum with spots blacked out. (**d**) Restored image without horizontal lines; inverse FFT. (**e**) The mask alone. (**f**) The difference between (**a**) and (**d**), multiplied 15× to make the removed lines visible. (**g**) and (**h**) are a phase-contrast image from an arranged diatom slide and its power spectrum; in this case, spots in the spectrum represent very real, repeating features of the specimen

that one needs a new laser or better cooling, but the contaminated data collected in the meantime can be corrected with a straightforward procedure.

This method is based on the premise that all images are really a superposition of a series of waves which vary in frequency, direction, phase, and relative power—a Fourier series [4]. Any periodic pattern within the image, whether a real feature or an artifact, means that the waves with frequency and direction that add up to

create that pattern have more power than other waves. ImageJ can display and manipulate the "power spectrum" of an image: with any image window active, choose **Process → FFT → FFT**. This command produces a two-dimensional image which is actually a plot in which the brightness of each pixel indicates the power assigned to waves of each frequency (the horizontal and vertical axes). The lowest frequencies—corresponding to the broadest gradients—are at the center, whereas the highest—the finest details—are at the periphery. (The phase information, which is required to reconstruct the image, is not displayed.)

The power spectrum *is* the image, just represented in the frequency domain. The familiar spatial-domain version is recovered by reversing the transformation: with the power spectrum window active, choose **Process → FFT → Inverse FFT** to re-create the original. One can modify pixel intensities—the relative powers—in the frequency-domain version of the image to accomplish changes that would be difficult in the spatial-domain version. In this case, to get rid of the artifactual horizontal banding, we want to suppress—that is, reduce the relative power assigned to—a certain subset of waves which do not belong. These show up in the power spectrum as distinct spots (Fig. 7b). In the crudest possible way, one can simply blot them out: using a selection tool or the pen, cover them with black (Fig. 7c). This is equivalent to applying the mask in Fig. 7e to the power spectrum. Inverting the Fourier transform now re-creates the image without this pollution (Fig. 7d).

In ImageJ, doing this to an entire stack of images simultaneously requires slightly more steps, and of course it must be the case that the *same* periodic pattern is present in all frames:

1. Choose a frame from the stack to be processed in which it is obvious which is artifact and which is real. Use **Process → FFT → FFT** to display the power spectrum.
2. Once you have guessed which spots correspond to the contaminant, use selection tools to draw the smallest circles or rectangles which cover those spots and as little else as possible; hold down the shift key to add successive selections. It helps to zoom in on the power spectrum image to do this accurately. The selected areas should be as close to symmetrical around the center as possible.
3. Choose **Edit → Selection → Create Mask** to produce a new image window which contains black background with white areas corresponding to the selected spots. We want the opposite however, so choose **Edit-Invert**.
4. With the copied test frame as the active window, verify that this mask will eliminate the contaminant: choose **Process → FFT → Custom Filter…**, and choose the mask image for the filter.

5. Optional: Slightly better results will be achieved if the mask image is blurred (choose **Process → Filters → Gaussian Blur…** with a sigma of 3 or so) so that it consists of fuzzy spots instead of sharp-edged blots.
6. Assuming that the results are satisfactory, make the window containing the stack active, and revisit **Process → FFT → Custom Filter…**, selecting the option to process the entire stack.

(Not all spots in the power spectrum are pollution! For example, *see* Fig. 7g and its power spectrum, Fig. 7h.)

3.10 Encoding Movies Using QuickTime

Currently the QuickTime 7 Pro player remains the easiest and most reliable way to turn rendered frames into efficiently presentable, high-quality movie files. Apple's documentation for QuickTime Pro covers simple editing features; the procedures below are appropriate for encoding the kind of frame sequence created in Subheading 3.1 (*see* **Notes 12** and **13** on compression and key parameters).

Because of ongoing changes in both ImageJ and QuickTime, directly exporting QuickTime movies from ImageJ is presently troublesome. Therefore, once you are ready to create a display version:

1. In ImageJ, convert the image sequence to 8-bit or RGB, and use **File → Save As → Image Sequence…** to export a series of numbered files in TIFF format (no compression).
2. In the QuickTime player, choose **File → Import Image Sequence…**. At this point you must choose a frame playback rate; I recommend using 15 or 30 (*see* **Note 13**). Changing it later within QuickTime means dropping or interpolating frames, not speeding up or slowing down the video.
3. Choose **File → Export**, and select "Movie to QuickTime Movie" from the pop-up menu; click "Options."
4. In the Options dialog, the buttons arranged under the "Video" heading all need attention; the "Filter…" button just needs to be clicked to make sure that "None" is selected.
5. QuickTime does high-quality image scaling to arbitrary dimension, so if resizing is necessary, this is a good place to do it; if not, make sure that "Current" is chosen; otherwise, QuickTime retains the last values used.
6. Click "Settings…" to specify compression parameters. Select "H.264" from the drop-down menu at the top. Under "Motion," make sure that "Current" is chosen for frame rate. Anything else drops or interpolates frames—remember that the frame rate should be set on import (**step 2**). Unless experiment suggests otherwise, use "Automatic" for key frame frequency.
7. One can either constrain the data rate or the image quality. If one chooses to use the image quality control, for H.264 "best" is usually nearly flawless but inefficient; "medium" is often

acceptable. However it is usually better to specify the data rate: for standard-size video (640×480) it should be possible to achieve acceptable image quality somewhere between 3 and 10 Mbit/s.

Your eyes are the best judge of whether compression has lost anything important, but with hundreds of frames they could use some help. To compare compressed video to the original using ImageJ:

1. Open the original frames into ImageJ as one stack, e.g., by choosing **File → Import → Image Sequence…** to import the series used as input to QuickTime.
2. ImageJ can open QuickTime movies, but it is presently unclear how much this depends on operating system, Java version, etc.; if your ImageJ declines to open QuickTime files directly, then within the QuickTime player choose **File → Export…**, specify "Movie to Image Sequence," and save the frames as TIFFs. Check that the exported frame rate is the same as the movie's playback rate.
3. Open the sequence of encoded frames in ImageJ using **File → Import → Image Sequence…**.
4. Use **Process → Image Calculator…** to compute a new stack consisting of the difference between the original and encoded frames.
5. With the window resulting from **step 4** active, Choose **Analyze → Histogram**, clicking "Yes" when prompted whether to include the whole stack. If the histogram shows that the maximum pixel value in the difference image is smaller than 32 with a distribution skewed strongly to the left, then it would be very difficult to spot the difference between compressed and original frames.
6. For compressors like H.264 that use inter-frame compression, it can be informative to examine the difference stack by making a Z profile: select a region (or the entire frame), and choose **Image → Stacks → Plot Z-axis Profile**; if key frame frequency and data rate are poorly matched, the Z profile of the difference stack will exhibit large periodic oscillations.

QuickTime's file format is a container which coordinates the timed display of various kinds of media, not just video. This makes it straightforward to add simple graphic overlays—scale bars, pointers, or captions—nondestructively. One needs only to make the overlay as a graphic file, add it to the QuickTime file as a separate track, and set a few options:

1. Open the movie in the QuickTime player and move to a convenient reference frame (the first one or the principal one on which the overlay is to appear).

2. Choose **Edit → Select None**—this ensures that only the current frame is selected—and then **Edit → Copy**.
3. In PhotoShop, create a new file the exact size of the movie frame, selecting "Transparent" for the background fill. Paste in the copied movie frame.
4. Draw the desired graphics and text as layers over the movie frame. If some graphics—like a scale bar—are to appear throughout the movie but others—like an explanatory caption—are only to appear for some portion of the movie, one should segregate them into different layer groups; this will make it convenient to save these sets as separate files.
5. To all graphics and text layers, it helps to apply a drop shadow effect. This ensures that a white overlay shows up almost as well when placed over lighter areas of the image (same for black on dark areas). Select the layer in the Layers palette, and then choose **Layers → Layer Style → Drop Shadow…**; to make a tight-fitting shadow, specify 100 % opacity, distance of 1 or 2 pixels, spread of 0 pixels, and size of 1 or 2 pixels.
6. Hide the layer containing the reference movie frame by clicking the layer visibility icon in the Layers palette. One should see only the overlays against a transparent background. Use **File-Save As…** to export the currently visible layers as a PNG file (this preserves transparency). If making several overlays that will be visible at different times, save the relevant layers as separate files.
7. Open the PNG file in the QuickTime player—it will look strange; ignore this—and choose **Edit → Select All** and then **Edit → Copy**.
8. Go to the movie window; choose **Edit → Select All** if the overlay should be added to the entire movie. If the overlay is to show for only a part of the movie, then instead select the appropriate portion using the draggable selection controls on the movie playback bar, just below the pointer which indicates the current position.
9. Then choose **Edit → Add to Selection and Scale**.
10. **Step 9** blocks out the movie; to blend them, first choose **Window → Show Movie Properties**. This brings up a window in which all tracks within the file are listed. Select the new track (which is of type PNG), and click the heading for "Visual Settings." At the lower left is a "transparency" control; from the menu choose "Straight Alpha." It may be necessary to jog the movie from frame to frame to see the effect. (Layer order matters in this step: in the Movie Properties dialog, it is set within the Visual Settings set, next to the transparency option, and the overlay should have smaller/more negative numbers than the movie).

4 Notes

1. When using ImageJ's 3D projection function, if the starting data are larger than about 800 square pixels, it is tempting to scale the stack down before rendering: both rendering and movie playback are cumbersome with large frames. However, because of the way ImageJ's 3D project function works, pre-reduction can reduce the quality in a surprising way: if the axial distance between frames is about the same as the pixel width, the frames adjacent to the straight-on view (small angles) will be little different from the straight-on view itself, resulting in jerks during playback. The same quirk leads to stereo pairs that appear to have only a few distinct layers. Therefore, if available memory allows, it is better to render first and then scale. For stereo pairs, it sometimes helps to scale *up*, then render, and scale down.
2. The "Lower Transparency Bound" in the 3D projection function can be useful to eliminate a background haze; anything below the specified value will not contribute to the projection.
3. When rendering a 3D animation, if you only want to rock the view in one axis, you could stop after **step 2** in Subheading 3.1. ImageJ will animate the frames in reverse as well as forward. However, if you intend to save the animation as a QuickTime or an AVI movie, it is a good idea to conduct **step 5** as well: although QuickTime can loop movies back and forth, other players cannot, and it imposes difficulties for all but the simplest video compressors. Duplicating frames in reverse order avoids any trouble at the cost of "merely" doubling file size.
4. The appropriate angle of separation for a stereo pair is somewhere between 5° and 8°. It depends on how far the screen or the page is from the viewer. Trial and error, with a specimen whose aspect ratio you have an intuitive feel for, will tell you approximately the "correct" angle. Too great an angle will accentuate the depth artificially.
5. It would be great to render movies in stereo. Using ImageJ's built-in functions it is only possible to create a swing around the Y-axis in stereo: render two 30° swings using **Image → Stacks → 3D Project…**, one starting from –18° and the other from –12°, and then use **Image → Stacks → Combine…** to place them side by side.
6. The diverged/wall-eyed and convergent/cross-eyed methods for viewing stereo images have contrasting intrinsic virtues. The former seems to involve less strain, but the latter is less dependent on the physical size of the images to be fused. For this writer, divergent viewing (which I do almost without

meaning to) leads to an apparently larger, and consequently more detailed, image compared to cross-eyed viewing (which I do with difficulty).

7. The gamma function multiplies every pixel value by one over the maximum value, raised to the specified power (the gamma value): a gamma of 1.0 means no change—a linear look-up table—whereas a gamma of 0.5 makes a look-up table shaped like the square root function on the interval 0–1.0, and a gamma of 2.0 corresponds to the equivalent part of the parabola.
8. ImageJ can use color look-up tables for display, and if the same color coding scheme is used repeatedly, the necessary RGB values can be computed using a spreadsheet (columns R, G, and B), saved as a CSV table, and imported using **File → Import → LUT…**. This requires more forethought but saves time and memory.
9. When reslicing stacks to make a kymograph, the "Avoid Interpolation" option is suggested to make one pixel in the resultant stack correspond to a single time point and to ensure that all pixels of the original are represented once and only once. However with short sequences or rapid motion it may be useful to stretch time, which can be done by manipulating the voxel depth (e.g., make it 2× the pixel width and height) and then instructing the reslicer to use a slice spacing equal to one of the original pixels, unchecking "Avoid Interpolation."
10. "Kymographs" in difficult cases: If objects do not move on straight lines and yet one must quantify just the *speed* of a few recognizable objects, then ImageJ's ability to straighten an arbitrary path will be useful. Use the segmented path selection tool (right-click or control-click on the straight line selection tool), and follow objects of interest, one at a time, setting down additional points while using the keys < and > to move through the stack; then use **Image → Stacks → Reslice…** to create a plot of path length over time.
11. Background subtraction and noise reduction require care. Noise and background both represent extremes: the highest and lowest frequencies, which, if excessive, distract perceptually from the detail we care about. If it is more than just a distraction, one cannot usually do much about the noise or the background and still recover detail unaltered. Trying to keep the operator's opinion out of it (by relying on canned routines) is foolish because informed judgement is essential. Whatever filtering is done should be rationalized and repeatable, and one must be able, in principle, to put a sharp pencil on all features of interest in the original and find them again in the product.

12. QuickTime offers many different compressors. For short sequences (<100 frames), use Photo JPEG. It is virtually lossless at "best" quality, and nearly as good at "high" quality, and it plays backwards and forwards smoothly and edits without complication. For longer sequences or to achieve the very smallest file size, use H.264; of all the true video compressors in QuickTime, which use both inter- and intra-frame compression, H.264 usually yields by far the best image quality for a given compression ratio. It suffers from one sporadic annoyance, which is that the color table is sometimes slightly different from the input frames.
13. Key parameters for movie encoding are the frame rate, frame size, file size, and data rate. These are tightly coupled. There is little point encoding video frames at greater than 30 frames per second; humans perceive that to be smooth motion. If one needs to speed up the video, eliminate the extra frames to save space. At the other end, below 15 frames per second, an image series looks like just that: one still picture after another.

    File size is more or less proportional to frame size—to detail level, actually—and playback quality depends on whether the computer can read the data and decode it fast enough to keep up with the specified frame rate; a comfortable range at the time of writing is between 320×240 and 1,024×768, leaning toward the smaller end of that range for Internet playback.

    For presentation within PowerPoint or Keynote, compression should achieve a data rate of less than 20 Mbit/s to ensure smooth playback. For Web presentation, compression should either achieve a small enough file that the average user will not give up while waiting to download (10 MB or so) or a data rate that allows video to stream while downloading over a moderately fast connection (3–6 Mbit/s). Note that one can halve the data rate (for streaming presentation) without dropping a single frame by choosing to play back at 15 fps instead of 30 fps.

Acknowledgement

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Chapter 7

TEM Analyses of Chaetognath Reproductive Organs

George L. Shinn

Abstract

Transmission electron microscopy (TEM) enables analysis of subcellular organization. It is especially useful for describing the diverse array of cell types in the gonads and embryos of marine invertebrates. Here, I describe methods for preserving and embedding the reproductive organs of marine arrow worms for TEM, including procedures for staining thick sections for light microscopy and thin sections for TEM.

Key words Embedding, Fixation, Karnovsky's fixative, Lead citrate, Staining, Uranyl acetate

1 Introduction

Transmission electron microscopy (TEM) provides high-resolution images of biological structures, including the types and arrangement of organelles within a cell and the locations of plasma membranes of complexly shaped cells (Fig. 1). This information allows the characterization of the diverse cell types within, for example, the gonads and embryos of marine invertebrates, as well as the structural changes that occur during cellular processes such as gametogenesis [1] and fertilization [2].

Preparation of specimens involves chemical preservation, commonly referred to as fixation, in toxic chemicals that simultaneously arrest enzymatic activity and cross-link molecular components in order to stabilize their positions [3]. The preserved tissues are then infiltrated with and embedded in a plastic resin that physically supports the internal structures of specimens so that they can be cut into slices, called sections [4]. Typically, stained "thick sections" are first examined by light microscopy in order to gain an understanding of the general cellular organization of the specimen. Targeted parts of the specimen are then "thin sectioned" for more detailed study by TEM. Thin sections are stained with heavy metal stains to enable visualization of components. The image produced by TEM is essentially a shadow of the thin section. The electron beam passes through lightly stained "electron lucent" parts of the

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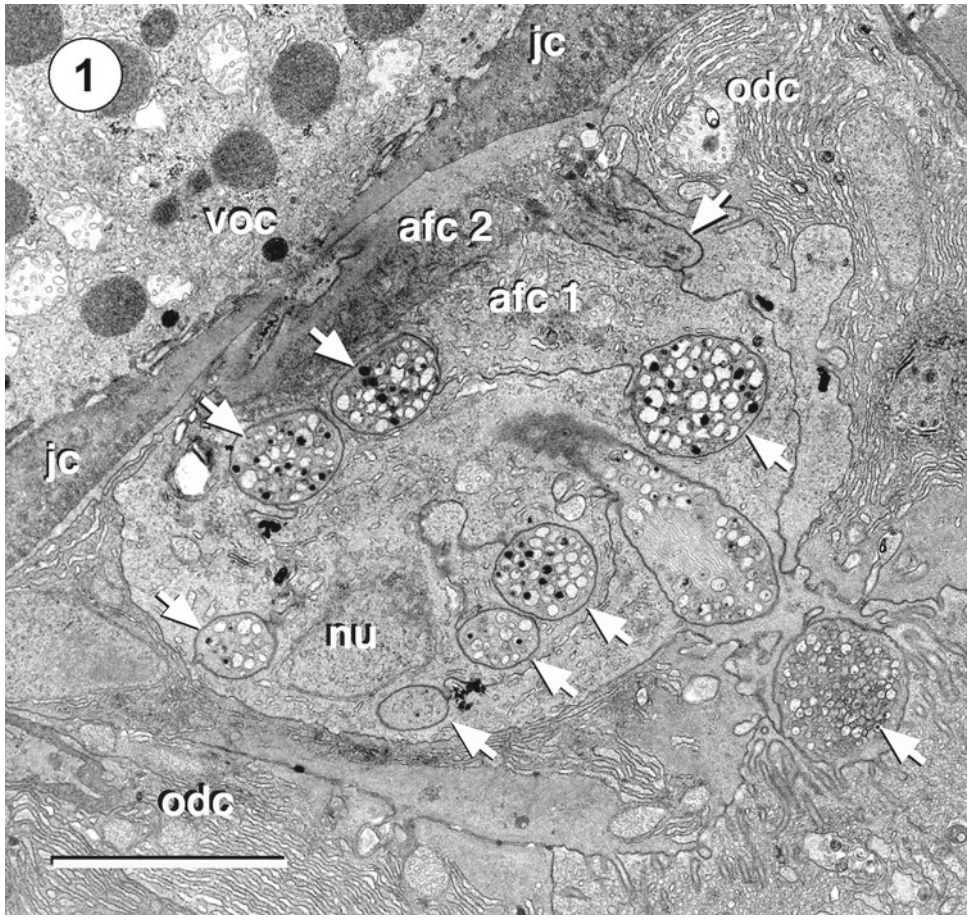

Fig. 1 Transmission electron micrograph showing accessory fertilization cells (afc1, afc2) of the arrow worm *Adhesisagitta hispida. Arrows* indicate sectional profiles of the coiled cytoplasmic process extending from afc2 through the fertilization canal in afc1. *jc* jelly coat of vitellogenic oocyte (voc), *nu* nucleus of afc1, *odc* cellular oviduct. Scale bar = 5 μm

specimen, showing up as light parts of the image; the heavily stained "electron dense" parts of the specimen show up as darker parts of the image.

2 Materials

Use only "EM-grade" reagents. Volumes can be adjusted proportionally to suit the amount of material being used (*see* **Note 1**). Wear gloves and use a fume hood when working with fixatives (glutaraldehyde, paraformaldehyde, osmium tetroxide), propylene oxide, and the components of epoxy resin (NMA, DDSA, DMP-30, EMbed 812). Osmium tetroxide is a particularly volatile and potent fixative and should never be held near the eyes or nose. Similarly, avoid contact with the uranyl acetate and lead citrate

used for staining thin sections. Carefully follow institutional regulations for disposal of chemical wastes.

2.1 Fixation and Embedding Components

1. 0.2 M buffer stock solution A: 2.76 g of monobasic sodium phosphate dissolved in 100 mL distilled water in a 125 mL flask. Store covered at room temperature.
2. 0.2 M buffer stock solution B: 53.65 g of dibasic sodium phosphate heptahydrate dissolved in 1,000 mL distilled water in a 1 L flask. Store covered at room temperature.
3. Sorensen's phosphate buffer (0.2 M, final pH 7.4): 19 mL buffer stock solution A and 81 mL buffer stock solution B.
4. 8 % Paraformaldehyde: in a fume hood, pour 50 mL distilled water into a 100 mL beaker and heat to 60–70 °C (with stir bar on a hot plate). Add 4 g paraformaldehyde to heated distilled water, stirring until dissolved. Add 1 N NaOH dropwise until solution clears (*see* **Note 2**). Cool solution to room temperature, keep covered, and use within 24 h.
5. 50 % Glutaraldehyde (EM grade): Store 10 mL sealed ampoule (Electron Microscopy Sciences, Hatfield, PA, USA) in original container in refrigerator. Just before use, warm to room temperature; shake well and wipe exterior of ampoule before opening.
6. Modified Karnovsky's primary fixative: 50 mL 8 % paraformaldehyde, 10 mL of 50 % glutaraldehyde, and 40 mL of 0.2 M Sorensen's phosphate buffer in a 4 oz amber glass bottle.
7. Secondary fixative: 2 % osmium tetroxide (OsO_4) in 0.1 M Sorenson's buffer: combine in a scintillation vial 5 mL 0.2 M Sorenson's buffer with 5 mL of 4 % OsO_4 from 5 mL sealed ampoule of 4 % OsO_4 (EMS) that had been kept in original shipping canister, refrigerated, until needed. Open only in a fume hood.
8. Epoxy resin: 11.5 g EMbed 812, 3.5 g DDSA, 0.4 mL DMP-30, and 7.4 g NMA from EMbed 812 Resin Kit with DMP-30 (EMS) (*see* **Note 3**).
9. 4 oz amber glass bottles with leak-proof lids (Fisher Scientific) (*see* **Note 4**).
10. 8 mL Wheaton "Snap-Cap" specimen vials (EMS or Ted Pella, Inc.).
11. 20 mL glass scintillation vials with leak-proof lids.
12. 95 % Ethanol (reagent grade).
13. 100 % Ethanol (EM grade).
14. Propylene oxide.
15. 4 oz plastic specimen containers (EMS).
16. Silicon embedding molds (Ted Pella, Inc.).
17. Dedicated benchtop oven, set to 60 °C.

2.2 Components for Staining Thick Sections

1. 1 % Methylene blue stock: 1 g of methylene blue added to 100 mL distilled water in a screw-top jar. Shake to dissolve, store at room temperature.
2. 1 % Azure II stock: 1 g of azure II and 1 g sodium tetraborate added to 100 mL distilled water in a screw-top jar. Allow to dissolve, store at room temperature.
3. Richardson's stain: 10 mL of 1 % methylene blue stock stain added to an equal volume of 1 % azure II stock stain; filter mixture into a 100 mL flask just before use.
4. Wire loop for collecting thick sections during sectioning (*see* **Note 5**).

2.3 Components for Staining Thin Sections

1. Uranyl acetate stain: 2 g of uranyl acetate added to 100 mL of boiled, room-temperature ultrafiltered distilled water. Adjust pH to 5 with 1 N NaOH. Store in brown bottle, wrapped in aluminum foil, in refrigerator. Shelf life approximately 5 weeks (*see* **Note 6**).
2. Lead citrate stain: 1.33 g lead nitrate (reagent grade), 1.76 g sodium citrate (reagent grade), and 30.0 mL boiled, room-temperature distilled water in a 50 mL volumetric flask. Stopper and shake vigorously for 1 min. Allow to stand with intermittent shaking for 30 min. Adjust pH to 12 by adding 8.0 mL 1 N NaOH (prepared from carbonate-free 10 N NaOH, Fisher Scientific Co.). Add distilled water to make 50 mL of solution. Mix by inversion; precipitate will dissolve. If solution is turbid, check water source for dissolved CO_2, NaOH for carbonates or the lead nitrate for age. Store in brown bottle in refrigerator. Shelf life is approximately 5 weeks.
3. NaOH pellets.
4. Stainless steel, fine-tipped reverse-action forceps, as many as five pairs (Ted Pella, Inc.).

3 Methods

3.1 Fixation and Embedding

The entire procedure spans parts of four days, including nearly all of the second day (*see* **Note 7**).

1. Prepare the modified Karnovsky's primary fixative.
2. Pour a small amount of primary fixative into a suitable shallow container, and introduce the specimens into the fixative with as little seawater as possible. Immediately replace the diluted fixative with fresh fixative (*see* **Note 8**).
3. After the specimens have hardened for a few minutes, cut them into pieces, being careful to minimize damage due to handling (*see* **Note 9**). Transfer specimens to Snap-Cap vials, along with more than enough fixative to keep the specimens completely

covered (*see* **Note 10**). Use multiple vials if needed to avoid stacking of specimens in the bottom of the vial. Fix for 1–2 h.

4. While waiting for primary fixation to occur, prepare a 0.1 M phosphate buffer (for rinsing during **steps 5** and **8**) by mixing 10 mL 0.2 M Sorensen's phosphate buffer with 10 mL distilled water. Also, prepare the secondary fixative.
5. Replace primary fixative with 0.1 M phosphate buffer rinse, and leave specimens for 5–10 min.
6. Replace buffer rinse with the secondary fixative; leave specimens in secondary fixative for 1 h.
7. While waiting for secondary fixation to occur, prepare a first batch of epoxy resin by very thoroughly mixing components. Then, prepare the propylene oxide/epoxy resin mixtures for use during **steps 11** and **12** and create "spacers" in the embedding molds (*see* **Notes 11** and **12**).
8. Replace secondary fixative with 0.1 M phosphate buffer rinse; mix gently and leave for 2–5 min.
9. Dehydrate specimens by transferring them through a graded ethanol series. Specimens should remain for 10 min in each dilution of ethanol: 25, 50, 75, 95, and 100 %. Replace the 100 % ethanol twice with fresh 100 % ethanol (*see* **Note 13**).
10. Replace 100 % ethanol with propylene oxide: two changes for 10 min each (*see* **Note 14**).
11. Replace propylene oxide with a 2:1 mixture of propylene oxide/epoxy resin. Very gently rotate containers to mix residual propylene oxide with the resin mixture. Leave capped for 4 h, with gentle occasional stirring (*see* **Note 15**).
12. Replace propylene oxide with a 1:2 mixture of propylene oxide/epoxy resin. Leave vials capped, with occasional gentle stirring, for 12 h (e.g., overnight).
13. Prepare a fresh batch of epoxy resin by very thoroughly mixing 11.5 g EMbed 812, 3.5 g DDSA, 0.4 mL DMP-30, and 7.4 g NMA. Use a clean dry pipette to fill embedding molds with resin (*see* **Note 16**).
14. Transfer specimens to embedding molds. Allow specimens to settle to bottom of embedding molds, and then orient specimens within mold (*see* **Note 17**). Cure epoxy resin by placing embedding molds into 60 °C oven for 24 h.
15. Store embedded specimens at room temperature in well-labeled containers.

3.2 Staining Thick Sections

1. "Thick sections" for light microscopy are cut at a thickness of 850–1,000 nm (*see* **Note 18**).
2. Slides to receive sections should be pre-cleaned with soap, thoroughly rinsed with hot water, and dried quickly while still hot so that they do not accumulate lint from the paper towels.

3. Prepare Richardson's stain.
4. Use a clean wire loop to transfer sections to a small pool of filtered, distilled water on a clean glass microscope slide (*see* **Note 19**).
5. Dry thick sections down onto the microscope slide by placing the slide on a hot plate set to a relatively low temperature. To ensure that the sections are firmly attached to the slide, continue heating the slide for 30 s after the water has fully evaporated (no adhesive is used).
6. While the slide is still being heated, add several drops of freshly mixed, freshly filtered Richardson's stain directly onto the sections; stain for approximately 1 min (*see* **Note 20**).
7. Wash away all excess stain by holding the slide under gently flowing tap water, followed by immersion in distilled water for 1 min. Dry the bottom of the slide on a paper towel and place the slide back on the hot plate to evaporate the remaining rinse water.
8. Add a very small amount of warmed, high viscosity (HV) immersion oil as the mounting medium, and then carefully lower a clean No. 1 cover glass over the sections; firmly press the cover glass down to ensure that the immersion oil is distributed as an ultrathin layer (*see* **Note 21**).

3.3 Staining Thin Sections

1. Allow stock solutions of uranyl acetate and lead citrate to come to room temperature. Do not shake bottles and pipette carefully so as not to suspend any precipitate.
2. Stain grids with uranyl acetate as follows: for each grid to be stained, dispense three drops of uranyl acetate as a small pool on a Parafilm-lined Petri dish. Using reverse-action forceps, float a grid, section side down, on top of each pool. Cover Petri dish; stain for 20 min (*see* **Note 22**).
3. Retrieve the grids and very gently but thoroughly rinse with distilled water. Dry the grids with filter paper (*see* **Note 23**).
4. Stain grids with lead citrate as follows: avoiding contamination due to CO_2, carefully dispense drops of lead citrate onto Petri dish containing pellets of NaOH (*see* **Note 24**). Stain grids for 10 min. Rinse grids thoroughly and dry them.

3.4 Examining Stained Thin Sections by TEM

The ultimate goal of TEM is to obtain high-quality photographs of specimens. Images must be evenly illuminated, precisely focused, and have an optimal balance between contrast and resolution of image details. The areas to be photographed should have well-preserved membranes visible as thin continuous lines that unambiguously reveal the edges of cells and parts of cells. The following procedures apply to a JEOL JEM-100SX that has been retrofitted with an AMT digital camera, but they should be broadly applicable to other models of TEM.

1. Turn on TEM and achieve a working vacuum, but leave both the accelerating voltage and filament set to off.
2. Using reverse-action forceps, load a stained grid into specimen exchange rod from TEM. Insert specimen exchange rod into its port on side of column, and evacuate antechamber; move exchange rod through inner airlock of antechamber, but keep exchange rod and grid out of the beam path (*see* **Note 25**).
3. With room lights off (*see* **Note 26**), turn on high voltage (stepwise to 80 kV then back to 60 kV), slowly turn up filament to saturation (*see* **Note 27**), and align column (*see* **Note 28**).
4. With brightness (condenser lens) at the lowest setting (electron beam maximally spread), insert grid into beam path and warm all sections on the grid by using the stage controls to systematically move all sections through the beam path (*see* **Note 29**). At the same time, assess orientation and quality of sections (*see* **Note 30**). Select a single section to examine in detail and warm it further with gradually higher settings of the brightness until the parts of the specimen can be studied.
5. Focus by viewing the fluorescent screen using the binoculars mounted by the viewing port. Before focusing the objective lens on the image of the specimen, the individual user must focus the binoculars, including both eyepieces, on the fluorescent screen. To focus the objective lens, engage the "wobbler" function of the microscope. Focus exists when the doubled offset images created by the wobbler are superimposed as one (*see* **Note 31**).
6. Frame area to be photographed (*see* **Note 32**), double-check focus, adjust brightness as needed, and lift fluorescent screen out of the beam path to capture image.
7. Before saving an image, adjust the black, white, and gamma values as needed to enhance brightness and contrast. Save the captured digital image as a TIFF file at the highest allowed resolution. Record essential information about the photograph, including magnification, grid number, and identity of the subject.
8. Remove grid from beam path, return scope to standby conditions, remove specimen exchange rod from column, and remove grid from specimen exchange rod. Save backup copies of digital image files on a separate storage device.

4 Notes

1. Through each step of the fixation process, the volume of fluids should be 5–10 times the volume of tissues.
2. When first dissolved, the paraformaldehyde solution is opaque white; when sufficient NaOH is added, the solution becomes

abruptly more transparent, but a small amount of white precipitate commonly persists in suspension.

3. Except for the DMP-30, I use a top loading scale, placed in a fume hood, to weigh the components of the resin into a single 4 oz disposable plastic specimen container; zero ("tare") the scale before adding each subsequent chemical. The chemicals are quite viscous and easily dispensed by pouring from their original containers. I very carefully measure out the DMP-30 with a 1 mL disposable syringe. Small variations in the amount of DMP-30 will affect the harness and brittleness of the hardened resin. Layering the DMP-30 between other ingredients ensures that it gets well mixed with other components of the resin. I stir the combined ingredients using two wooden applicator sticks, counting 200 strokes. This introduces thousands of minute bubbles, which I eliminate by placing the mixed resin in the 60 °C oven for a few minutes. I like to use a relatively hard formulation of the embedding medium. By changing the proportions of ingredients, softer formulations of the resin are possible: 11.2 g EMbed 812, 4.7 g DDSA, 0.4 mL DMP-30, and 6.3 g NMA are considered "medium" hard; and 10.9 g EMbed 812, 5.8 g DDSA, 0.4 mL DMP-30, and 5.3 g NMA are considered "soft."
4. Containers used for dissection and fixation must be completely free of particulates that might adhere to specimens and interfere with sectioning. Thoroughly rinse containers, including new ones, with distilled water but do not dry them with paper towels. Containers to be used for the embedding process must be kept perfectly dry; clean dust out of these using compressed air.
5. I make wire loops using 0.2 mm diameter palladium/gold wire (it is durable and does not rust). A four-inch piece of wire is folded in half around the tapered end of a Pasteur pipette, and the two ends of the wire are twined around each other. This twined stem is taped to the end of a wooden applicator stick.
6. Avoid contact with the uranyl acetate and lead citrate, in either solution or powdered forms. Always wear latex or vinyl gloves throughout the entire staining procedure and be extremely careful not to spill the stains or let stray drops of the stains fall on the counter top. Avoid breathing dried stain powder. Properly dispose of used stains in the appropriate waste container in a fume hood.
7. The entire procedure typically spans 4 days. On the first day, preparation of buffers and the 8 % glutaraldehyde solution takes 1–2 h. Fixation occupies most of the second day. I typically arrange events so that the 12-h second step of the infiltration process occurs overnight. Specimens will need to be placed into embedding molds early on the morning of the third day; this step typically takes 1–2 h.

8. When I collect arrow worms using a plankton net, I keep them alive for 1–2 days in 1 L jars of seawater; surviving specimens heal, yielding improved preservation of epidermal structures. For the initial step of fixation, I use the bottom part of a 3 cm plastic Petri dish. Replacing the fixative eliminates the cloudy white precipitate that forms when the phosphate buffer in the primary fixative contacts seawater.
9. Cutting the arrow worms transversely enables the fixative to penetrate the body quickly and is essential for high-quality preservation. In general, specimens to be embedded for electron microscopy should be cut down to 1 mm per side. I make my cuts near recognizable anatomical landmarks, so that internal organs will be more easily targeted for sectioning after the body has become opaque due to secondary fixation in OsO_4. For example, I cut a short distance anterior to the ovary and just anterior to the ciliated funnel of the sperm duct. I create scalpels by attaching pieces of double-edged razor blades to wooden applicator sticks. The individually wrapped razor blades are bent lengthwise to snap them in half, and each half is broken into 4–5 smaller pieces. One end of the applicator stick is incised; the blade fragment is inserted and then glued into place with fingernail polish.
10. During all steps, it is essential that the specimens be protected from physical damage that would be visible as artifacts when viewed by TEM. I transfer specimens using a plastic pipette that has been cut to enlarge the opening; I never use forceps to handle preserved specimens. During subsequent steps, specimens are left in the Snap-Cap vials, and the fluids are exchanged. Care should be taken to avoid damaging specimens with the tip of the pipette. Also, I leave a very small amount of the original fluid in the vial during each exchange of fluids to ensure that the surfaces of specimens do not desiccate. This is especially important for steps involving volatile fluids such as 100 % ethanol and, especially, propylene oxide.
11. A "spacer" is thin layer of epoxy resin, pre-hardened in a well of an embedding mold. Introduce a very small amount of fluid resin into the mold, spread the resin with the tapered end of a broken applicator stick, remove air bubbles adhering to the sides of the well, and then harden the resin by placing the mold into the 60 °C oven until needed the next day (approximately 24 h). Without spacers, elongate specimens such as arrow worms will not be surrounded by enough resin for effective cross-sectioning.
12. I prepare the mixtures of propylene oxide/epoxy resin in 20 mL glass scintillation vials capping them tightly, mix by shaking, and keep covered until needed during later steps; I approximate amounts of each component added to the vials,

rather than contaminating other containers for making more precise measurements.

13. The dehydration in ethanol must be complete since any water molecules remaining in the specimens will prevent the epoxy resin from infiltrating and hardening evenly.
14. Propylene oxide is extremely volatile. In humid environments, rapid evaporation of propylene oxide will cool the container, leading to condensation of water on its walls. Keep the container of propylene oxide covered as much as possible to minimize evaporation, and dry the condensate to avoid reintroducing this water to the dehydrated specimens.
15. Gentle mixing is achieved by holding the vials at an angle and slowly rotating them, being careful to keep the resin away from the lid.
16. Examine the empty molds with a dissecting microscope to ensure that they are free of debris; use compressed air to blow dust, lint, dog hairs, or other contaminants out of the molds. After adding the resin, place the molds into the 60 °C oven for a few minutes to force air bubbles to the surface. If necessary, use the tapered end of a broken applicator stick to lift residual bubbles out of the resin and away from the top. I do this while looking through a dissecting microscope.
17. After specimens have been introduced to embedding molds, I put the molds back into the oven to accelerate evaporation of residual propylene oxide. The specimens gradually settle to the bottom of the molds. A broken applicator stick is used to center the specimens in the wells and ensure that they are oriented parallel to the sides of the wells.
18. Section thickness is determined empirically to minimize curling of sections as they come off the sectioning knife. Thinner sections yield more delicate staining.
19. The loop must be cleaned by holding the looped end in a flame until the wire becomes red hot; the loop must be thoroughly rinsed with filtered distilled water to remove any ash that may adhere to the loop. I do this once and then keep the loop clean by avoiding touching it with my fingers or any other potentially contaminated surface. Distilled water is filtered using a 0.02 μm syringe filter attached to a 50 mL disposable syringe.
20. Filtering the stains before use and thoroughly rinsing sections after staining are essential steps for removing stain debris that would ruin otherwise good sections. The problem of removing stain debris is exaggerated if the stain is allowed to dry down. Staining time is determined empirically. Optimally, all parts of the specimen can be distinguished on the basis of differential intensity of staining.

21. Once it cools to room temperature, the HV oil serves as the mounting medium, requiring no drying time. If the layer of HV oil is too thick, or if the cover glass used is too thick (e.g., No. 1½), sections will appear out of focus when viewed at high magnifications with a compound light microscope. Slight heating of the slide as the cover glass is being added helps to ensure a thin layer of mounting medium, but extreme care must be taken, because the heated oil soon begins to dissolve the stain out of the sections, ruining them.
22. Extreme care should be taken to avoid contaminating reverse-action forceps, containers, and other materials used during staining. Because the risk of contamination from either external sources or stain precipitates is fairly high, I never stain all the grids from one sectioned block at a single time. Examining stained grids with the TEM is the only way to verify that there have been no problems with the procedure. To dispense the stains from the stock bottles, wipe the outside of a glass Pasteur pipette with a Kimwipe, and then use the pipette to draw a small volume of stain from the middle of the jar, avoiding the surface stain, bottom stain, and the sides of the jar, as these areas may include precipitates.
23. Grids are retrieved from the stains and rinsed one at a time. I do three rinses of 1 min each. Three clean, dust-free beakers are filled with distilled water. A single grid, held by the reverse-action forceps, is immersed into a beaker and moved about continuously using a slicing motion. To avoid losing sections, the grid is never moved with a paddle-like motion that would force the water through the meshes of the grid. One quick way to dry a grid without allowing the sections to contact a surface is to touch the thin edge of the grid to a piece of filter paper. Alternatively, a small piece of filter paper can be inserted between the tines of the forceps and slowly moved towards the grid, until water begins to wick up on the paper, but pushing the paper too far will cause the forceps to release the grid.
24. Avoid contaminating the lead citrate with CO_2 by holding your breath during each step that the lead citrate is exposed to air. Before adding the stain to the Petri dish, arrange 10–20 NaOH pellets around the perimeter of the Petri dish. Once the stain has been added, keep the dish covered except when adding or retrieving individual grids.
25. If the specimen is not introduced into the column at the outset, the beam will have to be turned off to later accomplish this step.
26. One challenge when using a TEM is that the viewed image, formed by a fluorescent screen, is very dim. Room lights must be turned off and the operator's eyes adjusted to darkness.

27. Turning the filament up quickly or operating the scope with the filament setting higher than necessary will shorten the life of the filament. The filament has burned out if the current meter reading fails to rise as the filament is turned up (assuming accelerating voltage is on).
28. Exact alignment of the column is essential for achieving even illumination and optimal contrast and resolution during photography, but not for general viewing of sections. I typically work through **step 4**, evaluating the quality of new sections and allowing the electronics of the scope to warm up and stabilize for 30–60 min, before aligning the column. During alignment, selection of a small-diameter objective aperture (e.g., 30 μm) enhances contrast, but slightly reduces resolution of detail compared to a larger aperture (e.g., 50 μm).
29. These processes should never be bypassed as gradual warming at low brightness stabilizes the embedding medium so that sections can be examined with a more condensed beam (higher brightness) as magnification is increased. It also functions to anchor sections to grid bars and lighten the embedding medium so that stained details of the specimen are more conspicuous. In general, with dark-adapted eyes, use the lowest brightness necessary to see the desired parts of the specimen. This should always be below the level at which the edge of the beam is evident on the fluorescent screen. If the specimen moves by itself, brightness has been increased too quickly; decondense the beam and warm the section more thoroughly. Even modest brightness settings can cause physical damage to parts of specimens that are not supported on all sides by a grid bar or that have holes in the embedding medium.
30. For complex multicellular subjects, it is essential to know the histological structure of the specimen from light microscopic examination of stained thick sections. Sections vary with respect to how they land on grids; for some, the important parts will be obscured by grid bars or will not be supported on all sides by grid bars. When viewed at low magnification, sections commonly vary in overall shade. The lightest sections are the thinnest. These typically yield lower contrast images, most evident at low magnification, but better resolution of detail, most evident at high magnification.
31. TEMs are not parfocal and must be refocused whenever magnification is changed. For a microscope that has been retrofitted with a digital camera, I focus the specimen on fluorescent screen of the TEM. Focusing live digital images on the computer monitor is difficult because of the slow refresh rate and graininess of the live camera mode. If final (captured) digital images are blurry, then the digital camera itself must be focused relative to the fluorescent screen.

32. Take low-magnification photographs first. These typically reveal groups of cells and provide a context for understanding the positional setting of higher-magnification images. Medium-magnification photos typically show characteristic structures of single whole cells, including the nucleus, and high-magnification images reveal important subcellular details. Because it would contain information about the relative sizes and positions, a single photograph that clearly shows three parts is more useful that three photographs each showing just one part.

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Chapter 8

SEM Analysis of Marine Invertebrate Gametes

John Buckland-Nicks

Abstract

Scanning electron microscopy (SEM) enables a close-up investigation of topographical features at the cellular level down to a resolution of about 3 nm. In terms of gametes this allows for examination of minute surface details and changes that occur during fertilization. Sometimes these features are hidden from view and must be carefully exposed during preparation in order to be made visible in the SEM. This chapter describes methods that can reveal surface details of gametes for observation, as well as fix them permanently, while keeping fixation artifacts to a minimum.

Key words Chiton gametes, Fixation artifact, Osmolarity, Transitional solvent, Immunogold, Critical point drying, Sputter coating, Remounting, Stereo pairs, Back scatter, Secondary electrons

1 Introduction

In scanning electron microscopy (SEM), a focused beam of electrons scans the sample, interacting with its surface and releasing a variety of signals. Such signals in turn can provide detailed topographical information that complements structural information revealed by transmission electron microscopy (TEM). For example, low-energy "secondary electrons" detected by SEM are used to form an image of surface features at about 100× better resolution than is obtainable by conventional light microscopy (Figs. 1, 2, 3, 4, 5, and 6). Other forms of electromagnetic radiation, including back scattered electrons, X-rays, and cathodoluminescence, may also be captured by specific detectors and used to reveal more information about the sample. In most situations this requires that the cells and tissues be stabilized as close to their natural state as possible with fixatives that maintain osmolarity, cross-link proteins, and impregnate cells with heavy metals. These procedures prevent the electron beam from penetrating too deeply and instead facilitate interactions with the sample's surface. Cells and tissues usually must be fully dehydrated without causing drastic changes to cell volume, shape, or fine structure. Fine details that

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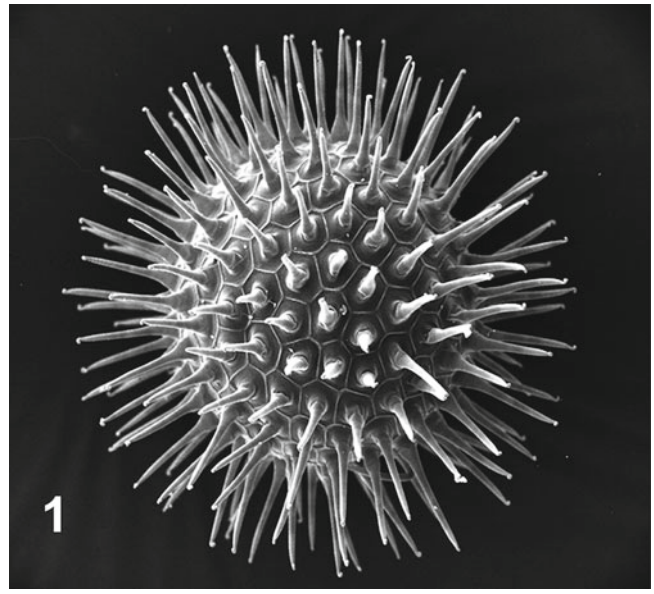


Fig. 1 Egg of the white chiton, *Stenosemus albus*. Note: removing eggs soon after they are spawned avoids debris. Filtering solutions that contact these eggs also reduces particulate contamination on the hooked spines. Scale bar = 50 μm

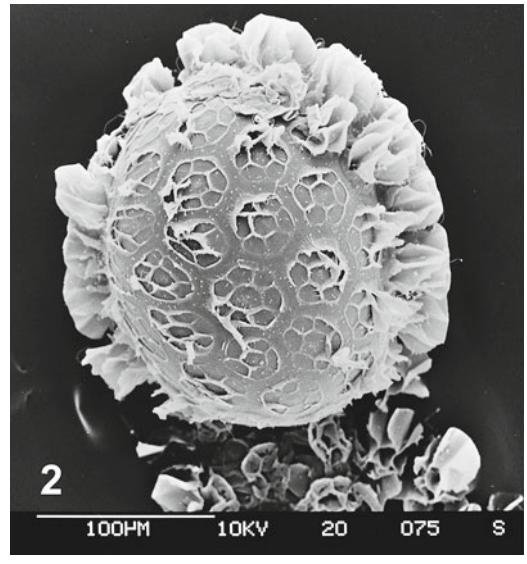


Fig. 2 Egg of the chiton *Mopalia muscosa* rolled on sticky tab to remove cupules and allow interior views. Scale bar = 100 μm

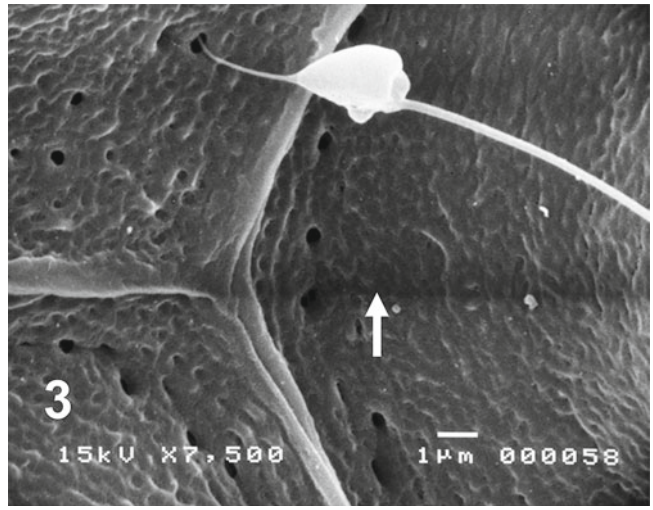


Fig. 3 Photograph of sperm fertilizing egg of *Stenosemus albus* taken in 2007. Note: small charge line (*arrow*). Scale bar = 1 μm

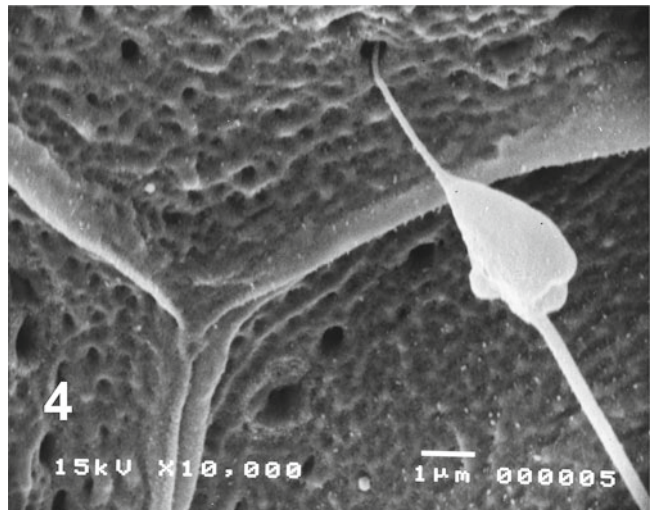
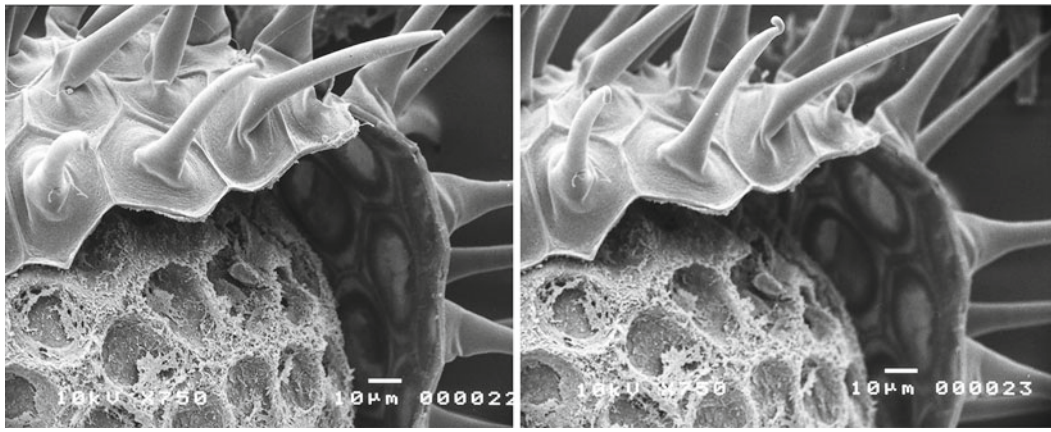


Fig. 4 Same sperm as in Fig. 3 photographed in 2010 after storage in desiccator for 3 years. Recoating with gold has removed charge line. Resolution has been improved by reducing working distance and spot size. Scale bar = 1 μm

are hidden from view must be revealed by removing layers or dissecting away parts that confuse or confound the key targets of the study. All of these techniques require meticulous attention to detail for best results. Fortunately, since the inception of SEM in the 1960s, various helpful SEM techniques have been described [1–4], including reliable methods for processing gametes and embryos



Figs. 5 & 6 Stereo pair of SEMs of cracked egg of *Stenosemus albus*. Scale bars = 10 µm

[5–13]. This chapter takes advantage of the discoveries of these SEM pioneers and adds new details on how to process gametes for SEM, while making these techniques accessible to first time electron microscopists.

2 Materials

SEM reveals both important structural details and unwanted debris or unnatural form. Thus it is imperative to keep all experimental solutions free from particulates by filtering them. Moreover, to minimize contamination even when animals spawn in dishes, replace natural sea water (SW) with filtered SW (*see* **Note 1**). Furthermore, manipulations of fixed samples to reveal inner layers or remove contaminating debris, crucial as these steps are, should be kept to a minimum. Most solutions used in SEM are toxic and must be disposed of correctly. In addition, the majority of procedures involve volatile or otherwise hazardous materials that should be carried out wearing double gloves under the fumehood. All materials listed are available from EM Sciences Ltd. unless otherwise stated.

2.1 Obtaining Gametes

1. Filtered sea water (FSW): 0.22 µm FSW. Vacuum filter 500 mL sea water with a 0.22 µm Millipore filter kit.
2. 100 mm petri dishes.
3. 20 mL glass scintillation vials with poly-seal lined screw caps.

2.2 Fixation

1. Primary fixative buffer: 0.2 M sodium cacodylate buffer. Weigh 4.28 g sodium cacodylate and transfer to 100 mL distilled water (DW) in a beaker. Mix and adjust pH to 7.4.
2. 10 mL ampoule of 25 % EM grade glutaraldehyde.

3. Isotonic primary fixative: 2.5 % glutaraldehyde, 0.1 M sodium cacodylate buffer, FSW, 0.06 M sucrose, pH 7.4. Add 10 mL 25 % glutaraldehyde to 40 mL FSW in a beaker. Add 50 mL primary fixative buffer. Weigh and dissolve 2.74 g sucrose. Refrigerate (*see* **Note 2**).
4. Isotonic buffer rinse: 0.1 M sodium cacodylate, FSW, 0.26 M sucrose, pH 7.4. Mix 50 mL FSW with 50 mL primary fixative buffer in a beaker. Weigh and dissolve 8.9 g sucrose.
5. Osmium tetroxide (OsO_4) stock solution: 5 % OsO_4. Break 1 g ampoule of OsO_4 along the score line and upend into 20 mL FSW in new scintillation vial, cap tightly, seal with Parafilm. Leave in fumehood overnight to dissolve (*see* **Note 3**).
6. Isotonic secondary fixative: 1.25 % OsO_4, 0.1 M sodium cacodylate buffer, FSW, 0.21 M sucrose, pH 7.4. Mix 5 mL OsO_4 stock solution with 5 mL FSW in a new vial. Add 10 mL primary fixative buffer. Weigh and dissolve 0.72 g sucrose. Cap the vial, seal with Parafilm, and store in fumehood until use.
7. Uranyl acetate solution: 0.5 % uranyl acetate. Weigh 0.1 g uranyl acetate and add to 20 mL DW in scintillation vial. Cap, cover with aluminum foil, mix, and leave for 24 h to dissolve.
8. Teflon® microporous specimen capsules (78 μm pore size) (EMS Cat# 70187-10).
9. 5 × 7 mm silicon wafers (Ted Pella Inc, Cat#16007).
10. Wet chamber: place two layers of filter paper in lid of petri dish and wet with DW.
11. Overlay with a strip of 2″ Parafilm cut to fit.
12. 0.1 % Poly-L-Lysine hydrobromide 70,000–150,000 MWt.
13. G-gard-ER or Formalex (*see* **Note 4**).
14. Corn oil (*see* **Note 5**).

2.3 Dehydration

1. Anhydrous ethanol (100 %).
2. Ethanol series: 10, 30, 50, 70, 90, 95 %. Made from anhydrous ethanol and DW and run through 0.22 μm filter, before use.
3. Powder free vinyl or nitrile gloves.
4. Acetone.
5. Chloroform.
6. Amyl acetate.
7. 60 mm Pyrex® petri dishes.
8. 35 and 60 mm disposable petri dishes.
9. 50 mL Pyrex® beakers.
10. Vacuum desiccator with dry desiccant.
11. Critical point dryer (CPD).

2.4 Mounting

1. SEM stubs, 12.7 mm diam (*see* **Note 6**).
2. 12 mm double-sided carbon sticky tabs.
3. Carbon cement or silver cement.
4. SEM stub storage box.
5. DuMont #5 fine forceps.
6. Dumoxel SEM specimen mount forceps.
7. 000 gauge insect pins (Fine Science Tools Cat# 26001-10) (*see* **Note 7**).
8. Chloroform glue (*see* **Note 8**).
9. Breakable double edge carbon steel razor blades (*see* **Note 9**).
10. Wooden applicator sticks 15 cm × 2 mm.
11. LePage's 5 Minute® epoxy glue.

2.5 Immunogold Labeling

1. 250 mL glass media bottles with screw caps.
2. 10 mL ampoule 10 % paraformaldehyde solution.
3. Tris buffer: 20 mM Tris, 150 mM NaCl, pH 7.6. Weigh 2.42 g Tris base, dissolve in 800 mL deionized DW, adjust pH to 7.6 with 1 N HCl. Weigh and dissolve 8.7 g NaCl. Adjust to IL with DW.
4. 0.4 M Millonig's phosphate buffer: weigh 11.8 g Sodium phosphate (monobasic) and dissolve in 200 mL DW. Weigh and dissolve 2.85 g NaOH. Adjust pH to 7.4.
5. Buffer wash: 0.1 M Millonig's phosphate buffer, 3 % NaCl, pH 7.4. Mix 25 mL 0.4 M Millonig's phosphate buffer with 75 mL DW. Weigh and dissolve 3 g NaCl.
6. Immunofixative: 4 % paraformaldehyde, 0.1 M Millonig's phosphate buffer, 3 % NaCl, pH 7.4. Mix 7 mL DW, 8 mL 10 % paraformaldehyde, and 5 mL 0.4 M Millonig's phosphate buffer. Weigh and dissolve 0.6 g NaCl.
7. 5 mm × 7 mm silicon wafers (Pelco). Scratch labels with diamond pencil on "back side" of each wafer (*see* **Note 10**).
8. Aldehyde blocking buffer: 100 mM glycine, Tris buffer, pH 7.4. Weigh 0.75 g glycine and dissolve in 100 mL Tris buffer.
9. Antibody blocking buffer: Tris buffer, 1 % fish skin gelatin (Sigma), pH 7.4. Weigh and dissolve 2 g fish skin gelatin in 200 mL Tris buffer.
10. Primary antibody: dilute 1:50 in "antibody blocking buffer" (*see* **Note 11**).
11. Secondary antibody conjugated to 15 nm gold. Dilute 1:50 in "antibody blocking buffer."
12. Whatman filter papers, 55 mm circles.
13. Wet chamber: 60 mm petri dish containing two layers of wet filter paper overlaid with strip of Parafilm cut to size.

3 Methods

It is best to observe fertilization in real time with light microscopy (Phase contrast or Nomarski) before deciding how to process unfamiliar gametes for SEM. Eggs such as those of chitons [11], and ascidians [15] have elaborate egg envelopes (Fig. 1) that may obscure fine surface details or fertilization events. Eggs of echinoderms and some mollusks, including basal chitons, have a jelly coat that must be removed prior to fertilization in order to examine sperm–egg interaction at the vitelline layer. Methods for obtaining eggs and removing jelly coats are well established for echinoderms [5–7, 14] and ascidians [15] (*see* **Notes 12** and **13**) but not for mollusks.

3.1 Obtaining Gametes

1. Bring animals into the lab from the field and isolate individuals overnight in separate petri dishes containing FSW, either on a running sea water table or held at room temperature (RT). Observe frequently to detect first signs of spawning (*see* **Note 14**).
2. If spawning does not occur, add to these dishes two drops of sperm concentrate that have been dissected from gravid males (*see* **Note 15**).
3. Remove any spawning animals, wash in sea water then FSW and place in clean separate dishes with FSW. Collect any freshly released, unfertilized eggs (*see* **Note 16**).
4. Invertebrates with internal fertilization usually require special handling (*see* **Note 17**).

3.2 Fixation and Dehydration of Eggs

1. Using a Pasteur pipette (*see* **Note 18**), gently transfer less than a monolayer of eggs into scintillation vials containing FSW. Wash two times by gently aspirating most of the solution and replacing it with fresh FSW (*see* **Notes 19** and **20**). Keep vials on a thin layer of crushed ice in the fumehood.
2. Take one vial of about 30 unfertilized eggs and fix as in **step 5**.
3. To a second vial of 30 eggs, add dilute activated sperm to induce natural fertilization and development and label. Rinse eggs with FSW after 5 min and fix after 1 h as in **step 5**.
4. To a third vial containing about 100 eggs, add activated sperm concentrate (*see* **Note 21**) to induce polyspermy. Pipette aliquots of 20 eggs to primary fixative in labeled vials, at timed intervals of 10 s, 30 s, 1 min, 5 min, and 1 h. This series is likely to reveal the key events of sperm entry. However, be prepared to vary times.
5. For each vial, aspirate the solution and replace it with 5 mL cold primary fixative by dribbling it down the side of the vial, to avoid mechanical damage to delicate eggs.

6. Fix all samples for 1 h on ice and then move vials onto a dry surface and allow fixation to continue for another 2 h, or overnight, as temperature rises to RT.
7. Aspirate most of the fixative by tipping the vial at an angle and highlighting it against a black background, while carefully observing the aspirations to avoid losing any eggs. Discard primary fixative (*see* **Note 22**) and gently replace with buffer rinse that is dribbled via a clean pipette down the side of the vial.
8. After 10 min repeat this process with a second buffer rinse and incubate 10 min.
9. Replace buffer rinse with isotonic secondary fixative in the same way. Fix for 1 h at RT.
10. Aspirate secondary fixative against a white background, as eggs will now be black, and replace with DW. After 10 min, repeat this process with fresh DW (*see* **Note 23**).
11. Replace half the DW with 10 % ethanol. 10 min later replace half of this solution with 30 % ethanol. Repeat this with 50, 70, 80, 90, 95, 100 % ethanol (*see* **Note 24**). Aspirate most of the 100 % ethanol and replace with 20 mL 100 % ethanol. Repeat two times.
12. Transitional solvent (*see* **Note 25**). Add ten drops of amyl acetate to each vial in the fumehood. Gently swirl to mix. Ten minutes later remove 1 mL and add 1 mL of amyl acetate. Gently swirl to mix. Repeat this with 3, 5, 10 and 15 mL with 10 min gaps. Finally replace most of the solution with fresh amyl acetate and repeat this after 10 min.
13. Gently suck up eggs into a Pasteur pipette already containing some amyl acetate and transfer them to open 78 μm Teflon® capsules sitting in a 60 mm Pyrex® petri dish half-filled with amyl acetate in the fumehood. In general pipette a maximum of 50 eggs per capsule. Label outside of capsule lid with a graphite pencil and with gloves on, cap the capsules (Fig. 7).

3.3 Fixation and Dehydration of Sperm

There are several excellent ways to prepare sperm for SEM. As opposed to the method described below, others have used round 12 mm coverslips (*see* **Note 26**) or have found that 25 mm nucleopore filters in combination with a Millipore "Swimmex" filter holder (Fisher Cat# 09300-6) [3] or Teflon® capsules [9] also work well.

1. With fine forceps, remove each 5 × 7 mm silicon wafer from the plastic backing and label the back with a diamond pencil. Dip each wafer into Poly-L-Lysine solution before air drying on filter paper in a petri dish.
2. Transfer dried wafers to a wet chamber and pipette drops of activated sperm concentrate (*see* **Note 21**) onto front side of wafers and let sit for 5 min with the chamber closed.

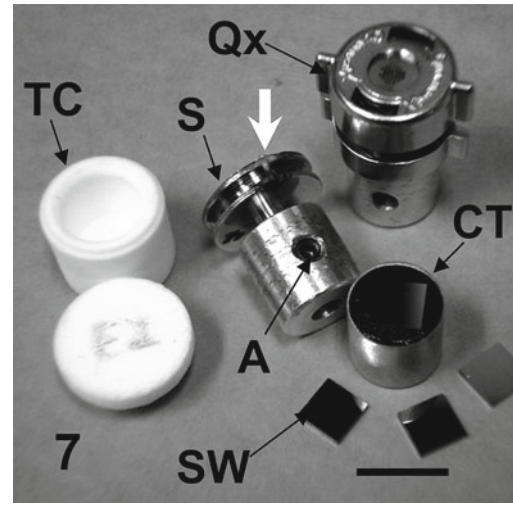


Fig. 7 Teflon® microporous specimen capsule (TC) with lid labeled E2 in pencil; 12.7 mm Cambridge stub (S) adapted to JEOL 9 mm stub with central hole and Allen key (A) on side. Coverslip has been grounded to metal stub with carbon cement (*white arrow*). QuantomiX 102 capsule (Qx) adapted to JEOL stub; silicon wafers (SW) one on carbon tab (CT) on JEOL 9 mm stub. Scale bar = 6 mm

3. Pick up each wafer with fine forceps and place under cold primary fixative in a 35 mm petri dish on ice for 3 h.
4. Wash each wafer in two 10 min changes of phosphate buffer rinse and transfer to secondary fixative in a clean 35 mm petri dish for 1 h at RT.
5. Transfer each wafer through DW for 10 min. Insert one wafer into each Teflon® capsule sitting in DW in a petri dish and close caps. Label caps as before.
6. Transfer capsules through an ethanol series up to 100 % ethanol, using a glass petri dish with 10 min between each change.
7. Run capsules through an amyl acetate series in a Pyrex® petri dish, as for eggs, then transfer quickly to the CPD (*see* **Note 27**).

3.4 Critical Point Drying of Specimens

Specimens are best dried before mounting by using a CPD in which the solvent is replaced with liquid CO_2. This procedure minimizes shrinkage artifact that is introduced by evaporation of liquids during air drying. The solvent can be 100 % ethanol, but in general first replacing ethanol with amyl acetate gives the best results.

1. Quickly transfer each Teflon® capsule of eggs or sperm samples to the open chamber of the CPD, stacking them efficiently. Close the chamber, tighten the hold down nuts and quickly fill chamber with liquid CO_2 to limit evaporation of solvent (*see* **Note 28**).

2. Alternately fill and partially vent the chamber (into the fumehood) while keeping the temperature below 8 °C with the cooling cycle (*see* **Note 29**). This procedure gradually replaces all the amyl acetate or ethanol with liquid CO_2.
3. Arrange a white card at the edge of the fumehood so that after a few minutes of venting the stream of gases can be directed at it. When most of the solvent has been replaced, a white cone of dry ice will develop on the card. When this is flicked off it will leave "wet" spots of solvent. Continue flushing with liquid CO_2 until no wet spots appear.
4. Close all control knobs to the chamber, turn off the CO_2 tank, and turn on the heating cycle.
5. The temperature and pressure rise slowly in the chamber until the critical point is reached (about 31 °C and 1,072 psi), when the chamber should automatically vent excess pressure but should be left closed for another 5 min.
6. Undo the purge control to vent the chamber manually so that it slowly loses pressure (100 psi/min) down to atmospheric pressure, about 10 min.
7. Once at atmospheric pressure again, the samples should be removed and transferred to a desiccator, or they can be mounted on SEM stubs right away.
8. Dried silicon wafers can be mounted directly onto 12 mm carbon tabs on Cambridge stubs or 9 mm carbon tabs on JEOL stubs (Fig. 7). Label each STUB with a Sharpie® marker pen.

3.5 Mounting of Specimens

1. Eggs: If there are 20 or more eggs in a capsule, remove the cap and touch the edge of the capsule to the edge of the carbon tab on a labeled specimen stub (*see* **Note 30**). Then in one motion roll the capsule onto the stub, thus tipping the eggs out. Tap the bottom of the capsule to free up stuck eggs and remove the capsule with the same rolling motion.
2. Under a dissecting microscope check for any eggs remaining in the capsule. Use a 000 gauge needle dipped in chloroform glue to pick up these individual eggs and place them at the perimeter of a separate labeled stub. Similarly, when there are less than 20 eggs in a capsule, they can be handled individually and placed evenly around the perimeter of a stub.
3. In placing individual eggs with a needle, touch the needle to the carbon tab and roll it under and away from the egg, leaving the egg behind with any damaged surface beneath it.
4. View the stub with >20 random eggs stuck to it. Under the microscope see if there is any way to improve their arrangement. Use the mounted needle to gently knock apart stacks of eggs; pick up and place individual eggs that are not firmly

attached; and remove any hairs or debris that can easily be attracted to an eyelash brush or needle dipped in chloroform glue, or picked off with fine forceps (*see* **Note 31**).

5. Use the 000 gauge needle to roll individual eggs on the sticky carbon tab, which strips off the outer envelopes (Fig. 2) (*see* **Note 32**). Repeat this with about half of the eggs, always choosing those eggs that are already damaged or have debris stuck to them that can be removed in this way. The cleanest and best preserved eggs are reserved for examination intact.
6. A few of the best eggs can be cut in half with a 15° microsurgical knife or homemade razor knife (Fig. 9) (*see* **Note 9**). Working with eggs at the edge of the stub, aim the knife to the middle of the egg and cut through it into the carbon tab. If successful, gently pry one way, then the other, to separate the two pieces of the egg and then remove the knife (*see* **Note 33**).
7. Once operations on one stub are completed use the proper forceps to transfer it to the specimen box and then into the desiccator until ready to sputter coat.

3.6 Immunogold Labeling of Specimens

Molecules of interest on the surface of gametes can be labeled for SEM by using immunogold techniques that attach gold particles of known size to exposed antigens. The samples are then viewed in the backscatter mode of the SEM, which detects differences greater than 3 in the atomic numbers of elements. This enables the operator to spot large (15 nm) gold particles against a background of low atomic number elements, such as C, N, P, O, and identify the locations of each antigen [16, 17]. The molecular cross-linking caused by most primary fixatives may mask delicate antigens and in general alters immunological characteristics. To overcome these problems one can use short exposures to non-coagulating, dilute fixatives prior to immunolabeling, followed by a stronger post-label fixation, as described below, or one can quick freeze sperm or eggs [9], or do wet SEM [18, 19] with the aid of QuantomiX capsules (EMS Cat# SP-202-24) (Fig. 7). Not covered in this chapter but dealt with elsewhere are methods for working with fractions of eggs or sperm, such as mitochondria, membranes or flagella [20, 21] and high resolution (1 nm) Field Emission Gun-SEM of immunolabeled samples for viewing back scattered electrons or cathodoluminescence [16, 17].

1. Attach sperm or egg samples to labeled silicon wafers for 30 min as in Subheading 3.3. Dry back of wafer by blotting on filter paper, but keep front surface wet.
2. Immerse wafers under immunofixative in a 60 mm petri dish at RT for 30 min.
3. Rinse wafers in three changes of phosphate buffer wash, 10 min each.
4. Immerse wafers in aldehyde blocking buffer for 10 min.

5. Rinse wafers in three changes of Tris buffer, 10 min each.
6. Immerse wafers in antibody blocking buffer for 30 min.
7. Rinse wafers in three changes of Tris buffer, 10 min each, blot back of wafer.
8. Place wafer in wet chamber and add 10 μL primary antibody onto sample.
9. Incubate 2–3 h in closed wet chamber.
10. Rinse wafers in three changes of Tris buffer, 10 min each. Dry back of wafer.
11. Place wafer in wet chamber and pipette on 10 μL secondary antibody.
12. Incubate 1–2 h in closed wet chamber.
13. Rinse wafers in two changes of Tris buffer, 10 min each, followed by primary fixative buffer for 10 min.
14. Immerse in isotonic primary fixative (*see* Subheading 2.2) for 2 h at RT.
15. Rinse wafers in two changes of primary fixative buffer, 10 min each.
16. Immerse in isotonic secondary fixative for 1 h at RT.
17. Rinse in DW, place vertically in Teflon® capsule sitting in petri dish with DW. Close capsule and run quickly through ethanol series (2 min per change), then CPD as before (*see* Subheading 3.4).
18. Remove dried samples and mount on SEM stubs as in Subheading 3.5.
19. Sputter coat with carbon (*see* **Note 34**).
20. Observe samples either right away in SEM or store in desiccator.

3.7 Sputter Coating of Specimens

Stubs that are ready for observation by SEM must first be sputter coated with an inert conductive metal such as gold (*see* **Note 35**) so that excess electrons from the electron beam can be effectively grounded without causing overheating (*see* **Note 36**) or charging problems on the specimen surface (Fig. 3).

1. Sputter coat eggs twice with 1-min exposures at a distance of 5 cm from the gold target with 18 mAmp plasma current (*see* **Note 37**). This provides a gold coating about 30 nm thick (*see* **Note 38**).
2. After coating, release vacuum slowly so as not to disrupt any delicate structures.
3. Coated stubs can be viewed in the SEM immediately or stored in a desiccator. Sometimes, even after years there is little change in surface morphology if desiccant is maintained (Fig. 4) (*see* **Note 39**).

3.8 Introduction to Using SEM

Most SEMs can be operated in either SEI (secondary electron image) mode or BEI (back scatter electron image) mode. In SEI mode, low-energy secondary electrons emerging from the surface of the specimen are captured by a positively biased detector placed to one side and reveal topographical features of the specimen. In BEI mode, high-energy primary electrons are captured by a separate detector placed above the specimen and encircling the electron beam (*see* **Note 40**). These electrons, emerging from deeper in the specimen, reveal information about the distribution of high atomic number elements in the sample, distinguishing best between elements that differ by more than 3 in atomic number. This mode can be used also to detect immunogold labeled antigens on the specimen surface. In most SEMs the SE signal can be combined with the BSE signal often permitting identification of the exact location of labeled antigens in the sample (*see* **Note 41**).

1. Insert specimen stubs into stub holder and tighten nuts that properly ground the stubs.
2. Vent the SEM, open the access door and attach stub holder to specimen platform. Close door and evacuate column.
3. Choose basic settings: 10 kV, 20 mm working distance, mid-range spot size (i.e. if the spot size has an arbitrary scale of 0–30, choose 15). Whole eggs, roughly 300 μm in diameter, can be photographed effectively with these settings, up to a magnification of about 1,000× (*see* **Note 42**).
4. Once working vacuum has been reached, turn on HT and increase filament current until filament is saturated (*see* **Note 43**).
5. In TV mode, focus at lowest magnification and find a specimen to examine.
6. Focus on an area at two or three steps higher in magnification than you intend to use and correct any astigmatism in the beam (usually two adjustment knobs), then refocus. Decrease magnification to where you want to observe and photograph, this will produce the best image.
7. If charging occurs at a sharp edge of the specimen ("edge effect"), one can try reducing the voltage and put up with a slight loss of resolution. If this does not work it may be necessary to check that the stub is properly grounded with carbon cement and recoat the specimen.
8. Specimens can be viewed from any angle by rotating the stage and by tilting it to as much as 90° in some SEMs, in order to make visible, parts that are hidden from view. Increasing the "detector bias" will capture electrons from the "dark side" and can be used to illuminate those areas in the final image.
9. In BEI mode one can view samples that have been immunolabelled with gold. Begin with settings at 15 kV, medium to

large spot size, 20 mm working distance and focus with *Z*-axis control (working distance control).

3.9 Taking Stereo Pairs for 3D Viewing

In the case of eggs and examining sperm–egg interaction it is often useful to take stereo pairs, by tilting the specimen between two photographs, to provide 3D visualization of the target area.

1. Focus and adjust contrast and brightness of the first stereo image in the normal way and photograph it (*see* **Note 44**).
2. Tape a piece of clear acetate to the video screen and trace the outline of the specimen with a wax pencil, noting one key point of focus near the center that can be found again.
3. Tilt the specimen +8° and recenter it onto the wax outline. Then refocus using the *Z*-axis control (working distance adjustment) (*see* **Note 45**).
4. Finally adjust the brightness and contrast to match the first micrograph and photograph its stereo image [3, 8].
5. Arrange the printed images side by side, view them with stereo glasses and adjust them to exactly overlap features and see the 3D effect (Figs. 5 and 6).

3.10 Reusing Rare SEM Specimens for TEM

Sometimes excellent results can be obtained by reusing a sample for TEM that has already been observed with SEM. This is most useful with rare specimens (Fig. 8).

1. Use a mounted needle to gently knock eggs off SEM stub into 100 % ethanol in petri dish.

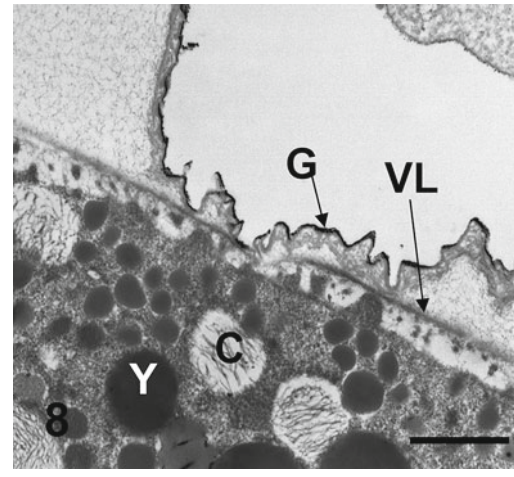


Fig. 8 TEM of oocyte of the chiton *Acanthopleura granulata* from a recycled SEM sample. Note surface coating of gold (G), vitelline layer (VL), cortical granule (C), and yolk granule (Y). Preservation is adequate even though this SEM sample was in the desiccator for 10 years before processing for TEM. Scale bar = 1 μm

2. Eggs should sink to the bottom when fully infiltrated with ethanol. Pipette eggs into a scintillation vial containing 5 mL fresh 100 % ethanol.
3. Follow directions in Chapter 7 by Shinn for infiltration and embedding, but use a 1:1 mixture of Spurr's and Epon resins for best results with these specimens (*see* **Note 46**).

4 Notes

1. Plastic 0.22 μm vacuum filtration units (EMS Cat# 67046-13) are convenient for filtering sea water and alcohols but not acetone or amyl acetate. Use only glass Pasteur pipettes to dispense these solvents. Disposable 0.22 μm filters on syringes can be used to dispense primary fixative or heavy metal stains but usually not osmium tetroxide solutions.
2. Osmolarity of fixatives and buffer rinses is critical in SEM. Preferably the total osmolarity of a solution should be isotonic or very slightly hypotonic to cells and tissues in the sample. The osmolarity of sea water varies, but the accepted standard is about 1,000 mOsm [9].
3. OsO_4 solutions can be prepared quickly by running the ampoule under hot water until crystals melt (wear double gloves, use fumehood). Tap liquid beads of OsO_4 into large end and cool ampoule under cold water to solidify them. Dry ampoule then break off top at score mark and push open end into new scintillation vial containing 20 mL DW. Cap and seal with Parafilm, otherwise vapors of OsO_4 leak out. Heat with hot water until crystals melt again and then swirl to mix for about 15 min. Solution turns pale yellow as beads disappear. Ready to use.
4. Waste solutions of formaldehyde and glutaraldehyde should be neutralized with G-Gard-ER or Formalex. Manufacturers claim that treated solutions are safe for septic systems.
5. OsO_4 solutions can be reduced and kept safer by pouring them into a glass jar containing corn oil, in the fumehood. Full jars can be sealed and sent for recycling.
6. 12.7 mm "Cambridge" stubs are an optimal size of specimen viewing area and can be easily adapted to other SEMs. For example, many JEOL SEMs use a 9 mm stub. One of these can be adapted by drilling in the center with a 3 mm drill bit and tapping in a 3 mm Allen screw on the side, to hold a 12.7 mm stub (Fig. 7).
7. Insect pins can be mounted on sharpened applicator sticks with 5 min epoxy resin to make delicate tools for manipulating eggs or cracking them open (Fig. 9). The pins can be sharpened even finer on a moonstone.

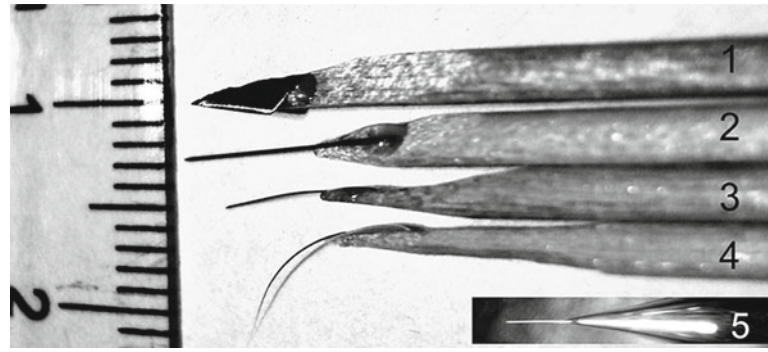


Fig. 9 Instruments made with 5 min epoxy resin and applicator sticks: *1*. Carbon steel razor fragment. *2*. 00 gauge insect pin, *3*. 000 gauge insect pin, *4*. Eyelash brush. *5*. glass electrode, double-pulled to give sharp, strong point. Scale at *left* in mm

8. Chloroform glue is made by dissolving either ten adhesive tabs (EMS Cat# 76760) or 30 cm clear Scotch tape in 10 mL chloroform in a scintillation vial in the fumehood. The backing tape or paper tabs can be removed after glue has dissolved. A needle or eyelash brush (Fig. 9) dipped in this solution dries sticky and can be used to pick up dried eggs and embryos.
9. Carbon steel razor blades can be broken with fine pliers to yield small pieces of blade that can be mounted with 5 minute® epoxy glue to make fine dissecting knives (Fig. 9).
10. Wafers of silicone are sold in 4" discs already scored into 5 × 5 mm squares or 5 × 7 mm rectangles on a plastic backing. 5 × 7 mm is the best size for processing in Teflon® capsules.
11. Primary and secondary antibodies used for SEM analysis should be more concentrated than in TEM protocols. A 1:50 dilution in antibody blocking buffer is recommended as a starting point.
12. Gametes of gravid sea urchins may be obtained by intracoelomic injection of 2 mL 0.53 M KCl (40 g/L) using a #23 needle inserted into the perioral membrane. Gametes are collected by placing the animal aboral side down over a beaker with slightly smaller diameter and full of FSW. If milky sperm are released the animal can be removed, dried and placed on a 60 mm Pyrex® petri dish to collect "dry sperm" concentrate, or else sperm can be pipetted directly from the gonopores as it is extruded. Eggs are more granular, sometimes colored, and should be rinsed with FSW soon after spawning. Eggs can be fixed with jelly coat intact or it can be removed to reveal events or details at the vitelline layer by washing the eggs through a Nitex nylon cloth with a mesh size 1.6× the diameter of the egg [14]. De-jellied eggs can be fertilized by exposure to a

solution of just activated sperm (two drops of dry sperm to 100 mL FSW) and fixed at intervals as for chiton eggs.

13. Gametes of gravid sea stars may be obtained by injecting each arm with 0.4 mL 1-methyladenine solution. Sperm concentrate may be pipetted directly from gonopores of males. The female can be placed in a large dish of filtered sea water and eggs pipetted into fresh FSW as they are released.
14. It is generally accepted that the stress associated with these changes often causes the animals to release gametes. If not, one can try introducing heat stress by raising water bath temperature to 10 °C above ambient for 30 min, then return to sea water at RT.
15. Eggs of chitons that are dissected out are rarely in a condition to be fertilized by sperm, due to presence of follicle cells or poor elevation of cupules. Natural spawning of eggs is usually a prerequisite for examination of fertilization events.
16. Marine invertebrate eggs, particularly chitons, rapidly pick up debris on their surface. Thus it is important to remove eggs from dishes as soon as they are spawned and wash them in FSW.
17. Mature sperm of most invertebrates, including internal fertilizers such as snails, can be dissected out from the gravid testes or sperm storage organs of the properly euthanized animal by pricking the organ several times with a 00 gauge needle and aspirating the extruded sperm. Release of eggs may sometimes be stimulated in gravid snails, such as *Littorina* sp., by placing the animal in a FSW solution of 5 μM serotonin [12].
18. The diameter of the pipette tip should be larger than the eggs to prevent unwanted breakage of spines or jelly coat. If Pasteur pipette is too small use plastic disposable pipettes which can be cut to best size.
19. Removing most of the previous solution provides an opportunity to eliminate tiny particles that could contaminate gamete surfaces. However, when removing volatile solutions, such as 100 % ethanol, propylene oxide, or amyl acetate, always leave some solution covering the gametes, so that they do not dry out prematurely.
20. Highlight vial with a bright fiber optic light to illuminate all the contents.
21. To obtain activated sperm for natural fertilization of eggs, add 1 drop of sperm concentrate to 100 mL FSW with stirring (provides 1×10^7 sperm/mL). To obtain an activated sperm concentrate for inducing polyspermic eggs that in turn provide more chances to find a penetrating sperm, use 1 drop of sperm concentrate in 10 mL FSW. Check under microscope for

intense activity before using. If sperm activity is poor, sample a different male.

22. Double fixation with glutaraldehyde then osmium tetroxide enables both SEM and TEM to be done on specimens. Furthermore, samples that have been viewed with SEM can be removed from the stub and effectively processed for TEM with reasonable results (Fig. 8) (*see* Subheading 3.10). If TEM is not planned, an excellent simpler alternative fixative for all gametes is 0.4 % OsO_4 in FSW for 1 h on ice followed by rinsing in FSW and dehydration in an ethanol series (Ron Koss personal communication), also *see* [9].
23. If you have excess eggs or embryos it is worth transferring some through 0.5 % uranyl acetate that has been filtered with a 0.22 μm filter syringe to remove any particulates. This further impregnates the cells with a heavy metal, which decreases beam penetration, prevents damage and enhances resolution by releasing more secondary electrons.
24. If at any time the solution begins to turn dark, replace it with fresh solution of the same concentration.
25. Eggs and sperm can be critical point dried from 100 % ethanol, but better results are obtained if samples are first passed through amyl acetate. This should be done in a stepwise manner, gradually increasing the concentration of amyl acetate until 100 % is reached.
26. Silicon wafers are conductive and provide a better substrate than glass for observation of gametes in SEM. However, 12 mm coverslips cleaned then dipped in Poly-L-Lysine have been used successfully to image sperm samples in the SEM. Ten coverslips can be stacked in a CPD coverslip holder kit (EMS Cat# 70193-01) and processed at once but must be kept wet with solvent at all times. Otherwise, the samples will dry out prematurely.
27. Teflon® capsules (Fig. 7) can be closed and transferred quickly enough to the CPD chamber without significant evaporation. Thus amyl acetate does not need to be added to the chamber. This is important as amyl acetate can take 10× longer to purge with liquid CO_2 than ethanol.
28. CO_2 tanks come with about 45–50 lb of CO_2 in them. Place the new tank on a heavy duty bathroom scale and take initial weight. When weight has dropped by 40 lb, after several uses, change to a new tank. Also use an in-line filter to prevent contaminants near the bottom of the tank from entering chamber. Change filter once per year.
29. While the solvent is being displaced prior to refilling with liquid CO_2, try not to let the liquid level fall below the specimens to avoid premature drying.

30. Label each stub underneath with a fine Sharpie® permanent marker before mounting specimens.
31. Any surface is easily damaged and any damage is readily visible in the SEM, so manipulation must be kept to the practical minimum.
32. Currently this is the best method for removing the hull of chiton eggs. Care must be taken to roll in one motion about 90° and then leave that egg alone (Fig. 2), because rolling too far can strip off both outer layers and some of the egg itself.
33. Eggs may be cracked in two also by piercing with a mounted insect pin or glass electrode (Fig. 9). If eggs are rare, cutting them in half can be postponed until after the initial photography of intact eggs is completed.
34. Many sputter coaters come equipped with a separate chamber that can deposit carbon or metals such as chromium [16, 17] onto specimens. For carbon evaporation, specimens need to be about 6 mm away from the carbon rods. A small piece of white ceramic tile with a drop of oil on it is placed in the chamber. The oil stops carbon deposition and so one can see how much is deposited next to it on the ceramic tile. Coating is finished when the area around the oil droplet turns light brown, which takes about 1 min.
35. Gold/Palladium targets form finer particles than gold alone and can improve resolution, but they are more expensive. Gold is good for routine work (Figs. 1–6).
36. A cooled specimen stage can prevent overheating of specimens but usually is not essential.
37. Plasma current is produced in the sputter coater when argon gas molecules are ionized by electrons flowing from cathode to anode. These positively charged argon gas molecules are forcefully attracted to the cathode, where they smash into the gold target releasing a cloud of gold particles that "sputter" over all exposed surfaces in the chamber.
38. A 1-min exposure (15 nm thickness) is usually enough for sperm on silicon wafers or coverslips to ensure that surface details are not obscured.
39. Before using specimens that have been stored for more than a couple of months it is a good idea to recoat with gold for 60 s at 18 mAmp of plasma current.
40. Recent development of easy to operate, compact, bench-top analytical SEMs, such as the Hitachi TM3000, will make SEM analysis available to inexperienced users, such as undergraduates. These instruments can operate with variable vacuum pressures enabling examination of robust, uncoated, hydrated specimens, such as insects and plants. They also come equipped

with an energy dispersive X-ray spectrometer (EDS) that enables elemental analysis of any sample. Furthermore, 3D topographical analysis may be performed using a combination of the four backscatter electron detectors.

41. Often the BSE and SE signals can be combined in the SEM to create a composite image that allows one to see exactly where the gold particles are located on the specimen surface.
42. When examining details of sperm and sperm–egg interaction from 5,000 to 50,000× it is best to reduce spot size to 5 on the arbitrary scale, reduce working distance to 10–15 mm (increases resolution but reduces depth of field) and increase voltage to 15 kV to provide more secondary electrons in the final image. For helpful discussion of the relationship between resolution, working distance, apertures, depth of field, and voltage, consult [3].
43. If there is not a preset stop for filament current, use "linescan" to show increasing filament current and enable correct increase of Y-axis peaks up to their maximum (saturated) and not beyond (oversaturated).
44. Stereo pairs can be captured digitally or on film. A photographic negative is more permanent and allows both for printing of a micrograph as well as scanning the image at high resolution (600–1,200 dpi) and working with it digitally.
45. This method retains the same magnification and orientation, which would not be the case if focused as usual with the final condenser lens.
46. It is recommended to include the lower viscosity Spurr's resin in the embedding medium to ensure that gold-coated samples become properly infiltrated with resin. Follow manufacturer's instructions for mixing "standard" hardness.

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Chapter 9

Imaging Neural Development in Embryonic and Larval Sea Urchins

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Abstract

Imaging is a critical tool in neuroscience, and our understanding of the structure and function of sea urchin nervous systems owes much to this approach. In particular, studies of neural development have been facilitated by methods that enable the accurate identification of specific types of neurons. Here we describe methods that have been successfully employed to study neural development in sea urchin embryos. Altering gene expression in part of an embryo is facilitated by injection of reagents into individual blastomeres, which enables studies of cell autonomous effects and single embryo rescue experiments. The simultaneous localization of an in situ RNA hybridization probe and a cell type specific antigen has enabled studies of gene expression in specific types of neurons. Fixatives and antibodies can be capricious; thus, we provide data on preservation of antigens with commonly used fixatives and buffers.

Key words Blastomere, Fixation, Morpholino, Oligonucleotide, Immunofluorescence, In situ hybridization

1 Introduction

Interest in deuterostome evolution, embryonic signaling, and larval biology continues to stimulate studies of neural development and the structure of the nervous system of sea urchins. Echinoderm nervous systems are not easily analyzed by conventional electrophysiological approaches, and identification of neurons from ultrastructural features or histochemical dyes has met with only limited success [1–4]. However, antibodies and RNA hybridization probes that are specific for proteins and genes that are expressed only in neurons have enabled detailed studies of neuroanatomy and neural development [5–7]. Many of these probes have resulted from access to genomic tools derived from the sequencing of the sea urchin genome [8, 9]. A powerful approach to understanding developmental mechanisms is obtained when these neuroanatomical methods are combined with methods for altering expression

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levels of specific genes, such as morpholino antisense oligonucleotides or in vitro transcribed mRNA [10–12].

We are fortunate in having a number of excellent reviews of imaging methods that can be applied to sea urchin eggs and embryos [13–17]. As well, methods for injecting eggs have been well described [18–20]. The objective of this chapter is to describe methods that have proven useful in studying neural development in sea urchin embryos. Inhibiting translation or expressing a specific gene in part of the embryo has facilitated studies of signaling; this we do by injecting individual blastomeres. Identifying expression of specific genes in neurons has been accomplished by combining fluorescent in situ methods with immunofluorescence. Choice of fixative for imaging studies is critical and guided by the need to preserve specific combinations of antigens and details of cellular structure. Thus, we compare several fixatives commonly used in studies of sea urchin embryos.

2 Materials

Gametes should be prepared and cultured following conventional procedures previously described [21–25] (*see* also Chapter 1 in this volume). Prepare all solutions using deionized, distilled water, and analytical grade reagents.

2.1 Blastomere Injections and Double Injection Components

For injection of blastomeres there are differences in how embryos of different species respond to divalent cations. *Strongylocentrotus purpuratus* has a thick hyaline layer, and embryos can be treated with calcium and magnesium-free seawater without dissociating. *Hemicentrotus pulcherrimus* embryos can tolerate only calcium-free seawater without dissociating.

1. Natural seawater: taken from 2 to 3 m depth and filtered through qualitative filter paper (FSW) for culturing embryos. Store at 15 °C.
2. MBL-artificial seawater (ASW): 423 mM NaCl, 9.0 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.14 mM $NaHCO_3$ (ASW is satisfactory for many applications).
3. Calcium- and magnesium-free artificial seawater for *S. purpuratus* ($Ca^{2+}Mg^{2+}$-free ASW): 500 mM NaCl, 9.0 mM KCl, 2.0 mM $NaHCO_3$, 29 mM Na_2SO_4. Add 700 ml water to a graduated cylinder or a glass beaker, and stir with magnetic stirrer. Weigh and add the following reagents in turn: 29.22 g NaCl, 0.67 g KCl, 0.168 g $NaHCO_3$, 4.12 g Na_2SO_4. Mix well and make up to 1 L with water. Store at 15 °C.
4. Calcium-free artificial seawater (Ca^{2+}-free ASW) for *H. pulcherrimus:* 435 mM NaCl, 9.3 mM KCl, 24.5 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$ [2]. Add 700 ml water to a

graduated cylinder or a glass beaker, and stir with magnetic stirrer. Weigh and add the following reagents in turn: 25.43 g NaCl, 0.69 g KCl, 2.33 g $MgCl_2$ (anhydrous), 3.07 g $MgSO_4$ (anhydrous), and 0.18 $NaHCO_3$. Mix well and make up to 1 L with water. Store at 15 °C.

5. FSW containing 3-amino-1,2,4-triazol (ATA): 100 mM ATA-FSW stock (100×); use 1:100 dilution in FSW as a working solution [3]. Store at 4 °C.
6. Protamine sulfate solution: 1 % (w/v) protamine sulfate in water; aliquot in 1.5 ml tubes. Store at −20 °C until use.
7. Injection solution 1: To alter gene activity, morpholino oligonucleotide (MO) or in vitro transcribed mRNA is used. MO is commercially available from Gene Tools (www.gene-tools.com). mRNA is synthesized following the instruction of the mMessage mMachine kit (Life Technologies; www.lifetechnologies.com). Mix the mRNA or MO at optimal concentration in 24 % glycerol, filter the mixture through 0.22 μm membrane filters (Millipore), and back-fill injection needles (*see* **Note 1**).
8. Injection solution 2: For blastomere injections to be followed by immunological detection of an injected cell lineage tracer, several options have been used successfully. We have used myc mRNA because a reliable specific antibody (9E10) is available [26]. A stock solution of myc mRNA at 1.0 μg/μl in 24 % glycerol is filtered and stored at −20 °C until use. Prior to loading, dilute this stock with MO or mRNA solution 1:10, to a final concentration of 100 ng/μl in the injection needle. For live imaging, alternatives include GFP mRNA, DSRed mRNA, or fluorescent dextran. GFP or DSRed can be detected after prolonged fixation with specific antibodies, whereas fluorescent dextran is lost after fixation. Most fixatives readily preserve myc protein translated from injected mRNA.

2.2 Immunological Methods: Fixatives and Components

1. Paraformaldehyde in Seawater (PFA): For a hot water bath, put 175 ml distilled water in a 250 ml beaker and heat to boiling. Put 25 ml of filtered seawater in a 50 ml plastic tube, add 30 mg Tris base. Dissolve Tris by vortexing the solution. Loosen cap completely and heat the solution in a microwave to 80 °C, being careful not to overheat. In a 1,200 W microwave this takes 15–20 s. Immediately add 1.0 g paraformaldehyde and vortex. Immerse in hot water bath, remove the tube and shake vigorously or vortex every few minutes. The solution will gradually clear. Do not heat the paraformaldehyde solution in the microwave. When the solution is completely clear, cool the solution to room temperature in an ice bath, and adjust pH to 7.0 using pH paper. Use immediately or aliquot and freeze at −20 °C.

2. Methanol (MeOH, analytical grade): Store at –20 °C and use while ice-cold.
3. Modified microtubule stabilization buffer (PEM): 100 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, 0.2 % Triton X-100. Adjust the pH to 7.0 and store at room temperature (stable for several months). Immediately prior to fixation, combine nine parts PEM buffer with one part formalin (37 % formaldehyde solution). Use immediately.
4. Blocking buffer: "Superblock" (Pierce Chemical), 0.03 % Triton X-100. When using antibodies to phosphorylated proteins, add NaF (10 mM) or Na_3VO_4 (1 mM) and incubate 45 min at room temperature.

2.3 Co-localizations with Anti-serotonin and In Situ Hybridization Components

Prepare all solutions using deionized, distilled water and analytical grade reagents.

1. Fixative: 4 % paraformaldehyde in FSW.
2. MOPS buffer: 0.1 M MOPS, pH7.0, 0.5 M NaCl, 0.1 % Tween-20. Mix from the following stock solutions. 1 M MOPS (10×): Add 350 ml water to a graduated cylinder or a glass beaker, and stir with magnetic stirrer. Weigh and add 104.7 g MOPS and adjust pH to 7.0 with 5 N KOH. Make up to 500 ml and sterilize it with 0.22 μm filter. Store at room temperature (RT) in dark. 5 M NaCl (10×): Add 600 ml water to a graduated cylinder or a glass beaker, and stir with magnetic stirrer. Weigh and add 292.2 g NaCl and make up to 1 L and autoclave to sterilize. 10 % Tween-20 (100×): Add 2.0 ml Tween-20 to 18 ml sterilized water. Mix well and store at RT.
3. Blocking buffer: 5 % Lamb serum in MOPS buffer, prepare immediately before use.
4. Antibodies: Dilute rabbit anti-serotonin antibody (Sigma) 1:1,000 in blocking buffer. Dilute goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) 1:2,000 in blocking buffer. Sheep anti-digoxygenin (DIG) antibody conjugated with peroxidase (Roche) was used in 1:1,000 dilution in blocking buffer.
5. Tyramide signal amplification (TSA) system: TSA Fluorescein system and TSA tetramethyl-Rhodamine (TMR) system were obtained from Perkin Elmer.
6. Hybridization buffer: 70 % formamide, 0.1 M MOPS, pH 7.0, 0.5 M NaCl, 1 % bovine serum albumin (BSA), 0.1 % Tween-20 [5].
7. RNA probes: DIG-labeled RNA probe encoding antisense nucleotides against a target gene is made from plasmid or PCR product. Choose the RNA polymerase that binds the antisense

strand 5′ to the start of translation. Following the Takara T7 RNA polymerase instructions: mix the following reagents in a 0.5 ml plastic tube; 1.0–1.5 μg plasmid DNA template (or 200 ng/μl PCR product) in 6.0 μl sterilized pure water, 1.0 μl 10× RNA polymerase buffer, 1.0 μl 50 mM DTT, 1.0 μl 10× DIG RNA labeling mix (Roche), 1.0 μl T7 polymerase. Incubate it at 37 °C for 3–3.5 h. Add 1.0 μl TurboDNase (Life Technologies) and incubate it at 37 °C for 15 min. Mix 1.5 μl lithium solution (from mMessage mMachine kit) and 38 μl 100 % ethanol. Store at −20 °C overnight. Centrifuge at 14,000 × *g* at 4 °C for 15 min with a conventional high-speed centrifuge. Wash the pellet with 75 % ethanol–water. Centrifuge at 14,000 × *g* at 4 °C for 5 min and remove ethanol. Air-dry briefly and dissolve it with molecular biological grade water at 100 ng/μl.

3 Methods

The gametes of sea urchin are collected by intra-coelomic injection of 0.5 M KCl. Eggs are stored in a glass beaker filled with FSW at 4 °C, and sperm are collected as dry-sperm and stored in a 1.5 ml tube at 4 °C. Eggs can be stored at 4–15 °C for up to 2 days. Adding antibiotics (penicillin or streptomycin) helps to keep eggs longer. In contrast, the sperm can be stored stably for a week at 4 °C.

3.1 Blastomere Double Injections

The following double injection method permits injection of one reagent into the fertilized egg, and after the first cleavage, another reagent is injected into one blastomere of 2-cell stage. This type of experiment facilitates examination of cell autonomous processes and single embryo rescue experiments. The basic injection methods are derived from previous reports [19, 20, 27].

1. Fill injection dish, coated with 1 % protamine sulfate, with 1 mM ATA-FSW. A row of eggs is settled on the bottom of an injection dish with a mouth pipette. After 1 min, remove any eggs that do not stick to the dish or stick outside of the registration lines marked on the dish. The number of eggs for double injection should not be large; we normally put 100–200 eggs on one dish. This dish should be kept on an ice-chilled tray, not directly on ice, for *S. purpuratus*. For *H. pulcherrimus*, the dish should not be stored on ice-chilled tray, but at 15 °C.
2. Inseminate eggs with a few drops of 1:100 diluted sperm in FSW. Wait until fertilized envelope is obvious. Avoid using excess sperm, as polyspermy and putrefaction can be a problem.

3. Inject the first reagent (morpholino, mRNA, protein, antibody, or chemical) into the aligned eggs. Un-injected eggs should be removed after injecting all other eggs on one dish.
4. After washing four or five times with FSW to remove ATA and extra sperm, culture the injected eggs at 15 °C until first cleavage is complete (about 2 h). To ensure cytokinesis is complete, wait longer until just before the second cleavage starts.
5. Remove FSW and add $Ca^{2+}Mg^{2+}$-free ASW for *S. purpuratus* or Ca^{2+}-free ASW for *H. pulcherrimus*, and repeat 3–5 times.
6. After 5–10 min, the embryos will be ready for the second injection. Inject the reagent into one of the blastomeres. To penetrate extraembryonic layers and the membrane, set the injector with constant flow, press the tip of the needle on the surface of the blastomere to be injected and gently tap the manipulator or the base of the microscope to vibrate the needle.
7. The embryos will only tolerate divalent cation-free seawater for about 20 min, after which blastomeres will dissociate.
8. Remove $Ca^{2+}Mg^{2+}$-free ASW and add FSW gently, and repeat 3–5 times. Culture them at 15 °C until the stage at which they will be analyzed (Fig. 1).

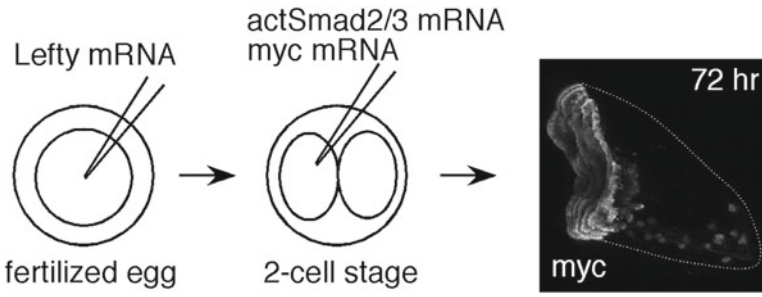

Fig. 1 Double injection strategy and image of an embryo in which a dorsal–ventral axis has been induced without Nodal signaling. The secondary (dorsal–ventral) axis in the sea urchin embryo depends on Nodal, which is expressed in prospective oral ectoderm during cleavage [28]. In this experiment we initially blocked endogenous Nodal activity by injecting mRNA encoding Lefty, a Nodal antagonist, into the unfertilized egg. After the first cleavage, mRNA encoding a constitutively activated Smad2/3 (actSmad2/3), which is an intracellular mediator of Nodal signaling [29], and myc mRNA were simultaneously injected into one of the blastomeres. At 72 h embryo, the myc-injected cells are detected following immunolocalization procedures. This embryo has lost the endogenous diffusible Nodal, which by itself would produce an embryo without a dorsal–ventral axis [28]. However that cells expressing Smad 2/3 behave as though they are activated by Nodal and differentiate as oral ectoderm, which established the ectopic dorsal–ventral axis. In this image, the Myc-positive side of the embryos is a thickened oral ectoderm surrounded by a ciliary band, and the oral-half of the gut. The *dashed-line* outlines aboral ectoderm, which is not visible in this image

3.2 In Situ Hybridization and Immunofluorescence

In some experiments it is necessary to simultaneously detect an mRNA and an antigen, such as a protein or a neurotransmitter, in the same specimen. We have successfully combined immunofluorescence and in situ hybridization methods with the TSA system (Perkin Elmer). A major limitation of this method is that mRNAs require prolonged fixation with the result that some antigens are undetectable. This is the case with anti-synaptotagmin (1E11), a neural marker that is sensitive to formaldehyde cross-linking. However, anti-serotonin has proven very useful in this type of experiment.

1. Fix embryos or larvae with 4 % PFA-FSW overnight in a well of 96-well plate (4 °C). All subsequent steps, except for TSA-treatment, are carried out in this flat-bottom 96-well plate using a dissecting microscope and an aspirator. Alternatively, 0.5 ml clear microcentrifuge tubes can be used; although specimen loss can be a problem, rinsing steps can be reduced in number from 5 to 3.
2. Remove PFA-FSW and add MOPS buffer. Discard the used fixative safely. This MOPS buffer rinse is repeated at least five times for 7–10 min each.
3. Treat samples with 5 % Lamb serum in MOPS (blocking buffer) for 1 h at room temperature (RT).
4. Remove blocking buffer and add 1:1,000 diluted rabbit anti-serotonin antibody in blocking buffer, and incubate it for 1–2 h (RT).
5. Wash with MOPS buffer five times for 7–10 min each.
6. Add 1:2,000 diluted goat anti-rabbit IgG antibody conjugated with horseradish peroxidase in blocking buffer and incubate it for 1–2 h at RT.
7. Wash with MOPS buffer five times for 7–10 min each.
8. Transfer all samples to 0.5 ml clear tubes and remove MOPS buffer.
9. Add diluted TSA-TMR in diluent and incubate it for less than 10 min in the dark.
10. Remove TSA reagent and wash with MOPS buffer once. Protect the samples from light for the following steps.
11. Transfer samples to 96-well plate and wash it with MOPS buffer five times for 7–10 min each. Check a few of samples with a fluorescent microscope.
12. Remove MOPS buffer and add 100 μl hybridization buffer per well and mix well by tapping the plate. Incubate it for 15 min. Change the hybridization buffer three times.
13. Incubate it for 1–3 h at 50 °C (pre-hybridization) with adhesive PCR sheet on wells to avoid evaporation of the buffer.

14. After pre-hybridization is finished, mix 0.4 µl DIG-labeled probe per 100 µl pre-warmed hybridization buffer. Remove the pre-hybridization buffer from the well and add hybridization buffer with probe.
15. Incubate the sample for 7 days at 50 °C with wells covered by an adhesive PCR sheet.
16. Remove the hybridization buffer and wash sample with MOPS buffer five times for 7–10 min each at RT.
17. Wash sample with MOPS buffer three times for 1 h each at 50 °C.
18. Wash sample with MOPS buffer three times for 7–10 min each at RT.
19. Add 5 % Lamb serum in MOPS buffer (blocking buffer) and incubate sample for 1 h at RT.
20. Remove the blocking buffer and incubate with 1:1,000 sheep anti-DIG antibody in blocking buffer for 1 h at RT.
21. Wash the sample with MOPS buffer at least five times for 7–10 min each. Longer is better. An overnight wash sometimes gives a better signal.
22. Transfer all samples to a 0.5 ml clear tube and remove MOPS buffer.
23. Add diluted TSA-Fluorescein and incubate it for 10 min.
24. Remove TSA reagent and wash samples five times for 7–10 min each.

3.3 Immunological Localizations: PFA Fixation

Transfer seawater containing embryos to an appropriate sized vessel and centrifuge. Pellet embryos by gentle centrifugation (<1,000 × *g*) in all steps. A manual, crank-driven centrifuge with a swinging bucket rotor is convenient. A mini-microcentrifuge will also work, however we find the fixed angle and high *g* forces on most models result in embryos being damaged during processing. Once embryos have been fixed they will settle to the bottom of tube without centrifugation and are readily resuspended without damaging embryos. The volumes presented assume 1 ml of embryo suspension containing about 50 µl pelleted embryos. Smaller volumes, with fewer embryos, in 0.5 ml tubes save reagents.

1. Remove the seawater supernatant and resuspend embryos in 1 ml PFA fixative (*see* **Note 2**). Incubate at room temperature for 15 min.
2. Centrifuge, remove supernatant, and resuspend in 1 ml PBS. Incubate for 5 min at room temperature, centrifuge, remove supernatant, and repeat this wash once more.
3. Remove the supernatant and resuspend embryos in 500 µl of blocking buffer.

4. Add antibodies or anti-sera at appropriate dilutions, mix gently by slowly rocking the tube and incubate overnight at 4 °C or for 4 h at room temperature.
5. Centrifuge embryos, remove supernatant and rinse three times each with of 1 ml PBS.
6. Centrifuge, remove supernatant and resuspend embryos in 500 µl PBS containing appropriate concentrations of secondary antibodies or fluorescent conjugates. Incubate in a dark place for 1 h at room temperature.
7. Centrifuge, remove supernatant, wash three times with PBS, and resuspend in 20 µl PBS. 0.01 % (w/v) Sodium azide can be added to this final resuspension, allowing storage of fixed embryos for up to three weeks.
8. Alcohol-clean a glass slide and fix two strips of double-sided tape across the width, spaced 15 mm apart. Place 10 µl of SlowFade reagent between the strips of tape. Resuspend embryos by gentle agitation/trituration and place 10 µl onto the slide and mix with the SlowFade reagent, carefully mount a cover glass so that it spans the two strips of tape and creates a fluid filled space containing the specimens. Seal the edges with a small amount of vacuum grease to prevent leakage and evaporation.

3.4 Immunological Localizations: MeOH Fixation

1. Protocol is carried out as described as in Subheading 3.3.
2. At **step 1**, substitute 1 ml of ice-cold methanol for PFA.
3. Incubate embryos in methanol for 5 min at room temperature (*see* **Note 3**).
4. Remove methanol and carrying out all remaining steps described in Subheading 3.3.

3.5 Immunological Localizations: PEM Fixation

1. Protocol is carried out as described for Subheading 3.3.
2. At **step 1** substitute 1 ml of PEM fixative for PFA (*see* **Note 4**).
3. Fix embryos in PEM for 15 min at room temperature.
4. Remove PEM and carrying out all remaining steps described in Subheading 3.3.

3.6 Immunological Localizations: PFA/PEM Fixation (See Note 5)

1. Protocol is carried out as described for Subheading 3.3 except that following the 15 min incubation in PFA (**step 1** in Subheading 3.3), embryos are post-fixed in PEM.
2. Follow the instructions for PFA fixation for 15 min at room temperature, then centrifuge and remove the PFA.
3. Resuspend the embryos in 1 ml PEM buffer and incubate at room temperature for 15 min.
4. Centrifuge, remove PEM and carry out all remaining steps as described in Subheading 3.3 (*see* **Note 5**).

3.7 Preparing Small Numbers of Embryos

Often experiments yield only a few embryos that are to be processed for immunofluorescence. In these situations, all steps for removing buffer or reagents are performed in a 96-well plate under a dissecting microscope. A mouth pipette consisting of a length of latex tubing with a mouth piece and a PVDF in-line filter enables precise control of pressure, yet prevents accidental aspiration. To facilitate complete rinsing, the number of rinse steps is increased.

1. Collect embryos in a well of 96-well plate. Remove the seawater. Add fixative (PFA, MeOH, PEM) and fix samples for 15 min at RT.
2. Remove fixative and wash samples with PBS five times for 10 min each.
3. Block sample with blocking buffer: Superblock (Pierce Chemical), 0.03 % Triton X-100.
4. Remove blocking buffer and add mixture of diluted antibodies in blocking buffer. Incubate the sample overnight at 4 °C.
5. Remove antibody solution and wash samples with PBS five times for 10 min each.
6. Add secondary antibody mixture and incubate samples for 2 h at RT.
7. Remove antibody solution and wash samples with PBS five times for 10 min each.

4 Notes

1. The glycerol permits visualization of flow from the tip of the injection needle and reduces evaporation.
2. Preservation of cell surface antigens is enhanced with PFA fixation and the cilia are clearly presented, however detail on the basal cell surface is lacking in some instances and cytoskeletal architecture is not preserved. Preservation of serotonin is excellent; however, the epitope recognized by anti-synaptotagmin B (1E11) is only preserved by fixing embryos for about 15 min (Figs. 2 and 3).
3. Methanol provides effective immunofluorescent labeling in select cases but is not suitable in preserving several cell-surface, cytoskeletal and nuclear features (Figs. 2 and 3). Serotonin is not preserved with methanol fixation; however, the epitope recognized by anti-synaptotagmin (1E11) remains intact with methanol fixations up to 30 min.
4. PEM-buffered formaldehyde enhances visualization of many internal structures at the expense of structures on the surface of the embryo. Preservation of cytoskeletal details is excellent. Nucleoplasm and strands of chromatin are well resolved and

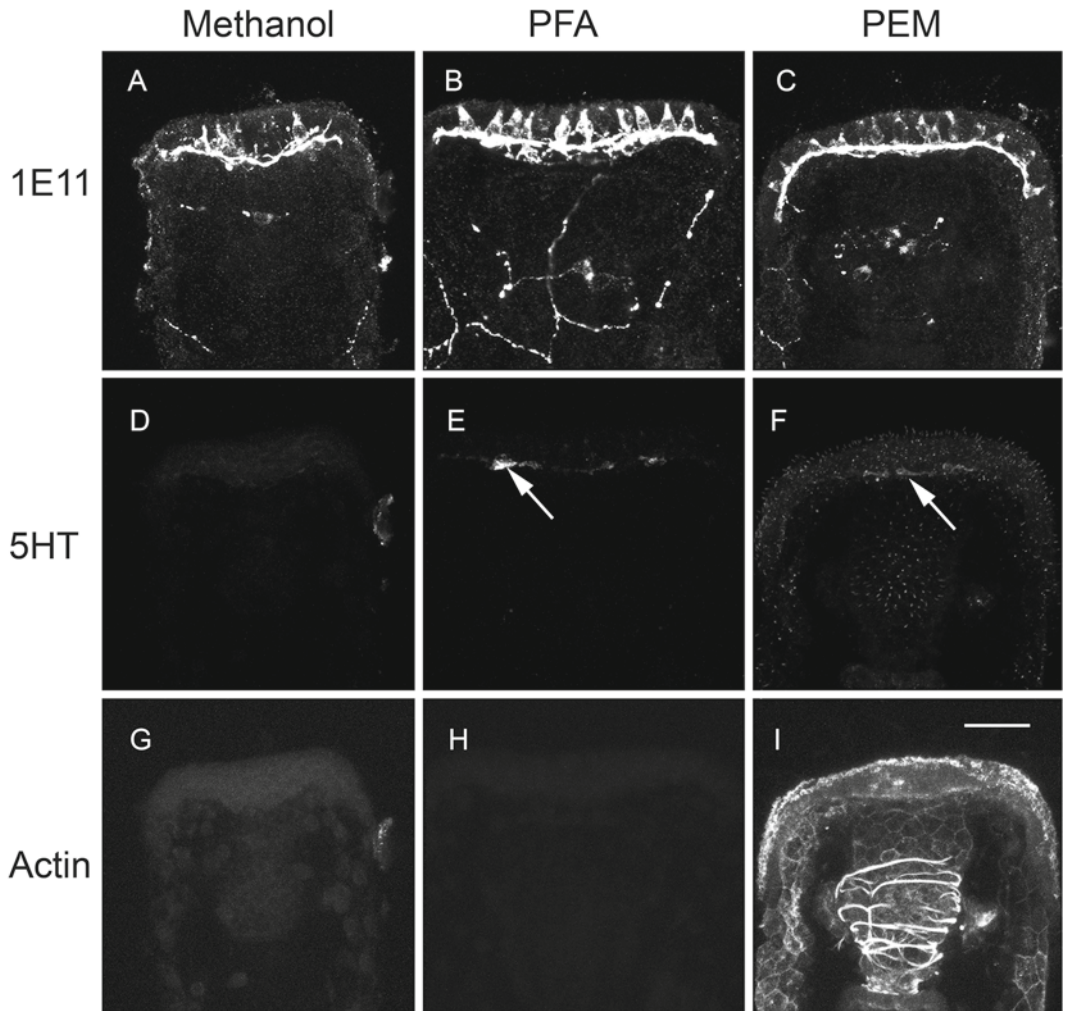

Fig. 2 Confocal images of a portion of the apical organ of 5-day embryos prepared for the localization of synaptotagmin (1E11), serotonin (5HT), and actin. Embryos were fixed with methanol, PFA, or PEM and processed for immunofluorescence as described. All the fixatives preserve the synaptotagmin epitope (**a**–**c**), however fixations longer than 15 min produced poorer results. Serotonin is well localized with PFA, not at all with methanol, and PEM fixation produces a less intense signal with higher background (**d**–**f**). In these preparations laser power and photomultiplier voltage were set at the highest values and satisfactory localization of 5HT can be obtained with PEM. Actin is reliably preserved only with PEM (**g**–**i**). Bar = 20 μm

protein complexes associated with the inner apical membrane (PAR6, aPKC) are well preserved (Fig. 3n, o) and cytoplasmic background fluorescence is reduced significantly compared with PFA fixation (Fig. 3n, o and h, i). The cell-surface antigen αC integrin is well preserved on the basal surface (Fig. 3p) but antigens on the apical surface show only weak fluorescence. Visualization of cytoskeletal detail is enhanced with PEM and actin arrays in regions of apical constriction are easily identified (Fig. 3r) although fine detail in membrane blebs and

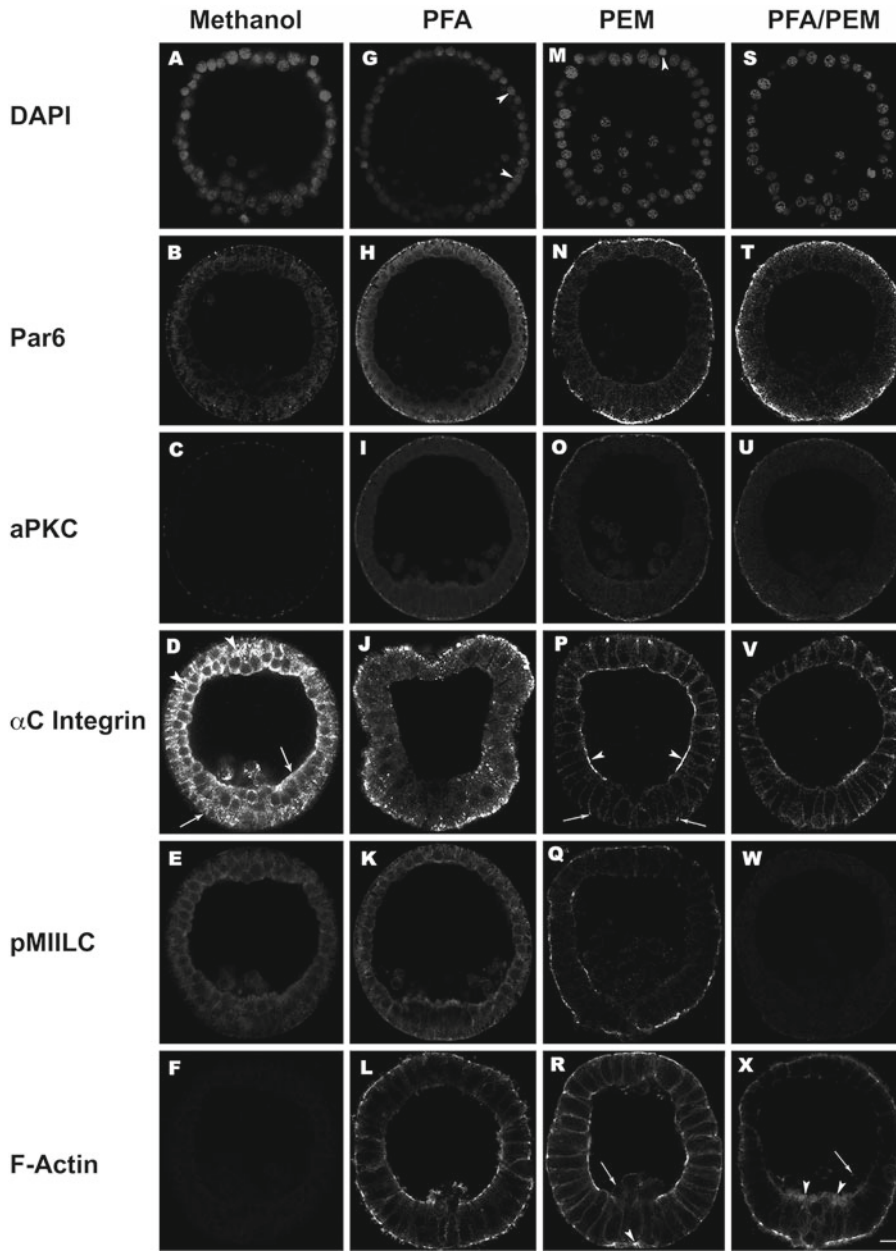

Fig. 3 Confocal images of 28 h embryos prepared using different fixation methods and their effects on preservation of cytoskeletal, nuclear, cell-surface, and membrane-associated protein complexes. Embryos are fixed with methanol (**a**–**f**), paraformaldehyde (PFA) (**g**–**l**); or PEM buffered formalin (**m**–**r**), or PFA/PEM (**s**–**x**). (**a**, **g**, **m**, **s**) DAPI stained nucleoplasm shows the most clarity and preservation of detail when PEM and PFA/PEM fixation methods are used. Antibodies against Par6 (**b**, **h**, **n**, **t**) and aPKC (**c**, **i**, **o**, **u**) illustrate PEM is the best fixative for preserving these membrane associated apical complexes. Methanol fixation causes abundant background fluorescence in the cytoplasm with many of the antibodies used (**b**, **d**, **e**) and although aPKC (atypical Protein Kinase C) shows some specificity at cell–cell junctions (**c**), this is poorly presented in comparison with other fixation methods. (**d**, **j**, **p**, **v**). Preservation of cell-surface antigens is illustrated using αC integrin. PEM buffer provides the best preservation of basal structures while PFA provides the best preservation of apical structures. (**e**, **k**, **q**, **w**) Phosphorylated myosin II light chain (pMIILC) is preserved best with PEM buffer (**q**). (**f**, **l**, **r**, **x**) Filamentous actin structures are preserved with the most clarity and preservation of structure using PFA/PEM fixation. Cytoskeletal preservation with methanol is not satisfactory as f-actin is not preserved (**f**) and labeling of pMIILC is absent (**e**). Bar = 20 μm

filopodia associated with mesenchyme internalization is lacking. Localization of pMyo is also satisfactory with PEM.

5. The combination of PFA and PEM yields a fixation that has the benefit of preserving cytoskeletal architecture with a high level of detail while enhancing the preservation of internal structures on the apical membrane. These benefits are at the expense of cell surface antigen and pMyo preservation. Nucleoplasm and chromatin are highly resolved (S) and fluorescence is similar to that seen with PEM alone (Fig. 3m, s). The apical proteins, PAR6 and aPKC are clearly preserved, with little cytoplasmic background fluorescence (Fig. 3t, u). Preservation of cell surface antigens appears poor and αC fluorescence on both apical and basal surfaces is reduced significantly by this double fixation (Fig. 3v). The actin cytoskeleton is preserved in fine detail (Fig. 3x), allowing the visualization of basal structures normally difficult to preserve. These include filopodia, membrane blebs (arrowheads), and filaments associated with ingression of mesenchyme cells into the blastocoel.

Acknowledgment

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Chapter 10

Preparation and Use of Sea Urchin Egg Homogenates

Anthony J. Morgan and Antony Galione

Abstract

Cell homogenates provide a simple and yet powerful means of investigating the actions of Ca^{2+}-mobilizing second messengers and their target Ca^{2+} stores. The sea urchin egg homogenate is particularly useful and almost unique in retaining robust Ca^{2+} responses to all three major messengers, i.e., inositol 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose, and nicotinic acid adenine dinucleotide phosphate (NAADP) (Lee and Aarhus. J Biol Chem 270: 2152–2172, 1995). It is not only invaluable for probing the pharmacology and mechanism of action of these messengers, but can also be used to assay Ca^{2+} uptake mechanisms (Churchill et al. Cell 111: 703–708, 2002), second messenger production (Morgan et al. Methods in cADPR and NAADP research. In: Putney JW Jr (ed) Methods in calcium signalling, CRC: Boca Raton, FL, 2006), and dynamics of luminal pH (pH_L) changes within acidic Ca^{2+} stores (Lee and Epel. Dev Biol 98: 446–454, 1983; Morgan and Galione. Biochem J 402: 301–310, 2007). Here, we detail the protocols for preparing and using egg homogenates, wherein eggs are shed and collected into artificial sea water (ASW), dejellied, washed several times in Ca^{2+}-free ASW, and then finally washed and resuspended in an intracellular-like medium. Homogenization is effected with a Dounce glass tissue homogenizer (at 50 % (v/v)) and aliquots frozen and stored at –80 °C. For Ca^{2+} (or pH_L) measurements, homogenate is thawed and sequentially diluted in an intracellular-like medium and the fluorescence of Ca^{2+}- or pH_L-sensitive dyes monitored in a standard fluorimeter or plate-reader.

Key words IP_3, cADPR, NAADP, Ca^{2+}, pH, Fluorescence

1 Introduction

Ca^{2+}-mobilizing second messengers are hydrophilic, membrane impermeant molecules, and their introduction into intact cells requires micropipettes or chemical derivatization to yield membrane-permeant forms [1]. In the case of sea urchin eggs, microinjection is feasible [2] but hardly high-throughput, and the usual trick of synthesizing esterified "pro-drug" analogues of second messengers [1] is not applicable since many sea urchin species appear to lack sufficient cytosolic esterases to generate the parent messenger [3]. As a result, egg homogenates allow free access to the "cytosol" in a simple, inexpensive, and potentially high-throughput preparation that can be stored well over many years.

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Moreover, no specialized equipment is required other than a standard cuvette-based fluorimeter (or plate reader).

2 Materials

All solutions should be prepared with ultrapure water (~18 mΩ cm) and analytical grade reagents to minimize contamination by Ca^{2+} and heavy metals. They should also be prepared and stored in plasticware to circumvent Ca^{2+} leeching and contamination from glass.

2.1 Buffers

1. Artificial sea water (ASW): 435 mM NaCl, 10 mM KCl, 40 mM $MgCl_2$,15 mM $MgSO_4$, 11 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 7 mM Tris base, 13 mM Tris–HCl, pH 8.0. Microadjust pH if necessary with HCl or NaOH. Store 4 °C, ≤3 months.
2. EGTA-ASW: As for ASW but substitute 11 mM $CaCl_2$ with 1 mM EGTA.
3. Ca^{2+}-free ASW: As for ASW but without $CaCl_2$ (or EGTA).
4. Glucamine intracellular medium (GluIM): 250 mM potassium gluconate, 250 mM *N*-methyl-D-glucamine, 20 mM Hepes (acid), and 1 mM $MgCl_2$, pH 7.2. NMDG does not dissolve readily without adjusting the pH with glacial acetic acid (~13 ml/l) (*see* **Note 1**).
5. Final GluIM: on the day of requirement, supplement with the ATP regenerating system and Complete™ EDTA-free protease inhibitor cocktail tablets (Roche) (*see* **Note 2**). Typically prepare 50–100 ml. Store on ice.

2.2 Miscellaneous Stock Solutions

1. 0.5 M KCl, in water. Store 1 l at room temperature, ≤6 months.
2. ATP regenerating system: 0.1 M ATP (Mg^{2+} salt), 1,000 U/ml Creatine phosphokinase (CPK), 1 M Phosphocreatine (PCr). All dissolved in water, store at –20 °C (≤6 months) in 100 µl aliquots.
3. Fluorescent Ca^{2+} dye: 3 mM Fluo-3 (K^+ salt). Dissolve in water, store at –20 °C (≤6 months) in 10 µl aliquots. Light sensitive.
4. Fluorescent pH_L dye: 10 mM acridine orange. Dissolve in water, store at –20 °C (≤1 month). Can be freeze-thawed several times. Light sensitive.
5. 1–10 mM NAADP. Dissolve in water, store at –20 °C (≤6 months) in 5–50 µl aliquots.
6. 5 mM IP_3. Dissolve in water, store at –20 °C (≤6 months) in 5–50 µl aliquots.

7. 10 mM cADPR. Dissolve in water, store at −20 °C (≤6 months) in 5–50 µl aliquots. cADPR is particularly sensitive to multiple freeze–thaw cycles.
8. 100 mM β-NAD^+. Dissolve in water, store at −20 °C (≤6 months) in 5–50 µl aliquots.
9. 500 mg/ml heparin (~3,000 kDa, low MW) in water, store at −20 °C (≤3 months).
10. 3 mM 8-Br-cADPR in water; store at −20 °C (≤3 months).
11. 5 M nicotinamide in water; store at −20 °C (≤3 months).
12. 5 mM ionomycin (acid form) in DMSO; store at −20 °C (≤6 months) in 10 µl aliquots.
13. 10 mM nigericin in DMSO, store at −20 °C (≤6 months) in 10 µl aliquots.
14. 10 mM valinomycin in ethanol; store at −20 °C (≤6 months) in 10 µl aliquots.
15. 1 M NH_4Cl in water; store at room temperature ≤1 month.
16. 0.5 M EGTA (K^+ salt, pH 7–8) in water; store at 4 °C ≤3 months (−20 °C ≤6 months).

2.3 Filtration Units

To dejelly large volumes of eggs, we make our own filtration units (Fig. 1; *see* **Note 3**). Take a 50-ml Falcon tube and saw-off the

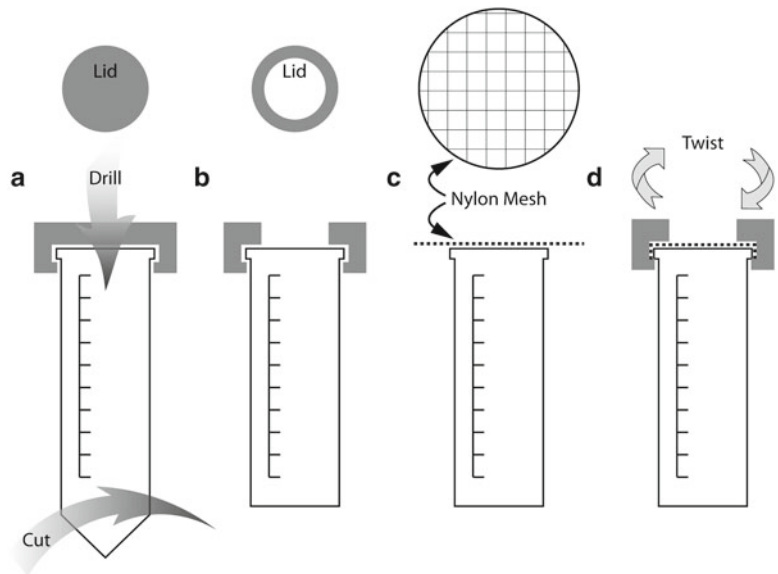

Fig. 1 Preparation of a filtration unit to dejelly eggs. A 50-ml Falcon tube (**a**) is modified by drilling a 20–25 mm diameter hole in the lid and sawing off the *bottom* (**b**). Holding the tube body upright, a 100-µm nylon mesh gauze (47 mm diameter) is placed and aligned centrally on *top* (**c**) and then the modified lid is screwed on to secure and tauten the mesh (**d**)

bottom to make an open tube. Drill out a hole in the screw-on lid (e.g., a 20-25 mm hole in a cap of ~32 mm diameter). Place centrally a 47-mm diameter nylon net filter (100-μm mesh; Millipore—*see* **Note 4**) over the threaded end of the tube and carefully screw on the annular lid. The trapped gauze across the aperture should be taut. Make at least four for a large-scale preparation. The units are washed and reused indefinitely; the filters are changed almost daily.

3 Methods

3.1 Egg Collection

For large-scale preparations, we recommend several people work together as a "factory," assigned to their particular tasks. This shortens the time enormously and ameliorates the homogenate quality. The natural temperature for *Lytechinus pictus* (Californian Blonde) is 17 °C so maintain room temperature as close to this as is feasible. Ensure that the solutions have reached room temperature to avoid temperature shock. *See* Fig. 2 for a schematic flow diagram of the whole procedure.

1. *Lytechinus pictus* (Marinus Inc., Long Beach, CA, USA) sea urchin eggs are collected during the gravid season (May to September). For large-scale homogenate preparations, we normally harvest eggs immediately upon receipt of the shipment (typically 500 animals of which 300 might be female).
2. 20-ml universal tubes are filled to the brim with ASW. We use old scintillation racks to hold them and avoid adjacent places to allow sufficient space between animals.
3. Gamete shedding is stimulated by five intracoelomic injections of 0.5 M KCl solution (up to 1 ml total volume) into each chamber on the oral face using a 1-ml syringe fitted with a 25G needle. From the aboral surface, yellow-orange eggs (or white sperm from males) will begin to appear. The spawning female is inverted and placed upon the filled universal tube. Eggs tend to be shed over a period of 5–20 min with volumes of 1–5 ml (*see* **Note 5**).
4. Once shedding is complete, the sea urchin carcasses are discarded, and the process of pooling the eggs together begins (eggs are precious—take care at each stage to retain them). To reduce dilution and minimize volumes, the excess (clear) supernatant above the settled eggs in each universal tube is carefully discarded. Gently swirl (*not* invert) each tube to resuspend the remaining loose egg pellet (~5–10 ml) and pool into a single large volume (e.g., 2 l) beaker (gently decant the suspension down the tilted beaker's side—like pouring a beer). Do *not* discard the "empty" tube that still contains eggs! To collect the residual eggs, rinse the first "empty" tube with

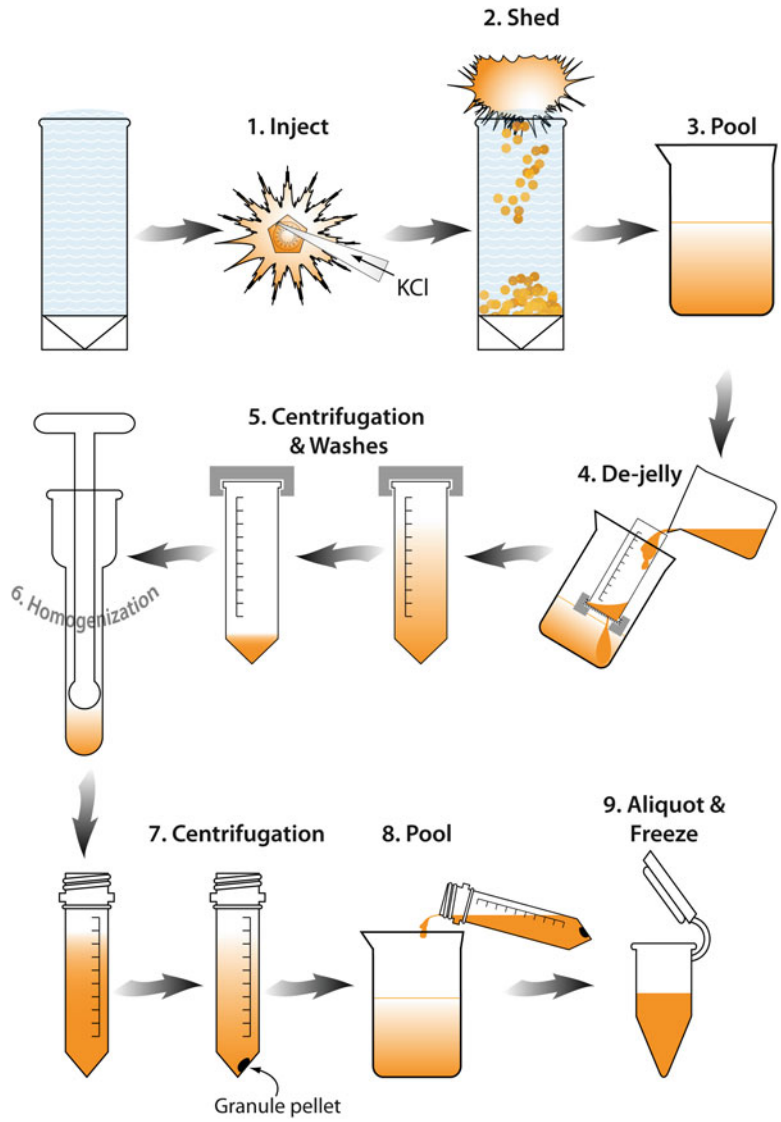

Fig. 2 Flow diagram of egg homogenate preparation. 20-ml Sterilin tubes are filled to the brim with ASW. Each animal is injected intracoelomically with 1 ml of 0.5 M KCl (*1*), and once eggs start to appear from the gonopores on the aboral surface, the animal is inverted and placed on the tube (*2*). After shedding is completed, the eggs are pooled in a large beaker, the tubes rinsed to collect residual eggs (*3*), and dejellied, by passing the egg suspension through the filtration unit (*4*). Ca^{2+} and ASW are removed by sequential rounds of centrifugation and resuspension (*5*), and egg pellets are finally resuspended in an equal volume of intracellular-like medium (GluIM). A Dounce homogenizer is used to break open the eggs (*6*), and the homogenate is spun briefly in 2-ml uncapped tubes to pellet granules (*7*) before collecting the supernatant in an ice-chilled beaker (*8*). Finally, the homogenate is dispensed as 500-μl aliquots in microfuge tubes and stored at –80 °C (*9*)

10–15 ml of ASW. Swirl, and gently decant this straight into a second "empty" tube. Repeat for 5–10 consecutive tubes (depending upon the egg carry-over) and finally add these washes to the "pooled" beaker.

5. Once all the eggs are collected, you can end up with at least 1–2 l of egg suspension. Seal the beaker with Parafilm and immerse in a chilled 17 °C water bath and allow the eggs to settle for ~45 min.

3.2 Egg Preparation and Homogenization

1. Carefully aspirate/decant any clear supernatant from the pooled eggs.
2. Eggs are mechanically dejellied by filtering through a mesh slightly smaller than the egg diameter. This "nicks" the egg jelly coat as they pass through. For *L. pictus* (120-μm eggs) we routinely use a 100-μm mesh (*see* Subheading 2.3). Filter units are pre-wetted with ASW and held by hand inside another large volume beaker. Approximately forty microliters of egg suspension are poured into the filtration unit at a time, and the dejellied eggs allowed to drain into the second beaker. Always keep the filter in superficial contact with the receiving suspension. In between filtrations, clogging of the mesh is countered by washing deionized water across the mesh (in both directions—do *not* collect these cleaning washes!). However, large volumes of suspension will ultimately block the filters which need to be replaced several times during the course of the day. Repeat steps until all the eggs are dejellied.
3. Dejellied eggs are then washed twice to remove Ca^{2+}. The dejellied egg suspension is transferred into (and fills) multiple 50-ml Falcon tubes and eggs pelleted by centrifugation at 300 × *g* for 1–2 min at 10 °C. We use a centrifuge with buckets that hold 16 tubes at a time. Decant (or aspirate) as much supernatant as possible (without losing eggs) and discard. The egg pellet should be compact but not too firm and no more than 15 ml per tube. Resuspend up to ~50-ml with EGTA-ASW (gentle inversion and flicking of the tube bottom with the thumb may help). Centrifuge as before and remove supernatant (*see* **Note 6**). Repeat steps above.
4. Eggs are then washed twice to remove EGTA. Resuspend pellets in ~40-ml Ca^{2+}-free ASW. Centrifuge as before and remove supernatant. Repeat.
5. Eggs are then washed twice in intracellular medium. Resuspend pellets in ~40-ml GluIM. Centrifuge as before and remove supernatant. Repeat. After the last spin, aspirate as much of the supernatant as possible.
6. Estimate the volume of each pellet and add an equal volume of ice-cold Final GluIM to each tube just before homogenization (50 % v/v).

7. Resuspend the pellet in Final GluIM (≤ 10 ml) by gentle pipetting and swirling and transfer to a pre-chilled, tight-fitting Dounce homogenizer.
8. Use the *minimum* number of strokes to homogenize (*see* **Note 7**).
9. With a pipettor and a 10-ml tissue-culture pipette, rapidly aliquot the homogenate into 2-ml capless tubes and spin in a cooled microfuge (as brief as possible (<30 s), pulsing up to ~13,000 × *g* and then immediately stopping). This should only form a small pellet of dark granules (a few mm across); too vigorous a spin and you will form a large, orange pellet containing the actual vesicles you want (*see* **Note 8**).
10. Rapidly collect the supernatant by pouring it out and tapping the tube into a small plastic beaker kept on ice.
11. Once all the homogenate has been collected, use a repeater pipette to rapidly dispense 500 µl aliquots in Eppendorf microfuge tubes which have been pre-placed in freezer storage racks/boxes.
12. Cap and promptly store at −80 °C. Snap-freezing is not necessary.
13. From 500 animals, a good homogenate preparation will yield 300 aliquots; a poor one as few as 50. Stored at −80 °C, where they last for many years (*see* **Note 9**).

3.3 Fluorimetry to Measure Ca^{2+} Release

To measure Ca^{2+} (or pH_L) changes, concentrated (50 %) egg homogenate is diluted *gradually* 20-fold over ~3 h (to facilitate optimal Ca^{2+} loading of the stores), fluorescent dyes are added and fluorescence changes monitored in a stirred cuvette in a standard fluorimeter (summarized in Fig. 3).

1. On the day of the experiment, equilibrate 10 ml of GluIM (*see* **Note 10**) in a 15-ml Falcon tube at 17 °C in a chilled water bath and when ready, thaw the following: a 500 µl aliquot of homogenate, one tube each of the ATP regenerating system components. All the steps below are carried out at 17 °C.
2. Add 100 µl each of ATP, CPK, and PCr to the GluIM and mix (=GluIM+).
3. In another 15-ml Falcon tube, carefully pipette the 500 µl of homogenate directly to the bottom of the tube and similarly add and mix 500 µl GluIM+. Incubate in the water bath for 45–60 min.
4. Add 1 ml GluIM+, incubate for 30 min.
5. Add 2 ml GluIM+, incubate for 30 min.
6. Add 3 ml GluIM+, incubate for 30 min.
7. Add 3 ml GluIM+, incubate for 30 min.
8. The 10 ml of 2.5 % (v/v) homogenate is now ready to use.

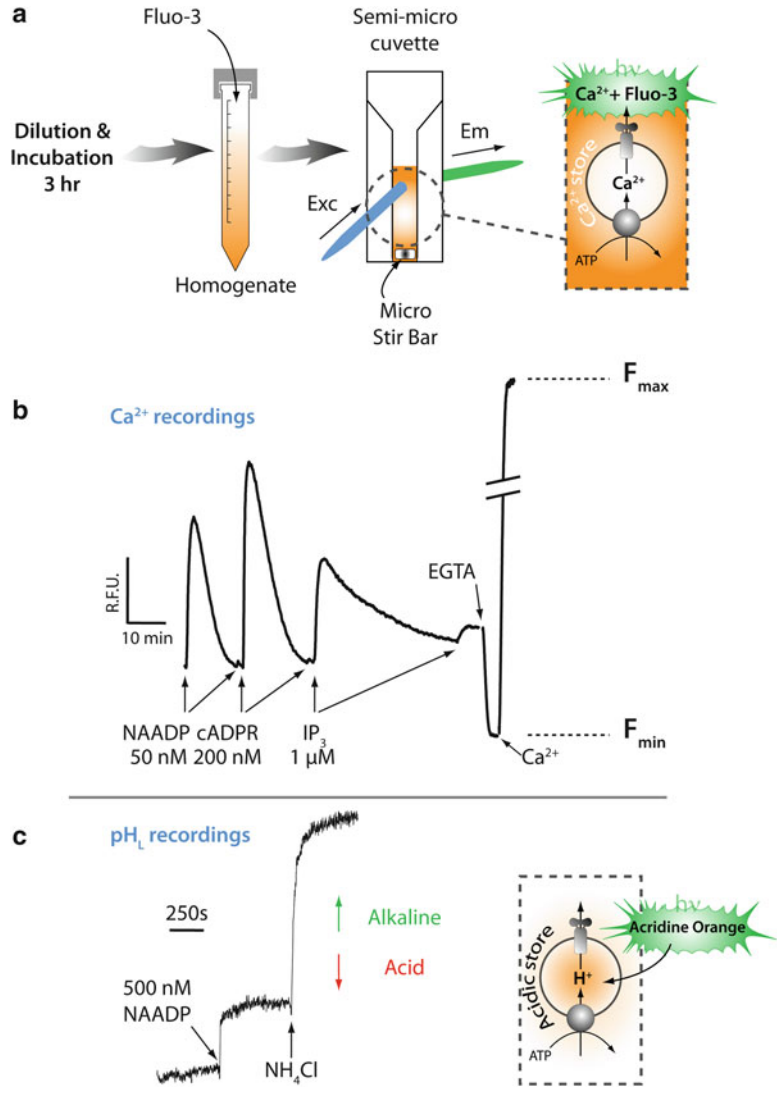

Fig. 3 Monitoring Ca^{2+} and pH_L using fluorimetry. (**a**) Homogenate is diluted 20-fold (2.5 % v/v final) over 3 h at 17 °C in a 15-ml tube. When measuring Ca^{2+}, 3 μM fluo-3 is added to the 10 ml homogenate and 600 μl added to a semi-micro cuvette complete with a micro stir bar and placed in the chilled sample block of a fluorescence spectrometer. Fluo-3 monitors Ca^{2+} in the extravesicular ("cytosolic") compartment. Drug additions are made in 0.6–6.0 μl aliquots. (**b**) Sample trace of Ca^{2+} signals in response to addition of second messengers; note their homologous desensitization. To calibrate fluorescence in terms of absolute [Ca^{2+}], the minimum and maximum fluorescence (F_{min} and F_{max}) are determined at the end of the run by addition of 0.5 mM EGTA and 10 mM $CaCl_2$ respectively (note the break in the trace after Ca^{2+} addition). RFU = relative fluorescence unit. (**c**) To monitor pH_L changes within acidic vesicles, fluo-3 is omitted and either 1–10 μM acridine orange or a Lysosensor is added to the cuvette 5–10 min before recording. Sample trace of acidic vesicle alkalinization in response to NAADP and 10 mM NH_4Cl

9. For Ca^{2+} measurements, 10 µl of fluo-3 (3 µM final) is added to the entire 10 ml of homogenate.
10. We use an LS-50B Perkin Elmer fluorescence spectrometer whose cuvette holder is water-cooled to 17 °C (fed from the same water bath for the homogenate dilution) and which controls a magnetic stir bar (*see* **Note 11**).
11. Place a micro-flea in the bottom of a semi-micro cuvette (*see* **Note 12**), add 600 µl of homogenate (or a suitable volume for the light path) and place in the cuvette holder.
12. Fluo-3 is excited at 506 ± 3 nm (526 ± 2 nm emission) and fluorescence recorded ≥1 Hz.
13. Generally, compounds are added in 6 µl volumes (from 100× stocks). However, to minimize the concentration of solvents (e.g., DMSO or ethanol), 1,000× stocks are prepared for 0.6 µl additions. In either case, add to the bottom of the cuvette to ensure efficient mixing by the flea.
14. Fluo-3 fluorescence can be calibrated in terms of absolute Ca^{2+} concentration using the following equation: $[Ca^{2+}] = K_d \times (F - F_{min})/(F_{max} - F)$, where $[Ca^{2+}]$ is in the same units as the K_d of fluo-3 (e.g., 0.4 µM or 400 nM), F is the fluorescence at any time, F_{min} and F_{max} are the fluorescence in the "absence" of Ca^{2+} and presence of saturating Ca^{2+}, respectively (*see* **Note 13**).
15. At the end of each run, F_{min} and F_{max} are empirically determined by the sequential addition of 500 µM EGTA and 10 mM Ca^{2+}, respectively (6 µl of 50 mM EGTA; 6 µl of 1 M $CaCl_2$). *See* Fig. 3.
16. Plate-readers can be excellent for high-throughput screening. With sea urchin egg homogenates, however, there are some additional considerations. For example, maintaining 17 °C is difficult (or impossible) for many models. With manual drug additions, temporal resolution is often inferior to the cuvette system due to delays between addition and recording as well as a lower sampling frequency throughout the experiment. The former can be reduced with multichannel pipettes. Mixing can be problematic. Each well of a 384-well plate holds 50 µl of homogenate. Adding 0.5 µl of sample requires physical "stirring" with the pipette tip. We also purchase low retention volume pipette tips to increase accuracy when using such small volumes.

3.4 pH_L of Acidic Ca^{2+} Stores

When NAADP evokes Ca^{2+} release from acidic Ca^{2+} stores the pH_L of these target vesicles increases [2, 4, 5]. Indeed, the pH_L of acidic vesicles is regulated by NAADP and fertilization (in intact eggs) in overlapping ways which underscores its physiological importance [2, 5, 6].

1. The best signal for pH_L monitoring is achieved with acridine orange although Lysosensor family members can also be used [2, 4, 6] (*see* **Note 14**).
2. Dilute and prepare homogenate as above (Subheading 3.3, **steps 1–8**).
3. To a flea and 600 µl of homogenate in a cuvette, 0.6 µl of acridine orange is added (1–10 µM final) and fluorescence monitored at the same wavelengths as for fluo-3,although probably with narrower slit widths since acridine orange is brighter than fluo-3.
4. As the acridine orange accumulates into the acidic vesicle lumen, it self-quenches and the fluorescence falls. This takes about 5–10 min.
5. Once a stable plateau is reached, test compounds can be added. Note that the dynamic range of acridine orange is less than that of fluo-3. The acridine orange fluorescence is proportional to the pH_L (an increase in fluorescence reflects an increase in pH_L) although non-linearly.
6. Calibration in terms of absolute pH_L is difficult and so relative changes are recorded. We frequently normalize the fluorescence changes to that observed at the end of the run with a maximal concentration of NH_4Cl (10 mM; 6 µl of 1 M stock) [7].

3.5 Discontinuous Bioassay of Secondary Messenger Production

Although there are multiple methods to assay cADPR and NAADP concentration or ADP-ribosyl cyclase (ARC) activity [8], a simple yet sensitive method utilizes egg homogenate and Ca^{2+} release as a "bioassay" for messenger synthesis [8]. In the first mode, second messengers are generated in other cells (intact or broken), extracted, and assayed for Ca^{2+} release activity. In the second mode, ARC activity in the egg homogenate itself can be monitored as the conversion of substrate, β-NAD^+, into product cADPR (and Ca^{2+} release) [9]; we routinely use the latter to screen compounds against ARC activity [10].

1. Egg homogenate is prepared as above and Ca^{2+} measured with fluo-3 (Subheading 3.3, **steps 1–9**).
2. Cytosolic, de-proteinated extracts from other cells should be pH-neutral before addition to egg homogenate. Neutralization of acid extracts is best effected with bicarbonate (rather than hydroxide) which has a broader equivalence point and reduces the likelihood of overshooting the pH (note: NAADP is hydrolyzed at alkaline pH); *see* [11] for details.
3. 6–60 µl of extract is added to homogenate in a fluorimeter and Ca^{2+} release measured (*see* **Note 15**).
4. Which messenger(s) is present in the extract should be confirmed by blockers added to the cuvette several minutes

before the extract [8]: IP_3 is blocked by 1 mg/ml heparin (*see* **Note 16**), cADPR by 30 μM 8-Br-cADPR, and NAADP by a self-inactivating concentration of NAADP (5–10 nM) [3]. Always verify that the inhibitors are active (and appropriately selective) against maximal concentrations of *bona fide* messengers.

5. Once identified, the absolute amount of messenger produced can be calibrated by comparison with a detailed (half-log) concentration–response curve to pure, commercial messenger. Approximate EC_{50} in egg homogenate: 0.2 μM (IP_3), 40 nM (cADPR), 30 nM (NAADP).
6. In the case of cADPR, assay sensitivity can be further improved by adding 2 mM caffeine 1 min before the extract/messenger: this sensitizes the target ryanodine receptor and decreases the cADPR EC_{50} fourfold (to 10 nM) [8].

3.6 Measuring ADP-Ribosyl Cyclase Activity

1. Egg homogenate is prepared as above and Ca^{2+} measured with fluo-3 (Subheading 3.3, **steps 1–9**).
2. The reaction is initiated by addition of 50–150 μM β-NAD^+ to the cuvette (*see* **Note 17**). There should be a significant delay before Ca^{2+} begins to rise (representing the time taken to generate cADPR product)—an immediate rise with β-NAD^+ suggests contamination by Ca^{2+} or product.
3. The requirement for ARC activity can be confirmed by its inhibition with 5 mM nicotinamide [9].
4. The rate and amplitude of Ca^{2+} release is an index of ARC activity.

4 Notes

1. The presence of the sugar derivatives greatly reduces the GluIM storage time due to bacterial/fungal growth, even at 4 °C (≤2 months). This period can be extended by sterile (0.22 μm) filtering or storage at −20 °C (≤6 months).
2. The final GluIM will be diluted 1:1 with eggs. Therefore, supplement with the ATP system and protease inhibitors at twice their final concentration, i.e., 2 mM ATP, 20 U/ml creatine phosphokinase, 20 mM phosphocreatine, 2 protease tablets per 50-ml GluIM.
3. For smaller scale preparations, we make the units from 15-ml Falcon tubes.
4. Each sea urchin species has its own characteristic egg diameter (*L. pictus* 120 μm, *S. purpuratus* 80 μm, *P. californicus* 200 μm), and therefore, the mesh pore size will have to be changed accordingly.

5. Healthy eggs are a vibrant yellow-orange and sink in broken strands; unhealthy eggs appear dull-colored and/or settle as unbroken elongated, "spaghetti" (presumably due to egg lysis releasing sticky DNA).
6. Egg lysis can occur when placed into Ca^{2+}-free media, and this is manifested as cloudy supernatants. This is greatest with poor quality (or handling) of eggs and can lead to a substantial loss (≤50 %) of the cells in the worst instances.
7. Successful homogenization is heralded by a marked increase in the resistance to a Dounce stroke (presumably when intracellular DNA is released). Only give 1–2 more strokes and avoid over-homogenization. If unsure, check a drop of homogenate on a petri dish on a light microscope—there should be no, or very, few intact eggs remaining.
8. Historically, this final spin was designed to remove cortical granules since they contain damaging proteases. However, quite a lot of precious homogenate is wasted in the process. Therefore, we recently omitted this step and observed no obvious change in the Ca^{2+} response to the three second messengers. We do not yet know whether the long term storage of this homogenate batch will be compromised.
9. Occasionally you come across a rogue aliquot that does not pump Ca^{2+} down after thawing (<1 % of all tubes). We do not know why this occurs; perhaps it is due to different freezing times compromising vesicular integrity.
10. In our hands, GluIM can often be contaminated with an unknown substance that inhibits NAADP-dependent Ca^{2+} release. We have found that pretreating GluIM with activated charcoal removes this factor [3]: stir 50–100 ml GluIM with 10 mg/ml charcoal for 60 min at room temperature; to remove the charcoal, centrifuge at ~3,000 × *g* for 5 min, carefully pour off and collect the supernatant and finally filter with a 0.2 μm filter unit attached to a 50-ml syringe. Store in plastic.
11. Stirring is essential for two reasons: to prevent the homogenate settling and to ensure rapid mixing of additions particularly with non-aqueous solvents such as DMSO. Individual micro fleas differ greatly in their efficiency of mixing; some merely "vibrate" and do little; good fleas spin around. Each flea should be examined by eye in an empty cuvette and non-spinners discarded.
12. Ensure that the semi-micro cuvettes are suitable for fluorimetry. They should transmit at all wavelengths required and they must not be "frosted" on any face.
13. Ca^{2+} calibration is recommended and is essential when comparing signals across different machines; their absolute fluorescence changes will not be the same.

14. With pH_L reporters, chronic loading of the entire homogenate in one fell swoop must be avoided since these compounds continue to partition into acidic vesicles and will eventually alkalinize them [12]. Instead, acute addition to each cuvette is recommended.
15. The volume of extract required will be determined by how much messenger is present. The smaller the volume, the better.
16. Some heparin preparations can be contaminated with Ca^{2+} and addition evokes a large Ca^{2+} spike. Allow this Ca^{2+} to be re-sequestered by the homogenate before addition of IP_3 or extracts.
17. Different batches of homogenate convert β-NAD^+ to cADPR with different efficiencies. It is more reassuring to see a slight lag before Ca^{2+} rises in keeping with the conversion time, so determine the optimal β-NAD^+ concentration for each batch.

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Chapter 11

Microinjection and 4D Fluorescence Imaging in the Eggs and Embryos of the Ascidian *Phallusia mammillata*

Alex McDougall, Karen Wing-man Lee, and Remi Dumollard

Abstract

Time-lapse 4D imaging of fluorescently tagged proteins to follow the dynamics of cellular structures (chromosomes, microtubules, actin, centrosomes, cortical structures like the CAB in ascidians, etc.) combined with targeted gene knockdown during embryonic development is a powerful technique to understand the mechanisms of embryonic development. The eggs and embryos of the primitive marine chordate *Phallusia mammillata* are an excellent model system for combining live cell imaging with gene knockdown experiments. Here we describe simple methods for microinjecting *Phallusia* eggs with mRNA encoding fluorescent fusion proteins combined with 4D time-lapse imaging techniques we use to follow all of embryonic development from the egg to late tailbud stage.

Key words Ascidian, *Phallusia mammillata*, 4D imaging, Fluorescent fusion proteins, Gene knockdown, Microinjection

1 Introduction

A systems-level approach using in toto imaging of all cells during embryonic development is a major goal of imaging studies aimed at deciphering how different cell behaviours are generated during embryonic development [1]. Control of cell division orientation plays a major role in early embryo body plan specification [2]. This is typified by simple marine chordate embryos such as ascidians where both the cell division orientation and cell cycle duration are precisely controlled [3]. Cell division axis reorientation is most dramatic in two posterior vegetal cells at the 8-, 16-, and 32-cell stages. In these cells the mitotic spindle is attracted towards a cortical structure, causing the cell to divide in a precise orientation while also cleaving unequally to generate daughter cells of different sizes [4]. Although these cells are the most studied examples of unequal cleavage in ascidian embryos, many other cells divide unequally. Cell cycle duration is also precisely controlled in ascidian embryos. For example, at the 16-cell stage cycle duration is shorter in the

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8 vegetal blastomeres generating a brief 24-cell stage. Further asynchrony in the vegetal blastomeres yields an embryo at the time of gastrulation composed of precisely 112 cells [5]. Importantly, each blastomere in the gastrula has a distinctive size, shape, and position relative to its neighbors. One of our goals is to decipher the mechanisms regulating unequal cleavage, oriented cell division and cell cycle duration which together create the distinctive morphology of the 112 cell gastrula-stage embryo, and for this we use live cell imaging of fluorescently tagged proteins coupled with gene knockdown in the ascidian *Phallusia mammillata*.

Ascidians embryos have been used for more than a century to study embryonic development. Their embryos display the typical chordate body plan but are composed of an extremely small number of cells. Gastrulation occurs about 3–4 h after fertilization at the 112-cell stage, and a simple chordate type larva (tadpole) forms approx. 12 h after fertilization composed of only about 2,600 cells [3]. *Ciona intestinalis* was the first ascidian to have its genome sequenced [6] and has provided an exceptional wealth of information concerning gene expression patterns during development. Excellent Web-based resources exist housing genomic and transcriptomic data sets: [7] Aniseed (http://aniseed-ibdm.univ-mrs.fr/), [8] GHOST (http://hoya.zool.kyoto-u.ac.jp/SearchGenomekh.html#CDNA) as well as proteomic data CIPRO [9] (http://cipro.ibio.jp/2.5/) and a 3D atlas of embryonic stages FABA [10] (http://chordate.bpni.bio.keio.ac.jp/faba/1.3/top.html). Some ascidian species like *Phallusia mammillata* are ideal for imaging studies (see film archive at http://biodev.obs-vlfr.fr/recherche/biomarcell/), not only because their eggs and embryos are completely transparent [11], but also because their unfertilized eggs translate well exogenous mRNA, which is not the case for *Ciona intestinalis* [12]. This is important since eggs and many early cleavage stage embryos including ascidians are transcriptionally inactive. Until transgenic lines that express in the egg become available (transgenic tadpole and juvenile lines of *Ciona intestinalis* are available. See: http://marinebio.nbrp.jp/ciona/imageListAction.do?strainType=All&stage=ALL), a simple means of introducing molecular markers into the egg is required. Since protein production is time-consuming, mRNA injection is the simplest method for producing fluorescent fusion protein constructs in unfertilized ascidian eggs. The recent availability of extensive EST databases for *Phallusia* (currently 120,000 ESTs from mixed embryonic stages) has made it possible to obtain the nucleotide sequence around the first ATG, making it straightforward to design morpholino oligonucleotides to perform gene knockdown experiments. We have recently exploited this in *P. mammillata* to perform the first 4D fluorescence confocal imaging study coupled to morpholino knockdown during early cleavage cycles in *P. mammillata* [12]. Here we describe in detail the procedures for microinjecting mRNA into *P. mammillata* eggs and for performing 4D fluorescence imaging of embryos expressing fluorescently tagged proteins.

2 Materials

2.1 Solutions, Plasticware, and Glassware for Microinjection

1. mRNA: mix at around 2 μg/μl in distilled water and store in 1 μl aliquots at −80 °C. We use mMessage mMachine and follow the manufacturer's protocol which we do not detail here (Ambion). It is important to note that the only mRNA that translates well has a poly A tail (*see* **Note 1**).
2. 1 % GF: in a 50 ml Falcon add 0.5 g Gelatin (Sigma Type A from porcine skin) to 40 ml distilled water and heat in a water bath at 60 °C for 10 min to dissolve. Once the gelatin has dissolved, leave it to cool and then add 1.35 ml of 37 % Formaldehyde and bring the total volume to 50 ml by adding more distilled water. We call this stock solution 1 % GF (*see* **Note 2**).
3. 0.1 % GF: dilute the 1 % GF with distilled water to make a 0.1 % GF solution. Coat plasticware, coverslips, and slides on one side with 0.1 % GF and leave to air-dry. Briefly, add enough 0.1 % solution to each petri dish to coat the bottom or apply 40 μl 0.1 % GF to one side of a coverslip and spread with a pipette tip then stand to air-dry. Once dry, wash all coated surfaces with distilled water and leave to air-dry before storing.
4. VaLaB: mix 1:1:1 Vaseline, lanolin, and beeswax. We use this to prepare the mounting chamber or "Wedge" for microinjection (*see* Fig. 1) (*see* **Note 3**).
5. To prepare the mounting chamber. The mounting chamber is based on the design of Kiehart [13]. First use one GF-coated glass coverslip and one uncoated coverslip. Cut the GF-coated coverslip into small pieces using a diamond knife (approx. 10 mm by 3 mm) and cut larger pieces of glass from the non-coated coverslip (approx. 15 mm by 4 mm) which we term "spacers." Cut about ten pieces of each and store as GF and Spacers (*see* Fig. 1).
6. Sea water: prepare 500 mM TAPS pH buffer (pH 8.2) and 100 mM EDTA (pH 8.0) both in distilled water. Filter natural seawater through 0.2 μm filter to remove bacteria and debris. Add 5 ml of 500 mM TAPS and 0.5 ml of 100 mM EDTA to 500 ml filtered sea water and pH to 8.2 with NaOH and HCl. Store sea water at 16 °C (*see* **Note 4**).
7. 1 % Trypsin solution (bovine pancreas): mix in TAPS-buffered filtered sea water and store as 1 ml aliquots at −20 °C.
8. Cut capillary free microinjection needles (GC100T10) into 8 mm long pieces and keep for later use as "filling tubes" for mRNA microinjection (*see* Fig. 1).

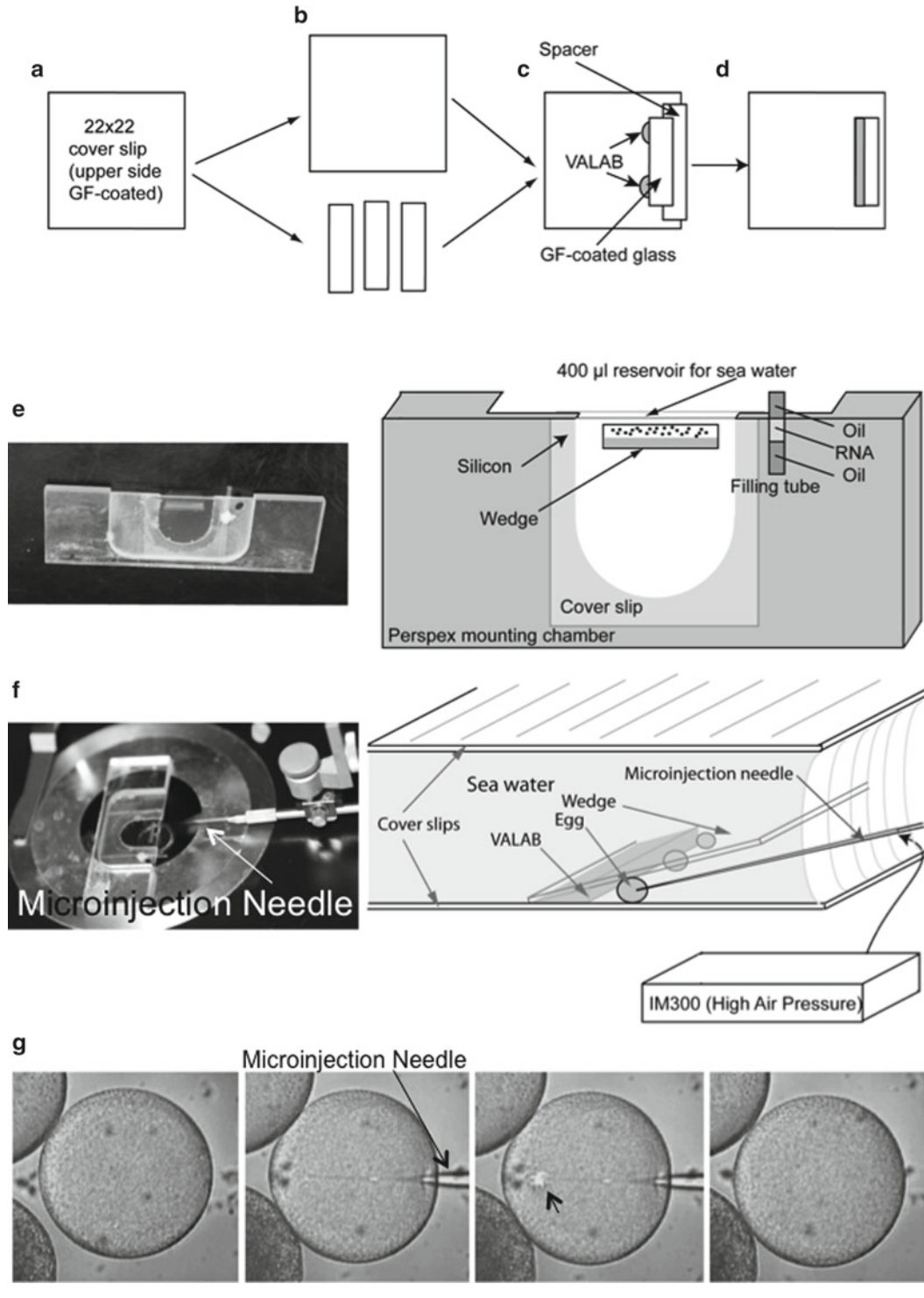

Fig. 1 Microinjection of *Phallusia* eggs. (**a**) Coat one side of coverslip with 0.1 % gelatin/formaldehyde (formaldehyde cross-links the gelatin). (**b**) Cut pieces with diamond knife (approx. 10 mm by 4 mm). (**c**) Heat on hot plate until VaLaB melts. (**d**) Remove from heat and once VaLaB is set, remove spacer. (**e**) Mounting chamber with wedge and filling tube attached. Model showing mounting chamber and filling tube. Wedge containing eggs fixed to mounting chamber with Dow Corning vacuum grease. (**f**) Mounting chamber on microscope stage.

3 Methods

3.1 Preparing Dechorionated Eggs

1. Remove a tube of 1 ml 1 % Trypsin to defrost (*see* **Note 5**).
2. Prepare eggs. Cut through the hard protective tunic (composed of a type of cellulose) of the ascidian making a cut from the oral siphon to the atrial siphon with a razor blade or scissors. Use thumbs to pry open the tunic and remove the soft-bodied ascidian. Lay animal so that eggs and sperm are facing up on a paper towel to absorb water. Remove eggs first by puncturing oviduct with needle then aspirate the eggs with a 1 ml disposable plastic pipette and add directly to 4.5 ml fresh filtered sea water in a 5 cm petri dish. Next collect the sperm in a similar manner but store the sperm "dry" in a 1.5 ml Eppendorf (*see* **Note 6**).
3. Dechorionation (removal of the outer layer of the egg by trypsin treatment). Add 1 ml 1 % trypsin solution to the 4.5 ml eggs and agitate on an orbital shaker for 1 h 30 min at 20 °C (or until approx. 10 % of the eggs begin to lose their outer layer) to dechorionate the eggs.
4. Once approx. 10 % of the eggs begin to dechorionate gently pipette approx. ten times without making air bubbles to dechorionate the remaining eggs.
5. Wash eggs to remove trypsin, chorionated eggs and dead eggs. Gently swirl in a circular motion to collect dechorionated eggs and remove the excess sea water. Add fresh TAPS filtered sea water and repeat four or five times or until most of the dead or chorionated eggs are removed leaving only dechorionated eggs.
6. Transfer the dechorionated eggs to a larger a fresh petri dish which has been GF-coated and store at 16 °C (*see* **Note 7**).
7. With a mouth pipette or a handheld pipette transfer approx. 50–100 eggs to an injection chamber or "wedge" (*see* Fig. 1).
8. Proceed to microinjection of *Phallusia* eggs.

Fig. 1 (continued) The microinjection needle tip is first broken on the side of the filling tube. The microinjection needle is then tip-filled using negative pressure (be sure to use filament-free capillaries (e.g., GC100 T-10)). Keep the meniscus in view to check balance. Inject egg at around 10–20 psi. (**g**) Microinjection of eggs in the wedge. The strip of VaLaB is to the left and the opening of the wedge is to the *right* as in (**f**). The microinjection needle is inserted into the egg so that the tip is near the center (the far side of the egg is also fine but inserting the tip at the near side kills the egg more readily). The egg is injected near the center or even near the opposite side of the egg an in this example. Displacement of the egg cytoplasm reveals the site of microinjection (*arrow*). Typically for mRNA injections 1–3 % egg volume is injected. The microinjection needle is then removed from the egg

3.2 Microinjecting Phallusia Eggs

1. We use an inverted Olympus IX70 with left mounted stage control, right-hand mounted three-way Narishige hydraulic micromanipulator, and an air compressor providing the compressed air for a Narishige IM300 injection box.
2. Our microinjection method is as described previously with some small modifications [13]. The mounting chamber is made of Perspex (*see* Fig. 1). Briefly, the injection chamber is 73 mm long, 25 mm wide and 4 mm thick. The central reservoir is 1 mm thick and has a 15 mm by 15 mm piece removed that will make a reservoir for approx. 400 μl of seawater once the coverslips are attached (*see* Fig. 1).
3. Prepare the wedge first as shown in Fig. 1 (verify that GF-coated glass surfaces are pointed towards each other).
4. Dow Corning vacuum grease is applied to both sides of the injection chamber where the coverslips will later be attached.
5. The coverslip with the wedge is attached first to the flat side of the mounting chamber with the "wedge" facing what will be the seawater reservoir (*see* Fig. 1).
6. Next using a binocular microscope 50–100 eggs are transferred into the wedge by mouth pipette (capillary action pulls the seawater and eggs into the wedge so long as a small enough volume is applied).
7. Then a coverslip is applied to the other side to make a sandwich with the wedge and eggs in the center (*see* Fig. 1).
8. Next the reservoir between both coverslips is filled up with TAPS filtered sea water carefully so as not to dislodge the eggs.
9. Then place the mounting chamber into a large enough petri dish on two pads of water-soaked tissue paper to create a humid environment so that the 400 μl reservoir does not evaporate and store at 16 °C until required.
10. Once the eggs have been prepared for microinjection, prepare the mRNA Filling tube. Pipette 1 μl RNAase free mineral oil into a "Filling Tube" prepared previously (**step 8** of Subheading 2.1). Then pipette 1 μl RNA into the same Filling tube then pipette a further 1 μl oil making an oil-mRNA-oil sandwich. Attach this to the underside of the injection chamber with a little VaLaB (*see* Fig. 1). *Note, we keep these Filling tubes with mRNA in them for up to about 6 weeks at 4 °C and can reuse the same 1 μl of mRNA several times over that 6 week duration.*
11. Place the mounting chamber with eggs and wedge onto the stage and advance the microinjection needle towards the injection chamber (*see* Fig. 1). We use filament-free glass needles to prepare the microinjection needles (pulled from GC100T10 pipettes) using a Narishige PN-30 horizontal puller.

12. First break the tip of the needle against the Filling tube then suck up some mRNA from the Filling tube into the injection pipette using negative pressure. (We inject using the 10× objective and suck up enough mRNA to fill the field of view, keeping the meniscus in view at all times.)
13. Set the balance on the IM300 so that the meniscus is stable.
14. Advance the microinjection needle towards the egg (*see* Fig. 1).
15. Insert the needle into the center of an egg and apply a brief 10 ms pulse of negative pressure to break the plasma membrane. Then inject the mRNA into the center of the egg (*see* Fig. 1).
16. Remove the injection needle slowly until it is almost out of the egg and then remove it very rapidly to avoid killing the egg (we use the left-hand stage control for the fast removal).
17. Push those injected eggs out the edge of the wedge using the microinjection needle. Once sufficient eggs have been injected collect them with a mouth or handheld suction pipette and transfer to a GF-coated petri dish containing TAPS filtered seawater and store at 16 °C until required (we sometimes keep injected eggs overnight but not for longer).

3.3 4D Imaging

1. For imaging events in the egg such as meiotic spindle behavior leave mRNA-microinjected eggs overnight to accumulate sufficient fluorescent fusion protein. If monitoring fluorescence from the 16-cell stage onwards, eggs can be fertilized 2–3 h after microinjection, and there will be fluorescent fusion protein the embryo at the 8–16-cell stage (*see* Fig. 2).
2. To fertilize eggs sperm are first activated. Add 2–4 µl "dry" sperm to 500 µl pH 9.5 sea water and agitate for 20 min (*see* **Note 8**) then add approx. 10 µl of this mixture near some eggs in a petri dish containing about 5 ml TAPS filtered sea water.
3. Once eggs have fertilized wash them with fresh sea water to dilute out remaining sperm to avoid polyspermy.
4. Mount the eggs or embryos for 4D fluorescence imaging between GF-coated slide and coverslip sealed with Dow Corning vacuum grease making an airtight chamber. Be careful not to compress the embryos.
5. We use either an objective mounted Piezo or an x, y, z stage to perform 4D fluorescence imaging.
6. A typical low light level imaging protocol using a 75 W Xenon lamp and a filter wheel would consist of ten z steps approx. ranging from 1 µm spacing to 10 µm spacing depending on what we want to monitor, 200 ms exposure at each fluorescence wavelength and 20 ms bright field every 1–2 min. We typically use a 2×2 bin with our cooled CCD camera which has 6.7 µm square pixels (*see* **Note 9**). For longer duration experiments (up to 15 h

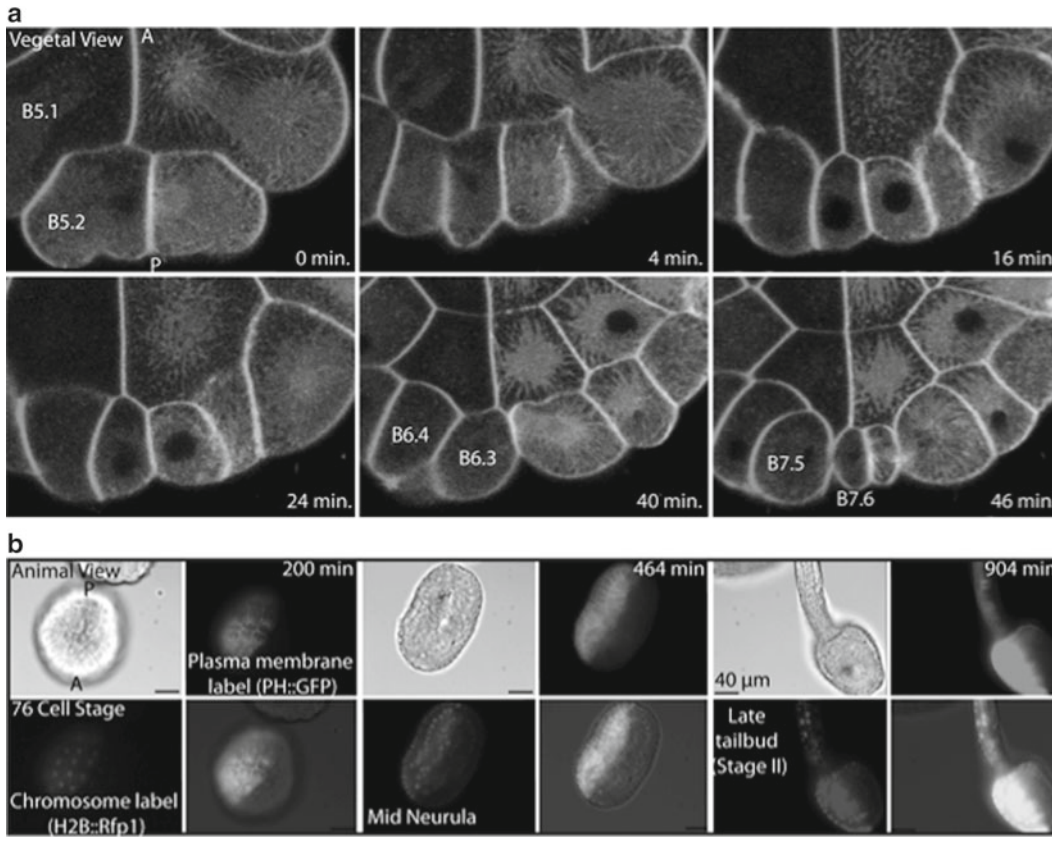

Fig. 2 Fluorescence imaging in *Phallusia* embryos. (**a**) Confocal images of *Phallusia* embryo at the 16–44-cell stage. These embryos are bilaterally symmetrical. This offers the possibility of performing an internal control for embryo quality by injecting only one of the two blastomeres at the 2-cell stage (as has been performed here). mRNA encoding MAP7::EGFP was injected at the 2-cell stage into the right-hand blastomere. The plasma membrane was then labeled at the 16-cell stage with the red lipophilic dye FM4-64. Confocal microscopy was performed with a Leica SP5. Ten z sections were collected over a 60 min period during which time the germ lineage precursor blastomeres (B5.2) at the 16-cell stage divided unequally a further two times resulting in two small posterior vegetal cells (B7.6). (**b**) Epifluorescence images taken from a 4D data set of a *Phallusia* embryo that was previously microinjected with a mixture of three different mRNAs (encoding PH::GFP to label the plasma membrane, MAP7::EGFP to label microtubules and HH2B::Rfp1 to label the chromosomes). Three images from the 76 cell, neurula, and late tailbud stages are shown. For each time point the bright-field image and two fluorescence images are shown together with the overlay of all 3. Note that the embryo forms normally indicating that neither the exogenous mRNA nor the 4D fluorescence imaging protocol hindered development to the late tailbud stage (*bottom right-hand* image shows the late tailbud stage II following more than 10 h of imaging (ten z sections every 2 min)). P is posterior and A anterior

of embryonic development), this protocol works well (*see* Fig. 2). However, in order to improve the image quality, we also perform 4D confocal microscopy, but here we can image for approx. 90 min with a similar protocol before embryonic development is adversely affected (*see* Fig. 2).

7. In both examples shown in Fig. 2 one blastomere at the 2-cell stage was injected. Ascidian embryos display bilateral symmetry (the first cleavage creates one left-hand and one right-hand blastomere that will form the left- and right-hand sides of the embryo). Microinjection of mRNA encoding fluorescent fusion proteins into one blastomere at the 2-cell stage therefore allows us to compare development in the injected and non-injected halves of the embryo (*see* Fig. 2). A typical 4D confocal imaging experiment can be seen in Fig. 2a. An example of a 4D low-light level imaging experiment can be seen in Fig. 2b—this embryo was imaged from the 76-cell stage to the late tailbud stage II [9] without development being adversely affected.

4 Notes

1. To make mRNA we use mainly pSPE3 but also sometimes a modified pRN3 to make C ter. PH::GFP fusion constructs. One important point is that the mRNA has a 3′poly A tail of 30 or more adenines. For example, mRNA prepared from other vectors such as pCS2 only becomes translated efficiently when we polyadenylate the mRNA product before microinjection.
2. 0.1 % GF prevents the dechorionated egg from sticking to the glassware or plasticware. The formaldehyde cross-links the gelatin creating a thin layer (approx. 1 μm thick) on the coverslip/plastic. For microscopy this is convenient since it does not increase appreciably the thickness of the coverslip and is completely transparent. 1 % GF is stored at room temp.
3. Heat the VaLaB mixture on a hotplate until it melts then mix and cool to solidify. VaLaB can be kept for years.
4. EDTA is used to remove potential contaminating heavy metals. This improves embryonic development.
5. Be sure to use a form of trypsin that is not inactivated by calcium—which is 10 mM in sea water. The most common problem for poor dechorionation is that the pH drifts. If dechorionation is poor try adding more TAPS pH buffer during the dechorionation reaction.
6. Take a look at the dechorionated eggs with a binocular microscope. If there are more than 20 % dead eggs (dead eggs appear opaque) discard and use another animal.
7. The dechorionated eggs should be smooth in appearance. If the eggs have been left too long in the trypsin solution they will form cytasters (the eggs take on a golf ball texture). Eggs like these should be discarded.

8. Change the pH with NaOH, not KOH which is spermicidal.
9. Apparent pixel size is an important consideration. For ideal imaging pixel size should not limit the objective resolution and one should strive for pixels that are two or even three times smaller than the objective resolution. However, when embryo viability takes precedence over resolution this means limiting light exposure. Having maximized light capture by using your best objective lens (the 40×/1.3 NA oil obj. gives the brightest images of all standard objectives) and a good quality sensitive back-illuminated cooled CCD camera, if embryo viability is still compromised then pixel binning is an option. A 2×2 bin increases the light per pixel by a factor of 4 and a 3×3 bin by a factor of 9 and so on. However, binning also increases the apparent pixel size, thus limiting resolution. We find that a good compromise between objective resolution and apparent pixel size is a 2×2 bin. With 6.7 μm pixels the apparent pixel sizes with the different objectives (20×/40×/60×) and binning protocols are as follows:

Objective lens	Fluorescence resolution (0.61 λ/na when λ is 500 nm) (nm)	Apparent pixel size (6.7 μm/obj. mag) (nm)		
		1×1 bin	2×2 bin	3×3 bin
20×/0.75 na	407	335	670	1,005
40×/0.90 na	339	167	334	502
40×/1.3 na	235	167	334	502
60×/1.20 na	254	112	224	335

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Chapter 12

Isolating Specific Embryonic Cells of the Sea Urchin by FACS

Celina Juliano, S. Zachary Swartz, and Gary Wessel

Abstract

Isolating cells based on specific gene expression enables a focused biochemical and molecular analysis. While cultured cells and hematopoietic cells, for example, are routinely isolated by fluorescence activated cell sorting (FACS), early embryonic cells are a relatively untapped source for FACS applications often because the embryos of many animals are quite limiting. Furthermore, many applications require genetic model organisms in which cells can be labeled by fluorescent transgenes, or antibodies against cell surface antigens. Here we define conditions in the sea urchin embryo for isolation of embryonic cells based on expression of specific proteins. We use the sea urchin embryo for which a nearly unlimited supply of embryonic cells is available and demonstrate the conditions for separation of the embryo into single cells, fixation of the cells for antibody penetration into the cells, and conditions for FACS of a rare cell type in the embryo. This protocol may be adapted for analysis of mRNA, chromatin, protein, or carbohydrates and depends only on the probe availability for the cell of interest. We anticipate that this protocol will be broadly applicable to embryos of other species.

Key words Sea urchin, FACS, Embryonic cells, Antibody, Vasa

1 Introduction

Lineage-specific gene expression is a major theme of developmental biology. However, the dynamics of differential gene expression may be obscured by experimentation in which the whole embryo is tested. Techniques that fractionate the embryo into its constituent lineages are therefore desirable for performing focused analyses. In the sea urchin embryo, protocols are available for isolating several cell types. For example, micromeres, the skeletogenic cells produced at the fourth embryonic cleavage, can be collected en masse by sucrose gradients due to their small size relative to other blastomeres [1, 2]. McClay and colleagues devised a procedure for separating the ectoderm from the endoderm based on differential adhesion, which was then adopted for applications of cell adhesion studies and mRNA comparisons [3–5]. Primary mesenchyme cells,

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the derivatives of micromeres could also be isolated by differential affinity to lectins which enabled their isolation en masse following ingression into the blastocoel [6]. However, all of these methods target a limited number of cell types at particular developmental time points.

Fluorescence activated cell sorting (FACS) affords great flexibility in isolating cells of interest. Traditional FACS uses antibodies against cell surface proteins to label specific live cells for isolation from a suspension, and more recently, of fluorescent protein labeling of cells by genetic means. Such approaches yield viable populations of isolated cells; however, they require antibodies against surface antigens or a model organism in which genetic manipulation is possible [7, 8]. Alternatively, cells may be fixed, permeabilized, and labeled with antibodies against cytoplasmic antigens (Juliano and Swartz, unpublished observations). However, this approach has typically precluded further downstream analysis of the cell isolates, such as gene expression profiling.

We present a method that combines the flexibility of FACS using antibodies to antigens inside of cells with fixation conditions that allow downstream extraction of RNA for qPCR. In brief, cultured blastula stage embryos are disaggregated into a suspension of single cells, methanol fixed, immunolabeled, and FACS isolated (Fig. 1). Fixed, dissociated cells maintain their morphology and can be effectively immunolabeled (Fig. 2). By titrating different amounts of YP30-labeled labeled cells (a ubiquitous cytoplasmic protein) into unlabeled cells, we find that positive cells representing as little as 1 % of the total population can be effectively FACS isolated (Fig. 3). We go on to show isolation of the small micromeres of the sea urchin embryo, a rare (approximately 4 cells within an embryo of 1,000 cells) multipotent cell population with germ line competency (Fig. 4) [9, 10]. With antibodies specific to small micromere proteins Vasa and Nanos2, the small micromeres can be distinguished and FACS isolated from other cells in the suspension (Figs. 2 and 4).

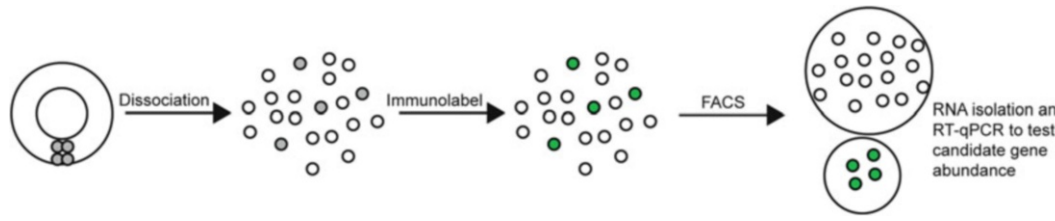

Fig. 1 Scheme for isolating specific *S. purpuratus* embryonic cell populations by FACS. *S. purpuratus* embryos are dissociated, fixed, and immunolabeled with an antibody that recognizes your cell population of choice. Positive cells are sorted by FACS and RNA is isolated for RT-qPCR analysis of candidate genes

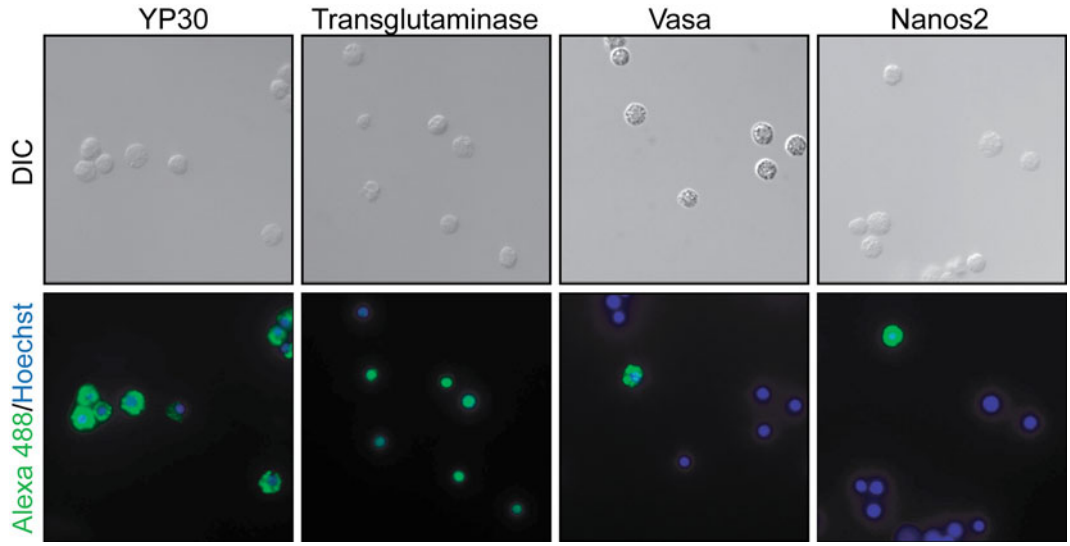

Fig. 2 Immunolabeled single cells from mesenchyme blastula embryos Mesenchyme blastula stage embryos were dissociated, fixed, and immunolabeled with antibodies that recognize YP30 (ubiquitous, cytoplasmic), Transglutaminase (ubiquitous, nuclear), Vasa (small micromere specific, cytoplasmic), or Nanos2 (small micromere specific, cytoplasmic) [9, 11, 12]. Cells were uniformly labeled with antibodies against transglutaminase and YP30. Approximately 1–2 % of cells were labeled with the small micromeres-specific antibodies

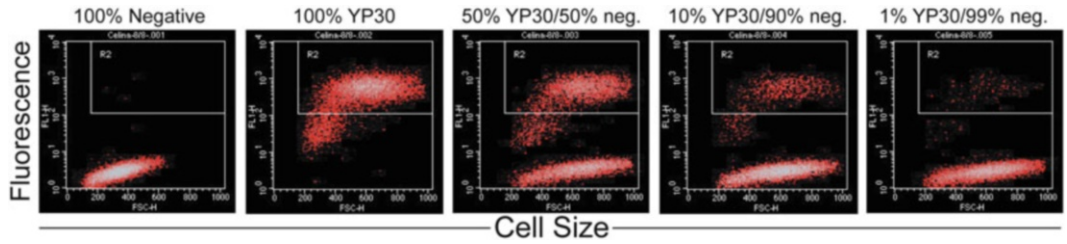

Fig. 3 Separation of YP30 immunolabeled from unlabeled single embryonic *S. purpuratus* cells by FACS. FACS plots demonstrate successful separation of dissociated mesenchyme blastula cells based on YP30 immunolabeling (*see* Fig. 2). First, cells immunolabeled with YP30 or a secondary alone negative control were run separately followed by the successful sorting of mixed populations containing 50, 10, or 1 % YP30 immunolabeled cells. The R2 population was gated as YP30-positive cells

The following protocol is adaptable to a number of different cell types and perhaps different species. The most critical aspect of this method is the selection of an antibody that targets the cells of interest and is compatible with methanol fixation, which in our hands best preserves RNA.

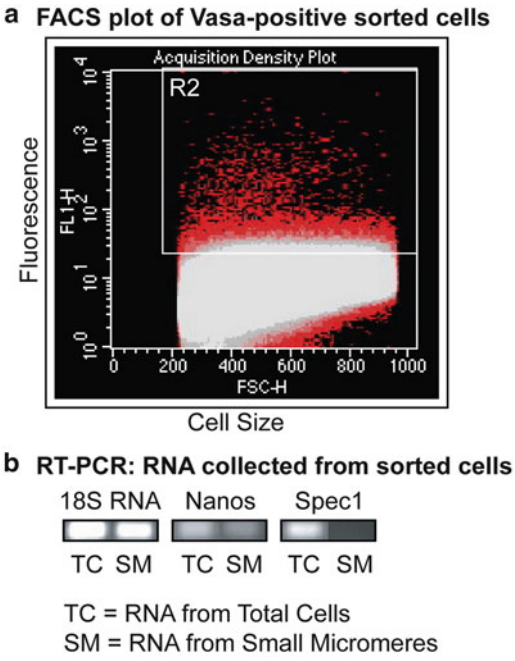

Fig. 4 Separation of *S. purpuratus* small micromere descendents by FACS. (**a**) Single cells dissociated from mesenchyme blastula were immunolabeled with a Vasa antibody (*see* Fig. 2) and separated by FACS on the basis of fluorescence. 270,000 cells were collected from the sort (*box* denoted by *R2*), which represented 0.75 % of the total cells sorted. (**b**) 50 ng of total RNA was isolated from the Vasa-positive small micromere descendents (SM). RT-PCR demonstrates that these cells contain the small micromere-specific gene *nanos*, but not the ectoderm specific gene *spec1*. As a control for the RT-PCR, total RNA was isolated from the total cell population before FACS (TC)

2 Materials

2.1 Dissociation of Embryos into Single Cells

1. Materials: 4 L glass beaker; Stirring paddle; 50 and 15 mL conical tubes; Plastic transfer pipettes; 45 and 20 μM Nitex® mesh.
2. Artificial Sea Water such as Coral Life Scientific Grade Marine Salt (Carson, CA), follow manufacturer's instructions.
3. Calcium-free Sea Water: to 900 mL ddH_2O, add 29.3 g NaCl (0.5 M), 0.75 g KCl (0.01 M), 0.21 g $NaHCO_3$ (1.5 mM), 2.5 g NaOH (0.06 M), and 7.6 g EGTA (0.02 M). Adjust pH to 8.0 with HCl and then fill to 1 L with ddH_2O.
4. Dissociation Buffer: 1 M Glycine, 25 mM EDTA, pH 8.0. Store at 4 °C.

2.2 MeOH Fixation of Single Cells

1. Materials: 15 mL conical tubes, 100 mm × 15 mm petri dishes.
2. 100 % MeOH. Chilled at −20 °C.

3. 1 L 1× PBS–Tween: In 800 mL of RNase-free water dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO. Adjust pH to 7.4 with 1 M HCl. Add 1 mL Tween-20 (0.1 % final concentration). Add RNAse-free water to 1 L and filter-sterilize through 0.22 μM filter.

2.3 Immunolabeling of Single Cells

1. Materials: 15 mL conical tubes, PBS–Tween (see above for recipe), primary and secondary antibodies of choice.

2.4 FACS Sorting and RNA Extraction for RT-qPCR

1. FACS collection tubes.
2. Trizol® LS, Catalog #10296028 (Invitrogen; Carlsbad, CA).
3. Glycogen, Catalog #10901393001 (Roche; Indianapolis, IN).
4. RQ1 DNase, Catalog #M6101 (Promega; Madison, WI).
5. Acid Phenol–Chloroform, Catalog # AM9720 (Ambion; Austin, TX).
6. 5 M ammonium acetate, pH 5.2.
7. 100 % EtOH.
8. High Capacity cDNA Reverse Transcription Kit, Catalog # 4368814 (Applied Biosystems; Foster City, CA).

3 Methods

3.1 Dissociation of Embryos into Single Cells

1. Fertilize between 1 and 5 mL of *S. purpuratus* eggs and culture for 24 h (*see* **Note 1**) in artificial seawater (ASW) at 16 °C in a 4 L beaker with stirring.
2. Collect and concentrate embryos into two to four 50 mL conical tubes using 45 μM Nitex® mesh.
3. Spin down the embryos in a clinical centrifuge at ~130 × *g* for 5 min and remove seawater.
4. Resuspend the embryos in up to 50 mL of calcium-free seawater per conical tube.
5. Spin down the embryos in a clinical centrifuge at ~130 × *g* for 5 min and remove calcium-free seawater.
6. Resuspend the embryos in approximately 20 mL of dissociation buffer per 1 mL of embryos and incubate on ice for 10 min.
7. Pipette the embryos up and down using a plastic transfer pipette, which will dissociate the embryos into single cells. Monitor the progress of dissociation by periodically examining a drop of the suspension under a compound microscope. When approximately 95 % of suspension is single cells, the dissociation step is completed.

8. Strain the dissociated cells through a 45 μM Nitex® mesh and collect the flow through. Strain this sample now through a 20 μM Nitex® mesh and collect the resulting flow through. This removes all whole embryos and clumps of cells from the single-cell suspension.
9. Transfer the single-cell suspension into 15 mL conical tubes. The number of tubes depends on the volume of your suspension.
10. Spin the single-cell suspension at ~255 × *g* for 5 min and remove the dissociation buffer.
11. Resuspend the single cells in calcium-free seawater.
12. Repeat **steps 10** and **11** twice.

3.2 MeOH Fixation of Single Cells

1. Collect approximately 0.5 mL of single cells into a 15 mL conical tube and spin for 5 min at ~255 × *g* (*see* **Note 2**).
2. Remove all but 2 mL of the calcium-free seawater and resuspend the cells.
3. Add 8 mL of ice-cold 100 % MeOH drop-wise while slowly vortexing (*see* **Note 3**).
4. Pour the suspension into a 100 × 15 mm petri dish and incubate at –20 °C for 1 h.
5. Collect the cells into a 15 mL conical tube.
6. Spin for 5 min at ~255 × *g*.
7. Remove MeOH and add up to 15 mL of PBS-Tween.
8. Repeat **steps 6** and **7** twice.

3.3 Immunolabeling of Single Cells

1. Prepare antibody-labeling solution by diluting antibody in 2–10 mL of PBS-Tween per 500 μL of single cells (*see* **Notes 4–6**).
2. Spin single cells for 5 min at ~255 × *g* and remove last PBS-Tween wash.
3. Add antibody solution to the single cells and incubate overnight at 4 °C with end-over-end rotation (*see* **Note 7**).
4. Spin the single cells for 5 min at ~255 × *g* and remove supernatant.
5. Add up to 15 mL of PBS-Tween.
6. Repeat **steps 4** and **5** twice.
7. Prepare secondary antibody solution in 2–10 mL of PBS-Tween. Choose a conjugated fluorochrome that is compatible with the Flow Cytometer that you will use. Dilute secondary antibody as per manufacturer's instructions.
8. Add secondary antibody solution to the single cells after the last wash is removed.

9. Incubate for 1 h at room temperature with end-over-end rocking.
10. Spin the single cells for 5 min at ~255 × *g* and remove supernatant.
11. Add up to 15 mL of PBS-Tween.
12. Repeat **steps 10** and **11** twice.
13. Check labeling under fluorescence microscope (Fig. 2).

3.4 FACS Labeling and RNA Extraction for RT-qPCR

1. Bring labeled cells immediately to FACS machine for sorting (Figs. 3 and 4a).
2. Sort the cells directly into Trizol® LS. Fill an appropriately sized tube (depending on the instrument) with 750 μL of Trizol® LS and sort the cells into tube until the volume reaches 1 mL. To collect more cells, use multiple tubes. Both the positive and negative populations can be collected if this is desired for downstream applications. If necessary, after sorting adjust the final sample volume with nuclease free or Trizol LS water such that the final ratio is three parts Trizol LS to one part aqueous sample.
3. Isolate RNA from sorted cells following the Trizol® LS manufacturer's protocol. During isopropanol precipitation, add 0.5 μL of glycogen to help visualize the pellet.
4. Resuspend the pellet in 10 μL of DNase solution (8 μL nuclease-free water + 1 μL 10× buffer + 1 μL DNase) (*see* **Note 8**).
5. Incubate at 37 °C for 30 min.
6. Bring volume up to 300 μL with nuclease-free water.
7. Extract RNA with acid phenol–chloroform following manufacturer's instructions.
8. EtOH-precipitate RNA overnight at −20 °C (0.1 volumes of ammonium acetate and 2.5 volumes of 100 % ethanol).
9. Resuspend pellet in 10 μL of nuclease-free water.
10. Use entire volume for cDNA synthesis.
11. Proceed with qPCR to test the relative abundance of candidate genes between distinct cell populations (*see* **Note 9**).

4 Notes

1. The time of embryo culture depends on the desired stage and cell type that you wish to collect. The dissociation works well for the morula stage through mesenchyme blastula. Dissociation of the gastrula stage is still possible, especially if ectodermal cells are desired. Once the embryos reach the larval stage, isolation of endodermal and mesodermal cells becomes more challenging.

2. All volumes can be scaled up if necessary.
3. Although single cells can be fixed using other methods, such as 4 % paraformaldehyde in seawater, we found that the RNA is better preserved using MeOH fixation. Furthermore, the quantity of RNA obtainable is much higher from MeOH fixed cells versus paraformaldehyde. Be aware that different fixing conditions will likely have a direct impact on the effectiveness of your antibody labeling. We suggest testing your antibody labeling with different fixes on whole embryos before choosing a condition to use for single-cell labeling.
4. Ideal concentrations for labeling differ greatly between different antibodies. The concentration used for a specific antibody should be determined empirically.
5. The volume used for antibody labeling should be as high as possible to reduce the possibility of cell clumping. To conserve antibody, consider saving the solution and reusing for future experiments.
6. We find that using blocking reagents such as Bovine Serum Albumin (BSA), including those certified as RNAse free, will cause severe RNA degradation and should thus be avoided if possible. This could affect the quality of the antibody labeling, and these conditions should be tested in whole embryo labeling before single-cell labeling is done. In general, all incubation times and centrifugations should be minimized, and whenever possible, performed at 4 °C to minimize RNA degradation. RNAse-free reagents should be selected whenever available.
7. If it does not decrease the quality of the antibody labeling, consider decreasing the labeling time, which may increase the quality of the RNA recovered from the sorted cells.
8. Alternatively, the pellet can be resuspended in 10 μL of nuclease-free water and can be used directly for cDNA synthesis (**step 10**). In this case though, there will likely be contaminating genomic DNA in the sample. If primer pairs are designed to span an intron for use in RT-qPCR, contaminating genomic DNA should not be a problem.
9. In our hands, the RNA collected from this method is not of high enough quality for microarray or deep sequencing. Therefore this method cannot be used for gene discovery, but is better suited for testing if candidate genes are enriched in your cell population by RT-qPCR. This would be particularly useful for testing genes of low abundance that cannot be detected by whole-mount in situ RNA hybridization. However, it is possible that further improvements to this protocol could yield sufficiently high quality RNA for gene

discovery. Many new experimental applications would be possible if an investigator were capable of isolating specific cell populations. The most direct application of the defined protocol herein is in mRNA analysis though improvement of the mRNA fidelity will be important in order to take full advantage of the protocol. Alternatively, if antibodies are available that recognize a cell surface component, they can be used in live-cell immunolabeling and traditional FACS protocols. In this situation, the cells need not be fixed, and the mRNA will be protected as long as the cell remains intact. Chromatin Immunoprecipitation (ChIP) for the analysis of epigenetic modifications and for promoter occupancy tests would benefit from this FACS protocol. In this case, the DNA is sheared to small (500 bp) fragments following cell isolation and EDTA can be added to the fixed cells to minimize nucleases that may chemically cleave the DNA. In this application the isolated cells present a more homogeneous population of chromatin that may be more effective than use of the whole embryo and may enable a more refined condition for analysis of DNA or protein modifications, and for trans-factor analysis of promoter occupancy. With new proteomic technologies enabling whole cell analysis, this FACS protocol will enable analysis of protein products. In many cases, cells will accumulate an mRNA but not translate the protein, so such analysis will inform what proteome the cell is working with at the time of isolation. This approach will also enable more effective identification of post-translational modifications of specific proteins including phosphorylation, glycosylation, ubiquitylation, and sumoylation. Since the protein samples are trypsinized prior to analysis, fixation with methanol or even formaldehyde will not prevent protein identification, and for phosphorylation and glycosylation, a pre-purification column (e.g., Fe^{3+} IMAC enrichment of phosphopeptides) will enable more focused analysis of the biochemical question at hand [13].

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Chapter 13

Biotinylation of Oocyte Cell Surface Proteins of the Starfish *Patiria miniata*

Samantha Cihal and David J. Carroll

Abstract

Understanding the signal transduction processes that occur during oocyte maturation and fertilization requires knowledge of the constituent proteins from the cell surface to relevant intracellular compartments. To identify starfish oocyte and egg cell surface proteins, a biotinylation method was adapted from prior protocols using B cells, leukocytes, mouse oocytes, and sea urchin eggs (Cole et al. Mol Immunol 24:699–705, 1987; Flaherty and Swann NJ. Mol Reprod Dev 35:285–292, 1993; Haley and Wessel. Dev Biol 272:191–202, 2004; Hurley et al. J Immunol Methods 85:195-202, 1985). This method utilizes the water-soluble Sulfo-NHS-Biotin, which does not cross the egg plasma membrane. The process of biotinylation does not appear to have any effect on the process of oocyte maturation or fertilization. Furthermore, it can be used with either vitelline-intact or vitelline-free oocytes and allows the proteins to be visualized successfully through immunoblotting, immunoprecipitation, or by scanning confocal microscopy.

Key words Biotin, Cell surface proteins, Streptavidin, Oocyte, Echinoderm

1 Introduction

Fertilization involves the fusion of a single sperm with a single egg. The sperm receive signals from the oocyte in the form of peptide chemoattractants that direct sperm towards the oocyte [5]. Once the sperm reaches the oocyte, the gametes interact via cell surface molecules and fusion of the gametes can occur [6]. Intracellular Ca^{2+} is released in the form of a wave, which acts as a polyspermy block and allows the oocyte to resume the cell cycle [7, 8]. The Ca^{2+} wave that sweeps across the entire oocyte that begins at the site of sperm fusion is a universal feature of egg activation in all species that have been examined [7, 9].

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In echinoderm oocytes, the egg activation pathway involves many signaling molecules, including several Src family kinase (SFK) members and phospholipase Cγ (PLCγ) [10–13]. SFKs can act as upstream regulators of PLCγ, which is located in the plasma membrane of echinoderm eggs [12–17]. Once PLCγ is activated, it travels from the cytosol to the plasma membrane where it has better access to PIP_2 [11–13, 18]. Src and PLCγ are both required for egg activation in echinoderms [14, 15, 18–21]. However, other molecules hypothesized to be involved in the fertilization signal transduction pathway (such as a hypothetical egg cell surface receptor for sperm that interacts with either Src or PLCγ) are not very well characterized, except for the sea urchin egg receptor for sperm that appears to be involved in species-specific gamete interaction [22–24, 26, 27]. Therefore, identification of oocyte and egg cell surface proteins remains an important component for eventual understanding of these mechanisms.

Biotin can be used to label the surface proteins on the starfish oocyte. Labeling of cell surface proteins has been successfully accomplished in the past by radioactive iodine labeling; however, because of the concerns regarding radioactivity, biotinylation has become the preferred alternative labeling method [1, 4, 23]. Biotinylation has been successful in labeling human B-cell line BALM-1, leukocytes, and sea urchin and mouse oocytes [1–4, 31], Flaherty and Swann 1993; Haley and Wessel 2004). Biotin labeling has also been accomplished in other cell types such as platelets, erythrocytes, and lymphocytes [25, 28–30]. Biotin is water soluble with a reactive pH between 7.0 and 9.0, which makes it convenient for use with gametes in vitro. Biotin forms a covalent bond between free primary amine groups via a stable amide bond. Sulfo-*N*-hydroxysuccinimide can be used to selectively label the cell surface because the sulfonate groups prevent the biotin from permeating the cell membrane, unless the cells are broken [1]. Avidin and streptavidin conjugates provide reliable systems for detecting biotinylated surface proteins [1–4]. Biotin has a very high affinity for avidin with a dissociation constant of 10^{-15} M [4]. This means that the detection system is very sensitive and stable.

In this chapter, a method for labeling surface proteins in the oocytes and eggs of the starfish *Patiria miniata* is described. Two different oocyte preparations are compared: those with an intact extracellular matrix (the vitelline coat) and those in which the extracellular matrix has been removed. Confocal microscopy utilizing fluorescein-conjugated streptavidin on living and fixed specimens is used to determine whether the labeling is restricted to the oocyte plasma membrane.

2 Materials

2.1 Protease Treatment

1. Pronase® Protease (Calbiochem; Gibbstown, NJ): store powder at 4 °C. Handle with gloves.
2. 9″ glass Pasteur pipette (*see* **Note 1**).
3. Disposable culture tubes (Fischer Scientific; Fair Lawn, NJ): 13×100 mm disposable borosilicate glass tubes are used for protease treatment, biotinylation, and maturation (*see* **Note 2**).

2.2 Biotinylation of Oocytes

1. Sulfosuccinimidyl-6-[biotin-amido]hexanoate (EZ Link® Sulfo- NHS-LC-Biotin) (Pierce; Rockford, IL) (*see* **Note 3**).
2. Filtered natural seawater (NSW): natural seawater was filtered using a Millipore filter apparatus with 0.4-μm nitrocellulose filter discs.
3. Glycine wash (0.1 M in NSW) is prepared fresh just before use and stored at 17 °C.
4. Labquake tube rotator (Thermo Fisher Scientific, Pittsburgh, PA).

2.3 Maturation of Oocytes

1. The hormone 1-methyladenine (1-MA; Acros Organics, Geel, Belgium) at a concentration ranging from 0.5to 5 μm (*see* **Note 4**).
2. Zeiss Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) for observation of oocytes and eggs.

2.4 Cell Lysis

1. Lysis buffer: 20 mM HEPES, pH 7.0, 150 mM NaCl, 10 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, and 1× Protease Inhibitor Cocktail (PIC). Prepare fresh for each use and keep on ice.
2. Protease Inhibitor Cocktail set III (Calbiochem/EMD Biosciences, Gibbstown, NJ).
3. Resuspension buffer: 20 mM HEPES, pH 7.0, 150 mM NaCl, 10 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, 60 mM Na-β-glycerophosphate, 1 % Triton X-100, and 1× PIC. Prepare fresh for each use and keep on ice.
4. Protein Assay Dye Reagent Concentrate (Bio-Rad; Hercules, CA): use as recommended and store at 4 °C.
5. Albumin standard (Thermo Scientific; Rockford, IL): store at room temperature until used for standard in Bio-Rad protein assay then store at 4 °C.

2.5 Immunoprecipitation with α-Phosphotyrosine

1. Immobilized protein A plus resin (Pierce; Rockford, IL).
2. α-Phosphotyrosine primary antibody (Exalpha; Watertown, MA): store at –20 °C, use at a 1:500 dilution.

3. 1X phosphate buffered saline plus 0.2 % Triton X-100 (PBS-T): phosphate buffered saline tablets (Sigma-Aldrich, St. Louis, MO) dissolved in DI water to give a 0.01 M phosphate buffer solution that was then autoclaved. Triton X-100 (Fischer Scientific; Fair Lawn, NJ) is added to 0.2 % of the final volume.

2.6 SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

1. Resolving gel: all resolving gels are prepared at 7 % in Bio-Rad mini gel format (7 % acrylamide/bis, 375 mM Tris–HCl, pH 8.8, 0.1 % SDS, 0.05 % APS and TEMED).
2. Stacking gel: all stacking gels are prepared at 5 % (5 % acrylamide/bis, 375 mM Tris–HCl, pH 6.8, 0.1 % SDS, 0.05 % APS and TEMED).
3. Thirty percent acrylamide/bis solution 29:1 (3.3%C; Bio-Rad; Hercules, CA): store at 4 °C.
4. Ammonium persulfate (APS; Bio-Rad; Hercules, CA) (*see* **Note 5**).
5. TEMED (Bio-Rad; Hercules, CA).
6. Running buffer (5×): 25 mM Tris-base, 192 mM glycine, and 0.1 % (w/v) SDS.
7. Precision Plus Protein All Blue standard (Bio-Rad; Hercules, CA): store at –20 °C; use 5 μl per lane of the gel.
8. Laemmli (2×) sample buffer: 0.125 M Tris, pH 6.8, 4 % SDS, 20 % glycerol, and <2 mg bromophenol blue (*see* **Note 6**). Mix and store in 0.9 ml aliquots at –20 °C. Just prior to use, add 2-mercaptoethanol to 10 % (v/v).

2.7 Western Blotting for Biotinylated Surface Proteins

1. Transfer buffer (5×): 25 mM Tris-base, 192 mM glycine, 0.1 % (w/v) SDS, and 25 % (v/v) methanol.
2. Trans-Blot® transfer medium pure nitrocellulose membrane 0.45 μm and blot filter paper were used from Bio-Rad, Hercules, CA.
3. TST: 20 mM Tris, pH 7.6, 150 mM NaCl, pH 7.6, add 0.001 % (v/v) Tween-20.
4. Bovine Serum Albumin, Fraction V (BSA; bio-WORLD; Dublin, OH): 3 % solution (w/v) in TST for each use.
5. ImmunoPure® streptavidin, horseradish peroxidase conjugated (Pierce Chemical, Thermo Scientific, Rockford, IL): long-term storage at –20 °C and short-term storage at 4 °C for up to 6 months.
6. α-Phosphotyrosine primary antibody (Exalpha; Watertown, MA): store at –20 °C; use at a 1:500 dilution.

7. Goat anti-mouse secondary antibody (Bethyl Labs; Montgomery, TX): store at 4 °C; use at a 1:5,000 dilution.
8. Blotto: 4 % (w/v) dry, nonfat milk in TST.

2.8 Confocal Imaging for Biotinylated Surface Proteins

1. Microscope slides (Fischer Scientific; Fair Lawn, NJ): plain, pre-cleaned, glass slides 3″ × 1″ × 1.0 mm. Clean with ethanol prior to use.
2. Microscope coverslips (Corning; Edison, NJ): glass 22-mm square, pre-clean with ethanol.
3. Phosphate buffered saline (PBS): phosphate buffered saline tablets from Sigma (St. Louis, MO). Dissolve in DI water to give a 0.01 M phosphate buffer solution; adjust pH to 7.4. Autoclave and store at 4 °C.
4. Paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA): prepare an 8 % (w/v) solution in 1× PBS, pH 7.4 (*see* **Note 7**).
5. Streptavidin–FITC (Invitrogen; Camarillo, CA): store stock solution at 4 °C and avoid light. Prepare fresh dilutions prior to each use and store at 4 °C.

3 Methods

3.1 Collection of Gametes

1. Dissect ovaries from adult *Patiria miniata* using a 3-mm coring tool and place in a 25-ml beaker with NSW (*see* **Note 8**).
2. Chop the ovaries using straight spring scissors with 8-mm blades to release the oocytes.
3. Gently pour the chopped ovaries through 210-μm Nitex mesh to remove the ovary pieces while allowing the 190-μm-diameter oocytes to pass through.
4. Rinse oocytes three times with NSW and place at 17 °C.
5. Periodically, check the oocytes via microscope to make sure they are healthy and not undergoing spontaneous maturation.
6. Dissect testis from adult *Patiria miniata* via a 3-mm hole in the dorsal body wall and placed in an Eppendorf tube on ice.
7. Release sperm from testis by mincing with fine scissors. Sperm were kept on ice and diluted in NSW as needed.

3.2 Pronase Treatment

1. Prepare stock solution of pronase in NSW (1 mg/ml) and store on ice until use.
2. Dilute oocytes in NSW as a 10 % suspension (in a 10 ml total volume approximately 1 ml volume is occupied by the oocytes).

3. Resuspend oocytes by swirling and transfer 0.9 ml of the 10 % suspension to a 13 × 100-mm glass culture tube. Place at 17 °C (*see* **Note 9**).
4. Dilute pronase 1/10 by adding 100 μl to the oocytes in the glass culture tubes. Final pronase concentration is 0.1 mg/ml.
5. Resuspend oocytes every 2–3 min after addition of pronase. Also, pipette oocytes up and down with a 9″ Pasteur pipette to help mechanically remove the vitelline coat. Observe oocytes under a microscope every 5 min to make sure the vitelline coat is lifting off. When the vitelline coat begins to lift away, begin NSW washes. Do not let oocytes sit in pronase for more than 22 min.
6. After 20–22 min, rinse the oocytes 1× with 4 ml NSW, then rinse 5× with 1 ml NSW. Now the vitelline-free oocytes are ready to biotinylate.

3.3 Biotinylation of Oocytes

1. Sulfosuccinimidyl-6-[biotin-amido]hexanoate (EZ Link® Sulfo-NHS-LC-Biotin; Pierce) should be taken out of –20 °C freezer and allowed to warm to room temperature before being dissolved in filtered natural seawater (NSW) at a 1.0 mg/ml concentration.
2. Adjust oocyte concentration to a 10 % suspension in NSW.
3. Incubate oocytes for biochemical analysis in 100 μg/ml Sulfo-NHS-LC-Biotin in NSW for 1 h at 17 °C with rotation on a Labquake tube rotator. For confocal imaging, incubate oocytes in 100 μg/ml Sulfo-NHS-LC-Biotin in NSW with rotation for 30 min at 17 °C.
4. Quench the biotin reaction by replacing the biotin solution with 0.1 M glycine in NSW. Wash the biotinylated samples three times with the 0.1 M glycine wash, followed by four washes with NSW.
5. The biotinylated oocytes can then be induced to mature with 1-methyladenine or immediately frozen in liquid nitrogen for later analysis.

3.4 Maturation and Fertilization of Biotinylated Oocytes

1. To initiate maturation, incubate oocytes in 2 μM 1-methyladenine in NSW at 17 °C. After 30 min, check the oocytes for germinal vesicle breakdown (GVDB) under a microscope.
2. Prepare a 1:1,000 dilution of sperm in NSW.
3. Replace the entire 1-MA solution with the sperm dilution. Check for the presence of a fertilization envelope under the light microscope and record % fertilization.

4. Freeze samples in liquid nitrogen and store at −80 °C for later biochemical analysis or prepare samples for visualization by laser scanning confocal microscope.

3.5 Preparation of Oocytes for Cell Lysis

1. Lyse biotinylated samples by repeated aspiration in a 0.5 or 1.0 ml volume of lysis buffer using a tuberculin syringe and 27½-gauge needle.
2. Allow samples to incubate on ice for 20 min for efficient protein solubilization.
3. Centrifuge samples at 12,127 × *g* for 30 min at 4 °C to pellet insoluble material.
4. Remove the soluble supernatant and place into a fresh, prechilled Eppendorf tube before immersing in liquid nitrogen.
5. The supernatant samples can be stored at −80 °C while processing the pellet.
6. Resuspend pellet in resuspension buffer using a syringe with a 25½-gauge needle to break the pellet up completely.
7. Allow the resuspended pellet samples to solubilize on ice for 20 min.
8. Determine the protein concentration per sample and equalize all samples to the lowest concentration using resuspension buffer.

3.6 Immunoprecipitation with α-Phosphotyrosine

1. Once the protein concentrations in each sample have been equalized, add α-phosphotyrosine antibody to each sample at an appropriate dilution. Final antibody concentrations can range from 0.1 to 10 μg/ml, depending upon your sample.
2. Incubate samples with antibody for 1.5 h on a rocker at 4 °C.
3. Add 20 μl immobilized protein A plus resin to each sample and incubate 1.5 h on a rocker at 4 °C (*see* **Note 10**).
4. Wash samples 4× with PBS-T. Solubilize proteins using equal volume of Laemmli sample buffer. The proteins can now be separated by SDS–PAGE.

3.7 SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

1. The SDS–polyacrylamide gels are run using the Mini-PROTEAN® 3 Cell (Bio-Rad; Hercules, CA) tank system.
2. Heat samples for 3–5 min at 95 °C before loading onto the gel.
3. Resolve proteins at 115 V on a 7 % gel.
4. Stop the electrophoresis run when the bromophenol blue reaches the bottom of the gel.

3.8 Affinity Blotting for Biotinylated Surface Proteins

1. Assemble the sandwich that is used to transfer the proteins from the gel to the nitrocellulose membrane with everything completely immersed in the transfer buffer. The sandwich should consist of the plastic support cassette, sponges, filter paper, and the gel and nitrocellulose membrane.
2. Following electrophoresis, remove the gels from the glass plates, one at a time, making sure to keep them wet at all times.
3. Gently lay the gel on top of the filter paper, which should be on top of a sponge and cassette and submerged in transfer buffer.
4. Wet the nitrocellulose membrane in transfer buffer and place on top of the gel. Keep in mind the orientation of the lanes of the gel. It is useful to clip the right upper corner of the nitrocellulose paper to mark the orientation of the gel.
5. Place the cassettes into the transfer tank with an ice block, stir bar, and transfer buffer, and transfer overnight using constant current for a total exposure of 1,000 mA h (i.e., transfer at 50 mA for 20 h).
6. Following the transfer, remove nitrocellulose membranes from cassettes and label appropriately.
7. Incubate membranes in 4 % Blotto for 1 h at RT with gentle rocking.
8. Prepare ImmunoPure streptavidin–HRP in 4 % Blotto. Appropriate dilution will have to be determined for your system. Our dilution factors range from 1:10,000 to 1:150,000.
9. Incubate membranes in the diluted ImmunoPure streptavidin–HRP for 1 h at RT with gentle rocking.
10. Wash four times with 100 ml TST.
11. Develop signal by enhanced chemiluminescence (ECL) as for an immunoblot (*see* **Notes 11** and **12**; Fig. 1).

3.9 Confocal Imaging for Biotinylated Surface Proteins

1. To determine localization of the biotinylated proteins in the starfish oocyte, either live or fixed oocytes were labeled with streptavidin–FITC for visualization by confocal microscopy.
2. In initial experiments, live biotinylated oocytes were incubated with streptavidin–HRP at a final dilution of 1:2,000 in NSW. Alternatively, oocytes were fixed in 4 % paraformaldehyde in PBS for 1 h at room temperature.
3. Fixed oocytes were solubilized using 1 % Triton X-100 with 3 % BSA for 1 h at room temperature.

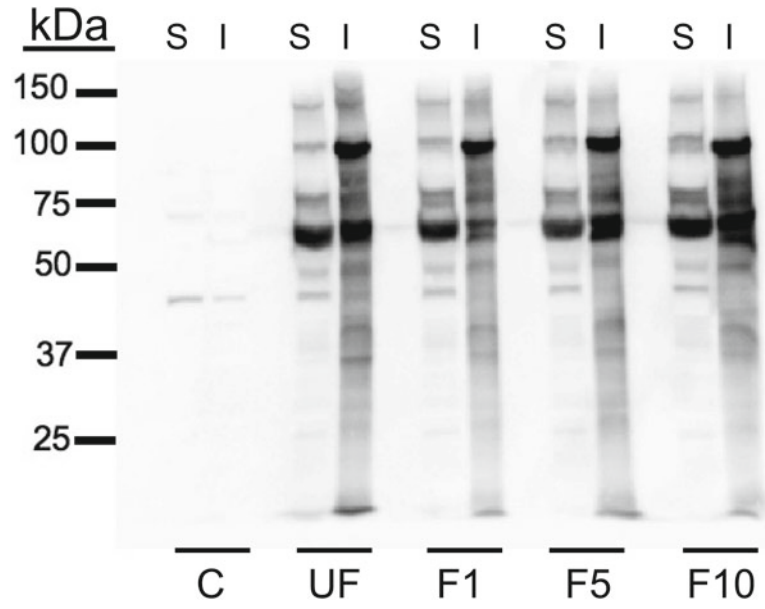

Fig. 1 Sulfo-NHS-Biotin labeling of egg and zygote proteins. Live immature starfish oocytes were incubated with Sulfo-NHS-Biotin prior to stimulation of maturation with 1-methyladenine. Unfertilized eggs (UF) or zygotes at 1 min (UF1), 5 min (UF5), or 10 min (UF10) after fertilization were treated as described in the protocol to separate soluble (S) from insoluble (I) proteins. Control samples (C) were prepared from unfertilized eggs that had not been incubated with Sulfo-NHS-Biotin. Proteins were resolved by SDS–PAGE and transferred to nitrocellulose for affinity labeling with streptavidin–HRP. Labeling was visualized by enhanced chemiluminescence (ECL) and the image captured on x-ray film. As seen, the majority of the labeled proteins partitioned to the insoluble fraction, but a sizable population was also observed in the soluble fraction. Based upon the immunolocalization of the biotinylated proteins via laser scanning confocal microscopy (*see* Fig. 2), the labeled proteins from both S and I fractions are localized to the cell surface

4. Oocytes were incubated in streptavidin–HRP for 30 min before washing twice in NSW for live oocytes or in PBS for fixed oocytes.
5. After rinsing, place oocytes on a clean glass microscope slide and pop any air bubbles that may have formed.
6. Place a coverslip that has had its edges supported with wax or clay feet onto the sample. Gently press the sides of the coverslip down, so the sample is gently compressed to improve clarity on the microscope.
7. Visualize using the appropriate filters and settings for your microscope (Fig. 2).

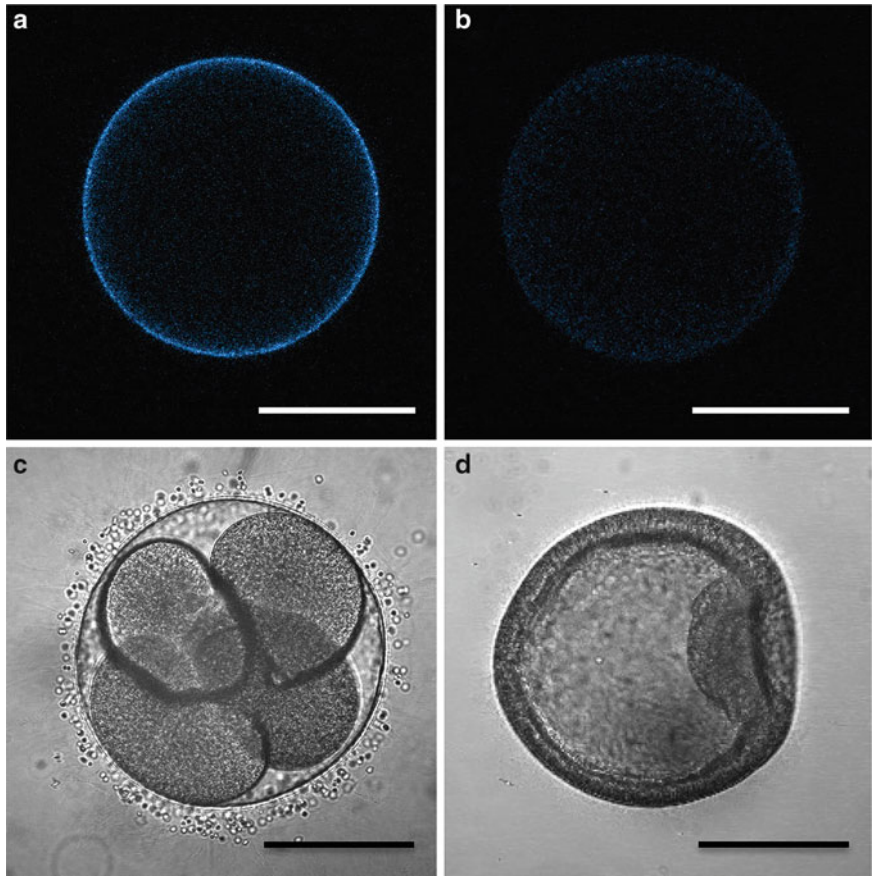

Fig. 2 Sulfo-NHS-Biotin labels the oocyte cell surface and has no effect on fertilization or early embryonic development in the starfish. (**a**) Live vitelline-free oocytes were incubated with Sulfo-NHS-Biotin before fixation with 4 % paraformaldehyde in filtered natural seawater. The oocytes were then incubated with streptavidin–FITC, washed, and visualized by laser scanning confocal microscopy under defined conditions. (**b**) Non-biotinylated oocytes were processed as for biotinylated oocytes, the only difference being lack of exposure to Sulfo-NHS-Biotin. (**c** and **d**) After exposure to Sulfo-NHS-Biotin, oocytes were stimulated to mature by exposure to 1-methyladenine. Following maturation, the eggs were fertilized and incubated at 18 °C in filtered natural seawater and allowed to develop through cleavage (**c**) and gastrulation (**d**). No developmental differences were observed between biotinylated and non-biotinylated embryos. Scale bar = 100 μm

4 Notes

1. Oocytes are pipetted through the 9″-long glass tip to help mechanically remove the vitelline coat prior to labeling, maturation, and fertilization. The shorter 5″-long Pasteur pipettes are not as effective at removing the vitelline coats.
2. The culture tubes are just the right size and shape to allow the oocytes to settle quickly by gravity and spread out in a single layer on the bottom of the tube.

3. A fresh solution of Sulfo-NHS-Biotin in either filtered natural seawater or artificial seawater should be prepared prior to each use. The EZ Link® Sulfo-NHS-LC-Biotin solid is moisture sensitive and stored at −20 °C. Acclimate to room temperature before weighing.
4. 1-Methyladenine is very slow to dissolve in solution. We prepared a stock solution of 1 M in deionized water by constant stirring at room temperature over a several hour period. Once dissolved, the 1 M 1-MA is aliquoted into smaller volumes, flash frozen in liquid nitrogen, and stored at −80 °C. A fresh working solution of 0.5–5 μm 1-MA is prepared prior to each use.
5. A fresh solution of 10 % ammonium persulfate should be prepared prior to each use because it starts to break down once dissolved in water. The free radicals that are formed from the persulfate with the aid of TEMED are needed for polymerization, and the efficiency decreases with the age of the solution. TEMED should also be relatively fresh. As the TEMED ages, polymerization time for the gels will increase significantly until they no longer polymerize.
6. Glycerol is very viscous and difficult to measure accurately at smaller volumes. To reduce variability, weigh the glycerol rather than measure the volume. Measure 2.582 g for the 2 ml of glycerol needed here.
7. Carefully heat solution using a hot plate and stir bar under a fume hood at 65 °C until paraformaldehyde dissolves. Filter solution to remove any particulates that may have been in the solution and store at 4 °C.
8. Tools for working with gonads and gametes can be purchased from Fine Science Tools, Inc. (Foster City, CA). The 3-mm sample corer works well to expose the ovary without too much damage to the adult starfish. Use the plunger to clean the coring tool after each use. The spring scissors with 8-mm blades are convenient for chopping the ovary without harming the oocytes.
9. The 13 × 100-mm glass culture tubes are highly recommended for working with the starfish oocytes. They allow the oocytes to settle quickly during washes, and the oocytes are maintained in a monolayer at the bottom of the tube. This allows for better exposure to the pronase than would be accomplished if an Eppendorf tube were used. It is also more gentle than rocking or rotating, which may damage the oocytes because the vitelline coat is being removed and the plasma membrane is exposed.

10. When using a P1000 Pipetman and standard 200–1,000-μl pipette tips for transferring the oocytes, the end of the pipette tip should be shaved to a larger size using a single-edge razor. Shave the tip so that the diameter of the opening is at least 0.5 mm.
11. A control consisting of sample incubated with the protein A beads alone (without antibody) is recommended. If the protein A beads bind to protein in your sample, it then becomes necessary to preclear the protein lysate with protein A before adding the immunoprecipitating antibody for each experiment.
12. The signal generated by the ImmunoPure streptavidin–HRP on the nitrocellulose membrane can be very strong, making it difficult to resolve the individual bands on the blot. Several strategies can be employed to combat this issue, including reduction of protein concentration loaded onto the gel, increase dilution of ImmunoPure streptavidin–HRP, decreased time of exposure, or even dilution of the ECL reagent before exposure to the sample on the nitrocellulose.

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Chapter 14

Perturbations to the Hedgehog Pathway in Sea Urchin Embryos

Jacob F. Warner and David R. McClay

Abstract

The Hedgehog pathway has been shown to be an important developmental signaling pathway in many organisms (Ingham and McMahon. Genes Dev 15:3059–3087, 2001). Recently that work has been extended to developing echinoderm embryos (Walton et al. Dev Biol 331(1):26–37, 2009). Here we describe several methods to perturb the Hedgehog signaling pathway in the sea urchin. These include microinjection of Morpholinos and mRNA constructs as well as treatments with small molecule inhibitors. Finally we provide simple methods for assaying Hedgehog phenotypes in the sea urchin embryo.

Key words Hedgehog, Patched, Smoothened, Cyclopamine, Sea urchin, Microinjection, Phalloidin

1 Introduction

The Hedgehog (Hh) pathway is a highly conserved signaling pathway involved in patterning germ layers, limbs, symmetry, organs, and other embryonically important components [1, 3–5]. Signal transduction in the simplest terms is as follows: in a non-Hh-responding cell, the Hh receptor Patched (Ptc) inhibits the activity of the co-receptor Smoothened (Smo). In the absence of Smo activity, the downstream effector Gli is phosphorylated and proteolytically cleaved to a short repressor form that translocates to the cell nucleus and represses target genes. In an Hh-responding cell, the ligand Hh binds to the receptor Ptc which relieves the repression of the co-receptor Smo. This allows Smo to promote the stabilization of the full-length activating form of Gli, which then translocates to the nucleus and activates target gene expression [6].

In the sea urchin genome, single homologous genes are present for each of the main Hh pathway components [7]. Functionally, Hh signaling was found to be required for proper patterning of mesodermal tissues [2]. In this chapter, we present several methods to perturb Hh signaling in the sea urchin embryo and methods to assay for mesodermal phenotypes. Hh signaling can be activated

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by microinjecting a mutated form of Smo mRNA (ActSmo) [8] or inhibited by injecting an *o*-methyl-substituted antisense oligonucleotide (MASO) targeted to the Hh translational start site. Both of these reagents, however, are specific to the species of interest. For situations where these reagents are not available, we describe below a protocol to treat embryos with cyclopamine: a small molecule inhibitor of Hh signaling [8, 9]. We then describe practical methods for assaying mesodermal phenotypes, including visualization of the skeleton with polarized light, and staining for muscle using phalloidin.

2 Materials

2.1 Generating an Activated Smoothened Construct

1. LB broth growth media: 10 g bacto-tryptone, 5 g yeast extract, and 10 g NaCl. Adjust pH to 7.5 and bring volume to 1 L with dH_2O.
2. Competent *E. coli*. We recommend DH5alpha-cells (catalog number 18258012) (Invitrogen).
3. LB agar plates with ampicillin prepared as described in Sambrook and Russell [10].
4. Full-length clone of Smo in an appropriate expression vector such as pCS2+.
5. Mutagenizing primers as described below (from Integrated DNA Technologies).
6. High-fidelity polymerase. We recommend Advantage 2 from Clontech (catalog number 639201).
7. DpnI restriction endonuclease (catalog number R0176L from New England Biolabs).
8. mMESSAGE mMACHINE kit (catalog number AM1344) (Ambion).
9. Tris-acetate-EDTA (TAE) agarose gel running buffer: 40 mM Tris–HCl, 20 mM acetic acid, and 1 mM EDTA in deionized water.
10. 1.5 % agarose gel: add 0.75 g agarose to 50 ml of TAE and heat until fully dissolved. Cast the gel per manufacturer's instructions of the gel running apparatus.

2.2 Microinjection of Sea Urchin Embryos

1. 0.25 % (W/V) protamine sulfate (catalog number P4380) (Sigma) solution in water.
2. Injection plates: coat the lid of a 60 × 15 mm petri dish with the 0.25 % (W/V) protamine sulfate solution. Wait for 1 min, then rinse the plates with water and allow to dry inverted overnight. Store long term in a dust-free container.
3. 1.0 mm × 152 mm glass thin-walled capillary tubes (catalog number TW120-6) (World Precision Instruments, Inc.).

4. Pasteur pipettes.
5. 105 μm Nitex mesh (this only applies if working with *Lytechinus variegatus* embryos).
6. Picospritzer injection apparatus with micromanipulator.
7. Artificial seawater (ASW): 437 mM NaCl, 9 mM KCl, 22.9 mM $MgCl_2 \cdot 6H_2O$, 25.5 mM $MgSO_4 \cdot 7H_2O$, 2.1 mM $NaHCO_3$, and 9.3 mM $CaCl_2$. Adjust pH to 8.0 (*see* **Notes 1** and **2**).

2.3 Cyclopamine Treatment of Sea Urchin Embryos

1. Dimethyl sulfoxide (DMSO).
2. Cyclopamine (catalog number BML-GR334) (Enzo Life Sciences).
3. Plastic 6-well tissue culture plates (catalog number 353046) (BD Falcon).
4. Artificial seawater as described above.

2.4 Assaying Sea Urchin Embryos for Hh Perturbation Phenotypes

1. 4 % (W/V) paraformaldehyde (PFA) in ASW: add 2 g PFA to 40 ml ASW in a 50 ml conical tube. Heat to 65 ° and vortex occasionally. PFA takes several hours to go into solution. Once most of the PFA is in solution, adjust the volume to 50 ml and continue heating/vortexing. Stock PFA is stored at –20 °C (*see* **Note 3**).
2. Phosphate buffered saline Tween-20 (PBST): dissolve the following in 800 ml of distilled H_2O: 8 g NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, and 1 ml of Tween-20. Adjust the pH to 7.2 and bring the volume to 1 l with dH_2O.
3. Microscope slides and glass coverslips.
4. Double-sided tape or modeling clay.
5. Rhodamine phalloidin (catalog number R415) (Invitrogen) (*see* **Note 4**).
6. Dimethyl sulfoxide (DMSO).
7. 50 % glycerol / 50 % PBST solution.
8. 0.25 % (W/V) protamine sulfate solution in water.

3 Methods

3.1 Generating an Activated Smoothened mRNA and Morpholino Design

The techniques associated with molecular cloning are beyond the scope of this protocol. A good reference for these techniques is *Molecular Cloning* by Sambrook and Russell [10]. To design Morpholinos, the sequence 5′ and 3′ of the ATG translation start site must be known for the species of interest. Alternatively the sequence of intron–exon boundaries can be used to design splice-blocking Morpholinos, but it should be noted that all of the *L. variegatus* Hh

pathway-specific Morpholinos published are designed against the translation start site [2]. Gene sequences can then be submitted to Gene Tools Inc (www.genetools.com) for design.

To create an activated Smo (ActSmo) construct, a full-length copy of this gene must be cloned or isolated from a cDNA library and subcloned in-frame into an appropriate expression vector with a T7, Sp6, or T3 promoter (e.g., pCS2+). The ActSmo construct contains a single base pair change from G to T results in an amino acid change of W to L. This mutation is analogous to the mouse ActSmo mutation W539L described in Taipale et al. [8].

1. Using a full-length Smo clone, design gene-specific primers containing the appropriate mutation analogous to the W539L mutation in mouse. These primers should include the specific point mutation as well as flanking gene sequences.
2. Perform a PCR reaction using high-fidelity polymerase such as Pfu (available from Promega) or Advantage 2 (Clontech), in which the primers amplify the entire plasmid.
3. Confirm PCR product sizes using agarose gel electrophoresis.
4. Clean up the PCR reaction using a PCR cleanup kit or phenol–chloroform extraction followed by ethanol precipitation.
5. Digest the product with the restriction enzyme Dpn1. This enzyme only cleaves the methylated DNA from the PCR template leaving the mutagenized plasmid intact.
6. Transform the plasmid, plate, and pick single colonies for overnight 5 ml cultures.
7. Mini-prep overnight cultures.
8. Verify the presence of the mutation by sequencing.
9. Cut the plasmid using a restriction endonuclease that cuts the vector singly and immediately 3′ of the ActSmo coding region. Phenol–chloroform extract, ethanol precipitate, and resuspend the reaction product in nuclease free water at 0.5 μg/μl.
10. Use this as a template for an mRNA synthesis reaction using an mMESSAGE mMACHINE kit (Ambion) or other mRNA transcription kits. Follow kit instructions.
11. Phenol–chloroform extract and ethanol precipitate the above reaction.
12. Resuspend mRNA in 10 μl nuclease-free H_2O.
13. Quantitate mRNA concentration using a spectrophotometer.
14. Verify the mRNA product by running 0.5 μl using electrophoresis with an RNAse-free gel (*see* **Note 5**). A single band should be visible.
15. Aliquot and store at −80 °C.

16. Just before use, thaw and dilute mRNA in 20 % glycerol/nuclease-free H_2O (*see* **Note 6**).
17. This mRNA can be microinjected into fertilized sea urchin eggs as described below.

3.2 Microinjection of Sea Urchin Embryos

Different echinoderms require different optimal temperatures for proper development. The experiments here describe perturbation of *L. variegatus* embryos for which the optimal growth temperature is 23 °C, although these experiments may also be carried out at room temperature. Additional information on the husbandry of other species of urchin as well as an extensive overview of the techniques associated with microinjection can be found in Methods in Cell Biology, vol. 74 [11, 12].

1. Induce spawning of adult *L. variegatus* urchins by injecting 1 ml of 0.5 M KCl into the body cavity and shaking the animal vigorously. Examine the top of the animal for the appearance of eggs (which will appear yellow) or semen (white). For males, collect the semen using a Pasteur pipette and place at 4 °C. For females, invert the spawning animal onto a glass beaker that is smaller than the diameter of the animal and filled with artificial seawater (ASW).
2. Allow the animal to complete spawning. Afterwards, discard the animal and remove the jelly coat of the eggs by passing them through a 105 μm Nitex mesh three times. Afterwards, wash the embryos three times by allowing them to settle, then removing and replacing the ASW (*see* **Notes 7** and **8**).
3. Transfer several thousand eggs to a petri dish and set the remaining aside for other experiments.
4. Make embryo transfer pipettes by flaming a Pasteur pipette just at the taper until soft, then removing it from the flame and quickly pulling it long and thin. Break off the excess glass and test it with the eggs. If the diameter is too small, the eggs will deform or sheer; if too large, the eggs will not be single file.
5. Make injection needles using the glass capillary tubes and a horizontal needle puller following the manufacturer's instructions (*see* **Note 9**).
6. Fill these needles with the appropriate injection solution (Morpholinos, ActSmo mRNA, etc.) by pipetting 1 μl of injection solution onto the end of the needle and allowing capillary action and gravity to draw it to the end (*see* **Notes 10** and **11**).
7. Using the transfer pipette, pick up several hundred eggs and gently expel them in a row onto an injection plate filled with ASW. Allow the embryos to settle then immediately fertilize them by adding a few drops of sperm diluted in ASW directly to the plates (*see* **Notes 12** and **13**).

8. After 1 min, check for the presence of fertilization envelopes. Add more sperm if needed.
9. Load a filled needle into a Picospritzer or other pressurized injection devices.
10. Position the injection plate beneath the micromanipulator holding an injection needle. The needle should approach the dish at approximately a 30° angle. Carefully move the needle into the light path and lower it to the seawater using the course adjustments. Using the joystick, break the needle tip by gently rubbing it along the scratch on the bottom of the injection plate. The flow can be observed just out of focus. Adjust the flow rate as needed (*see* **Note 14**).
11. To inject, adjust the needle height so it is equatorial to the egg to be injected. Inject the egg by pushing into the egg and fertilization envelope until the needle breaks through. At this point, the injection solution should enter the egg as you quickly and smoothly remove the needle.
12. Continue this process using the stage controls and joystick to inject all of the eggs desired. If the flow slows or stops, the needle can be cleared using the clear button on the Picospritzer or the needle can be re-broken to a larger diameter.
13. If a fluorescent dye was co-injected, un-injected, or over-injected, embryos can be removed using a dissecting microscope fitted with a fluorescent arc lamp and an embryo transfer pipette.

3.3 Inhibition of the Hh Pathway Using Cyclopamine

If genetic clones, sequences, or injection equipment is not available, the Hh pathway can also be perturbed using commercially available pharmacologic agents. The drawback from using this approach to inhibit the pathway is determining the proper dosage that will give an optimal phenotype without being toxic to embryos. Here we will focus on cyclopamine, a drug that inhibits Hh pathway by binding the co-receptor Smo [9]. There are also other commercially available drugs that modulate the Hh pathway [13] described in Subheading 4, but these have not been tested in sea urchin (*see* **Note 15**).

1. Resuspend the powdered cyclopamine at a concentration of 4 mg per ml in DMSO. This stock can be stored at –20 °C for up to 3 months (*see* **Note 16**).
2. Make several working dilutions of cyclopamine in DMSO.
3. Spawn animals and wash embryos three times as described in **steps 1** and **2** of Subheading 3.2 above. Dejellying the eggs is not necessary.
4. Fertilize embryos by adding 2 μl of sperm directly to the beaker and swirling.

5. Allow embryos to settle, and then wash once to remove the sperm. Streak 100 μl of the embryos onto a slide and check for complete fertilization. If needed, add more sperm and repeat this step.
6. Using a 100 μl of embryos as a representative density, count the number of embryos. Repeat this count three times to obtain the average embryo concentration.
7. Dilute the embryos in ASW to a concentration of less than 500 embryos per ml and add 5 ml of the embryo culture to each well in the 6-well plate.
8. Add the appropriate amount of cyclopamine to obtain a final volume of the desired dosage. It is highly recommended to perform a dose response when using cyclopamine as the dosage varies on the age of the stock solution, the quality of the animals, and the species of interest. For *L. variegatus*, the optimal dose is generally between 100 and 500 nM.
9. Mix the solutions by gently agitating the plates laterally. Avoid swirling as this concentrates the drug in the center of the well.
10. Grow embryos to the desired stage in a humid chamber.

3.4 Assaying Sea Urchin Embryos for Hh Phenotypes

Hh signaling is necessary for proper patterning of the mesoderm in sea urchin [2]. Two of these mesodermal tissues in particular, muscle and skeleton, are very easy to assess for patterning defects. The skeleton of the embryo can be visualized with polarized light. The musculature can be labeled with fluorescently conjugated phalloidin, a modified fungal toxin that binds to filamentous actin [14]. Alternatively, if a fluorescent or confocal microscope is not available, muscle function can be scored by an embryo's ability to swallow latex beads (*see* **Note 17**).

1. To visualize skeletal phenotypes, mount embryos on a slide. A coverslip is optional but will help immobilize the embryos. The slide can also be coated with 0.25 % protamine sulfate to immobilize live embryos. Rotate a polarizing filter between the light source and specimen until only the skeleton is visible. Embryos with inhibited Hh signaling will display a truncated skeleton (*see* Fig. 1) (*see* **Note 18**) [2].
2. To visualize muscle phenotypes, phalloidin staining can be employed. Fix embryos in 4 % PFA in ASW for 1 h at room temperature.
3. Wash 4 times with PBST for 5 min each.
4. Dissolve dry phalloidin or DMSO stock to 5 U/ml in PBST. Mix the solution by pipetting (*see* **Note 19**).
5. Transfer the embryos to the phalloidin solution and incubate for 30 min to 1 h.
6. Wash once with PBST for 5 min then 3× in PBS (*see* **Note 20**).

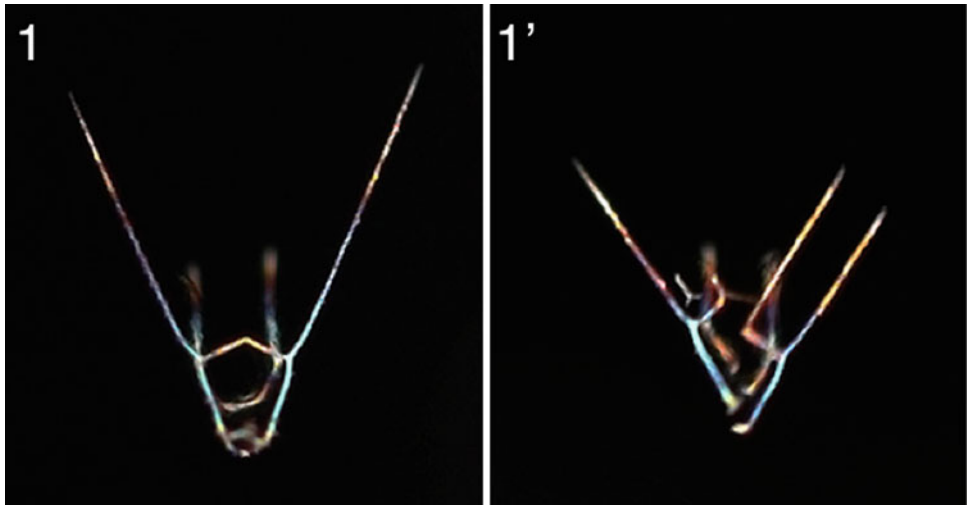

Fig. 1 Polarized light image of a pluteus stage sea urchin embryo showing the larval skeleton. Embryos injected with an ActSmo construct (**1'**) exhibit ectopic skeletal growths

7. Transfer into 50 % glycerol/ 50 % PBST.
8. Transfer a few embryos along with about 100 μl of 50 % PBST/50 % glycerol onto slides and apply coverslips supported with "feet" to avoid crushing the embryos (*see* **Note 21**).
9. Image with a fluorescent or confocal microscope, at an excitation wavelength appropriate for the conjugated fluorophore, soon after mounting as the staining will diminish if stored.

4 Notes

1. If available, high-quality filtered seawater can be used.
2. When making artificial seawater, add each of the salts, except the $CaCl_2 \cdot 2H_2O$, to a volume that is 10 % less than the desired final volume. Wait until everything else is in solution and add the $CaCl_2 \cdot 2H_2O$, then add water to the desired final volume. When all of the salts are in solution, the salinity can be tested with a refractometer and should be 34–36 parts per thousand.
3. Wear gloves and a protective mask when working with PFA to avoid accidental inhalation. Powdered PFA will dissolve in seawater more readily than granular PFA.
4. We have listed rhodamine phalloidin here, but there are a variety of different fluorescently conjugated phalloidins available.
5. Generally, a normal agarose gel will be sufficient to analyze the mRNA product. If needed an RNAse-free formaldehyde gel can be used.

6. Dilute Morpholinos or mRNA in 20 % glycerol in water. Generally several doses should be tested to determine the optimum injection concentration. 1:4, 1:10, 1:20, and 1:40 are generally good starting concentrations.
7. *S. purpuratus* eggs can be dejellied using acidic seawater. For all other species, a comprehensive guide to echinoderm egg handling can be found in chapter 3 of Methods in Cell Biology, Vol. 74 [11]. To dejelly eggs using acidic seawater, first add 30 μl of 0.5 M citric acid to 15 ml filtered seawater. The final pH of the seawater should be 4–5 and can be adjusted using a pH meter as needed. Transfer the eggs to a petri dish containing the acid seawater and incubate for 1–2 min. Swirl the plate to concentrate the eggs in the center, then using a Pasteur pipette, transfer the eggs to a petri dish containing normal filtered seawater carrying over as little of the acid seawater as possible. Next, transfer the eggs again to a third petri dish containing filtered seawater. The eggs are now dejellied.
8. Dejellied eggs do not keep very long and are best if used within an hour. Eggs that have yet to be dejellied however can be used up to 3 h later.
9. The settings of each needle puller need to be optimized for successful injections. We find shorter rigid needles easier to use than fine flexible needles. Some researchers note that the ideal needle will taper along a length of 65 μm and taper from a width of 10 μm to a width of about 10 nm [12]. In any case, experimentation with each needle puller is highly recommended.
10. You can make a needle holder using a line of modeling clay along the bottom of a petri dish.
11. Needle loading pipette tips can also be used to load needles if the outer diameter of the pipette tip is smaller than the inner diameter of the needle.
12. Less sperm is better as sperm can clog injection needles. We find it best to use 100 μl of a 1:1,000 sperm to seawater dilution added to the eggs.
13. *S. purpuratus* embryos can be fertilized in *para*-aminobenzoic acid (PABA) seawater to soften the fertilization envelope. To do this, add 150 mg of PABA (catalog number A6928) (Sigma) to 500 ml of filtered seawater for a final concentration of 2 mM.
14. Both continuous flow and spritzing can be used for injections. In both cases, a slight minimal back pressure needs to be set to avoid sucking contents from the embryos or the water into the needle.

15. There are many other molecules that have been shown to modulate Hh activity [13]. Three in particular that are commercially available from but have not been used in sea urchin are Jervine (*ALX-350-378*), SAG (*ALX-270-426*), and GANT61 (*ALX-270-482*). All of these are available from Enzo Life Sciences.
16. Once resuspended in DMSO, the efficacy of cyclopamine will gradually decline over the course of 3 months. Therefore, it is best to perform a dose response to determine the optimal concentration and then proceed immediately with planned experiments.
17. If the muscle is disrupted or mispatterned, then peristalsis of the foregut muscles will be impaired and the embryo will be unable to swallow. To test this, culture the embryos as described above for the drug treatment. Next, add 20 embryos to 1 ml of ASW. Add one drop of a 100 ml solution containing ASW and 50 μl of 10 μm polystyrene beads (Polysciences, Inc Warrington, PA cat# 17136). Allow the embryos to feed for 30 min then image the embryos under a light microscope to assay for the presence or absence of beads in the gut [2].
18. In order to obtain an image similar to Fig. 1, the embryo must be lying flat. Embryos that are resting at an angle can be "coaxed" by dabbing at the mounting media, or ASW, with a pipette tip; the resultant flow will often reorient the embryo. If a coverslip is being used, gently push an edge of the coverslip with a pipette tip to reorient the embryo.
19. Phalloidin is often shipped dry in a tube. Some suppliers recommend resuspending and storing it in methanol for long-term stability. Methanol, however, can disrupt staining; therefore, it is recommended to resuspend phalloidin in DMSO and keep frozen, thawing before each use. If you decide to resuspend and store the phalloidin in methanol, it is important to completely dry the stock before use. To do this, transfer the amount you intend to use to a microcentrifuge tube and dry using a SpeedVac or place the tube unsealed in a dark place and allow the methanol to evaporate.
20. PBS is used for washes as PBST can dissociate the phalloidin from the sample.
21. These "feet" keep the coverslip from crushing the embryo. Double-sided tape is very handy for mounting pluteus stage embryos. Simply cut two thin (~1–2 mm) strips and apply them to the slide are before transferring the embryos. Four small pieces of clay can also be used, especially for earlier stage embryos. To do this, "scrape" each corner of the coverslip along the clay to gather the clay. Only a small amount is needed on each corner (about the size of the tip of a ball-point pen). Transfer the embryos then lay the coverslip down carefully and depress the corners until the coverslip contacts the fluid.

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Chapter 15

Regulation of DNA Synthesis at the First Cell Cycle in the Sea Urchin In Vivo

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Abstract

Using fluorescent and non-fluorescent recombinant proteins has proved to be a very successful technique for following postfertilization events, in both male and female pronuclei during the first cell cycle of sea urchin in vivo. Proteins and dyes are introduced by microinjection into the unfertilized egg, and their function can be monitored by fluorescence or confocal/two-photon (2P) and transmitted light microscopy after insemination. Here, we describe expression and purification of GFP/RFP-tagged proteins involved in regulation of DNA replication. We also explain the techniques used to introduce recombinant proteins and fluorescent tubulin into sea urchin eggs and embryos.

Key words Fluorescent proteins, PCNA, GFP/RFP, Microinjection, Confocal/2P imaging, Male and female pronuclei

1 Introduction

Unlike vertebrates, insects, or *C. elegans*, sea urchin eggs are arrested in G1 after completion of meiosis with licensed replication origins in the maternal nucleus [1, 2]. The superior optical properties of sea urchin eggs and embryos make them a very good model to study the first round of DNA replication after fertilization in vivo [3, 4]. Sea urchins represent invertebrate system which nonetheless readily accepts cell cycle proteins derived from frogs, mouse, or humans. Although these proteins may differ at the level of both genetic code sequence and amino acid sequence, their cell cycle control functions are conserved [5, 6]. As protein expression from mRNA takes about 30 min [3], recombinant proteins proved to be a better tool for studying early postfertilization events [6]. For injected recombinant proteins to be active, the condition of their expression plays a major role in obtaining good quantities of soluble and properly folded protein. While purification is usually well described in instruction manuals provided with different vector systems (see Stratagene, Instruction Manual 'Affinity Protein

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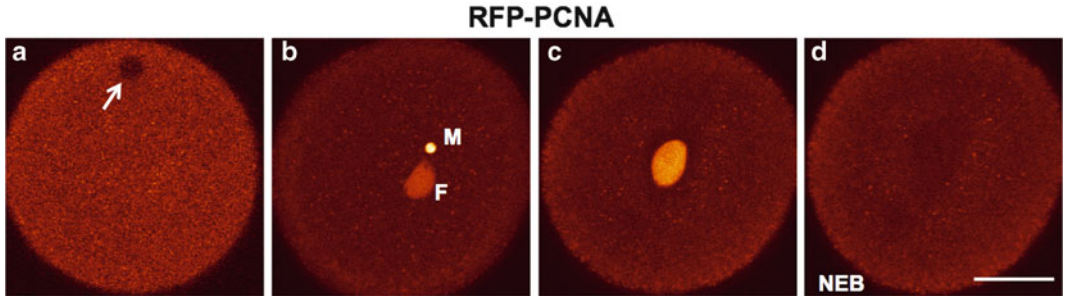

Fig. 1 GFP–PCNA pattern. The pattern of RFP–PCNA accumulation during the first S phase of *L. pictus* after fertilization. (**a**) Injected RFP–PCNA recombinant protein does not enter an unfertilized egg nucleus (*arrow*). (**b**) Accumulation of RFP–PCNA in male (*M*) and female (*F*) pronuclei indicating DNA replication before pronuclear fusion. (**c**) The end of S phase/prophase. (**d**) Nuclear envelope breakdown (NEB). Bar 50 μm

Expression and Purification System' and 'Affinity Protein Expression Vectors'; Novagen pET System, for more details and protocols), the expression conditions need to be adjusted individually to each protein.

The use of GFP–PCNA (green fluorescent protein fused with proliferating cell nuclear antigen) for visualization of replication foci in vivo was first described in mammalian cells [7]. This method has been adapted to the sea urchin model system in our laboratory. Microinjection of GFP/RFP–PCNA (green/red fluorescent proteins) allows the timing and degree of DNA synthesis in both male and female pronuclei to be established (Fig. 1) [3]. Because PCNA is involved in DNA synthesis and DNA repair, fluorescent recombinant PCNA may also be used to assess the DNA damage response [8, 9]. Use of proteins involved in the control process of licensing such as components of the pre-replicative complex (pre-RC) and geminin allows the determination of mechanisms that prepare DNA origins for replication [10]. Cell cycle fluorescent recombinant proteins such as cdk2 or cyclin E injected into sea urchin eggs allow their distribution and nuclear localization to be studied in vivo [3, 11].

Moreover, unlike *Xenopus*, sea urchin eggs and early embryos are permeable to most of the soluble inhibitors of MAP kinase, the proteasome and cdks, allowing the regulatory details of DNA synthesis after fertilization to be studied in vivo [4, 12].

In this chapter, we describe methods for expression and microinjection of cell cycle proteins involved in regulation of DNA replication. These approaches can be used for other regulatory proteins and their mutants or in combination with commercially available inhibitors, dyes, toxins, or antibodies. We provide some tips on expression conditions which can be applied to other less easily expressed proteins.

2 Materials

2.1 Vectors and Inserts

1. Recombinant proteins: Express and purify soluble proteins such as GFP/RFP–PCNA, GFP/RFP-cdk2, GFP-cyclin E, geminin–RFP, GFP-Orc6, and $p27^{Kip1}$ as described below.
2. Vectors: In our study, we used pCAL-n (Stratagene; *see* **Note 1**) and pET32a (Novagen; *see* **Note 2**) expression vectors, both ampicillin resistant.
3. Competent cells such as BL21(DE3)pLysS *E. coli* or Origami(DE3)pLysS (Stratagene; *see* **Note 3**).
4. Ampicillin stock in dH_2O, 100 mg/mL.
5. LB agar plates (*see* **Note 4**).
6. SOC medium: SOC medium can be purchased commercially from Stratagene or Sigma and also can be homemade as follows: 20.0 g tryptone, 5.0 g yeast extract, and 0.5 g NaCl. Add deionized water to a final volume of 1 L and autoclave. Add 10 mL of 1 M $MgCl_2$ and 10 mL $MgSO_4$ prior to use and filter sterilize. Add 2 mL of 20 % (w/v) sterile glucose solution to 100 mL of medium and filter sterilize. Store as aliquots in −80 °C.

2.2 Fusion Protein Expression

1. 2XYT broth medium: Dissolve 31 g of powdered medium (Melford or Merck) in 1 L of purified deionized water, divide to appropriate volume, and autoclave; at this stage, do not add ampicillin.
2. Terrific medium: 47.6 g of powdered medium (Melford or Merck) per 1 L deionized water with 4 mL of glycerol, pH 7.2; sterilize by autoclaving.
3. IPTG (isopropyl β-D-1-thiogalactopyranoside): 200 mM stock in dH_2O.
4. 2× Laemmli sample buffer (2× SB): 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromophenol blue, and 0.125 M Tris–HCl.
5. NuPAGE 10 or 4–12 % gradient Bis-Tris precast gels (Invitrogen).
6. 20× MOPS running buffer (Invitrogen).
7. Gel staining solution: for 1 L, 450 mL methanol, 450 mL dH_2O, 100 mL acetic acid, and 2.5 g Coomassie brilliant blue (Fluka).
8. Gel destaining solution: for 1 L, 450 mL methanol, 500 mL dH_2O, and 50 mL acetic acid.

2.3 Purification of Fusion Proteins

1. Protease inhibitor stocks as follows: 1 M benzamidine; 10 mg/mL leupeptin, pepstatin, and aprotinin.
2. DNAse I from bovine pancreas (Calbiochem), stock 20 mg/mL.
3. RNAse A from bovine pancreas (Sigma), stock 10 mg/mL.
4. Lysozyme 100 mg/mL (Sigma).
5. Triton X-100 10 % stock in dH_2O.
6. For purification with pCAL-n:
   - Calmodulin affinity resin (Stratagene).
   - Calcium-binding buffer ($CaCl_2$BB): 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, and 2 mM $CaCl_2$.
   - Elution buffer *I*: 50 mM Tris–HCl pH 8.0; 10 mM β-mercaptoethanol; 2 mM EGTA; and 150 mM NaCl (*see* **Note 5**).
   - Elution buffer *II*: 50 mM Tris–HCl pH 8.0; 10 mM β-mercaptoethanol; 2 mM EGTA; and 1 M NaCl.
7. For purification with pET32 vector, use His Bind purification kit (Novagen) and Ni-NTA His Bind Resin (Novagen).
8. Poly-Prep chromatography columns (Bio-Rad).
9. PD-10 columns (GE Healthcare).
10. Vivaspin columns (Sartorius Stedim Biotech): 10,000 or 30,000 MWCO.

2.4 Microinjection

1. Sea urchin injection buffer: 20 mM PIPES, 500 mM KCl, and 0.1 mM EGTA; pH 6.8 is for injection into unfertilized eggs and pH 7.2 into embryos.
2. Poly-L-lysine (Sigma), 1 or 10 % stock.
3. Micropipettes, GC150F-10 (Harvard Apparatus).
4. Imaging and microinjection chambers (*see* **Note 10**).

3 Methods

3.1 Transformation for All Vectors and Inserts

1. Prepare 1:10 dilution of your DNA construct, for example, pCAL-n GFP–PCNA, in DNAse-free water. Prepare a bucket of ice, 37 and 42 °C water baths/heaters, SOC medium, and LB agar plates supplemented with ampicillin (*see* **Notes 4** and **6**).
2. Take 20 μL of BL21(DE3)pLysS, *E. coli* (Stratagene; *see* **Note 3**) and thaw on ice (the volume of competent cells varies, and homemade cells can be used at 100 μL volume as their competency is usually lower).

3. Add 1 μL of 1:10 dilution of your plasmid into the bacteria solution and gently mix (flick with your finger, NEVER vortex competent cells). Leave on ice for 30 min.
4. After 30 min, apply the heat shock by placing the tube for 1 min in the 42 °C water bath. Although the duration of this step is critical and varies between 20 s and 1.5 min, we found the best transformation after 1 min.
5. Put the tube with the transformation reaction on ice for 2 min following by addition of 200 μL SOC medium and incubate for 0.5–1 h in the 37 °C water bath. Although mild shaking is recommended (250 rpm), we noticed that without shaking, we also obtain a high efficiency of transformation.
6. Instead of 200 μL of SOC, 0.9 mL of SOC medium can also be added, and this step is then followed by light centrifugation after the 37 °C incubation to concentrate the cells. The cell pellet is then resuspended in 200 μL of fresh SOC medium.
7. Spread the cells on LB agar plates (approximately 50–100 μL per plate) with a sterile spreader and incubate the plates at 37 °C overnight.
8. The next day there should be lots of colonies on the plates.

3.2 pCAL-n GFP–PCNA Expression and Purification Conditions

1. Prepare 200 mL of sterile 2XYT for overnight culture and few flasks of 500 mL sterile 2XYT for protein expression. A smaller volume of medium in the flask will make growth conditions better for the bacteria. Before use, add 1:1,000 dilution of ampicillin from the stock.
2. Divide 200 mL of 2XYT broth medium supplemented with ampicillin between 12 × 50 mL tubes. Each of the tubes should contain approximately 15 mL of medium.
3. Pick up a single colony with a sterile tip and inoculate the first tube leaving the tip inside. Each tube should be inoculated with a single colony. Put the tubes into a 37 °C orbital shaker overnight.
4. It is important to establish which colony is the best for large-scale expression. For this purpose, a small-scale culture should be undertaken. For each overnight culture, prepare two separate tubes with 0.9 mL of fresh 2XYT medium supplemented with ampicillin.
5. Add 0.1 mL of overnight culture to each of the two tubes and 2.5 μL of IPTG (final conc. 0.5 mM) to only one of them (*see* **Note 7**).
6. Put all tubes into the 37 °C orbital shaker for 2 h.
7. After this time, move 1 mL of each culture to the Eppendorf tubes and centrifuge at room temperature: 9,300 × *g* for 3 min.

8. Remove the medium and resuspend the pellet in 100 μL of 2× SB, and boil for 5 min in 95 °C.
9. Apply 10–15 μL from each tube, placing IPTG-induced sample next to the control sample. GFP/RFP–PCNA will run at ~80 kDa on 10 % Bis-Tris gel. We use NuPAGE precast gels (Invitrogen), but 10 % homemade gels are also as good as the precast ones. Precast gels usually run for 1 h at 180–200 V during SDS–PAGE electrophoresis.
10. For large-scale expression, use colonies that show the best ratio between induced and noninduced samples at the expected protein size level. Add 5–15 mL from overnight culture into 500 mL of 2XYT supplemented with ampicillin (0.5 mL of stock).
11. Grow bacteria in a 37 °C orbital shaker (180 rpm) until $OD_{600} = 0.5–0.6$. When the cells reach the appropriate density, add 1.25 mL of IPTG (final conc. 0.5 mM) and allow them to progress at the same temperature for the next 3 h.
12. After this time, place each 500 mL culture in a separate 500 mL centrifuge container and spin at 10,800 × *g* for 8 min in fixed angle rotor (we use Sorvall centrifuge RC-5B Plus and SLA-3000 rotor).
13. After centrifugation, the pellet should be green (in the case of a GFP fused protein). If you don't see clearly a green (or light green) pellet, it is possible that either the bacteria did not express the recombinant protein, usually due to defective DNA sequence or growth conditions; if the latter, these need to be adjusted see below for other proteins. If you see a clear greenish pellet, remove the medium and freeze the containers at −80 °C until purification. We often freeze the pellet at −20 °C for couple of days before purification.

3.3 Purification of pCAL-n GFP–PCNA

1. For GFP–PCNA purification, we follow the Stratagene protocol with some modifications.
2. Thaw containers containing the bacterial pellet on ice.
3. Resuspend the pellets with ice cold $CaCl_2$BB supplemented with protease inhibitors (2 μg/mL leupeptin, pepstatin, aprotinin, 1 mM benzamidine final concentration) and put the contents into sterile 50 mL tubes.
4. Use 30 mL of the buffer per 1 L of bacteria culture. This solution can be supplemented with lysozyme (final concentration of 200 μg/mL). The tubes are incubated for 15 min at room temperature on the roller. Alternatively, resuspended pellets can be put into the liquid N_2 for 15 min followed by thawing in a 30 °C water bath.
5. Sonicate the contents on ice for 30 s on the pulse setting and 40 % duty cycle (Cole-Parmer ultrasonic homogenizer 4710,

250 W, 20 kHz on control setting number 4). Check the viscosity of the contents. If the liquid contains flocculations, repeat the sonication step. Don't over sonicate as this can result in protein damage.

6. Add DNAse I and RNAse A to a final concentration of 5 μg/mL and leave on the roller at room temperature for 15 min. If the liquid is still thick and gluey, leave it for another 15 min.
7. After this time, the viscosity of the liquid should be low. Centrifuge the tubes at 15,000×*g* for 30 min at 4 °C (or 45 min at 10,500×*g*) (*see* **Note 8**).
8. Prepare the calmodulin affinity resin (Stratagene) on the column (we use BIO-RAD Poly-Prep chromatography columns, cat nr 731-15550).
9. Load the column with the resin (~1 mL of resin per 1 L of culture) and wash the resin with 50 mL of $CaCl_2$BB (no inhibitors).
10. The washed resin removed from the column should be combined with the supernatant from **step 7** in 50 mL tube/s and incubate on the roller at 4 °C overnight.
11. Next day, load the column with the content of the tubes. You should be able to see green resin (or another color depending on the fluorescent tag) on the bottom of the tube.
12. After removing unbound supernatant (it can be collected and used again if the expression yield was very high), wash the remaining resin in the column with at least 200 mL of $CaCl_2$BB. Ideally, the buffer will remove all unbound proteins leaving only these bound to the resin.
13. The eluate from the column has to be as pure as possible. Check the eluted liquid in glass cuvettes at OD_{280} during the buffer washing step. We usually proceed with protein elution when $OD_{280} < 0.03$–0.003. If the OD is still high ~0.8, extend the washing time of the column.
14. Depending on the scale of culture, prepare appropriate amount of collecting tubes for elution I and elution II.
15. For 2 L of culture, use ~8–12 tubes for elution step I and ~8 tubes for elution step II. In case of fluorescent proteins, the elution step is easy to see as it is obvious when the protein leaves the column.
16. Apply elution buffer I to the column and collect 1 mL of the fraction.
17. When a low concentration of salt does not elute the entire protein (as judging by color remaining on the column), use elution buffer II with higher salt concentration and collect samples in the remaining tubes.
18. Prepare twin tubes for each fraction with 3 μL of 5× SB.

19. Add 17 μL from each fraction to the corresponding tubes. Keep the eluted samples on ice.
20. Run SDS–PAGE electrophoresis of all your sample elutes to establish which sample contains the purest and the highest amount of GFP–PCNA.
21. Exchange the buffer as in Subheading 3.7.

3.4 pCAL-n p27^{Kip1} Expression Conditions

1. The construct has been transformed into high-efficiency Origami(DE3)pLysS expression strain. Because p27 is an inhibitor, we were interested in producing active protein in reasonable quantity. *E. coli* Origami-competent cells grow much more slowly than BL21 or Tuner allowing much better protein folding.
2. The small-scale culture is the same as in Subheading 3.2.
3. Grow large-scale culture at 20 °C until $OD_{600} \sim 0.6–0.8$.
4. Add IPTG to the final concentration of 0.2 mM and allow the expression for the next 6 h.
5. Alternatively decrease the temperature to 16 °C after induction and allow expression for the next 24 h. We noticed that in case of this protein slightly higher temperature decreases the amount of soluble protein by 50 %. On the other hand, using pET32a vector produces an extremely high yield of p27^{Kip1}, but with significant cleavage of the protein. Lower temperature allowed us to obtain 7 mg/mL of p27^{Kip1} from 2 L of bacterial culture.
6. For purification, follow the protocol for GFP–PCNA (Subheading 3.3).
7. CBP_p27^{Kip1} migrates at about 30 kDa marker on 10 % Bis-Tris gel.

3.5 Expression and Purification of pET32a GFP-Cyclin E and GFP/RFP-cdk2

For small-scale culture, follow the protocol in Subheading 3.2. The difference is that for pET32a GFP-cyclin E and GFP/RFP-cdk2, use Terrific broth medium supplemented with 1 % glucose instead of 2XYT medium:

1. BL21(DE3)pLysS are good for expression of cyclin E and cdk2. Grow the culture at 30 °C until $OD_{600} = 0.6$. We used 0.2 mM IPTG to induce the expression, but this could be adjusted depending on the expression yield expected.
2. After induction, decrease the temperature to 20 °C for 12 h allowing slower growth and better protein folding.
3. Purification of His-tagged proteins requires an Ni-NTA His binding resin. We used the His-tag purification kit from Novagen, but other companies also provide similar prepared buffers.

4. Bacterial pellets were thawed on ice and resuspended in binding buffer (Novagen) supplemented with protease inhibitors at the same concentrations as for $CaCl_2$BB and 0.05 % final concentration of Triton X-100. Use 40 mL of the buffer for 1 L of bacterial culture. This stage should be performed on ice.
5. Add 100 μg/mL of lysozyme and leave on a roller for 15 min at room temperature.
6. Follow the protocol in Subheading 3.3.
7. Sonicate the solution on ice as in Subheading 3.3 (**step 5**) and leave for 15 min on ice.
8. Check the viscosity. If the liquid is still viscous, add DNAse I and RNAse A as in Subheading 3.3.
9. Prepare His bound resin on the column washing with the buffers provided. This step should be followed according to the manufacturer's protocol.
10. Add washed resin to the supernatant and leave on the roller for 30 min at room temperature.
11. Apply the total content of the tube to the column and wash the resin with binding buffer (Novagen). We usually use three times more buffer at this step than recommended by manufacturer.
12. Usually, it is one-step elution, and the protein comes off the column within the first few fractions.

3.6 Expression Conditions for His Geminin–RFP

Geminin is a very soluble protein; however, the additional fluorescent tag at the C-terminus requires specific expression conditions. We found that RFP (or other fluorescent tags) attached to the C-terminus does not interfere with cell cycle activity of geminin:

1. Transform the construct into the Rosetta-gami *E. coli* strain (Novagen) as in Subheading 3.1.
2. For small-scale culture, follow the protocol at Subheading 3.2 including the use of 2XYT medium supplemented with ampicillin.
3. For the large-scale culture, grow bacteria at 20 °C until OD_{600} ~ 0.6–0.8, which can take up to 12 h as Rosetta-gami grows very slowly. The yield of the protein is lower, but the activity very high.
4. Induce bacteria with 0.2 mM IPTG and continue growing for the next 12 h at the same temperature.
5. After centrifuging as in Subheading 3.3, the pellet is usually very pink (for geminin–RFP).
6. For the purification, follow the protocol for His-tagged proteins above (Subheading 3.5).

3.7 Buffer Exchange and Protein Concentration

1. Choose the appropriate number of PD-10 columns sample concentrators you want to use for the buffer exchange remembering that for each column, 2.5 mL of sample can be used, for example, 2 columns for 5 mL of eluted protein.
2. Equilibrate the column with 25 mL of the sterile injection buffer pH 6.8 (or pH 7.2).
3. Apply 2.5 mL of your sample allowing it to settle on the resin and removing the column discharge.
4. Elute your protein with 3.5 mL of the injection buffer (collect the eluted protein!).
5. GFP/RFP–PCNA molecular weight is ~80 kDa, so to concentrate the protein, we use Vivaspin 6 columns with the membrane having a 30,000 molecular weight cutoff (MWCO); however, 50,000 will be still ok. For smaller proteins such as CBP_p27^{Kip1} with MW ~ 30 kDa, use a 10,000 MWCO membrane.
6. Apply your samples on the column sample concentrator and spin in swing-out rotor 4,000 × *g* for 20 min. Depending on how much final concentration is needed, extend the time of centrifugation. This step requires constant checking as if the centrifugation time is too long, your protein will be left on the membrane and you may not recover it!

3.8 Microinjection

For precise small micropipettes, we used a micropipette puller from Sutter Instrument CO., Model P-97. It gives a broad range of required tips. In our applications, we use different microinjection systems with oil or air pressure. For air pressure, we used either pneumatic PicoPump PV820 or integrated FemtoJet from Eppendorf. We also used Eppendorf micromanipulator InjectMan Ni 2 to introduce the microinjection needle into the egg/embryo.

1. Eggs or embryos have to be immobilized on 0.01–0.001 % poly-L-lysine [13]. Poly-L-lysine sticks very well to the glass bottom of the chamber (*see* **Note 9**); the excess of poly-L-lysine needs to be removed before sea water is applied into the chamber (*see* **Note 10**). Immobilization can be used for dejellied eggs or embryos without fertilization envelope as well as 72 h larvae.
2. It is important to place the pipette in the right angle to allow one sharp push into the egg (50–55°).
3. With your naked eye, place the needle above the chamber directly above the objective. Lower it slowly.
4. Looking at the eyepiece, you should easily see the shadow of the pipette. Place the micropipette end of the tips on the egg/embryo.

5. Either using a Piezo advance (PM 20, http://www.wpi-europe.com/en/products/microinjection/piezo.shtml) or with a sharp push, place the end of the pipette in the egg. Release your solution into the egg and slowly remove the pipette. This step requires a lot of practice. Depending on the system, it may easily damage or activate an egg.
6. Depending on the protein concentration in the tube, sharper tips are much better. Sea urchin eggs are very delicate and accept between 100 pL and 1 nL of injected volume. Higher volumes may easily damage the egg.
7. Unfertilized eggs are usually suitable for injection after dejelling for 30 min–1 h. It is a good idea to prepare a fresh batch of eggs after this time.
8. Injection of proteins usually requires slightly bigger diameter of tips. It is possible to overcome this problem by simple dilution of the protein. For example, typical concentration of GFP–PCNA should be no more than 2 mg/mL in the tube.
9. It is problematic to inject fluorescently labelled tubulin (Figs. 2 and 3). Usually, Cytoskeleton Inc. provides tubulin which can be injected into the sea urchin eggs or embryos; however, high concentration of this protein requires needles to be ice cold, and even if they are kept on ice, the protein blocks the pipette after just one injection. We found that simple dilution of tubulin by at least three or five times increases its flow through a pipette and also cells survive such a concentration much better.

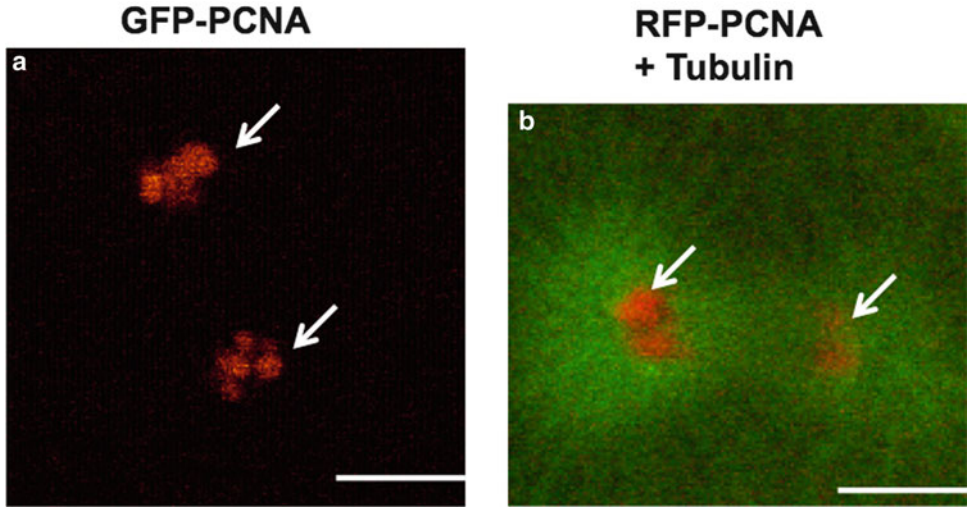

Fig. 2 FITC tubulin. Accumulation of PCNA at the beginning of the second S phase occurring before completion of the first cytokinesis. (**a**) GFP–PCNA in *L. pictus*; bar 50 μm. (**b**) Green tubulin injected together with RFP–PCNA into *P. miliaris*; 50 μm

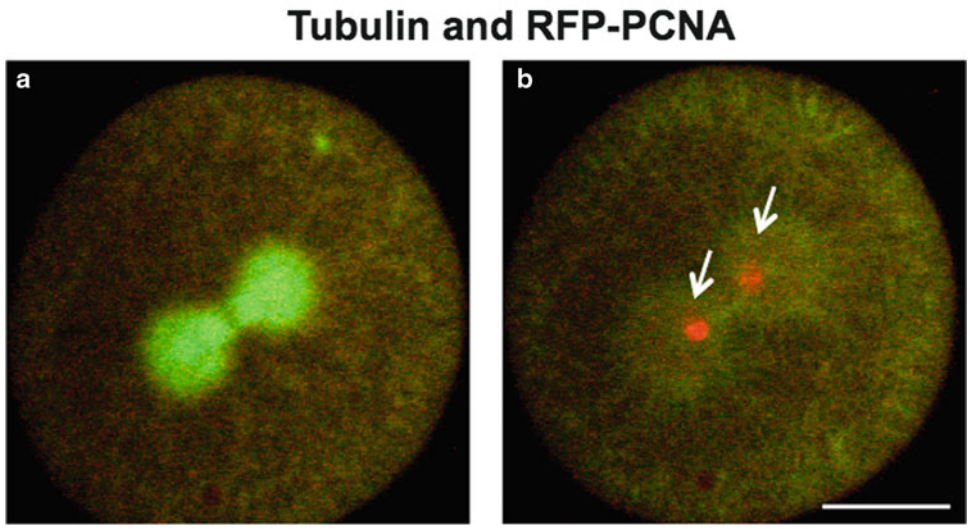

Fig. 3 Fluorescein-labelled tubulin injected together with RFP–PCNA into unfertilized eggs of *S. miliaris*. Tubulin was diluted 1:5 and injected together with recombinant PCNA. (**a**) Mitotic spindle at metaphase. (**b**) The second S phase begins before the first cytokinesis. Bar 50 μm

4 Notes

1. pCAL-n vector contains a CBP (calmodulin-binding peptide)-coding sequence upstream of the multiple cloning site. The presence of the T7 promoter and T7 terminator makes it easy to sequence the insert. We would not recommend this vector for in vitro transcription as the mRNA would also contain the sequence for CBP which makes the cellular expression of the insert inefficient. The pCAL-n vector is very efficient for highly soluble proteins such as GFP–PCNA, but based on our experience, it is less suitable for less soluble proteins such as pre-RC proteins.
2. The pET32a vector system was obtained from Novagen. Additional protocols associated with the system can be found at www.Novagen.co.uk (His Bind kit manuals). The vector used by us has a very broad spectrum of restriction sites allowing straightforward cloning strategies. For us, the advantage of this vector was the His-tag from N- and C-terminus together with the T7 promoter and T7 terminator. This vector also gives high level of expression in combination with the appropriate *E. coli* strain hosts and adjusted expression conditions. Using Novagen reagents, we modified the purification procedure.
3. *E. coli* expression strains can be commercially purchased or homemade and should be always aliquoted and stored in −70 °C. Avoid multiple freeze-thaw cycles as this decreases

strain competency and results in a loss of efficiency. BL21(DE3) pLysS-competent cells are used for general expression of soluble, nontoxic proteins. This strain can inhibit induced expression, and we do not recommend this strain for less soluble proteins.

4. LB agar plates 1 L: Dissolve 37 g of LB agar in 1 L of deionized water, autoclave, and store until use. Before preparation of the plates, boil agar in a microwave until the medium is melted. Allow the temperature of the medium to decrease enough for antibiotic to be added. After antibiotic addition, resuspend the medium on Petri dishes and allow setting in room temperature. The plates can be stored in 4 °C for many weeks covered with foil to protect drying.
5. For 20 mL of elution buffer I: Add 1 mL of 1 M Tris–HCl (pH 8.0), 15.0 μL of β-mercaptoethanol, 0.2 mL of 200 mM EGTA, and 0.75 mL of 4 M NaCl.

   For 20 mL of elution buffer II: Add 1 mL of 1 M Tris–HCl (pH 8.0), 15.0 μL of β-mercaptoethanol, 0.2 mL of 200 mM EGTA, and 5 mL of 4 M NaCl.
6. Ampicillin stock is 100 mg/mL in double deionized (dd) water, and we use 1:1,000 dilution, for example, 0.2 mL per 200 mL of medium.
7. Concentration of IPTG should be established. We found no difference between 0.2 and 0.5 mM.
8. Remember to take small samples of the suspension before centrifuging and after a sample of both the supernatant and the pellet. This will give an indication of how much protein remains in the pellet and how much is in a soluble form.
9. The bottom of the glass chamber is covered by poly-L-lysine (Sigma) up to the final concentration of 0.01 % in water. Higher concentration of poly-L-lysine results in embryonic death. Lower concentration then 0.001 % will not keep eggs in one place during microinjection. It may also result in detaching embryo during imaging.
10. Chambers: For in vivo imaging with inverted microscope, it is important that chambers have glass bottom. We use plastic edges covered with high-vacuum silicone grease (VWR international) to stick 22×64 borosilicate cover glass thickness No.1 (VWR).

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Chapter 16

Immunoblotting Analyses of Changes in Protein Phosphorylations During Oocyte Maturation in Marine Nemertean Worms

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Abstract

Immunoblotting analyses combined with phospho-specific antibodies can provide a powerful means for assessing protein activity states in various cellular extracts. This chapter describes a traditional, film-based immunoblotting method for monitoring the phosphorylation status of proteins in marine nemertean oocytes undergoing maturation. Similarly, with minor modifications, the protocol could potentially be applied to a wider variety of cellular processes and extract types that might be analyzed in other investigations of marine invertebrate development.

Key words Western blots, Kinases, Lysis buffer, SDS-PAGE, Enhanced chemiluminescence

1 Introduction

In order for development to proceed normally, oocytes must undergo a complex maturation process that is typically controlled by various protein kinases and phosphatases [1, 2]. Such kinases and phosphatases in turn modulate the phosphorylation status of several key regulatory proteins, thereby altering the activity states of these targeted regulators. In order to monitor the types of protein phosphorylations that occur during oocyte maturation, extracts of maturing oocytes can be subjected to immunoblotting analyses utilizing phospho-specific antibodies that determine whether or not critical sites on the assayed proteins are phosphorylated and thus modified to either up- or downregulate protein activity. Final processing and quantification of such immunoblots can be done with a turnkey system that allows high-sensitivity detection of signal over a relatively wide dynamic range without the need for film processing (e.g., the Odyssey Infrared Imaging System, www.licor.com). Alternatively, in the absence of an initial capital outlay for such automated instrumentation, it is also

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possible to conduct the immunoblotting via a more traditional method that involves the detection of blots on film.

This chapter describes a film-based protocol for assaying immunoblots probed with phospho-specific antibodies, as specifically applied to analyses of maturing oocytes obtained from marine nemertean worms [3, 4]. As such, the techniques covered here are suitable for use at marine biology stations that might not be equipped with automated immunoblotting detection systems [5–12]. Moreover, these methods should also be adaptable for applications with other types of eggs or with extracts of non-egg tissues that might be assessed during alternative investigations of marine invertebrate development.

2 Materials

Solutions should be mixed with high-quality (e.g., Milli-Q) water and, unless stated otherwise, prepared fresh from newly dissolved reagents or from stocks maintained at either room temperature or 4 °C (see specific comments next to the various recipes).

2.1 SDS Polyacrylamide Gel Components

1. Lysis buffer: 100 μL 0.5 M HEPES, pH 7, 750 μL 0.5 M NaCl, 1,250 μL 2 % NP-40, 150 μL 1 M β-glycerophosphate, 150 μL 0.25 M EGTA, pH 8, and 100 μL 1 M NaF. Just before vortexing and subsequent use on samples, add to the above the following: 4.5 mg sodium orthovanadate, 25 μL 100 mM PMSF (from aliquots dissolved in DMSO and stored at −20 °C), and ¼ tablet of Complete Protease Inhibitor Mini Tablet (Roche Diagnostics, Mannheim, Germany). Combine all nine components listed above and vortex for 2 min; let sit on ice for 10 min and transfer fluid component to microcentrifuge tube to avoid froth on top. Formulation is based on a recipe from ref. [13], using stocks stored at 4 °C, and makes 2.5 mL (i.e., enough for at least 50 protein assay samples).
2. 10 % resolving gel: 16.4 mL Milli-Q water, 13.2 mL premixed acrylamide solution (37:5:1, Bio-Rad Corp., Hercules, CA, #161-0158), 10 mL 1.5 M Tris–HCl resolving buffer, pH 8.8, 400 μL 10 % SDS (w/v), 200 μL 10 % ammonium persulfate (freshly made), and 20 μL TEMED. In a sidearm Erlenmeyer flask, add solutions in the order listed and gently swirl ~10 times in each direction to mix without generating bubbles.
3. 4 % stacking gel: 12.2 mL Milli-Q water, 2.6 mL premixed acrylamide solution (37:5:1, Bio-Rad Corp., Hercules, CA, #161-0158), 5 mL 0.5 M Tris–HCl stacking buffer, pH 6.8, 200 μL 10 % SDS (w/v), 100 μL 10 % ammonium persulfate (freshly made), and 20 μL TEMED. Mix as described above for the resolving gel. The above “quadruple-batch” gel recipes

make enough monomer for four 4 %/10 % gels that can be used to separate various proteins in the 20–250 kDa MW range when the four gels are run simultaneously as two pairs connected to two electrophoresis power packs. For running just two gels, halve the amounts, and when analyzing relatively high- or low-MW proteins that are not suitably separated with such a 4/10 formulation, gel concentrations can be altered to optimize protein separations (see Bio-Rad Instruction Manual for Mini-PROTEAN Tetra Cell, for further details). Note: Acrylamide is toxic and should be handled with care. Allow leftover monomer mixes to polymerize overnight so that the less toxic solid form of the gel can be disposed more safely.

4. Sample buffer: 3.55 mL Milli-Q water, 1.25 mL 0.5 M Tris–HCl, pH 6.8, 2.5 mL glycerol, 2 mL 10 % SDS (w/v), and 0.5 % 0.2 mL bromophenol blue (w/v). This formulation is based on a recipe in ref. [14], using stock solutions stored at room temperature. Just before use on samples, add appropriate amount of DTT (*see* Subheading 3.1, **step 3**).
5. 10× running buffer: 30.3 g Tris–HCl, 144 g glycine, and 10 g SDS. Bring all three to 1 L in Milli-Q water. Stock solution is stored at room temperature; 1× working solutions can also be used after several months of storage at 4 °C.

2.2 Transfer Components

1. Transfer buffer: 3.03 g Tris–HCl, 14.4 g glycine, 800 mL Milli-Q water, and 200 mL methanol (spectroscopy grade or higher quality). After mixing the above on a stirrer, add 0.5 g SDS and stir for at least an additional 15 min. For each run, make 1 L for two transfers or 2 L for four transfers.
2. 10× TBS (Tris-buffered saline) washing solution: 80 g NaCl, 24.2 g Tris–HCl, and ~800 mL Milli-Q water. Dissolve and pH to 7.6 with concentrated HCl before bringing up total to 1 L. Stock solution is stored at room temperature. Make at least 250 mL of 1× working solution for each run.
3. TTBS (Tween-Tris-buffered saline): To each liter of 1× TBS solution, add 500 μL of Tween-20 and stir for at least 15 min. Make 2 L of TTBS for each run. Stored at room temperature, this TTBS working solution is used for all washes of membranes except for the last (plain TBS) washes before conducting the enhanced chemiluminescence (ECL) protocol.
4. Blocking solution: 5 % nonfat dry milk freshly dissolved in TTBS.
5. Cold stripping buffer (made fresh just before use): 3 g glycine, 0.2 g SDS, and 80 mL Milli-Q water. Put on stirrer and pH to 2.2 with 6 N HCl (takes ~6 mL). Bring up to 98 mL in a graduated cylinder and add 2 mL Tween-20. Mix up and down numerous times to dissolve the Tween.

2.3 Supplies to Take to the Darkroom When Developing Film

1. Clear plastic wrap (e.g., Saran wrap).
2. White reflective screen (fold a 8.5 × 11 in. white paper in half, and have the 5.5 × 8.5 in. doubled sheet laminated at a print shop. Make several screens for multiple ECL incubations).
3. Forceps.
4. Laboratory tape.
5. Plastic transfer pipettes.
6. Book with a black non-reflective cover.
7. Timer.
8. Sharpie marker.
9. X-ray film, 5 × 7 in. for mini-blots (Sterling X-ray film from Life Science Products, Frederick, CO, has better sensitivity and costs less than comparable Kodak film).
10. Enhanced chemiluminescence solution (keep in dark and combine the two components of the ECL in the darkroom immediately before use on the blots).

3 Methods

3.1 Sample Processing

1. Make lysis buffer as directed in Subheading 2. Add 36 μL to each sample and vortex for 25 s so that froth is visible in each tube. Centrifuge samples at 4 °C and 16,000 × *g* for 12 min in a microcentrifuge. Maintain samples throughout the procedure on ice, and after protein assays have been completed, store at −20 °C (*see* **Note 1**).
2. After centrifugation, transfer 2 μL of the supernatant from each sample into a cuvette containing 798 μL of Milli-Q water for the protein assay. Set aside the cuvettes and continue processing the samples as described below.
3. After removing the 2 μL aliquot for a protein assay, add 26 μL of freshly made sample buffer (850 μL of stock solution from Subheading 2 plus 150 μL of 1 M DTT added just before use) to the remaining 34 μL of lysed sample. Mix by briefly vortexing and snap spin the tubes to bring down the sample. Boil the tubes for 4 min and subsequently store them at −20 °C (*see* **Note 2**).
4. After boiling the samples in sample buffer, add 200 μL of Bradford reagent (Bio-Rad, Hercules, CA) to each cuvette. Mix gently to avoid excessive bubbles by pipetting up and down with a plastic transfer pipette until the solution is a uniform color (*see* **Note 3**).
5. Read the absorption of each cuvette at 595 nm, using a cuvette containing 798 μL of Milli-Q water, 2 μL of lysis buffer, and 200 μL of Bradford reagent as the blank.

6. To convert absorbance into total protein per sample, construct a standard curve based on serial dilutions of bovine serum albumin.

3.2 Gels

1. Use either precast gels (e.g., Bio-Rad Mini-PROTEAN TGX) or pour gels as follows: first, prepare resolving gel solution according instructions in Subheading 2 (*see* **Note 4**).
2. Using a pipette, add the gel fluid slowly and steadily to the side of the glass plates, to avoid introducing bubbles. In order to leave room for the stacking gel, the resolving gel should extend to 1 cm below the level of where the comb of the wells will protrude once inserted into the overlying stacking gel (*see* **Note 5**).
3. After the gel has been poured, gently add a Milli-Q water cap until the water reaches the top of the short plate. Let the gel polymerize for at least 45 min at room temperature (*see* **Note 6**).
4. Following gel polymerization, pour out the water cap by tilting the glass plates, and use a paper towel to remove the remaining water without touching the gel.
5. Mix the stacking gel according to the recipe in Subheading 2 and pipette it on top of the resolving gel, filling to the top of the short glass plate. To avoid squirting out liquid acrylamide, tilt the comb and slowly insert from one side rather than rapidly putting it in straight down on the stacking gel.
6. Let stacking gel polymerize for 45 min, and then cover with plastic wrap and keep in the refrigerator for use within the next few days.
7. Mix up 1× running buffer and store in refrigerator overnight so it will be cold when used.

3.3 Electrophoresis

1. Thaw frozen samples that had been boiled in sample buffer and vortex them briefly to mix.
2. Place the gel into its holder ensuring there is a good seal (Fig. 1). Once assembled, put the holder with its two gels in a gel rig and add cold running buffer (*see* Subheading 2) into the central area between the two sets of gels. Pour remaining running buffer outside the gels to fill the container (*see* **Note 7**).
3. Based on the protein assays, load the equivalent of 25 μg of total protein per well using a gel-loading pipette with its tip placed against the edge of the short glass to ensure that the fluid flows into the well. It is important not to overload the wells. To determine well capacities in gels of varying thicknesses, consult the Bio-Rad Instruction Manual for Mini-PROTEAN Tetra Cell. Fill all wells; if one is to be left blank, add sample buffer. Also, add molecular weight markers to one or two of the lanes (*see* **Note 8**).

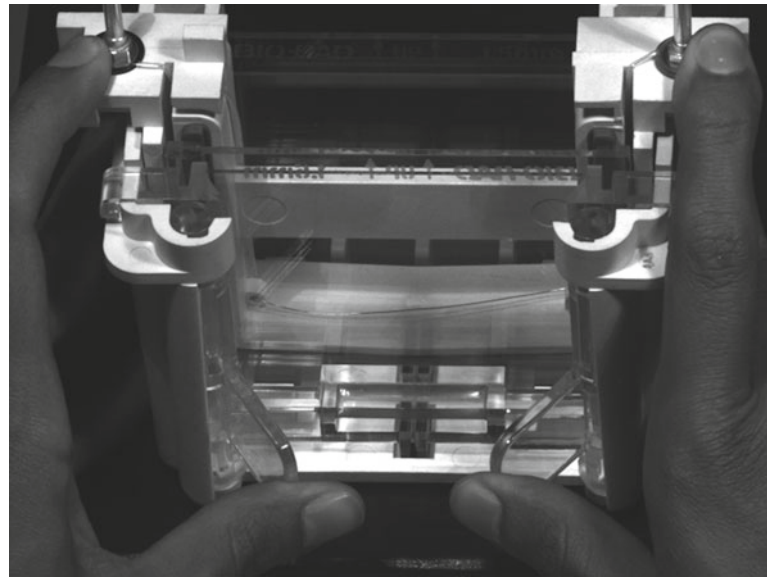

Fig. 1 Proper assembly of the gel apparatus showing downward pressure while clamping with thumbs to minimize chances of leakage

4. Plug the gel apparatus into an appropriate power supply that is set to a constant voltage of 100 V, and run the gel until the dye front is ~0.5 cm from the bottom (about 2–2.5 h) (*see* **Note 9**).

3.4 Transfer

1. After electrophoresis has been completed, turn off the power supply and carefully separate the gel plates. Using a clean razor blade, remove the stacking portion of the gel and rinse the remaining resolving gel in Milli-Q water for a few seconds.
2. After rinsing, equilibrate the gel in transfer buffer (*see* Subheading 2) for 15 min on an orbital shaker.
3. While the gel is equilibrating, manually agitate for 90 s in methanol PVDF membranes (Bio-Rad, Hercules, CA), which had been precut to ~5.5 × 8.5 cm rectangles for use in a mini-blot apparatus. Discard the methanol and equilibrate the membranes in transfer buffer for 15 min on an orbital shaker.
4. Load the gels into the transfer setup in the correct order. For example, with Bio-Rad mini-blot transfer setups, start with the black side of holder on bottom, followed by sponge, filter paper, gel, membrane, filter paper, sponge, and clear side of holder. It is important to assemble this "sandwich" submerged in transfer buffer in order to minimize trapped bubbles. To maintain the same order of the gel lanes on the blot, flip the gel horizontally so that the last lane of the gel is switched to the left side of the overlying membrane and thus will be on the right once the transfer is complete and the membrane is flipped over.

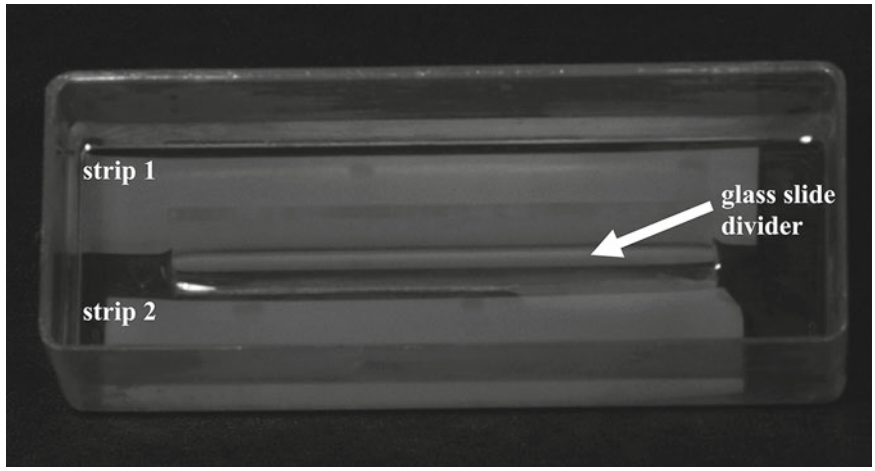

Fig. 2 A topside and oblique view of an incubation chamber with a glued-down glass slide divider allowing two blot strips to be probed simultaneously

5. Place the loaded sandwich into the transfer apparatus with the black side of the sandwich oriented toward the back of the chamber. Add a magnetic stir bar and place the transfer unit on a stirrer. Put in an ice pack and fill the unit with transfer buffer until the sandwich is completely covered.
6. Turn on the stirrer, snap on the lid, and run the transfer at 100 constant volts for 75 min. For two blots, the current should start around 230–270 mA and will gradually rise to 300–380 mA by the end of the run (*see* **Note 10**).

3.5 Antibody Probing

1. When the transfer is complete, peel away the membrane (you should see the molecular weight markers on the membrane). If probing with multiple antibodies for proteins of divergent MWs, it may be possible to cut the blot into horizontal strips that can be incubated individually in smaller containers (Fig. 2). In any case, with full or cut blots, put the membrane directly in blocking solution (*see* Subheading 2) with the side that had been next to the gel faceup. Shake at room temperature on an orbital shaker for 60 min. Do not block for shorter or longer periods as this might result in increased background or reduced signal (*see* **Note 11**).
2. Briefly wash the blocked membrane with TTBS (*see* Subheading 2) and add the primary antibody solution. Cover and agitate overnight at 4 °C (*see* **Note 12**).
3. Decant the primary antibody solution, and retain for future use after freezing at –20 °C. Rinse the probed membranes with TTBS for 30 s; then, wash three times with minimal agitation for 5 min each in TTBS at room temperature (*see* **Note 13**).

4. After washing, incubate with secondary antibody dissolved in blocking solution for 90 min at room temperature (*see* **Note 14**).
5. Remove the secondary antibody, rinse for 30 s in TTBS, and wash twice for 10 min each in TTBS. After washing in TTBS, perform two 15 min washes in TBS (*see* **Note 15**).

3.6 Film Developing

1. In addition to the probed membranes incubating in TBS, take to the darkroom items listed in Subheading 2, and set up two pieces (~6 × 8 in.) of clear plastic wrap. Place the washed blots faceup on one sheet of wrap and quickly incubate them for 90 s in the ECL solution. It is important to make sure that there are no dry spots on the blots.
2. Remove the ECL solution and place the membranes facedown on the other sheet of wrap. Put a white reflective screen on top of the membranes and neatly fold the wrap around the back of the screen before taping down the wrap. Make sure no bubbles or leaks are present.
3. Turn off the room lights, and turn on red safety lights. Take out unexposed film, keeping it a safe distance from the safety light, and place it on the ECL-loaded blots. Using a Sharpie, quickly make several small marks that extend across the edge of the film to the reflective sheet. This will allow subsequent orientation of the developed film relative to the molecular weight markers.
4. Cover with a book and expose. Exposure times vary depending on the intensity of the signal but are typically in the range of 15 s–5 min (*see* **Note 16**).
5. Place the film in an automated film processor or develop the film manually using Kodak D19 developer and fixer according to the manufacturer's instructions.

3.7 Re-probing Stripped Membranes

1. Keep the membranes in the refrigerator still wrapped in plastic on their white reflective screens in order to avoid drying of the blots. For re-probing, remove the blots and quickly place them in stripping buffer (*see* Subheading 2) with agitation for 30 min. Replace with fresh stripping buffer and agitate for another 30 min.
2. Give the membranes three washes with TTBS for 15 min each.
3. Incubate the membranes in freshly made blocking solution for 1 h, transfer to primary antibody solution, and repeat the rest of the protocol as described above to get a second set of signals from the blot (*see* **Note 17**).

3.8 Quantification of Blot Intensities

1. Create a digital image of the blot signal that was recorded on film.
2. Invert the contrast so that background is dark, and the blot bands are light (this will allow higher-density bands to be

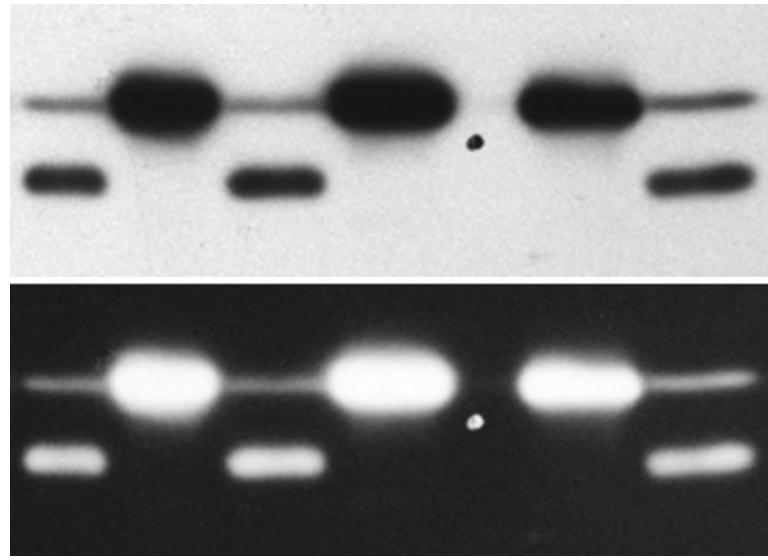

Fig. 3 Example of an original version of a blot (*top* image) and its inverted-contrast view (*lower* image) that in turn allows high-density bands to be recorded as high values during densitometric analyses

quantified as higher numbers, as compared to lower-density bands) (Fig. 3).

3. Place a region of interest around each band to be quantified and obtain an integrated intensity for that band using morphometric software (e.g., MetaMorph, Molecular Devices, Sunnyvale, CA).
4. After obtaining band densities, shift each region of interest to a background region of the film and obtain a background reading that is to be subtracted from the band intensity (thereby accounting for opacity in the film itself). Such quantification assumes that the blot signals are recorded in the linear range of the film and are not either saturated or below a threshold detection value for the film.

4 Notes

1. To adapt this protocol for non-oocyte cells or tissues, it may be possible to modify the lysis buffer and/or protein extraction steps. For example, longer incubations in lysis buffer or more stringent extractions employing sonication might be tried.
2. When boiling samples, set the Bunsen burner/hot plate to the minimum required to obtain weak boiling, thereby reducing the chance of getting water into the sample should a tube pop open.

3. The same pipette can be used for more than one sample, as long as very little, if any, fluid is transferred from one cuvette to the other. Also, the Bradford reagent loses signal over time, so the faster the readings are completed, the better.
4. To mix the gel solution, gently swirl the container; if bubbles continue to appear in the blots, try degassing gel solutions for 10 min in a vacuum prior to addition of the polymerizing agents (ammonium persulfate and TEMED).
5. To determine the appropriate height of the resolving gel prior to gel pouring, place a comb within the gel plate setup and mark 1 cm below the bottom of the comb.
6. Pour the water cap slowly to avoid mixing with the gel solution, which in turn can result in a curved gel front.
7. Smaller quantities of running buffer can be used (e.g., as little as 450 mL), but ~850 mL per gel setup ensures that any leakage from the central chamber will not lead to a significant current decrease and an ensuing stoppage of the gel run.
8. To reduce the amount of antibody solution needed, it is possible to cut the blot into strips that contain the proteins of the desired molecular weight for each antibody. These strips can fit in containers that require as little as 8 mL to cover the blots entirely (Fig. 2). In order to help cutting horizontal strips for multiple antibody incubations, load two lanes of MW markers with one near either end of the gel.
9. The current at the beginning of the run should be around 45–55 mA and by the end of the run, about half of starting value. Because of this decrease, the E9 safety feature of certain Bio-Rad power supplies should be disabled prior to the start of the run to avoid having the power turn off as the current drops.
10. It is possible to regulate the current by modulating the speed of the magnetic stirrer. Faster stirring will result in lower currents; adjust the speed until you reach the desired values.
11. Thoroughly mix the milk into TTBS at least 30–60 min prior to blocking in order to avoid uneven blocking.
12. Depending upon the strength of the primary antibody, try initial dilutions of 1:2,500–1:500 in TTBS containing 1–5 % bovine serum albumin as a blocking agent. For full blots, ~18 mL of solution is required to cover the entire blot (however, *see* **Note 8**).
13. It is possible to save some primary antibody solutions (e.g., phospho-ERK 1/2, phospho-Cdc2 Y15, phospho-AMPK T172) and freeze them at −20 °C for subsequent use on another blot. If the signal begins to fade, boost the solution by adding a few more microliters of concentrated antibody.

14. Following incubation in primary antibody, an appropriate secondary antibody conjugated to horseradish peroxidase (e.g., from Santa Cruz Biotechnology, Santa Cruz, CA). Initially, try a 1:5,000 dilution in blocking solution, and adjust accordingly.
15. TTBS is to be avoided in the final washes in order not to degrade the ECL signal.
16. Keep the book still over the blot and film in order to avoid blurring the signal. Use a book with a black non-reflective cover. The ECL solution fades over time, with optimal results being achieved within 25 min of mixing. Because of this, later exposures take longer to get equivalent results; so, whenever possible, do longer exposures first.
17. Strong bands usually do not get fully stripped. Thus, it is better to probe for a weak band first, and then probe the stronger band after stripping.

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Chapter 17

Multicolor Labeling in Developmental Gene Regulatory Network Analysis

Aditya J. Sethi, Robert C. Angerer, and Lynne M. Angerer

Abstract

The sea urchin embryo is an important model system for developmental gene regulatory network (GRN) analysis. This chapter describes the use of multicolor fluorescent in situ hybridization (FISH) as well as a combination of FISH and immunohistochemistry in sea urchin embryonic GRN studies. The methods presented here can be applied to a variety of experimental settings where accurate spatial resolution of multiple gene products is required for constructing a developmental GRN.

Key words TSA Plus, Digoxigenin, Fluorescein, Lineage, Signaling, Linkages, Marker, Perturbations

1 Introduction

In recent years, considerable progress has been made towards elucidating gene regulatory networks (GRNs) underlying embryonic development [1–6]. Functional connections within and between GRNs are typically established by examining the responses of regulatory factor expression to experimental perturbations. For maximum accuracy, GRN responses need to be examined at several levels. These include quantitative measurements of transcript abundance in the entire embryo [7], cis-regulatory analyses of genes encoding key factors [8, 9], and evaluations of gene function in specific blastomeres at defined developmental stages [10, 11]. The last type of analysis requires precise assessment of the spatial distribution of a gene product(s) of interest.

Optimum spatial resolution is critical when characterizing developmental GRNs for several reasons. First, during early embryogenesis, many key regulatory factors have dynamic expression patterns that evolve rapidly. Thus, within a few cell divisions, the same factor may be expressed in different blastomeres that give rise to distinct embryonic lineages [10, 12–16]. Important regulatory genes are also frequently expressed in precursors of multiple

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lineages at a single developmental stage, and the same gene can have very different roles in each of these lineages [10, 14]. Also, experimental perturbations targeting a specific gene can have both localized and general developmental consequences. In such situations, whole-embryo measurements of gene product abundance are of limited value unless supplemented by accurate spatial data. Second, at early stages, embryos show relatively few morphological features that permit reliable identification of specific blastomeres expressing a particular gene product(s). Consequently, they must be monitored at the molecular level. Third, during initial embryonic specification, more than one lineage-specific GRN can operate in a group of progenitors [2]. Understanding GRN function during lineage segregation at these early stages therefore requires accurate spatial information about multiple gene products in the same blastomeres [2, 17]. In each of these situations, the distribution of a gene product(s) of interest needs to be resolved with reference to expression of a well-characterized molecular "landmark."

Specific instances of GRN analysis where spatial assays are necessary for establishing accurate network connections include:

(a) *Identifying targets of transcription factors*: Direct targets of key GRN transcription factors are expressed/repressed within several hours of their upstream regulators in the same cells. Indirect targets, on the other hand, can be regulated by transcription factor activities or intermediate signaling events within or outside an upstream factor's expression domain. Spatial assays are therefore critical to locate both the upstream GRN factor and its putative target in order to establish GRN linkages. (b) *Lineage-specific regulation of target genes*: GRN factors frequently regulate distinct sets of target genes in precursors of multiple embryonic lineages. In these cases, whole-embryo assays of target abundance, by themselves, are incapable of resolving GRN responses within a specific lineage. This requires spatial assays of target gene responses relative to an appropriate lineage marker. (c) *Defining feedback loops*: GRN factors commonly autoregulate themselves or respond to inputs from their targets through feedback loops. To distinguish between cell-autonomous and non-autonomous feedback loops, it is essential to locate the upstream GRN factor, the target it initially regulates, and the site of the "feedback" response. Thus, responses to experimental perturbations that test the loop must be resolved in space.

In this chapter we describe two methods that permit accurate localization of a gene product relative to a known molecular "landmark" in sea urchin embryos. The first employs multicolor fluorescent in situ hybridization (FISH) to examine the spatial distribution of transcripts encoded by up to three genes of interest. The second method combines FISH with immunohistochemistry in whole embryos. In contrast to conventional alkaline phosphatase-mediated detection, fluorescent labeling allows for unambiguous resolution of multiple gene products, which is especially critical when they are

expressed in overlapping territories [2, 17, 18]. Furthermore, fluorescence-based approaches permit precise optical sectioning and volume projections of image stacks, thereby providing 3-dimensional descriptions of spatial expression patterns. By incorporating nuclear counterstaining, fluorescent labeling can easily be used to assess the numbers and distributions of cells expressing a gene product of interest.

The techniques described herein have been used to investigate a number of aspects of sea urchin GRN function. These include characterizing the expression of a regulatory factor during embryogenesis [18, 19] as well as understanding the effects of an experimental perturbation on the expression of a gene in specific blastomeres marked by a second gene product [20]. Additionally, these methods have been used to identify regulatory targets in blastomeres adjacent to cells that selectively receive a reagent to disrupt a signaling process [20, 21]. They can also be used to distinguish between a localized response to an experimental perturbation and a general developmental consequence such as toxicity caused by disruption of basic cellular function throughout the embryo [20].

2 Materials

2.1 Materials for In Situ Probe Synthesis

1. *Probe labeling*: 10× digoxigenin labeling mix (Roche) and 10× fluorescein labeling mix (Roche); ATP, CTP, GTP, and UTP (lithium salts, Roche); DNP-11-UTP (PerkinElmer); 10× DNP labeling mix consists of 10 mM each of ATP, CTP, and GTP, 6.5 mM UTP, and 3.5 mM DNP-11-UTP.
2. *In vitro transcription*: SP6 and T7 RNA polymerases (New England Biolabs and others) with included 10× RNA transcription buffers. Templates are discussed in Subheading 3.1, **step 2** and 4 **step 1**.
3. *Probe purification*: Turbo DNAse I (Ambion); ethylenediaminetetraacetic acid (EDTA); Qiaquick PCR purification kit (Qiagen) or size-fractionation columns (Illustra ProbeQuant G-50 Micro Columns, GE Healthcare); diethylpyrocarbonate (DEPC)-treated (nuclease-free) water.

2.2 Materials for Multicolor FISH and Immunostaining

1. *Labware*: 96-well flat-bottomed plates; thermocycler-grade sealing film; rubber tubing; glass Pasteur pipettes; standard suction line terminating in a 200 μL disposable pipette tip.
2. *Fixative*: 4 % paraformaldehyde (v/v, use 16 % EM grade paraformaldehyde solution, EM Sciences); 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS); 0.1 % Tween-20 in Artificial Sea Water (ASW: 0.461 M NaCl, 0.0024 M $NaHCO_3$, 0.009 M KCl, 0.026 M $MgCl_2{\cdot}6H_2O$, 0.032 M $MgSO_4$, 0.0097 M $CaCl_2$, pH 8.1).

3. *Wash and hybridization buffers*: Use nuclease-free molecular biology grade water. "MOPS wash buffer": 0.1 M MOPS, pH 7.0, 0.5 M NaCl, and 0.1 % Tween-20. "Hybridization buffer 1": 70 % formamide (v/v, use stock solution of commercial molecular-grade formamide, pH 7–9, Sigma-Aldrich), 0.5 M NaCl, 0.1 M MOPS, pH 7.0, 1 mg/mL bovine serum albumin (BSA), and 0.1 % Tween-20. "Hybridization buffer 2": 50 % formamide (v/v), 0.5 M NaCl, 0.1 M MOPS, pH 7.0, 1 mg/mL BSA, and 0.1 % Tween-20 (add formamide only after all other buffer components have mixed to allow BSA dissolution or use a stock solution of BSA at 100 mg/mL, stored at −20 °C).
4. *Probe detection*: Anti-fluorescein-HRP (horseradish peroxidase) antibody (PerkinElmer); anti-digoxigenin-POD (peroxidase) Fab fragments (Roche, reconstituted to give 150 U/mL stock), both antibodies stored at 4 °C; anti-DNP-HRP stored at 4 °C (part of TSA DNP-HRP kit, PerkinElmer); lamb serum/normal goat serum (stored at −20 °C); BSA; "blocking buffer 1": 10 % lamb/goat serum, 2.5 mg/mL BSA in MOPS wash buffer; "blocking buffer 2": 5 % lamb/goat serum in MOPS wash buffer; Tyramide Signal Amplification Plus (TSA Plus) system (PerkinElmer); 30 % hydrogen peroxide solution (v/v, Sigma-Aldrich, use protective labwear when handling hydrogen peroxide, store stock at RT away from light).
5. *Immunostaining and nuclear counterstaining*: "PBST wash buffer": 1× phosphate buffered saline (PBS) (use commercial 10× PBS) and 0.1 % Tween-20; Alexa series of secondary antibodies (Invitrogen), stored at 4 °C; 4′, 6-diamidino-2-phenylindole, dilactate (DAPI).
6. *Mounting and slide preparation*: 25 × 75 × 1 mm glass slides; 22 × 22 mm #1 coverslips; 13 or 20 mm inner diameter custom imaging spacers (Grace Bio-Labs, catalog #3181209 or 654006, respectively); custom MatTek 96-well flat-bottomed plate with 1.0 coverslip thickness (MatTek Corporation, catalog # P96G-1.0-5-F); glycerol.

3 Methods

3.1 In Situ Probe Synthesis

Take necessary precautions at each step of probe synthesis to maintain an RNase-free environment (gloves, dedicated labware for RNA work, and nuclease-free water as indicated). Also, refer to notes for comments on probe preparation.

1. Set up a 10 μL reaction for in vitro transcription of each probe.
2. Use 250–500 ng (plasmid) or 100–250 ng (PCR product) of template DNA in 3–5 μL of water. Templates can be appropriately restricted plasmids or PCR products containing full-length

or partial cDNA sequence of the gene of interest flanked by a suitable bacteriophage promoter sequence. Aim for sequence lengths between 300 and 1,000 nt. For rare targets, use as long a template sequence as possible. Alternatively, two PCR templates spanning different regions of the mRNA can be mixed either at the synthesis step, if they use the same RNA polymerase, or after synthesis and before hybridization. Use of two probes also allows one to hybridize both and each separately, providing confidence in the results.

3. Prior to use, template DNA should be purified using a column-based method (Qiaquick Gel Extraction or PCR purification kit, Qiagen) and eluted in nuclease-free water.
4. Add to the DNA 1 μL of 10× RNA transcription buffer, 1 μL (this can be reduced to as low as 0.3 μL per 10 μL reaction to save reagent) each of the 10× digoxigenin, DNP, or fluorescein labeling mix, nuclease-free water to bring the final volume to 10 μL and 0.5–1.0 U of RNA polymerase (SP6 or T7, usually 0.5–1.0 μL).
5. Incubate at 37 °C for 2 h.
6. Add 1–2 U (0.5–1.0 μL) of Turbo DNAse I directly to the transcription reaction (Turbo DNAse I is engineered to be highly tolerant of the salt concentration in transcription buffers) and incubate at 37 °C for 15 min to remove template DNA.
7. Add 1 μL of a 100 mM EDTA, pH 8.0 stock solution (make stock in nuclease-free water), incubate at room temperature (RT) for 5 min to stop enzymatic reactions. Addition of EDTA significantly improves binding of RNA to, and elution from, Qiagen columns (*see* **step 8**), resulting in substantially higher recovery of product yields.
8. Dilute reaction to 100 μL with DEPC-treated water.
9. Purify synthesized probe using the Qiagen Qiaquick PCR purification kit as per the manufacturer's instructions for purification of standard PCR products using a microcentrifuge (Qiagen). Alternatively, purification of synthesized RNA through size-fractionation columns (Illustra ProbeQuant G-50 Micro Columns, GE Healthcare; follow manufacturer's instructions) achieves comparable yields [15].
10. Elute RNA in 30–50 μL of DEPC-treated water if using the Qiagen PCR purification kit. Follow manufacturer's instructions for the Illustra ProbeQuant G-50 columns for elution. Quantitate product by spectrophotometry. Store probe at –20 °C between uses. For long-term use, store probes as ethanol precipitates or in hybridization buffer 1 containing 70 % formamide and yeast tRNA, both of which inhibit RNase.

3.2 Multicolor Fluorescent In Situ Hybridization (FISH) to Detect Endogenous Transcripts

1. Embryos are visualized with a dissecting microscope at 15–30× magnification and typically carried in 96-well flat-bottomed plates making solution transfers by mouth suction and pulled glass pipettes attached to rubber tubing. All steps from fixation through hybridization and staining are done in the wells of these plates (*see* notes for comment on choice of plates for this procedure). Add 50 μL of fixative and allow embryos to settle for 5 min at RT. Remove supernatant from well with suction line and replace with 150–200 μL of fixative; incubate at RT for 1 h. Embryos may also be fixed overnight at 4 °C, although this results in significantly lower signal. Fixative should be freshly made on the day of embryo collection and can be used for up to 24 h at RT.
2. Wash embryos five times with 200 μL of MOPS wash buffer at RT. If required, embryos can be stored in MOPS wash buffer at 4 °C for up to 7 days prior to further processing. Seal 96-well plates when storing embryos to minimize evaporation. If storing embryos longer than 2 days, add 0.01 % sodium azide (w/v) to the MOPS wash buffer to prevent bacterial contamination. For optimum signals and minimal background staining, MOPS wash buffer should be freshly made and used on the day of preparation if stored at RT. For long-term use, store buffer at –20 °C.
3. To equilibrate embryos in hybridization buffer 1, incubate them in 150–200 μL of each the following solutions for 30 min: 2:1 and 1:2 mix (v/v) of MOPS wash buffer and hybridization buffer, followed by hybridization buffer 1 alone. Store excess hybridization buffer at –20 °C.
4. Prior to hybridization, place embryos in 200 μL of hybridization buffer 1 for at least 1 h at 50 °C.
5. Replace hybridization buffer 1 with 150–200 μL of preheated hybridization buffer 1 (at 50 °C) containing 0.05–0.3 ng/μL of digoxigenin – and/or DNP – and/or fluorescein-labeled antisense RNA, as well as 500 μg/mL yeast tRNA to minimize nonspecific probe binding. Seal plates and hybridize at 50 °C for 3–7 days. Refer to Subheading 4 below for guidelines on probe conjugate selection.
6. Wash embryos five times with 200 μL of MOPS wash buffer at 50 °C for 3 h, followed by three washes with 200 μL of MOPS wash buffer at RT over a total period of 1 h.
7. Block embryos in 150–200 μL of blocking buffer 1 for 30–60 min at RT.
8. Incubate embryos overnight at either RT or 4 °C in 150 μL of a 1:750 dilution (v/v) of anti-fluorescein-HRP in blocking buffer to label fluorescein-containing hybrid RNA duplexes. Seal 96-well plate to minimize evaporation.

9. Wash embryos six times with 200 µL of MOPS wash buffer over 1.5–2 h at RT to remove unbound antibody.
10. Detect bound anti-fluorescein-HRP by adding 50 µL of freshly made Tyramide Amplification working solution (a 1:150 dilution of the TSA stock solution in the included 1× plus amplification diluent) for 2–8 min at RT (a 6-min incubation usually yields an optimum balance of signal intensity and minimal background staining). Ensure that all TSA kit solutions are at RT prior to use, and place embryos in the dark for all steps after addition of TSA Amplification working solution. Refer to the guidelines on fluor selection in Subheading 4.
11. Wash embryos six times each with 200 µL of MOPS wash buffer at RT for 5–10 min per wash to remove excess TSA detection reagent.
12. Quench the peroxidase activity of peroxidase-conjugated antibody by incubating embryos in 200 µL of a solution of MOPS wash buffer containing 3 % hydrogen peroxide (v/v, diluted from a 30 % stock solution) at RT for 1 h.
13. Remove excess hydrogen peroxide by washing embryos six times, each with 200 µL of MOPS wash buffer at RT over a total of 90 min.
14. Incubate embryos overnight at 4 °C in 150 µL of a 1:1,000 dilution (v/v using reconstituted antibody stock) of anti-digoxigenin-POD Fab fragments in blocking buffer 2 to label digoxigenin-containing hybrid duplexes. Seal 96-well plate to minimize evaporation.
15. Wash embryos six times, each with 200 µL of MOPS wash buffer over 1.5–2 h at RT to remove unbound antibody.
16. Detect bound anti-digoxigenin-POD by adding 50 µL of freshly made Tyramide Amplification working solution (a 1:150 dilution of the TSA stock solution in the included 1× plus amplification diluent) for 2–8 min at RT (6 min usually is optimal).
17. Repeat **steps 11** through **13**.
18. Incubate embryos overnight at 4 °C in 150 µL of a 1:250 dilution (v/v) of anti-DNP-HRP in blocking buffer 2 to label DNP-containing hybrid duplexes. Seal 96-well plate to minimize evaporation.
19. Wash embryos six times, each with 200 µL of MOPS wash buffer over 1.5–2 h at RT to remove unbound antibody.
20. Detect bound anti-digoxigenin-POD by adding 50 µL of freshly made Tyramide Amplification working solution (a 1:150 dilution of the TSA stock solution in the included 1× plus amplification diluent) for 2–8 min at RT (6 min usually is optimal).

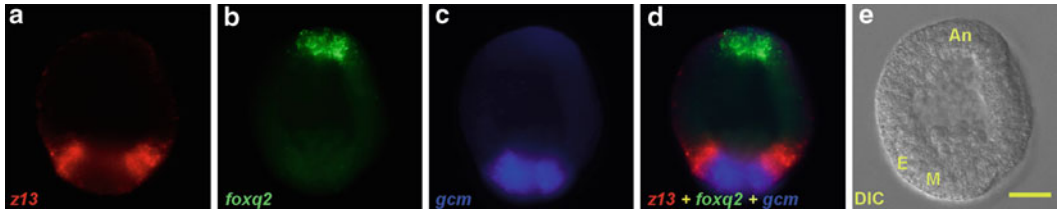

Fig. 1 Multicolor FISH to characterize the expression patterns of three endogenous mRNAs: *z13* (*red*, **a**, **d**), *foxq2* (*green*, **b**, **d**), and *gcm* (*blue*, **c**, **d**) mRNAs were hybridized with DNP-, digoxigenin-, and fluorescein-conjugated antisense probes, respectively, and detected sequentially as described in Subheading 3.2. Wild-type embryos were fixed at the mesenchyme blastula stage (22 h postfertilization) (shown in DIC in **e**). At this stage, *z13* mRNA is expressed in endoderm progenitors (*E*) in the vegetal region of the embryo, *foxq2* transcripts are restricted to the ectoderm near the animal (*An*) pole (**e**), and *gcm* mRNA accumulates in pigment cell precursors within the secondary mesoderm (*M*) (**e**). Scale bar in (**d**) represents approximately 20 μm

21. Wash embryos six times, each with 200 μL of MOPS wash buffer at RT for 5–10 min to remove excess detection reagent. Include an appropriate dilution of DAPI (based on specific stock purchased) in the first MOPS wash if performing nuclear counterstaining.
22. Equilibrate embryos in 50 % glycerol by successive washes through 15, 30, and 50 % glycerol made by diluting glycerol with MOPS wash buffer (v/v). Allow embryos to equilibrate at RT for 15 min between steps to minimize osmotic damage. Glycerol aids in embryo handling during slide preparation, and also provides a refractive index appropriate for microscopy. Alternatively, embryos can be transferred in MOPS wash buffer to 1.0 coverslip-thick flat-bottomed 96-well plates and photographed directly (*see* custom MatTek plates in **item 6** of Subheading 2.2).

An example of three-color FISH is shown in Fig. 1.

3.3 Multicolor FISH to Detect Transcripts Synthesized from an Exogenously Introduced DNA as Well as Endogenous Embryonic Transcripts

For this application, the stringency of the hybridization conditions is reduced to prevent denaturation of the introduced double-stranded DNA, thereby excluding formation of RNA/DNA hybrids. Post-hybridization high stringency washes are used to maximize the signal-to-noise ratio. Modifications to the multicolor FISH protocol from Subheading 3.1 follow (only modified steps with corresponding step numbers are described below; the rest of the protocol is identical to the one described for standard multicolor FISH):

1. To equilibrate embryos in hybridization buffer 2, incubate them in 150–200 μL of each of the following solutions for 30 min: 2:1 and 1:2 mix (v/v) of MOPS wash buffer and

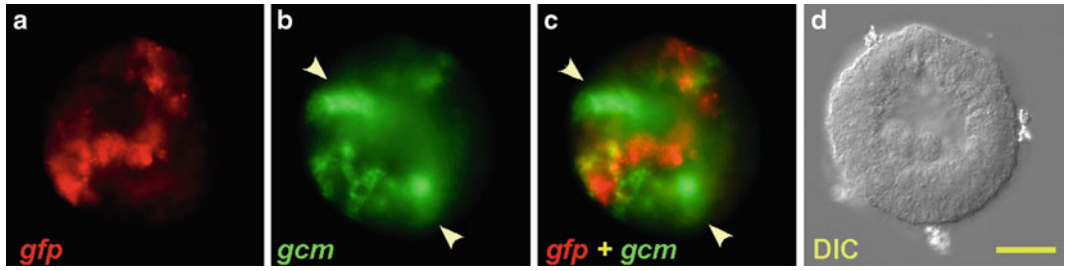

Fig. 2 Dual-color FISH to detect a secondary mesoderm GRN response to a transcription factor introduced exogenously in clones of cells in blastula-stage embryos: This experiment was designed to show induction of the secondary mesoderm specification gene, *gcm*, in cells adjacent to those expressing the transcription factor, Pmar1 [20]. DNA constructs were used to misexpress Pmar1 and GFP under control of the hatching enzyme (HE) promoter. When these constructs are co-injected, Pmar1 and GFP are expressed in the same blastomeres, traced here with a fluorescein-conjugated probe hybridized with *gfp* mRNA (*red*, **a**, **c**). A digoxigenin-coupled probe was used to detect ectopic induction of *gcm* (*green*, **b**, **c**) in cells adjacent to Pmar1 (and GFP)-misexpressing cells at the late hatching blastula stage (DIC in d) [20]. Endogenous *gcm* transcripts (*arrowheads* in **b**, **c**) accumulate in specific blastomeres (non-skeletogenic mesenchyme precursors) not immediately adjacent to Pmar1 (and GFP)-misexpressing cells. Hybridization conditions were adjusted as per the protocol in Subheading 3.3. Scale bar in (**d**) represents approximately 20 μm

hybridization buffer 2, followed by hybridization buffer 2 alone. Store excess hybridization buffer 2 at –20 °C.

2. Prior to hybridization, place embryos in 200 μL of preheated hybridization buffer 2 for at least 1 h at 45 °C.
3. Replace prehybridization buffer with 150–200 μL of preheated hybridization buffer 2 (at 45 °C) containing 0.05–0.3 ng/μL of digoxigenin-labeled antisense RNA, DNP-labeled antisense RNA, and/or fluorescein-labeled antisense RNA and 500 μg/mL yeast tRNA. Seal plates and hybridize at 45 °C for 3–7 days. Refer to Subheading 4 below for guidelines on probe conjugate selection.
4. Wash embryos five times, each with 200 μL of MOPS wash buffer at 50 °C for a total of 3 h, followed by two washes with 200 μL of hybridization buffer 1 (containing 70 % formamide) at 50 °C over an hour. Wash three times with 200 μL of MOPS wash buffer at RT over an hour (Fig. 2).

An example of two-color detection of transcripts synthesized from an exogenous template and an endogenous transcript is shown in Fig. 2.

3.4 FISH Followed by Immunostaining

To combine FISH with immunostaining, use the following protocol after completing the in situ hybridization procedure in Subheading 3.1 (i.e., after final MOPS wash buffer replacements in **step 18**). For a list of antibodies that have been found to give acceptable signals in sea urchin embryos using this approach, refer to Subheading 4 below.

Fig. 3 *FISH+ immunostaining*: Multicolor labeling was used to show the distribution of cells containing SoxB1 protein (*green*, **b**–**e**) compared to secondary mesoderm progenitors expressing the pigment cell gene, *gcm* (*red*, **a**, **c**, **e**). In this experiment, *gcm* transcripts were detected using a digoxigenin-conjugated antisense RNA probe and TSA-red, followed by immunohistochemical localization of endogenous SoxB1 protein and nuclear counterstaining with DAPI (*blue*, **d**, **e**) [20]. A stack of optical sections acquired with an axial tomography device (Zeiss) was used to generate a volume projection using Imaris 5.7.2 (Bitplane). Scale bar in (**e**) represents approximately 20 μm

1. Wash embryos twice, each with 200 μL PBST wash buffer at RT over 20 min.
2. Introduce an appropriate dilution of primary antibody(s) of interest in 150 μL of PBST blocking buffer (consisting of 5 % lamb or goat serum in PBST wash buffer). Incubate embryos in this blocking buffer containing primary antibody(s) overnight at 4 °C.
3. Wash embryos four times with PBST wash buffer at RT for a total of 1 h to remove unbound primary antibody(s).
4. Detect bound primary antibody(s) by adding 150 μL of a working dilution (determined separately for each secondary antibody used, usually 1:750 or 1:1,000, v/v) of an appropriate secondary antibody (i.e., specific for the species that each primary antibody was raised in and conjugated to a fluor different from the one(s) used in the FISH procedure).
5. Wash embryos three to four times, each with 200 μL of PBST wash buffer at RT for 5–10 min to remove unbound secondary antibody(s). Include an appropriate dilution of DAPI (based on the specific stock purchased) in the first PBST wash if performing nuclear counterstaining.
6. Equilibrate embryos in 50 % glycerol and PBST wash buffer as in **step 19** above.

An example of two-color detection of an RNA by FISH and a protein by immunostaining is shown in Fig. 3.

4 Notes

1. *Probe preparation*: A successful FISH experiment requires a pure antisense RNA probe of known sequence. It is essential to use a sequenced homogeneous DNA template (either a

completely sequenced highly purified plasmid preparation or a PCR amplicon generated from a cloned DNA fragment). We caution against the routine use of PCR products amplified from a heterogeneous source (e.g., cDNA synthesized from whole embryo RNA) unless the amplicon is sequenced to verify its identity. Also, a very important part of the probe preparation protocol is the column-based RNA purification protocol described above. Not only is it quicker than traditional RNA precipitation and isolation procedures, but, in our experience, it also yields in situ hybridization patterns with significantly superior signal intensity, specificity, and reproducibility.

2. *Choice of labware*: We strongly recommend the use of 96-well flat-bottomed plates for all protocols described in this chapter. Compared to microcentrifuge tubes, 96-well plates offer superior visibility and retention of embryos through numerous buffer replacements and a more even distribution of embryos during hybridization and staining procedures. This facilitates consistent probe and buffer penetration and affords highly reproducible in situ hybridization patterns. Additionally, embryos can be inspected under fluorescence after completion of each staining step without the need for any intervening mounting or slide preparation and consequent loss of sample. Hybridization and staining reactions can be sealed as effectively in plates as in microcentrifuge tubes by using thermocycler-grade sealing films. These perform reliably through weeklong hybridization procedures at 50 °C. Finally, the use of plates makes handling of large numbers of samples much more efficient.
3. *Probe conjugate selection*: We find that both digoxigenin and DNP-based labeling provide significantly superior sensitivity and signal specificity compared to fluorescein-based labeling. Furthermore, in our experience, digoxigenin and DNP-based detection give signals of equivalent intensity and specificity. Therefore, when performing dual-color FISH, detect transcripts of interest using digoxigenin and DNP-labeled antisense probes. When studying the distribution of three transcripts, rare/spatially diffuse transcripts should be detected using digoxigenin or DNP-coupled antisense probes, and the more abundant and/or highly localized mRNAs should be hybridized with a fluorescein-conjugated probe. Similarly, the relatively abundant transcripts synthesized from an exogenously introduced construct (e.g., a tracer sequence) are detected with a fluorescein-conjugated antisense probe and endogenous embryonic mRNAs with digoxigenin/DNP-coupled systems for optimum signals. If required, adjust the concentration of introduced DNA/RNA to maximize specific signals with the fluorescein-labeled probe and minimize embryo toxicity.

Although our current FISH protocol uses digoxigenin, DNP, and fluorescein labeling to detect up to three mRNAs of interest, additional probe conjugates such as biotin have been used in other model systems [17, 22] for multicolor FISH.

4. *Fluor selection*: In our experience, both Cy3- and fluorescein-TSA Plus detection systems give signals of equivalent intensity and specificity. With our current microscopy platform, the Cy5-TSA Plus kit gives signals of equivalent specificity to those of Cy3 and fluorescein, but significantly lower intensity. Nevertheless, Cy5-TSA Plus is a viable option with abundant, highly localized transcripts if Cy3 and fluorescein have already been allocated to other gene products in the same experiment. In addition to these, other TSA Plus fluors are available from PerkinElmer.
5. Order of probe detection: When performing three-color FISH, the fluorescein-conjugated probe must be detected before those labeled with DNP and digoxigenin. This specific sequence avoids the anti-fluorescein-HRP antibody binding with previously deposited TSA-fluorescein staining reagent and amplifying artifactual signals in successive fluor deposition steps. Subsequent detection of DNP- and digoxigenin-labeled transcripts can then be executed in any order with appropriately selected TSA Plus fluors. When detecting two transcripts of interest, fluorescein-conjugated probes should not routinely be used and hence digoxigenin and DNP-labeled transcripts can be detected in any order.
6. *Dynamic range*: Single and multicolor FISH have a narrower dynamic range of specific signal compared to traditional alkaline phosphatase-based whole-mount in situ hybridization. We believe this to be a function of the kinetics of the final staining reaction in each procedure. In the case of the FISH protocol described in this chapter, the final fluor deposition is extremely rapid (2–8 min to saturation) with limited user control. Conversely, alkaline phosphatase-based colorimetric deposition proceeds more slowly (1–24 h to saturation) and can be terminated whenever desired signal intensity has been achieved. This difference becomes significant when attempting to assess subtle changes in the levels of gene expression across a set of experimental conditions. Standard colorimetric detection would therefore be a superior choice in such situations. The restricted dynamic range of detection seen in FISH experiments is less significant when larger differences in transcript abundance are seen across perturbations. Both approaches are at best, semiquantitative, and ideally should be supplemented

with more direct quantitative measurements of transcript abundance (Q-PCR etc.).

7. *Signal specificity and background issues*: We have occasionally observed punctate background staining throughout the embryo when performing single or multicolor FISH. This is particularly evident when the target transcript is expressed at very low levels or has a broadly distributed expression pattern. Furthermore, sea urchin embryos at late mesenchyme blastula and gastrula stages tend to exhibit such nonspecific staining more frequently than at other developmental stages. This does not appear to be related to the choice of fluor or probe conjugate, since the incidence of nonspecific staining is comparable across different fluors and with digoxigenin-, DNP-, as well as fluorescein-coupled antisense RNA probes. A modest improvement in background staining can sometimes be achieved by lowering the concentration of the specific antisense probe. We have however, not encountered this issue with colorimetric in situ hybridization. Therefore, when characterizing a novel gene expression pattern, we strongly recommend an initial developmental series using both alkaline phosphatase and fluorescence-based detection. For mRNAs expressed ubiquitously or at very low levels, FISH may not be a viable option.
8. *FISH ± immunostaining*: Immunostaining following a FISH procedure is used to detect antigens whose epitopes persist through the FISH process. SoxB1, Myc, phospho-Smad1/5/8, Sm30, and Sm50 can all be detected reliably using the protocol described above after completion of a single or dual-color FISH procedure. Typically, epitopes that require methanol-based fixation are not amenable to a combination of FISH and immunostaining as described herein. Furthermore, compared to immunofluorescent detection performed in isolation, there is a significant loss of signal intensity of the protein epitope-antibody complex when a complete FISH procedure is followed by immunostaining. Modest improvements in staining intensity can sometimes be achieved with a higher concentration of primary antibody/longer incubations in the presence of the primary antibody but must be weighed against a concomitant increase in background labeling.

Acknowledgements

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Chapter 18

A 96-Well Plate Format for Detection of Marine Zooplankton with the Sandwich Hybridization Assay

Julio B.J. Harvey

Abstract

The sandwich hybridization assay (SHA) is a ribosomal RNA (rRNA) targeted molecular method used to detect specific target organisms from diverse communities found in environmental water samples. This sensitive, robust assay is particularly useful for detecting zooplankton, including copepod grazers or reproductive propagules from broadcast spawning invertebrates. Herein, I describe the most basic application of this flexible methodology—a 96-well plate format for analysis of water samples in the laboratory. A microarray format SHA is also available and uses the same basic chemistry for remote, robotically mediated, in situ target detection. Traditionally produced only in the laboratory, preassembled SHA reagents and consumables are now also available for purchase.

Key words Sandwich hybridization assay, Molecular probes, rDNA, rRNA, Invertebrate larvae, Copepods, Invasive species detection

1 Introduction

The SHA uses two DNA probes, one for target capture and another for immunological signaling (Fig. 1). Variations on the core SHA methodology are well documented [1–5]. Capture probes are typically designed from hypervariable regions of high copy number genes such as the small or large subunits of the ribosomal DNA (rDNA). Signal probes code for highly conserved regions of target genes and are located tens to hundreds of base pairs distant from capture probe annealing sites. Target capture and signaling steps are separated spatially and temporally resulting in consistent, remarkably robust presence–absence and quantitative detection results. It is important to note that the SHA detects total target rRNA and rDNA and not absolute cell numbers [6]. Filter collected samples are lysed in a guanidine thiocyanate (GuSCN) buffer. Biotinylated capture probes anchored via streptavidin to a polystyrene solid support are then exposed to sample lysates. After rRNA or rDNA target annealing to capture probes, detection is mediated

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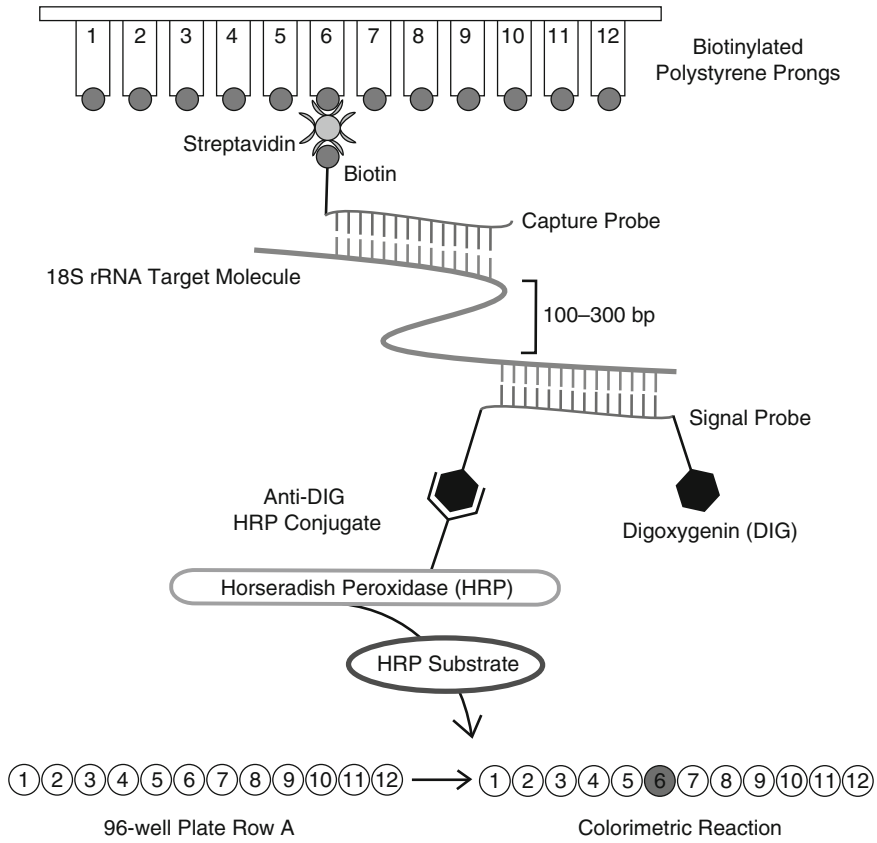


Fig. 1 Sandwich hybridization chemistry for 96-well plate assay. Biotinylated polystyrene prongs (solid support) first acquire a capture probe via the biotin-streptavidin interaction. The capture probe then acquires single-stranded target molecules present in the sample lysate solution. Subsequently, the signal probe attaches to target molecules and HRP enzyme catalyzes substrate cleavage, resulting in solution color change

by digoxigenin (dig)-labeled signal probes. The latter anneal to conserved regions of target molecules and subsequently, attach to anti-dig horseradish peroxidase conjugate, producing a colorimetric result upon substrate cleavage. The degree of enzyme kinetic response (signal strength) is spectrophotometrically quantified as optical density (OD) at 650 and 450 nm.

The 96-well plate format SHA has been successfully used to detect and monitor the progress of harmful algal bloom (HAB) species [3, 7–9] and marine bacterioplankton [10]. SHA probes for mussel, barnacle, polychaete, and invasive green crab (*Carcinus maenas*) larvae have also been developed and applied to environmental samples successfully [2, 11]. Additionally, a microarray format SHA has been extensively used on board the Environmental Sample Processor (Spyglass Biosecurity, Inc., Marina, CA), a fully automated robotic system that collects water samples and conducts the SHA in situ during month-long mooring deployments [1, 10–15]. SHA probes have also been successfully developed for major groups

of marine copepods and additional benthic invertebrate larvae (unpublished). Although the approach described below involves water samples, the SHA can theoretically be applied to any environmental sample from which genomic material can be extracted in solution.

2 Materials

All reagents are dispensed using sterile, individually packaged serological pipettes and autoclaved glassware subjected to pretreatment with RNAseZap (Applied Biosystems/Ambion, Austin, TX, USA) and four rinses with Sigma molecular reagent grade water (Sigma-Aldrich, St. Louis, MO, USA) immediately prior to use. Sigma water (or equivalent ultra-pure water) is used to make all reagents. All reagents are 0.22 μM filtered. Products and concentrations mentioned below are specific to zooplankton detection in some cases and may require modification to use with other groups of organisms. Store reagents in sterile containers at room temperature unless otherwise noted. If precipitate forms in any reagent, gentle heating to 22 °C (but <25 °C) followed by 0.22 μM filtration into a new, sterile container is recommended. Strictly adhere to proper handling, disposal, and safety guidelines when working with reagents described below. Table 1 contains supplier and pricing information for primary materials described herein.

2.1 Sample Filtration and Storage Components

1. Vacuum manifold: 3-Place PVC or stainless steel and 6-place stainless steel vacuum manifolds (Fig. 2a) are available for purchase (Millipore, Billerica, MA, USA). Multiple 3-place PVC manifolds can be connected with additional fittings (*see* **Note 1**). Alternatively, manifolds can also be constructed from widely available materials—schedule 80 PVC is recommended for its durability and low reactivity.
2. 200 mL polysulfone filter funnels with 25 mm filter holders (Pall Corporation, Ann Arbor, MI, USA): One filter funnel (Fig. 2b) is required per each manifold place. Filter funnels should be gently cleaned between samples with a bottle brush and minimal Micro-90 (Cole-Parmer, Vernon Hills, IL, USA), then rinsed thoroughly with warm tap water followed by deionized water. The above filter funnel product includes plastic filter frits necessary for proper filter installation.
3. Size #8 rubber stoppers: 1 hole (McMaster-Carr, Elmhurst, IL, USA).
4. Vacuum pump: A water-jet aspirator (Cole-Parmer) pump (Fig. 2c) or equivalent (*see* **Note 2**) and a pressure gauge with regulator capable of creating a gentle vacuum <10 in. Hg (0.033 Mpa) are highly recommended. PVC tubing: (1) 1/4″

Table 1
Materials suppliers and pricing

Equipment or reagent	Supplier	Catalogue number	Price (US $)
200 mL polysulfone filter funnels with 25 mm filter holders (total price for 3 needed)	Pall Corporation	4203	$434.00
PVC vacuum manifold 3-place	Millipore	XX2604735	$785.00
Water-jet aspirator pump	Cole-Parmer	EW-35031-00	$998.00
Pressure gauge and regulator	Cole-Parmer	EW-35031-16	$316.00
Saigene 96-well plate processor	Saigene, Inc.	–	$9,500.00
Saigene Plexiglas dust cover for plate processor	Saigene, Inc.	–	$850.00
VMAX Kinetic Microplate Reader	Molecular Devices	VMAX	$9,888.00
Evergreen 96-well microplates	Evergreen Scientific	222-8050-F1K	$84.00
Saigene 12-prong polystyrene solid support (20 pack)	Saigene, Inc.	SHA_PRG_20	$100.00
1.5 M lysis buffer, pH 9.0–1 L	Saigene, Inc.	SHA_LB1.5M_1000	$450.00
Wash buffer E—1 L	Saigene, Inc.	SHA_BE_1000	$200.00
HRP conjugate—1 L	Saigene, Inc.	SHA_CJ_1000	$2,200.00
1-Step Ultra TMB-ELISA (substrate)	Thermo fisher scientific pierce	34028	$116.00
2 M signal solution—1 L	Saigene, Inc.	SHA_SB2M_1000	$450.00
Signal solution diluent—1 L	Saigene, Inc.	SHA_SDIL_1000	$350.00
Probe assay wash—1 L	Saigene, Inc.	SHA_PAW_1000	$200.00
Streptavidin—5 mg	Thermo fisher scientific pierce	21125	$325.00
Signal probes	Oligos Etc.	–	Call for quote
Capture probes	Oligos Etc.	–	Call for quote

inside diameter, 5/8″ outside diameter, 3/16″ wall thickness and (2) 1/2″ inside diameter, 3/4″ outside diameter, 1/8″ wall thickness or equivalent (*see* **Note 3**). Quick disconnect fittings are recommended for easy separation of manifold(s) from the vacuum system if mobility is desired.

5. Filter membranes: For most zooplankton 25 mm, 5 μm Durapore PVDF disc filters or 30 μm nylon net filter membranes (Millipore) are sufficiently fine scale. Smaller organisms such as some phytoplankton and bacteria require finer scale filters. Additionally, 0.22 μm Millex 33 mm Durapore PVDF

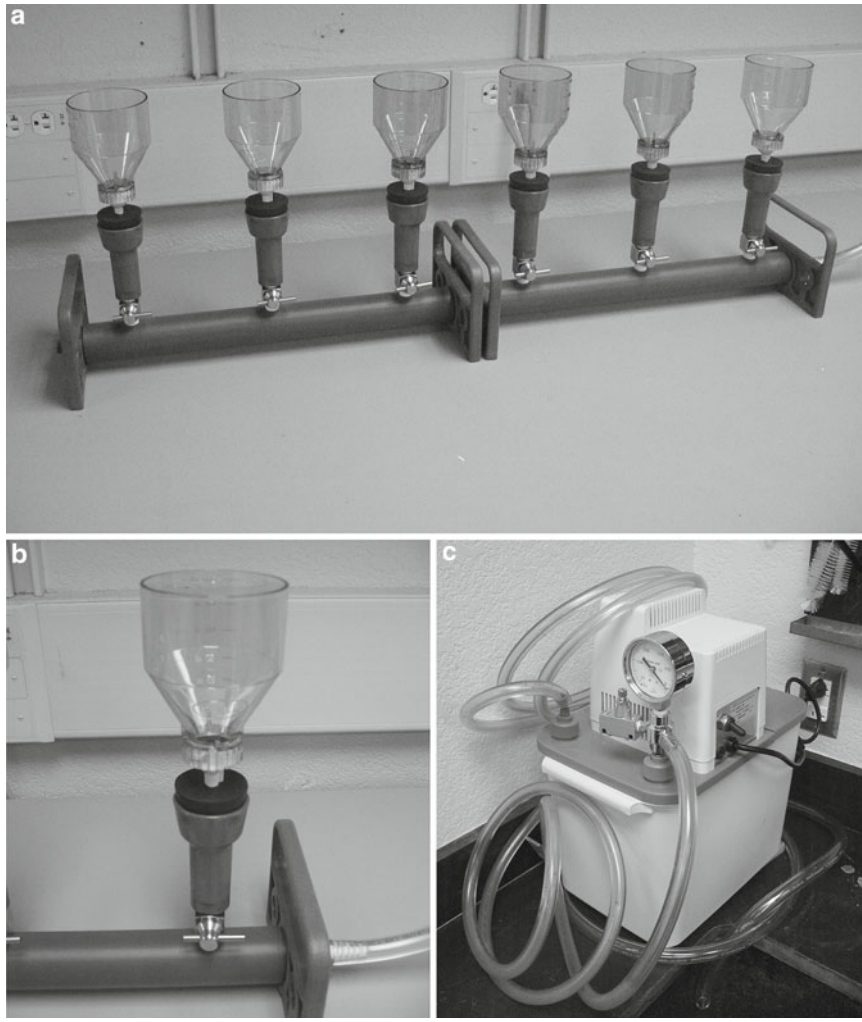


Fig. 2 Filtration equipment for water sample collection on 25 mm filters. (**a**) 6-Place PVC vacuum manifold for water sample filtration constructed by joining two 3-place manifolds together. (**b**) 200 mL polysulfone filter funnel atop a 25 mm filter holder. (**c**) Water-jet aspirator vacuum pump with pressure regulator and gauge

disposable filters (Millipore) and sterile, disposable luer lock syringes (10 mL or greater depending on sample lysate volume) are required for preparing sample lysates prior to analysis. Nalgene 2 mL screw-top cryotubes (Thermo Fisher Scientific, Rochester, NY, USA) are recommended for sample filter freezing, storage, and lysis.

6. Liquid nitrogen: An open mouth dewar (several liter volume) and a larger storage dewar are needed if samples will not be immediately processed. Sample storage must be in liquid nitrogen (–80 °C or other conditions are not recommended) or in RNAlater (Sigma-Aldrich).

2.2 Sample Lysis and 96-Well Plate Format SHA Chemistry Components

1. Guanidine thiocyanate (GuSCN) lysis buffer (Saigene Biotech, Inc., Monument, CO, USA): 1.5 M GuSCN, 50 mM Tris, 15 mM EDTA, 2 % Sarkosyl, and 0.2 % SDS (v/v) pH 8.9–9 [4, 11]. Stable up to 1 year or more at room temperature.
2. Evergreen 96-well microplates (Evergreen Scientific, Los Angeles, CA).
3. Wash buffer E (Saigene Biotech, Inc.): 50 mM Tris, 0.15 M NaCl, 0.05 % Tween-20, pH 7.6 [2, 16]. Stable up to 1 year or more at room temperature.
4. Digoxigenin antibody horseradish peroxidase (HRP) conjugate (Saigene Biotech, Inc.): ImmunoPure Peroxidase Conjugate Mouse Anti-dig IgG (Cat. No. 200-032-156; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1:400 in Guardian™ Peroxidase Conjugate Stabilizer/Diluent/Blocker (Cat. No. 37558B; Thermo Fisher Scientific Pierce, Rockford, IL, USA). Stable up to 1 year under dark, 4 °C conditions.
5. HRP Substrate (Thermo Fisher Scientific Pierce): 1-Step™ Ultra TMB-ELISA substrate for horseradish peroxidase enzyme. Stable up to 1 year under dark, 4 °C conditions.
6. Signal solution (Saigene Biotech, Inc.): 2 M GuSCN, 50 mM Tris, 10 mM EDTA, 0.5 % Tween-20, pH 8.6 [2, 4, 16]. Stable up to 1 year at room temperature. For zooplankton detection, the final signal solution concentration should be 0.5 M GuSCN.
7. Signal solution diluent (Saigene Biotech, Inc.): 50 mM Tris, 10 mM EDTA, 0.5 % Tween-20, pH 8.6 [2, 4, 16]. Stable up to 1 year at room temperature.
8. Signal probes (Oligos Etc., Wilsonville, OR, USA): Three signal probes EUK519, EUK915, and EUK1194 (Table 2) are used to create the signal solution cocktail [2, 11]. The signal probe sequence is flanked on both sides by Spacer9™ ethylene glycol backbone spacers with dig-labeled 5′ and 3′ terminal ends. Synthesis should be gel purified two times; both before and after dig labeling. The final concentration of signal probes in 0.5 M signal solution should be 100 ng/mL each probe (*see* **Note 4**). Preformulated signal solutions already containing signal probes at specified concentrations are also available (Saigene Biotech, Inc.).
9. ImmunoPure streptavidin (Thermo Fisher Scientific Pierce): dilute in Sigma water to approximately 10 mg/mL. After dilution, 10 mg/mL stock is stable for up to 1 month if parafilm sealed, under dark, 4 °C conditions.
10. Probe Assay Wash (Saigene Biotech, Inc.): 0.1 M sodium phosphate dibasic, 0.5 % Tween-20, pH 8.0. Stable for up to 1 year or more at room temperature.
11. Capture probes (Oligos Etc.): Design should be within roughly 100–250 base pairs of signal probe locations on the 18S rDNA

Table 2
Published zooplankton probes

Probe type	Name	Target organism	Reference	Constant (μg/OD)	5′–3′ probe sequence
Capture	B1066	Barnacles	[2]	28.49	GTT GCG GAT TGC TGG TCG AC
Capture	EUK338	Eukaryotes	[2]	29.79	CCT GCT GCC TTC CTT GGA TG
Capture	M2B	Mussels	[11]	26.42	AAG GGC GAA AAA CCG GGA GGT AGG T
Capture	P1022	Polychaetes	[11]	25.86	TCC AAC GAA TCG AGA AAG AGC TA
Capture	GCRAB	*Carcinus maenas*	[11]	28.53	GGT TTC ACC TTA TAT GGG CTT
Capture	OS433	*Osedax*	[11]	27.40	AGC ATC GTT AGA TAG CCC GCA TT
Signal	EUK519	Eukaryotes	[2]	28.59	CTG GAA TTA CCG CGG CTG CTG
Signal	EUK915	Eukaryotes	[2]	29.60	GTG CCC TTC CGT CAA TTC CTT
Signal	EUK1194	Eukaryotes	[2]	26.61	CAT CTA AGG GCA TCA CAG ACC

molecule [2]. The capture probe sequence has three 5′ Spacer9s™ with a biotin-labeled 5′ terminal end (*see* **Note 5**). Synthesis should be gel purified two times; both before and after dig labeling. *See* Table 2 for examples of currently published capture probes sequences for marine invertebrates.

12. Linker probes (Oligos Etc.) (optional): Linkers are synthetic oligonucleotides complementary to both capture and signal probes. Although not strictly necessary for performing SHA, linkers represent "perfect targets" and are invaluable for testing assay chemistry or diagnosing any problems that may arise [1, 2, 15].
13. Polystyrene prongs (Saigene Biotech, Inc.): Biotinylated solid support for attachment of streptavidin-labeled capture probes. Stable for up to 1 year under dark, 4 °C conditions, lint-free cloth wrapped and packaged in plastic bags with desiccant supplied by manufacturer.
14. Hydrosulfuric acid solution: A 10 % H_2SO_4 stock solution is necessary for stopping the enzymatic SHA signaling reaction after 96-well plate processing has completed. Dilute H_2SO_4 from manufacturer 1:10 with deionized water.

2.3 Specific Laboratory Equipment Required

1. Heating block (Fig. 3a): Must be capable of heating 12–13 mm outside diameter cryogenic vials to 85 °C (*see* **Note 6**).
2. A 300 μL 12-channel pipette and sterile, aerosol-free (barrier) tips.

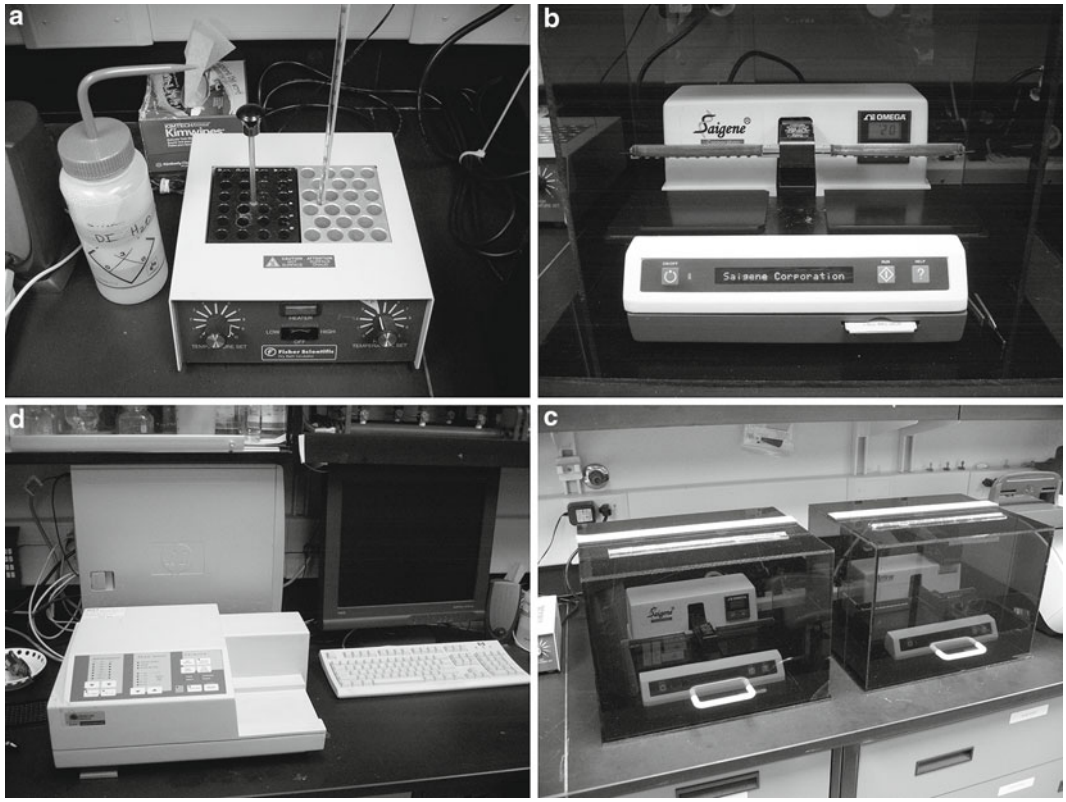


Fig. 3 Specific equipment required for 96-well SHA plate processing. (**a**) Heating block with cryogenic vial sized (12–13 mm outside diameter) wells (*right hand block*). (**b**) Saigene 96-well plate processor (*front view*). (**c**) Two Saigene plate processors with dust cover lids closed. (**d**) Microplate reader connected to a computer workstation running SOFTmax PRO data collection software

3. NanoDrop ND-1000 full spectrum UV/visible light spectrophotometer (NanoDrop, Rockland, DE, USA): Recommended for accurate measurement of DNA and protein concentrations in small (2 μL) volumes.
4. Saigene 96-well plate processor with Plexiglas dust cover (Saigene Biotech, Inc.): A bench-top device (Fig 3b, c) capable of processing up to two 96-well SHA plates simultaneously.
5. VMAX Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) or equivalent: A 96-well microplate reader (Fig. 3d) capable of making 650 and 450 nm wavelength measurements and a computer workstation running SOFTmax PRO software (Molecular Devices).

3 Methods

3.1 Sample Collection and Storage

1. Assemble vacuum manifold by first inserting each 25 mm filter holder into the center hole of a #8 stopper. It may be necessary to slightly lubricate filter holder stems with silicon-based lubricant

to facilitate installment. Insert filter holder/rubber stopper assemblies into manifold ports.

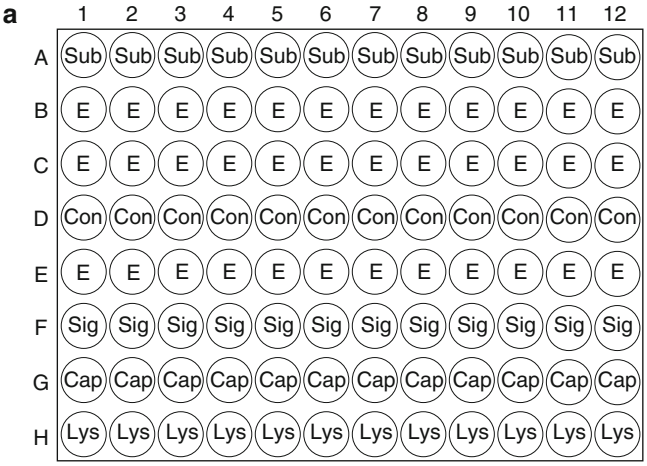
2. Assemble vacuum pump system including pressure gauge and regulator. If using a water-jet aspirator, add sufficient water to the reservoir and use tubing to connect the overflow drain valve to a sink or other drain. Prior to sample filtration, turn on pump system and adjust pressure regulator so that system vacuum pressure is just below 10 in. Hg (0.033 Mpa). Connect vacuum manifold to vacuum system with appropriate tubing and fittings.
3. Place 25 mm filters of desired porosity on filter holders, insuring that plastic filter frits are in place below them. Install 200 mL filter funnels onto filter holders, apply vacuum, and open manifold stopcocks. Apply samples to the manifold via filter funnels. Depending on sample contents, total volume, and filter porosity, multiple filters may be needed for a single sample due to filter clogging (*see* **Note 7**).
4. Immediately upon completion of sample filtration, turn stopcocks to cut vacuum pressure and gently remove filters with forceps, curl filter slightly inward, and insert each filter into a 2 mL screw-top cryogenic vial. Immediately immerse in liquid nitrogen (use proper protective equipment). Samples must remain in liquid nitrogen until analysis. Leave samples in liquid nitrogen for 3–5 min minimum before removal for lysis and SHA analysis. Typical record keeping includes filter porosity and sample volume.

3.2 96-Well SHA Plate Assembly, Sample Lysis, and Analysis

1. Resuspend 50 μg lyophilized signal and capture probes in 150 μL TE (10 mM Tris, 1 mM EDTA) and 200 μL TE, respectively. Let resuspended probes sit at room temperature for at least 10 min with occasional light vortexing (*see* **Note 8**).
2. Connect NanoDrop spectrophotometer to a computer with ND-1000 software installed, open software, and select the nucleic acid protocol. Initiate instrument as instructed with 2 μL Sigma water and make a blank measurement with TE. Set the constant field to the constant (number of μg/OD) provided on each probe synthesis data sheet. The OD constant is specific to each probe; therefore the constant field must be modified among probe measurements. Measure each probe three times, taking the average to arrive at final concentrations in ng/μL (*see* **Note 9**).
3. Dilute 2 M signal solution to 0.5 M with signal solution diluent (alternatively, make or order 0.5 M stock directly from Saigene). Determine how much signal probe must be added to arrive at a 100 ng/mL final concentration for each probe. Determine total volume of all signal probes to be added, and remove that volume before adding all signal probes to 0.5 M signal solution. Vortex on a high setting for 10 s and invert to mix.

4. Determine the approximate amount of 1 mg/mL streptavidin solution needed to achieve a 1.25 μg/mL final concentration for each capture probe solution. Use NanoDrop spectrophotometer on protein A280 setting to dilute 10 mg/mL streptavidin stock to 1 mg/mL (A280 reading = 3.44 OD) in a sufficient volume of Sigma water to make capture probe solutions while also allowing for multiple rounds of NanoDrop measurement. Continue to measure streptavidin solution, adding (with light vortex and spin down) either additional Sigma water or 10 mg/mL streptavidin stock (0.5–3 μL increments) until the average reading reaches 3.44 OD. Once diluted, 1 mg/mL streptavidin solution is only viable up to 24 h (parafilm sealed at 4 °C) and is best used within hours.
5. Determine volume of resuspended capture probe needed to arrive at a final concentration of 400 ng/mL for each capture probe solution final volume. Add capture probe to 1/2 final volume probe assay wash and mix by gentle swirling and inversion. Add 1 mg/mL streptavidin solution (to reach a final concentration of 1.25 μg/mL in 1 volume) to a second 1/2 final volume probe assay wash in another container with gentle swirling and inversion. Add the streptavidin fraction to the probe fraction by careful pouring between containers. Tightly cap final volume in a single container and invert and vortex on a high setting for 10 s. Capture probe solutions are stable under dark conditions at 4 °C for up to 1 year.
6. Assemble SHA plates (Fig. 4) by adding reagents in the following order (Fig. 4a) with a 300 μL 12-channel pipette and barrier tips (250 μL per well, all reagents): Rows B, C, and E, wash buffer E; row D, HRP conjugate; row A, HRP substrate; row F, signal solution; row G, capture probes; and row H, sample lysate (load just prior to processing). Avoid bubble formation when filling plate wells by not compressing pipette plunger beyond minimum volume setting. If filling more than one plate, estimate total reagent volumes needed in advance to facilitate dispensing (Fig. 4b). Generally, only two or three columns (replicates) contain the same capture probe, so 4–6 different capture probe (and corresponding signal probe) solutions can be loaded onto a single plate. All other reagents are identical across columns within each row. Plates can be sealed with a MiniSeal plate sealer (Porvair Sciences, Leatherhead, England) and are then stable under dark, 4 °C conditions for up to 1 year (*see* **Note 8**).
7. Turn on the Saigene plate processor, insuring first that its program card is properly installed. Adjust the temperature setting for the heated SHA plate stages to 30 °C. Just prior to plate analysis, install polystyrene solid support prongs on the Saigene plate processor's prong arm(s). Use forceps and take

a



b

Number of plates	Lysate	Wash Buffer E	Conjugate, Substrate or Signal Solution
1	3 mL	9 mL	3 mL
2	6 mL	18 mL	6 mL
3	9 mL	27 mL	9 mL
4	12 mL	36 mL	12 mL

Fig. 4 96-Well SHA plate assembly specifics. (**a**) Position of reagents in plate wells: *E* wash buffer E, *Con* HRP conjugate, *Sub* HRP substrate, *Sig* signal solution, *Cap* capture probes, and *Lys* sample lysate. (**b**) Reagent and lysate volumes needed for 1–4 SHA plates. When measuring out reagents to multichannel pipetting reservoirs, add 20–100 μL beyond listed volumes to avoid running short or introducing bubbles to plate wells

care not to contact prong tips during installation. One set of prongs should be installed for each plate to be processed (up to two plates per machine). Discard prongs after a single use. Do not leave unused prongs exposed or at room temperature, return promptly to dark, 4 °C conditions with desiccation.

8. Remove sample cryogenic vials from liquid nitrogen and allow to thaw for 1–3 min at room temperature before adding up to 2 mL of 1.5 M GuSCN lysis buffer per vial. Tighten vial caps and vortex on a high setting for 5 s. Place vials in 85 °C heating block for a total of 5 min, briefly removing at 2.5 min to vortex for 5 s. Attach a 0.22 μm Millex Durapore filter to a luer lock syringe and empty sample lysate into open syringe barrel end. Adjust total sample lysate volume to 3.5 mL per plate by adding additional lysis buffer to the syringe barrel, insert syringe plunger and push entire sample through filter into a 50 mL disposable reagent reservoir. Gently mix sample by slightly tilting reagent reservoir to move lysate back and forth.

9. Use 300 μL 12-channel pipette with barrier tips to add 250 μL sample lysate per well to row H of the SHA plate. Place each SHA plate squarely on one of the Saigene plate processor's heated stages, insuring that the SHA plate is properly centered. Press the Run button and insure that the prongs enter all wells without contacting the well sides (*see* **Note 10**). Monitor run time with the built-in countdown timer. Processing takes approximately 1 hour, not including set-up and post-run plate analysis. When processing is complete, press the Run button when prompted and wait for the prong arms to move to the home position.
10. Immediately after processing is completed, move the SHA plate(s) to the microplate reader and analyze at 650 nm. Immediately add 50 μL of 10 % H_2SO_4 to plate row A, killing the enzymatic signaling reaction. Read plate(s) at 450 nm.
11. In principle, SHA dose-response curves can be generated for any organism grown in pure culture or collected from nature [2, 3, 5, 6, 11, 15]. Examples of SHA dose-response curves generated for invertebrate larvae include barnacles, mussels, polychaete worms, and the invasive green crab, *C. maenas* [2, 11]. Dose-response curves can be used to calculate the number of individuals present per mL of lysate and subsequently per volume of water originally sampled [5]. Lower limits of detection can also be determined by comparison of positive control samples to 0.22 μm filtered seawater (FSW) negative control samples. FSW negative controls typically produce an average OD reading of 0.05 at both A650 and A450 nm when applied to the chemistry described above.

4 Notes

1. 1/4″ male NPT X 1/4″ nipple fittings can be used to join two or more manifolds together in series. It is best to use "close" fittings to eliminate additional space between manifolds once joined. Correctly apply Teflon tape to fitting threads. Prior to assembly, remove existing fittings if installed. Once fitting is threaded, simply turn adjacent manifolds in opposition (by hand) to tighten.
2. It is possible to use vacuum pumps that are not of the water-jet aspirator variety. Dry vacuum pumps require the addition of a water trap to the system so that manifold filtrate does not enter the pump. In this case, the water trap must be emptied periodically, prior to overflow.
3. If using the water-jet aspirator pump specified, 1/4″ ID tubing connects the manifold(s) to the vacuum system, and 1/2″ ID tubing connects the overflow hose barb fitting to a sink or

other drain. Other vacuum systems may have different fittings and therefore different tubing size requirements. Tubing wall thickness is important for the portion of the system under vacuum and should be sufficient to withstand vacuum pressure.

4. If ordering directly from the manufacturer, lyophilized probes should be resuspended in Sigma water, divided into 50 µg fractions among sterile 0.65 mL microcentrifuge tubes, dehydrated under vacuum in a centrifuge concentrator, and stored at −80 °C. Always remove gloves when handling lyophilized probes because static electrical charge can cause material to "jump" out of storage tubes before liquid is added.
5. Capture probes are typically 18–26 base pairs in length with a GC content of no more than 40–60 %. Test all probe designs prior to purchase using OligoTech analysis software (http://www.oligosetc.com/index.htm) or equivalent to check probe sequence stability, possible stem loop formation, and tendency to form homodimers.
6. Standard microcentrifuge tube heating blocks accommodate a tube diameter that is too narrow to accept 12–13 mm outside diameter cryogenic vials. For most heating units, replacement heating blocks that accept cryogenic vial sizes are available for purchase.
7. For example, 1.8 L seawater samples from Monterey Bay, California, usually require two or three 5 µM filters to complete filtration in 10–40 min depending on sample contents. High turbidity samples tend to slow or clog filters more quickly and require longer filtration times or additional filters. In contrast, a single 30 µM filter typically allows filtration of 1.8 L seawater in 5–10 min.
8. Alternatively, preformulated signal solution, capture probe solutions, or pre-sealed SHA plates containing all necessary reagents and custom probes made to user specifications can be purchased directly—contact Saigene, Inc. for a price quote. All reagents produced by Saigene are subjected to rigorous quality control testing, work that must otherwise be carried out by the end user.
9. Resuspended probes are stable up to 6 months in microcentrifuge tubes with parafilm sealed caps under dark, 4 °C conditions. Stored probes may experience some evaporation, and new concentration measurements should be made if more than a few days have passed.
10. Saigene plate processor prong arms (and therefore, solid support prongs, once installed) should be exactly parallel to SHA plate surfaces. Additionally, prongs should enter all plate wells the same maximum distance (about two thirds of the way from the well bottom) without touching the well sides or bottom at

any point. If these conditions are not met, the plate processor arm needs to be adjusted by a qualified technician before proceeding.

Acknowledgment

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Chapter 19

Isolation and Assessment of Signaling Proteins from Synchronized Cultures During Egg Activation and Through the Egg-to-Embryo Transition in Sea Urchins

Michelle M. Roux-Osovitz and Kathy R. Foltz

Abstract

Sea urchins are an excellent model system for investigating fertilization mechanisms and fundamental cell biological phenomenon such as release from quiescence, cell division, secretion, and basic signal transduction. The ease of gamete collection, fertilization, and culture is complemented by exquisite developmental synchronicity and the ability to carry out both large-scale biochemical studies and single-cell experiments. In particular, fertilization in echinoderms serves as a paradigm for a digital signaling event—a one-time only switch that launches the egg into the developmental pathway. Sperm-induced egg activation is dependent on the release of calcium from internal stores and subsequent effects on a myriad of cellular events such as exocytosis, cytoskeletal remodeling, and cell cycle reentry. Here we describe methods to investigate individual signaling proteins as well as global proteomic and phosphoproteomic changes involved in the initial steps of egg activation through the egg-to-embryo transition.

Key words Sea urchin, Fertilization, 2D electrophoresis, Phosphoproteins, Affinity chromatography

1 Introduction

The transition from a quiescent, unfertilized egg to an actively dividing embryo involves a myriad of cellular events collectively termed egg activation. A key mediator is calcium, which increases transiently in the egg cytosol at fertilization [1]. Most if not all of the proteins involved in egg activation are maternal and thus regulated posttranslationally by modifications such as phosphorylation or by sequestration or degradation. Therefore, organisms that are amenable to biochemical approaches coupled with ease of gamete isolation and fertilization are useful models for studying this phenomenon. Sea urchin gametes are easily obtained and in vitro fertilization is straightforward. Because fertilization and subsequent cellular changes are synchronous in a given batch, it is possible to evaluate signaling events on a rapid (seconds to minutes) time scale in a population of cells. A rich history of evaluating single proteins

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[1, 2] has recently been complemented by the ability to look at global changes in the sea urchin egg-to-embryo proteome [3, 4], made possible by the completion of the *Strongylocentrotus purpuratus* (California purple sea urchin) genome project [5]. Here we provide detailed methods for preparing protein fractions from eggs and synchronized cultures of fertilized zygotes over a rapid, tight time course. These samples can then be analyzed for enzymatic activity, protein–protein interactions, or phosphorylation state. These same preparative methods can also be used to generate samples for analysis by 2-dimensional gel electrophoresis (2DE) and subsequent protein identification via tandem mass spectrophotometry.

There are a number of concerns to take into account in terms of sample size, critical time point capture, buffer conditions to enhance a given subcellular fraction or to preserve an interaction, and stability of the protein sample, especially with regard to any posttranslational modifications. We provide detailed methods on how to prepare time course samples under non-denaturing conditions for evaluating affinity interactions with recombinant Src Homology 2 (SH2) domain proteins, co-immunoprecipitations, and immune complex Src family kinase (SFK) activity assays and for phosphoproteome-wide 2DE analyses.

An additional important consideration is whether the removal of the fertilization envelope (FE) is necessary. At fertilization, the calcium release triggers a massive exocytotic event in the egg [6]. The cortical granules fuse with the egg plasma membrane and release their contents, causing a permanent change to the egg surface—the slow, mechanical block to polyspermy. This elevated FE "hardens" after a few minutes due to cross-linking [6]. However, it is possible to prevent the hardening of the FE and to even remove the FE, though this can be a challenge to obtaining very tight time courses. In general, we have found it optimal to remove the FE and provide detailed methods for this here.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents and store at 4 °C unless indicated otherwise. For best results, use freshly made solutions and do not add sodium azide to any buffers.

2.1 Reagents Needed for Fertilization, FE Removal, and Culture

All solutions for use with living *S. purpuratus* eggs and embryos are kept and used at 12–15 °C (*see* **Note 1**).

1. Natural seawater (NSW): filter through a glass fiber filter and then a 0.22 μm filter and store in designated, detergent- and chemical-free carboys. Filter the seawater at least 12 h in advance of use and prechill to desired temperature.

2. MBL artificial seawater (ASW): 484 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$-H_2O, 29 mM $MgSO_4$-$7H_2O$, 27 mM $MgCl_2$-$6H_2O$, $NaHCO_3$, pH 8.0 with NaOH at specific experimental temperature.
3. To induce spawning: 0.55 M KCl and a 5 mL syringe fitted with a 20 G needle.
4. To prevent FE hardening: stock solution of 0.5 M 3-amino-1,2,4-triazole (ATAZ) (Sigma Aldrich, Inc; #A8056) dissolved in ASW. Just before use, dilute the stock into the desired volume of ASW to 5 mM.
5. 120 μm and 80 μm Nitex mesh (Tetko, Inc; #3-120/52 and #3-80/52) for stripping egg jelly coats and FEs, respectively. Cut the bottom out of a 50 mL orange cap tube. Secure a piece of the mesh across the bottom using a glue gun or a rubber band. Pre wet the mesh in ASW just prior to use.
6. Antibiotics for longer-term cultures: Penicillin–streptomycin (5,000 U/mL; Gibco, Inc; #15070).

2.2 Protein Preparation Buffers

All buffers should be prepared fresh and kept on ice. Protease and phosphatase inhibitors should be added just before use (*see* **Note 2**). All protein samples should be used immediately; degradation and loss of activity occur if sample are frozen and thawed for later use.

1. Lysis buffer for protein affinity interactions and immunoprecipitations (HNET): 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, 2 % Triton X-100 (10 μM Protease Inhibitor Cocktail III, 50 μM PEFABLOC SC, 0.5 mM Na_3VO_4, 1 mM NaF).
2. Lysis buffer for 2DE sample preparation (NP40 Buffer): 50 mM HEPES, pH 7.0, 150 mM NaCl, 15 mM EGTA pH 8.0, 1 % Nonidet P-40 (0.5 mM Na_3V0_4, 10 μM CalBiochem Protease Inhibitor Cocktail III, 50 μM PEFABLOC SC, 1 mM NaF, 60 mM Na β-glycerophosphate).
3. Protease inhibitors: Protease Inhibitor Cocktail III (CalBiochem, Inc; #539134) used at a final concentration of 10 μM; PEFABLOC SC (Fluka, Inc; #76307) used at a final concentration of 50 μM.
4. Phosphatase inhibitors: Sodium orthovanadate (0.5–1 mM final); NaF (0.1–1 mM final); Na β-glycerophosphate (60 mM final).
5. SDS solubilization buffer: 10 mM Tris, pH 8.0, 1 % SDS (passed through a 0.45 μm filter).
6. 2× kinase buffer (KB): 80 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 6 mM $MnCl_2$, 20 % glycerol, 2 mM DTT.
7. Kinase reaction buffer: For each reaction, dilute 0.7 μL γ^{32}P[ATP] (10 mCi/mL) in 25 μL of 1× KB.

8. 5× denaturing gel sample buffer: 10 % SDS, 20 % glycerol, 0.2 M Tris–HCl (pH 6.8), 0.05 % bromophenol blue, 12.5 % β-mercaptoethanol (added just before use).

2.3 Immunoprecipitation Reagents

1. Primary antibodies of choice.
2. Protein A-sepharose beads (Sigma, Inc; #P3391) or Protein G-sepharose beads (Sigma, Inc; #P3296), Anti-chicken IgY-agarose beads (Aves Labs, Inc; #P-1010), or specific secondary antibody of choice conjugated to beads. Before use, remove an aliquot of beads and wash several times with the buffer to be used in immunoprecipitation (here, HNET). Note approximate volume of settled beads. Final resuspension should be a 50 % slurry of beads in the buffer, kept on ice. Slurry can be stored at 4 °C for several weeks.

2.4 2DE Gel Components

1. Destreak Rehydration solution: 8 M urea, 0.5 % CHAPS, 0.01 M DTT, 2 % pH range-specific (e.g., pH 4–7) IPG buffer (added just prior to use), and 0.002 % bromophenol blue (IPG buffer pH 4–7; GE Healthcare, Inc, #17-6000-86).
2. Immobiline DryStrip: pH 4–7, 11 cm (IEF strip), (GE Healthcare, Inc; #18-1016-60). This pH range results reproducibly in the optimal resolution of the greatest number of egg proteins determined empirically [3, 4] but can be adjusted based on parameters for an individual experiment.
3. SDS equilibration buffer 1 (EQ1): 50 mM Tris–HCl pH 8.0, 6 M Urea, 30 % glycerol, 2 % SDS, and bromophenol blue containing 10 mg/mL DTT.
4. SDS equilibration buffer 2 (EQ2): 50 mM Tris–HCl pH 8.0, 6 M Urea, 30 % glycerol, 2 % SDS, and bromophenol blue 40 mg/mL iodoacetamide.
5. Criterion Tris–HCl: 8–16 % gradient gels, IPG + 1 well, 11 cm (Bio-Rad, Inc; #345-0105).
6. SDS gel running buffer (1× TGS): 25 mM Tris, pH 8.3, 192 mM glycine, 0.1 % SDS.
7. Agarose for overlaying strips: 0.05 % agarose in 1× TGS, dissolved by warming just before use; cool slightly before overlaying.
8. Gel fix solution: 50 % methanol, 10 % acetic acid.
9. ProQ Diamond stain for phosphorylated proteins (Molecular Probes/InVitrogen, Inc; #P-33300).
10. SYPRO Ruby stain for total protein (Molecular Probes/InVitrogen, Inc; #S-12000).
11. Gel destain for use with ProQ Diamond: 20 % acetonitrile, 50 mM sodium acetate, pH 4.0.
12. Gel destain for use with SYPRO Ruby stain: 10 % methanol, 7 % acetic acid.

2.5 Components for Protein Sequencing by Tandem Mass Spectrometry

1. Trypsin Gold, Mass Spectrometry Grade (Promega, Inc; #V5280).
2. Wash buffer 1 for in-gel trypsin digestion: 100 mM NH_4HCO_3.
3. Wash buffer 2 for in-gel trypsin digestion: 50 % acetonitrile/100 mM NH_4HCO_3.
4. Additional components to add to wash buffers: 45 mM DTT, 100 mM iodoacetamide, acetonitrile.
5. Additional extraction buffer: 60 % acetonitrile/0.1 % formic acid.

3 Methods

Carry out gamete collection and fertilization procedures at 12–15 °C unless otherwise indicated. All other procedures are at 4 °C unless otherwise indicated.

3.1 Preparation of Eggs and Synchronized Cultures

3.1.1 Obtaining and Washing Gametes

1. Rinse the adult urchin free of any debris using seawater. Use a 5 mL syringe fitted with a 20 G needle to inject ~1 mL of 0.55 M KCl directly into the coelomic cavity around the soft part of Aristotle's lantern (the mouth). Set the animal right side up until gametes begin to exude. Most species of sea urchin are not possible to sex by visual inspection of the adult animals. View the gametes as they are released from the gonadopores; eggs are yellow–orange and semen is white. The five gonadopores are located on the aboral surface (the top of the animal).
2. Place male urchin's aboral side up on a tray of ice. Collect sperm "dry" by pipetting it directly from the gonadopores as the semen is exuded. Use a pipettor with a yellow tip that has had the end cut off to make it wider. Put the semen in microcentrifuge tubes and keep on ice. Do not mix sperm from different individuals and do not dilute with seawater.
3. Invert spawning female sea urchin onto a beaker of NSW in a cool room (12–16 °C). Ensure that the gonadopores are immersed in seawater. Allow the eggs to fall into the beaker and settle. Note the approximate volume of settled eggs when the female has spawned completely. Transfer the eggs gently into a large glass beaker, and add a large (~500×) volume of filtered NSW. ASW can also be used for the initial collection and first wash of the eggs. Allow the eggs to settle again by gravity. Decant the NSW (by pouring or pipetting) and gently resuspend eggs in a large volume of ASW using a paddle. Allow the eggs to settle by gravity and decant.
4. Resuspend the eggs in ASW as a 10 % vol–vol suspension. Passage the egg suspension eight times through Nitex mesh

(120 μm for *S. purpuratus*). Allow eggs to settle by gravity, collect the jelly water using a pipette, and save it in a beaker or 50 mL tube at 12–16 °C.

5. Wash the dejellied eggs twice more by gravity settling in large volumes of ASW. Eggs will settle faster once dejellied.

*3.1.2 Fertilization Procedure (See **Note 3**)*

1. Decant the ASW and remove as much ASW as possible using a pipette or aspirator.
2. Resuspend the eggs in 500× volume ASW supplemented with 5 mM ATAZ. Allow the eggs to settle and remove as much ASW/ATAZ as possible.
3. Resuspend the eggs in ASW/5 mM ATAZ as a 10 % vol–vol suspension.
4. Set aside a sample of eggs as the "unfertilized" sample.
5. For each 100 mL of egg suspension, prepare 50 mL of jelly water + 50 mL ASW/10 mM ATAZ in a beaker. Dilute 20 μL of dry sperm into this, and mix gently with a paddle to disperse the sperm. Immediately pour this into the egg suspension and mix gently with a paddle. Set aside a small aliquot to be used for viewing FE elevation and early development later.
6. View a drop of the egg suspension under the microscope; when >90 % of the eggs appear to have elevated FEs (typically less than 1 min), passage them quickly through 80 μm mesh 4–6 times to strip away the "soft" FE.
7. If a long-term culture is desired, immediately dilute the fertilized eggs in a very large volume of ASW. Wash the fertilized eggs twice with large volumes of ASW using gravity settling to remove excess sperm.

3.1.3 Setting the Culture

1. Resuspend the washed, fertilized eggs in a final volume of ASW (or NSW) to give a 0.5–1 % (vol–vol) suspension.
2. Add penicillin–streptomycin for a final concentration of 5 U/mL (usually, a 1:1,000 dilution of the stock antibiotic).
3. Gently transfer the eggs to the culture dish and incubate at 12–16 °C. Monitor closely. First cell division typically will occur within 90 min of insemination and then roughly every 30 min thereafter until early blastula stage.

3.2 Small-Scale Isolation of Protein Samples Over a Tight Time Course

Follow the fertilization procedure as outlined above (Subheading 3.1; *see* **Note 3**). Pre-label and prechill all of the tubes for sample collection. A small mini centrifuge with pulse spin capability (such as a Fisher Scientific mini centrifuge, #05-090-128) is desirable for quickly collecting eggs. Have multiple pipettors available (with desired volume preset) and a ready supply of tips. For very rapid, tight time course sample preparation (secs to mins

post sperm addition), it is not feasible to strip the softened FEs from the fertilized eggs prior to solubilization (*see* **Note 4**).

1. For each time point to be collected, prepare 2 microcentrifuge tubes and a 1 mL tuberculin syringe fitted with a 27.5 G needle. Preload the syringes with 250 μL of lysis buffer (HNET). Keep on ice.
2. Transfer 1.5 mL of unfertilized egg suspension to one tube and pulse spin at 250–500 × *g* to collect eggs. Quickly remove the ASW using a pipette or an aspirator, and use the syringe to quickly resuspend and lyse the eggs. Triturate the sample to lyse the eggs, avoiding air bubbles. Keep on ice.
3. Fertilize the remainder (or a portion of) of the sample, starting a timer as soon as sperm are added to eggs (*see* **Notes 4** and **5**).
4. Repeat **step 2** for each time point sample, working as quickly as possible. The "time point" is the time of lysis, so collect and centrifuge the sample in advance, gauging how long it is taking to centrifuge and prepare the sample for lysis.
5. After the last time point is taken and solubilized, allow all samples to sit on ice for an additional 10 min.
6. Centrifuge all of the samples at maximum speed in a refrigerated microcentrifuge (4 °C) for 30 min.
7. Carefully transfer the supernatant ("total soluble protein lysate") to a new tube, prechilled on ice. The pellets ("insoluble") may be saved and solubilized in SDS buffer.
8. Determine the total protein concentration of each soluble lysate sample by desired method (*see* **Note 6**). Using the volumes recommended here, each sample should be roughly 50–200 mg/mL total soluble protein. This lysate can then be diluted in the appropriate buffer to the desired protein concentration for further analysis.

3.3 Large-Scale Isolation of Protein Samples Over a Broader Time Course

Follow the fertilization procedure as outlined above (Subheading 3.1; *see* **Note 3**). Pre-label and prechill all of the tubes and syringes for sample collection. A tabletop centrifuge (such as a Beckman RC50 or an old-style hand-crank centrifuge) is desirable for quickly collecting eggs. Have multiple pipettors available (with desired volume preset) and a ready supply of tips. Buffers (HNET or NP40 depending on the planned use) should be prechilled on ice and all inhibitors added just before starting the time point collections.

1. For each time point to be collected, prepare a 15 mL polypropylene tube and a 5 mL syringe fitted with a 27.5 G needle. Keep on ice to prechill.
2. Transfer 10 mL of egg suspension to one tube, and centrifuge at 250–500 × *g* for 1 min to collect eggs. Quickly remove the

ASW using an aspirator. Resuspend the eggs in 2 mL ice-cold lysis buffer using a pipette tip (*see* **Note 7**). Passage the sample through a 27.5 G needle fitted on a 5 mL syringe to lyse the eggs. Triturate the sample, avoiding air bubbles. Keep on ice. This is the "unfertilized sample."

3. Fertilize the remainder (or a portion) of the sample, starting a timer as soon as sperm are added to eggs. If feasible (depending on desired time points), remove softened FEs from fertilized eggs prior to taking samples (*see* **Notes 4** and **5**).
4. Repeat **step 2** for each sample, working as quickly as possible. The "time point" is the time of lysis, so collect and centrifuge the sample in advance, gauging how long it is taking to centrifuge and prepare the sample for lysis.
5. After the last time point is taken, allow all samples to sit on ice for an additional 10 min.
6. Aliquot the samples to pre-labeled and prechilled microfuge tubes on ice.
7. Centrifuge the samples at maximum speed in a refrigerated microcentrifuge (4 °C) for 30 min.
8. Carefully transfer the supernatants ("total soluble protein lysate") to new tubes, prechilled on ice. The pellets ("insoluble") may be saved and solubilized in SDS buffer.
9. Determine the total protein concentration of each soluble lysate sample by desired method (*see* **Note 6**). Each sample should be roughly 50–200 mg/mL total soluble protein (*see* **Note 7**). This lysate can then be diluted in the appropriate buffer to the desired protein concentration for further analysis.

3.4 Protein Affinity Interactions Using Recombinant SH2 Proteins as Bait

Consider controls carefully (*see* **Note 8**). The procedure described here has been optimized for recombinant SFK SH2 domain proteins prepared as N-terminal GST fusions but also works well with His-tagged SH2 domains [7].

1. Each affinity interaction (pull down) will use 1 mL of protein lysate adjusted to a concentration of 1 mg/mL in HNET buffer. Aliquot the lysate into 1.5 mL microcentrifuge tubes on ice. Be sure to save enough lysate for later assessment as "total input."
2. Add the appropriate volume of fusion protein on beads to give 5 μg of fusion protein to each sample (*see* **Note 9**).
3. Mix well and place on a wheel or rotator at 4 °C for 2–12 h. Timing may have to be optimized depending on the bait protein and conditions.
4. Centrifuge the samples at 6,000 × *g* for 1 min in a refrigerated microfuge (4 °C).

5. Carefully remove the unbound supernatant and transfer it to appropriately labeled tubes on ice (if it is to be analyzed) or discard it.
6. Add 1 mL of ice-cold lysis (HNET) buffer to each tube. Mix well, so beads are resuspended completely. Place back on the wheel or rotator for 10–15 min at 4 °C.
7. Repeat **steps 4–6**.
8. Do two additional washes, simply resuspending the beads and then centrifuging immediately (*see* **Note 10**).
9. After the final wash and removal of supernatant, pulse spin the samples in the microcentrifuge. Using a thin, elongated gel-loading pipette tip, remove as much buffer as possible from the bead bed.
10. Add 1 bead volume of 2× gel sample buffer and mix gently by flicking. Heat at 95 °C for 5 min. Pulse spin and load on a denaturing SDS polyacrylamide gel immediately, leaving behind the beads on the bottom of the tube.

3.5 Immunoprecipitations

1. For each immunoprecipitation (IP), use between 0.25 and 1 mL of soluble protein lysate adjusted to 1 mg/mL protein concentration. Aliquot in microcentrifuge tubes on ice.
2. Add the primary antibody to each sample and mix gently (*see* **Note 11**).
3. Incubate on a wheel or platform rocker at 4 °C for 4–16 h (*see* **Note 12**).
4. Pulse spin to remove liquid from cap of tube and place samples on ice.
5. Add 10 μL of a 50 % slurry of Protein A-sepharose to each sample and mix gently (*see* **Note 11**).
6. Incubate on a wheel or platform rocker at 4 °C for 2–4 h.
7. Centrifuge the samples at 6,000 × *g* for 1 min in a refrigerated microfuge (4 °C) to collect the beads.
8. Carefully remove the unbound supernatant and transfer it to appropriately labeled tubes on ice (if it is to be analyzed) or discard it.
9. Add 1 mL of lysis (HNET) buffer to each tube. Mix well, so beads are resuspended. Place back on the wheel or rotator for 10–15 min at 4 °C.
10. Repeat **steps 7–9**.
11. Do two additional washes, simply resuspending the beads in HNET and then centrifuging immediately.
12. After the final wash and removal of supernatant, pulse spin the samples in the microcentrifuge. Using a thin bore, elongated

gel-loading pipette tip, remove as much buffer as possible from the bead bed.

13. If samples are to be analyzed on gels and by subsequent immunoblotting, add 1 bead volume of 2× gel sample buffer and mix gently by flicking. Heat at 95 °C for 5 min. Pulse spin and load on gel immediately, leaving the beads in the tube. If samples are to be subjected to kinase assays, keep washed IPs on ice and proceed immediately to Subheading 3.6, below.

3.6 Immune Complex Kinase Assays

1. Prepare and wash immunoprecipitates (IPs) as in Subheading 3.5. Remove as much buffer as possible from the IPs using a thin bore gel-loading tip.
2. Wash each IP in ice-cold 0.5 mL 1× kinase buffer (KB; no ATP or substrate). Remove as much buffer as possible with gel-loading tip.
3. Resuspend each IP in 25 μL of 1× KB containing 0.7 μCi of γ-^{32}P[ATP] on ice. If using exogenous substrate, add this last. Mix gently and pulse spin (*see* **Note 13**).
4. Incubate at room temperature (or up to 30 °C) for 15 min (the temperature and time of incubation may need to be optimized; for echinoderm SFKs, 16–20 °C for 15 min typically is optimal).
5. Add 10 μL of 4× Laemmli gel sample buffer, mix, and heat at 95 °C for 5–10 min. Pulse spin and load on gels immediately (*see* **Note 14**).

3.7 2DE

The following 2DE protocol was designed to obtain and analyze the maximum resolvable components of the entire sea urchin egg proteome and phosphoproteome [3, 4]. Special consideration should be taken and adjustment made according to specifics of individual research questions. Many parameters can be altered including lysis buffer composition, pH range and profile of isoelectric focusing, and pH range and resolution of gels. All of these parameters can be easily adapted to the protocol outlined here. Typically, a minimum of four gels should be run for each sample:

1. For each time point sample to be analyzed, denature 100 μg NP40-soluble protein in Destreak Rehydration solution (including 2 % IPG pH specific buffer) to a final volume of 200 μL. Incubate for 30 min at 25 °C with occasional vortexing in order to reduce protein aggregates.
2. Immediately place each sample in a re-swelling tray, and carefully overlay with the 11 cm pH 4–7 IEF strip (*see* **Note 15**). Allow to rehydrate overnight at 25 °C.
3. Separate proteins in the first dimension by isoelectric point. For 11 cm IEF strips pH 4–7, the following program works well:

300 V 0:01 h, 3,500 V 1:30 h, 3,500 V 3:00 h (*see* **Note 16**). Samples here were focused using a Multiphor II flatbed (GE Healthcare, Inc) with these additional parameters: set 2 mA and 5 W total and run in gradient mode with current check turned OFF (*see* **Note 17**).

4. Prepare the EQ1 and EQ2 buffers, allowing enough time for the reducing agents to dissolve (do not heat).
5. Immediately after IEF is complete, the strips should be equilibrated for the second dimension. If absolutely necessary, the IEF strips can be stored in plastic conical tubes at –80 °C, but this is not recommended. Carefully remove IEF strips from focusing tray and place gel side up in a clean re-swelling tray or other clean tray of choice. Wash the focused IEF strips for 15 min at 25 °C with gentle agitation in EQ1 (use enough to cover strip in tray).
6. Wash the strips an additional 15 min in the same volume of EQ2 (*see* **Note 18**).
7. Separate the focused proteins in the IEF strips based on size in the second dimension by SDS PAGE using precast Criterion Tris–HCl 8–16 % gradient gels, IPG+1 well, 11 cm. If using the Dodeca Cell from Bio-Rad, prepare 7 L gel running buffer (1× TGS) and prechill to 4 °C prior to running the second dimension. Remove IEF strips from the re-swelling tray, dip them in cold 1× TGS, and place carefully in the Criterion IEF well with gel side facing forward. Overlay the strips with warm 0.05 % agarose in 1× TGS, being sure to avoid introducing bubbles. Run until dye front reaches end of gel. Again, all samples should be run in quadruplicate, and each dimension should be run simultaneously for each set to maximize reproducibility (*see* **Note 19**).
8. Remove gels from cases and place each one directly into 100 mL gel fix solution. Cover and incubate for 10–16 h with gentle agitation at 25 °C (*see* **Note 20**).
9. If phosphorylated proteins are to be analyzed, stain the gels first with ProQ Diamond stain using a volume equivalent to 10× volume of the gel. Incubate in the dark with gentle agitation for 90 min (do not stain overnight). To destain, carefully transfer the gel into a new container and incubate with 100 mL ProQ destain for 30 min in the dark with gentle agitation. Repeat destain step twice more (90 min total). Wash gels twice with ultrapure water for 5 min with gentle agitation. Replace water with fix solution. Image immediately always protecting gels from light (see below).
10. To stain for total protein using SYPRO Ruby, either skip the ProQ stain (**step 9**, above) or the ProQ gels can be subsequently stained with SYPRO Ruby after imaging them.

If staining gels directly following the 2D SDS gel run, incubate the gels in 100 mL fix solution in the dark for 30 min with gentle agitation. Repeat for a total of 60 min. Fixed gels can be stained with 60 mL SYPRO Ruby in the dark overnight. Alternatively, imaged ProQ stained gels can be placed directly into SYPRO Ruby stain and incubated overnight in the dark with gentle agitation at 25 °C.

11. Transfer SYPRO stained gels into a clean container, and destain with 100 mL 10 % methanol, 7 % acetic acid for 30 min in the dark with gentle agitation.
12. Immediately image the gels (*see* **Note 21**) using a Bio-Rad FX scanner with a ~600 nm band pass emission filter taking care to always protect gels from light. If using other imaging platforms, follow instrument specific instructions available from the manufacturer of the stains (Molecular Probes/InVitrogen, Inc).

3.8 Protein Spot Analysis, Isolation, and Identification

Image analysis, spot isolation, and protein identification will vary greatly depending on availability of platforms and software being used. The following provides one example of analysis using PDQuest (version 7.4) for spot detection and gel excision using the ProteomeWorks station (Bio-Rad, Inc) and quadrupole time-of-flight tandem mass spectrometry (Q-ToF MS/MS). Investigators are encouraged to explore all options and utilize a combination that is feasible given resources and will most effectively answer the research question being posed.

3.8.1 Image Analysis

1. Import images into PDQuest (version 7.4, Bio-Rad, Inc). Detect spots using the following filter parameters within PDQuest; noise smoothing filter-median 7×7, floating ball background detection filter set at default 21–27, vertical streak filter 43, and horizontal streak filter 53. Sensitivity should be set at 102.01 with a minimum size scale =5, minimum peak =63, and speckle detection =100.
2. Normalize matched spot quantities to total pixel density in respective gel images (reported in units = PPM). Organize images within PDQuest as "match sets." Group replicate ($n=4$) treatments and individually reconcile spots by eye within each group (very laborious, but necessary), then analyze for changes between treatment groups by establishing analysis sets.
3. Analysis sets should include the following: qualitative presence/absence and quantitative ≥ 1.5 increase/decrease. At least three of four gels must contain the spot meeting these criteria to be included for further analysis. For ProQ Diamond-stained gels, all spots must also be detected and matched in SYPRO Ruby image replicate groups in order to be successfully excised.
4. Perform quantitative analysis of selected spots as a confirmation of analysis set detection and visual determination of protein

dynamics. Measure relative spot intensity in PPM and calculate the coefficient of variance (CV) within each replicate group. Only spots whose replicate group CV is <30 % should be considered for quantitative differential detection analysis. The CV threshold is determined based on the combined amount of error induced by experimental variance and PDQuest software [8]. Quantitative differences between replicate groups are then calculated using Excel for both SYPRO Ruby and ProQ Diamond images. Set a threshold at ≥1.5 fold. Proteins reported having differential phosphorylation must not show a significant change in total protein detection using SYPRO Ruby, ensuring selection based on posttranslational modification and not protein abundance.

3.8.2 Isolation of Selected Proteins

1. Select proteins based on gel analysis (see above) and excise them using the ProteomeWorks robotic spot cutter and PDQuest Basic Excision Software (*see* **Note 22**).
2. Excised gel pieces will be in the bottom of microfuge tube. Wash the gel pieces for 20 min in 500 µL of 100 mM NH_4HCO_3 with gentle agitation. Discard the wash.
3. Add 150 µL of 100 mM NH_4HCO_3 and 10 µL of 45 mM DTT. Incubate at 50 °C for 15 min.
4. Cool to room temperature and add 10 µL of 100 mM iodoacetamide, and incubate for 15 min in the dark at room temperature.
5. Discard the solvent and wash the gel slice in 500 µL of 50 % acetonitrile/100 mM NH_4HCO_3 with gentle agitation for 20 min. Discard the solvent.
6. Add 50 µL of acetonitrile. After 10–15 min, remove the solvent and dry the gel pieces in a speed vac to remove excess acetonitrile.
7. Re-swell the gel pieces in 10 µL of 25 mM NH_4HCO_3 containing Promega Trypsin Gold (mass spectrometry grade) following the manufacturer's protocol to determine the amount of enzyme to add (if the amount of protein in the gel slices is not known, add 10–20 µL of 0.1–0.2 µg of trypsin in 10 µL of 25 mM NH_4HCO_3). After 10–15 min, add 10–20 µL of 25 mM NH_4HCO_3 to cover the gel pieces. Incubate overnight (8 h or more) at 37 °C.
8. Remove the supernatant (containing peptides) and transfer to a new microcentrifuge tube. Extract remaining peptides from the gel pieces twice with 50 µL of 60 % acetonitrile/0.1 % formic acid for 20 min. Add these extracts to appropriate tubes containing the initial supernatant of the sample. Speed vac to dry.

3.8.3 Identification of Excised Proteins by Quadrupole Time-of-Flight Tandem Mass Spectrometry (Q-ToF MS/MS)

1. Subject the trypsin-digested peptides to Q-ToF MS/MS.
2. Analyze peptide spectra using, for example, ProteinLynx Global Server (PLGS) software (Waters, Inc).
3. Design PLGS workflows to search the sea urchin genome on the NCBI database.
4. Assign putative protein identities based on the best hit assessed by E-value, and then cross-check by tBLASTn analysis (www.ncbi.nlm.nih.gov/BLAST/) and by BLASTp against the *S. purpuratus* gene predictions on Spbase (http://www.spbase.org/SpBase/).
5. Gene expression of each protein identified should be evaluated by manual inspection of the embryonic tiling array data to confirm expression [9]. Proteins selected for quantitative analysis reported should have at least two nonoverlapping peptide matches.

4 Notes

1. All glassware for use with gametes and embryos should be detergent- and chemical-free and kept separate from other glassware. Glassware should be kept clean using water and a paper towel to wipe the inside. Periodic treatment of the glassware with mild acid (such as 10 % nitric acid) followed by extensive rinsing in deionized and then ultrapure water aids in keeping the glassware clean. Finally, precool the glassware and then rinse the glassware in seawater of the desired temperature just prior to use. Large pyrex glass baking dishes are useful for large cultures. For smaller cultures, glass finger bowls work well. Seawaters and 0.55 M KCl solution can be stored at 12–15 °C for several weeks. *S. purpuratus* adults are gravid in the winter months; the methods described here can be readily adapted to other species of sea urchin (such as *Lytechinus pictus*) and to Asteroidea sea stars [10].
2. All buffers are used with the protease inhibitors listed in Subheading 2.2; phosphatase inhibitors also are included in these protocols, which are optimized for preserving phosphoepitopes. However, use of particular phosphatase inhibitors may be varied. All inhibitors should be added to buffer aliquots just before use. We have found it useful to prepare concentrated stock solutions (100–1,000×) of inhibitors, aliquoted and stored at −20 °C. For commercial stock solutions, aliquot after the first use. Avoid freeze thawing. In particular, a stock solution of 100 mM sodium orthovanadate should be prepared by dissolving in water and adjusting the pH with NaOH to pH 10. The color of the solution will be yellow. Heat in a

boiling water bath until solution clears. Cool to room temperature and readjust to pH 10. Use immediately or aliquot and freeze.

3. Prior to carrying out the fertilization procedure, determine the time points that are to be collected. All tubes, buffers, and centrifuges should be prepared in advance. This fertilization protocol is the basic one, designed to ensure synchronous fertilization. If <98 % fertilization occurs (as assessed by FE elevation), do not continue; start over with a new batch of eggs.
4. For extremely tight time courses over the first few min after sperm addition, it is not feasible to strip the FEs as this requires too much time. For short time courses, in fact, it is not even necessary to prevent FE hardening as the eggs will be lysed before the hardening occurs (we do, however, recommend stripping FEs whenever possible). For very tight time courses, multiple people are required to manipulate the samples. One way that has worked well in our laboratory is to designate an individual to collect the time point (place the sample into the tube and centrifuge), a second individual to pulse centrifuge and remove the overlying seawater, and a third individual to deliver the lysis buffer and triturate the sample. Having more than one person to carry out this last step can be very helpful. As eggs lyse, the color transitions from a milky orange to a clear, bright orange.
5. Always retain a small portion of the fertilized eggs so that the embryos can be monitored for normal cell division and early development. Follow the procedure outlined in the Methods section for setting the culture.
6. We typically use the BCA Protein Determination Assay (Pierce; #23225), setting a BSA standard curve and blanking with the lysis buffer being used (containing the protease and phosphatase inhibitors).
7. When preparing large-scale samples for 2DE analyses, samples can be resuspended and lysed in a slightly larger volume of lysis buffer. Roughly twice the volume of the egg pellet typically suffices to yield final soluble protein concentrations in the range of 10–50 mg/mL.
8. Always include a negative control. GST protein alone is a minimal negative control, but a GST fusion protein that is "inactive" or related to the experimental protein is better. For example, the Src superfamily of kinases includes ABL, which can serve as a negative control. The SH2 domain of the Abl kinase has no effect on Ca^{2+} release in sea urchin eggs, while specific SFK SH2 domains have strong inhibitory effect; similarly, the proteins that associate with the Abl SH2 domain are distinct from those that associate with those of the SFKs required for

sperm-induced Ca^{2+} release [7]. A further negative control that helps ensure specificity is use of a point-mutated, non-functional SH2 domain protein [11, 12]. If background is a problem, a preclearing step with GST alone or the control SH2 domain beads can be helpful.

9. Each affinity interaction requires 5 μg of fusion protein on sepharose or agarose beads. The protein concentration on the beads is one of the most critical aspects of the pull down. Too much protein per bead, the washes will be difficult and the interaction will not be efficient. Typically, adding at least 10 μL of beads to the reaction (50 μL is better) is required for pipetting ease. This necessitates optimizing the fusion protein expression and capture procedure. This will vary somewhat with each specific fusion protein. In our experience, most echinoderm SFK SH2 domain proteins express well in bacteria; adding 1.5 mL of a 50 % GT-bead slurry to the soluble lysate prepared from a 50 mL culture of bacteria captures roughly 1–5 μg of fusion protein per 10 μL of beads. In addition, most SH2 fusion protein beads can be stored at 4 °C for up to a week before use [7].
10. If high background is a problem, additional washes or adjustment to the components of the wash buffer (increasing salt or detergent concentration) may be required.
11. Follow the manufacturers' recommendations when using commercially available antibodies, unless it has been empirically determined that different amounts will work. It is important to avoid adding too much antibodies (generally no greater than 10 μg/mL) because the antibody can precipitate on its own, problems with background can arise, and quantitatively recovering the IgG can be problematic. Be sure to include controls (such as no antibody, pre-immune antibody, neutralized antibody, or a positive control antibody). The antibody capture step method will be dictated by your primary antibody. For most polyclonal antibodies raised in rabbit or mouse, Protein A or Protein G conjugated to Sepharose works well. Other antibodies, such as those raised in chickens (IgY) will necessitate a different secondary antibody conjugate. Please *see* [13] for excellent considerations of optimizing IPs.
12. The incubation time and temperature may need to be optimized. In our experience, we have had the best luck in terms of specificity with longer incubations at cooler temperatures.
13. We have found that using "driver free" (no unlabeled MgATP in the reaction) kinase assays [14, 15] increases the sensitivity in immune complex kinase assays for the egg SFKs. However, if using recombinant kinases and substrates in vitro, the 2× kinase reaction buffer can contain up to 200 μM MgATP.

14. If labeled proteins or added substrates are to be analyzed by autoradiography or phosphorimaging, follow this step. However, if exogenously added substrate is to be analyzed by other methods, follow the manufacturers' suggested protocol for substrate analysis.
15. Make sure the rehydration tray is cleaned with detergent, dried, and leveled. Load samples into the center of the well, with care not to introduce bubbles. Lay the strip gel facedown into the solution. Do not introduce bubbles, as this will lead to uneven rehydration. Gently overlay with mineral oil and leave overnight at room temperature to rehydrate. It is very important not to heat the sample over 37 °C. The high content of urea (6 M) in the rehydration buffer can cause carbamylation at elevated temperatures.
16. Be aware that IEF programs are often reported as times or kV hours. Optimization of focusing can be achieved by changing the last step in the focusing program. This will help to increase resolution and decrease smearing or streaking across the gel. A clean apparatus, careful technique, and uniform cooling of the flatbed will also ensure good focusing.
17. The dye front will run toward the anode. Always check this after 5–10 min into the run to ensure proper electrode connections. The power supply can be paused at any time during a run for any necessary adjustments. When running shorter strips, the maximum voltage will not be reached due to limits of conductivity.
18. Throughout all 2DE experimental procedures, it is crucial to strictly adhere to time of washes and incubations to ensure consistency between replicates and experiments.
19. Use of commercially available precast gels is not necessary but is recommended to ensure consistent results between gels and experiments. We have found the use of precast gels to be cost-effective with respect to both materials and time.
20. Fixed gels can be stored for extended periods of time (6 months +) prior to being stained. Use an airtight container and store in the dark. Staining containers must be polypropylene or polycarbonate; these high-density plastics adsorb minimal amounts of the dye (examples include Servin' Saver® and Stain Shield® containers from Rubbermaid). For best results, use containers dedicated for SYPRO® Ruby dye gel staining to minimize dye cross-contamination or other artifacts.
21. Following destaining of either ProQ or SYPRO Ruby stains, rinse the gels in ultrapure water for 2–5 min just before imaging. This will help prevent corrosion of the imaging instrumentation.

22. The number of spots excised from gels used to yield peptides will depend on relative protein abundance and the specific mass spectrometry technique used. In our experiments, we collected from a minimum of four gels over multiple experiments [3, 4].

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Chapter 20

Tools for Sea Urchin Genomic Analysis

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Abstract

The Sea Urchin Genome Project Web site, SpBase (http://SpBase.org), in association with a suite of publicly available sequence comparison tools provides a platform from which to analyze genes and genomic sequences of sea urchin. This information system is specifically designed to support laboratory bench studies in cell and molecular biology. In particular these tools and datasets have supported the description of the gene regulatory networks of the purple sea urchin *S. purpuratus*. This chapter details methods to undertake in the first steps to find genes and noncoding regulatory sequences for further analysis.

Key words Sea urchin, *Strongylocentrotus purpuratus*, Genome, Gene regulatory network, Cis-regulatory module

1 Introduction

Access to genomic information through a Web-based system has become an indispensible tool for laboratory research designed to reveal the molecular mechanisms that are the focus of studies in cell and developmental biology. Each new high-throughput technique, whether for genome and transcriptome sequencing or measurement of transcript abundance, adds exponentially to the data bits that need to be organized, analyzed, and archived in a manner amenable to human observation. A specific Web system (http://SpBase.org) for echinoderm genomics in general and purple sea urchin (*Strongylocentrotus purpuratus*) in particular fills this role [1].

Due in part to the ease of gene transfer and the broad base of experimental embryology at both the cell and molecular levels, the sea urchin system has proven to be an ideal platform to describe the gene regulatory networks (GRN) that control developmental processes [2]. Cis-regulatory elements that are hard-wired in the genome are the primary building blocks of GRNs and the sites through which regulatory genes that make up networks interact. A variety of sequence analysis and display tools are needed to

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accomplish these studies [3]. Here we will briefly describe the data types available and the tools to use them from the viewpoint of SpBase.

2 Sea Urchin Genome Data Resources (Materials)

1. The California purple sea urchin, *Strongylocentrotus purpuratus* (Sp), was the first echinoderm selected for sequencing in the early 2000s and the genome was completed in 2006 [4]. The genome of *S. purpuratus* is fairly well characterized for a non-chordate deuterostome. There are 42 chromosomes [5] and the mass of the diploid genome is 1.8 pg [6]. This corresponds to about 800 Mb of sequence. A complement of about 23,300 protein-coding genes was estimated from gene predictions and EST comparisons [4].
2. At the time of this writing, the newest sequences of echinoderm genomes and transcriptomes are derived from next-generation sequencing protocols and they continue to increase exponentially. Several additional sequencing rounds have provided additional information to significantly improve the assembly of the reference species, *S. purpuratus*. NIH has approved the genome sequencing of three additional species of echinoderms by the Human Genome Sequencing Center, Baylor College of Medicine (BCM-HGSC), in order to perfect new short read methods for the highly polymorphic genomes such as those possessed by these animals. The HGSC continues to sequence echinoderm genomes and transcriptomes for the following species: *Strongylocentrotus purpuratus*, *S. franciscanus*, *Allocentrotus fragilis*, *Lytechinus variegatus*, *Eucidaris tribuloides*, and *Patiria miniata* (*see* Table 1). These genome sequence resources are be added to SpBase as they come available.
3. There are a number of Web sites that present sea urchin data publicly (*see* Table 2). The archival site at NIH, the National Library of Medicine, National Center for Biotechnology Information (NCBI) is the first public repository for all federally funded sequencing project results. They also maintain information on the extent of various genome projects and link to many other kinds of information including their own independent gene annotation of the *S. purpuratus* genome assembly. As the short read sequencing, especially of transcriptomes, becomes more prevalent, additional sites will probably appear. It is likely that these will be represented by links at the SpBase and NCBI sites.
4. SpBase posts an extensive set of annotations along with genome browsers for different versions of the genome sequence. A variety of sequence objects are available as tracks on the

Table 1
The sequence resources currently available for echinoderms. All of the sequence sets from BCM-HGSC are in Genbank and available at SpBase

Species	Sequence type	Source	Assembled objects
S. purpuratus	Genome assembly	BCM-HGSC	32,0008 scaffolds
S. purpuratus	Transcriptome (20 samples)	Caltech	>30,000 transcripts
S. purpuratus	Transcriptome (2 samples)	University of Miami	ND
S. purpuratus	Transcriptome (2 samples)	MPIMG	ND
S. franciscanus	Genome sequence	BCM-HGSC	~12 million reads
A. fragilis	Genome sequence	BCM-HGSC	~12 million reads
L. variegatus	Genome sequence	BCM-HGSC	330,000 Scaffolds
L. variegatus	Transcriptome	BCM-HGSC	31,357 isotigs
L. variegatus	Transcriptome	Boston University	ND
E. tribuloides	Transcriptome	BCM-HGSC	39,802 isotigs
P. miniata	Transcriptome	BCM-HGSC	58,528 isotigs

Table 2
Web sites that display the sea urchin genome sequence and, in some cases, the associated annotations

Name	Contents	URL
SpBase	Genomic sequences, gene annotation (29,144), Genome browser, Biomart	http://www.spbase.org/SpBase/
HGSC	Genomic sequences, gene annotation (9,941 genes), Genome browser	http://www.hgsc.bcm.tmc.edu/
NCBI	Genomic sequences	http://www.ncbi.nlm.nih.gov/projects/genome/guide/sea_urchin
UCSC	Genome browser	http://genome.ucsc.edu/

genome browser. These include three versions of predicted gene models: the eBACS assembled by BCM-HGSC; ESTs mapped to the genome sequence by two methods; and assembly contigs and a number of computed features such as translations, restriction sites, and GC-content. In addition to the newest assembly version, the site retains the original version 0.5 genome assembly because this is the one from which the gene models were predicted for the presentation of the genome assembly in 2006 [4].

5. An original set of annotations for about 10,000 genes was produced by the sea urchin research community (various reports in Developmental Biology 300, 2006) and collected by BCM-HGSC. This database was moved to SpBase when it became active in 2008 and since extended. Nucleotide and peptide sequences for the 28,944 gene predictions constitute the original set of genes [7]. Community-supplied sequences not found in this set have also been added. A variety of information complements the sequence data (*see* Fig. 1). For example, the evidence for gene name assignment such as homologous gene identity is collected here.

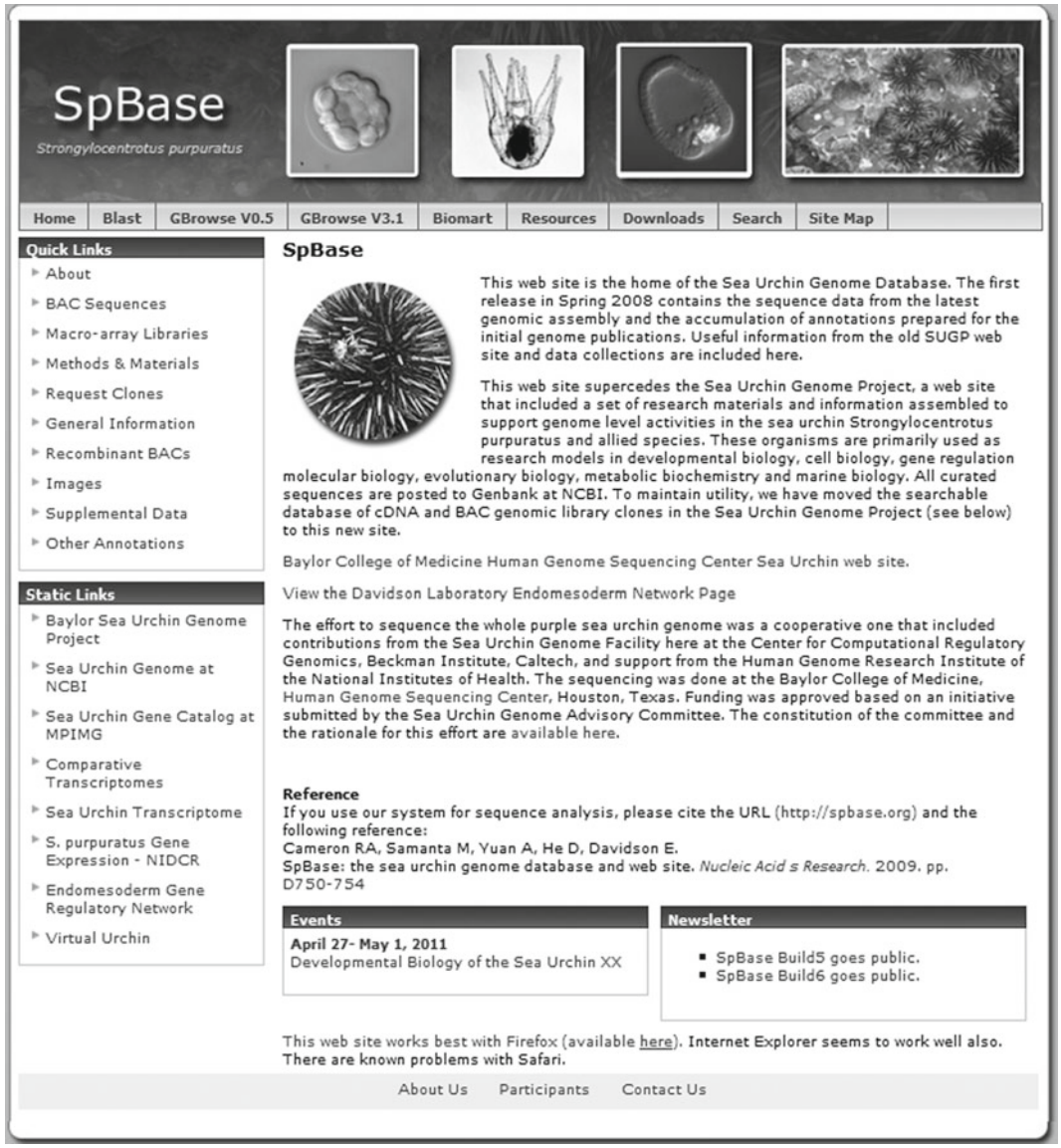

Fig. 1 The SpBase home page located at http://www.spbase.org/SpBase/

6. One data type of great interest to the research community is the temporal and spatial distribution of gene expression for characterized genes in the purple sea urchin which can be used to design experiments for the study of *cis*-regulatory modules and the gene regulatory modules into which they are assembled. In addition to data from research papers on gene function, there have been a series of high-throughput analyses of gene expression. In the embryo, these include a whole genome tiling array, a Genscan gene prediction array, a quantitative PCR and WMIS study of transcription factors and zinc fingers, and studies on individual gene families [8–18]. A medium-throughput procedure that counts individual RNA molecules, the NanoString nCounter [19] has been used to produce a high-density time course of the expression of 172 regulatory genes from 0 to 48 h of the embryonic stage [20]. The amount of information on expression for each gene in the set varies depending on the depth of study. The different classes of expression information are linked to an expression page for each gene (Fig. 2).

3 Methods

3.1 Finding Genes by Name

1. Annotated genes are those which have been assigned a name. The naming convention adopted by the sea urchin community specifies the use of the mouse version of the gene name wherever possible (*see* **Note 1**). The Gene Search page at SpBase (http://www.spbase.org/SpBase/search/index.php) serves as an entry into the database of information on annotated genes. Each search returns a list of matches that are linked to the gene information page as indicated. The query can take the form of a text fragment and wild card symbols, asterisk "*," to denote any alphanumeric text (bra* returns Sp-Bra or brachyury or Sp-Cobra). The question mark "?" denotes one alphanumeric character (br?=Sp-Bra or Sp-Fibropellin). The search page offers several fields to search (Fig. 3) and field entries can be combined in a Boolean search term.
2. The search categories (*see* **Note 2**) on the query page are Gene Official ID (SPU_######), Scaffold, Official gene name, Synonym, and PubMed ID.

3.2 Finding Genes by Sequence Comparison

1. Matching sequences to known items in a sequence database is a useful way to find out more about a gene of interest. At the SpBase BLAST page, we have installed a local version of NCBI Basic Local Alignment Search Tool (BLAST; [21]). This group of programs finds regions of local similarity between sequences. It compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches.

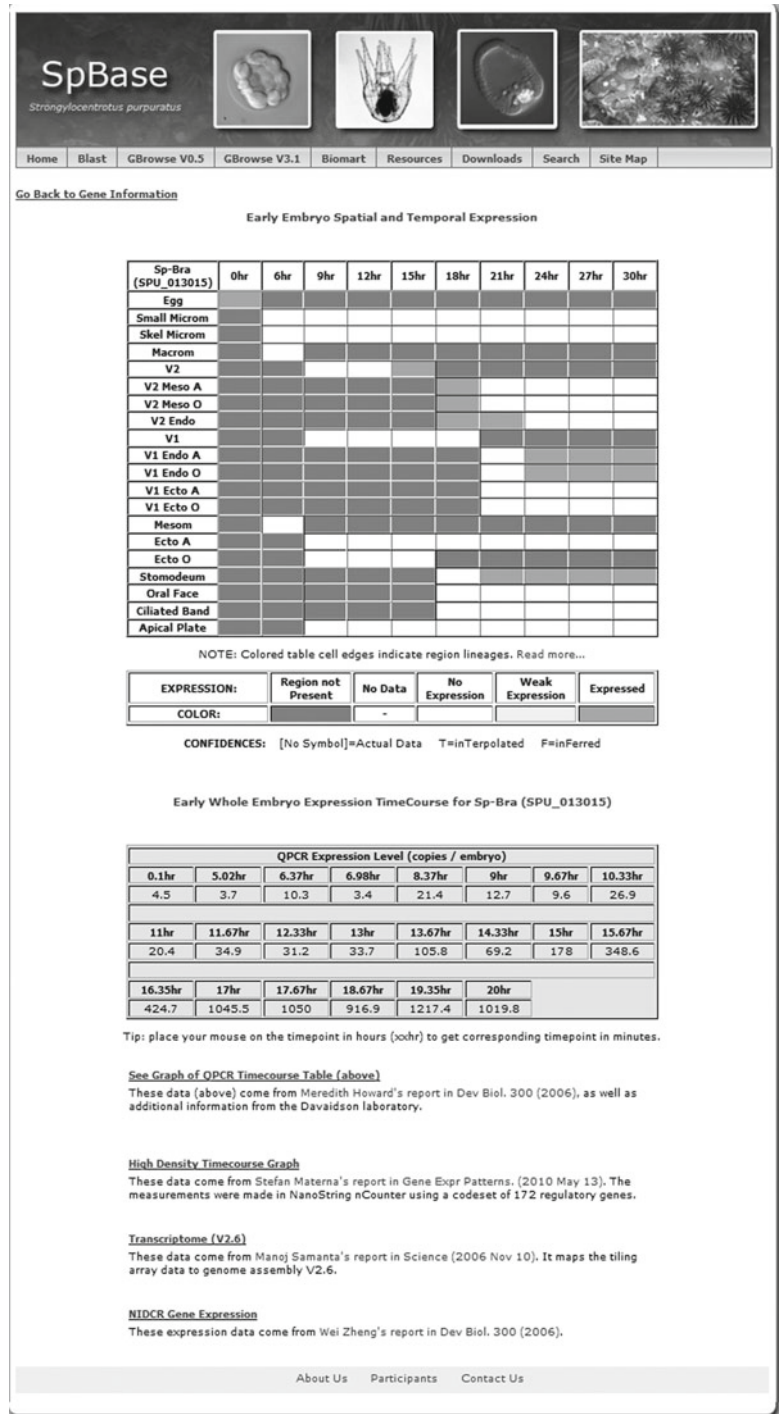

Fig. 2 An example of a gene expression page at SpBase. A variety of expression data is posted here and some is available through links to other Web sites

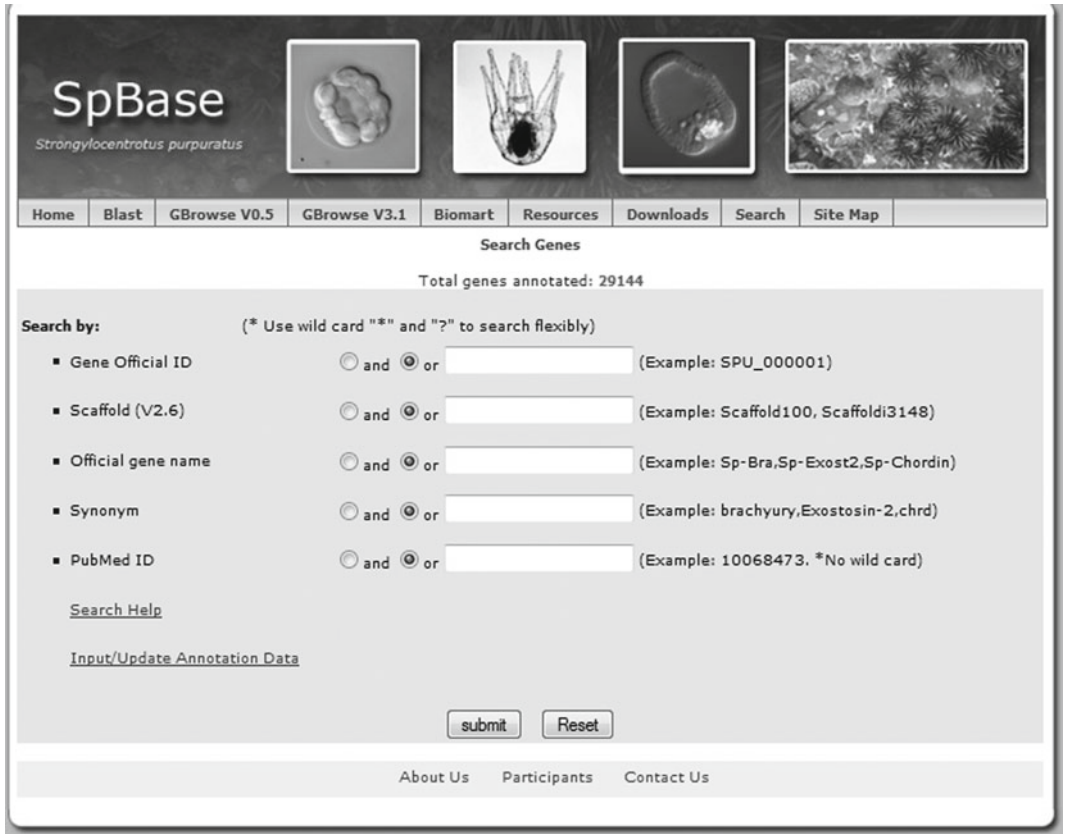

Fig. 3 The Gene Search screen at SpBase. A number of different fields in the annotation database are available to search

BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

2. There are several different BLAST databases available to search at SpBase. The genome assembly versions 0.5, 2.6, and 3.1 are available as well as three more specific ones: *SP genes* are nucleotide sequences of the entire gene set; *SP peptides* are the protein sequence of the gene set; and *clones* are the sequences in Genbank that correspond to the individual clones in the cDNA and BAC libraries maintained in the Center for Computational Regulatory Genomics. Many clones in these libraries were sequenced as part of the genome project [4].
3. The capacity to find clones by sequence allows "cloning by computer" for those situations where one has a sequence and wishes to find a biochemical reagent. This might be useful to, for example, construct a gene sequence in a plasmid for in vitro translation to obtain the protein. Please *see* **Note 3** for a description of the clone name conventions.

4. There are a number of search possibilities to use. Homologous gene searches are best conducted using protein sequences. If one has a protein sequence, blastp can be used with the Sp peptide database. Tblastn uses a protein sequence to search a translated nucleotide database. This strategy works well if the genome is suspected of containing an unannotated gene sequence. Lastly, blastx translates a nucleotide query to search a protein database. If you expect the sequence to be found in the genome, then blastn matches a nucleotide query to a nucleotide sequence database. A more complete discussion of BLAST program usage can be found in the NCBI Blast Handbook (http://www.ncbi.nlm.nih.gov/books/NBK21097/). Links from the BLAST result pages can be followed to gene annotation of Gbrowse genome viewer pages as needed.

3.3 Obtaining Gene Sequences with the Gbrowse Genome Viewer

1. Gene searches by available query term and sequence searches by BLAST are linked, directly or indirectly, to the Gbrowse genome viewer. Here one can display a number of genome feature tracks aligned with the genome assembly (Fig. 4). The Gbrowse display page has the usual controls for navigating along the sequence and zooming in or out to display the features on interest. There are also two ways to download sequence elements from the sequence in view. This is valuable if one wants to design PCR primers within one exon of a gene of interest or use PCR to obtain genomic sequence near a gene under study.
2. The first way is by region: Choose the region to be downloaded in the "Region" subpanel. It is the second subpanel from the top of the sequence track panel. Choose the region by dragging the cursor along the track.
3. In the search panel, a variety of data forms can be specified. The one we are discussing here is "Download Decorated FASTA File." Under the "Configure" button, a number of formatting selections are available. The "Go" button produces a text file to be downloaded and saved.
4. The second way is by feature: Clicking on an individual feature in the sequence subpanel will transfer the view to the information from the database for that feature, including the sequence. The sequence portion and FASTA-formatted header are easily copied to the clipboard from that window. Please note that the sequence displayed is the portion of the assembled genome sequence that is mapped to that feature.
5. If the feature has a slightly different sequence (e.g., due to the extreme polymorphism of this species), it can only be obtained by going to another site using the sequence reference.
6. If one clicks on an EST track feature, the resultant window includes an NCBI linkout to the EST sequence in Genbank. Most of the EST sequences are identical to the assembled

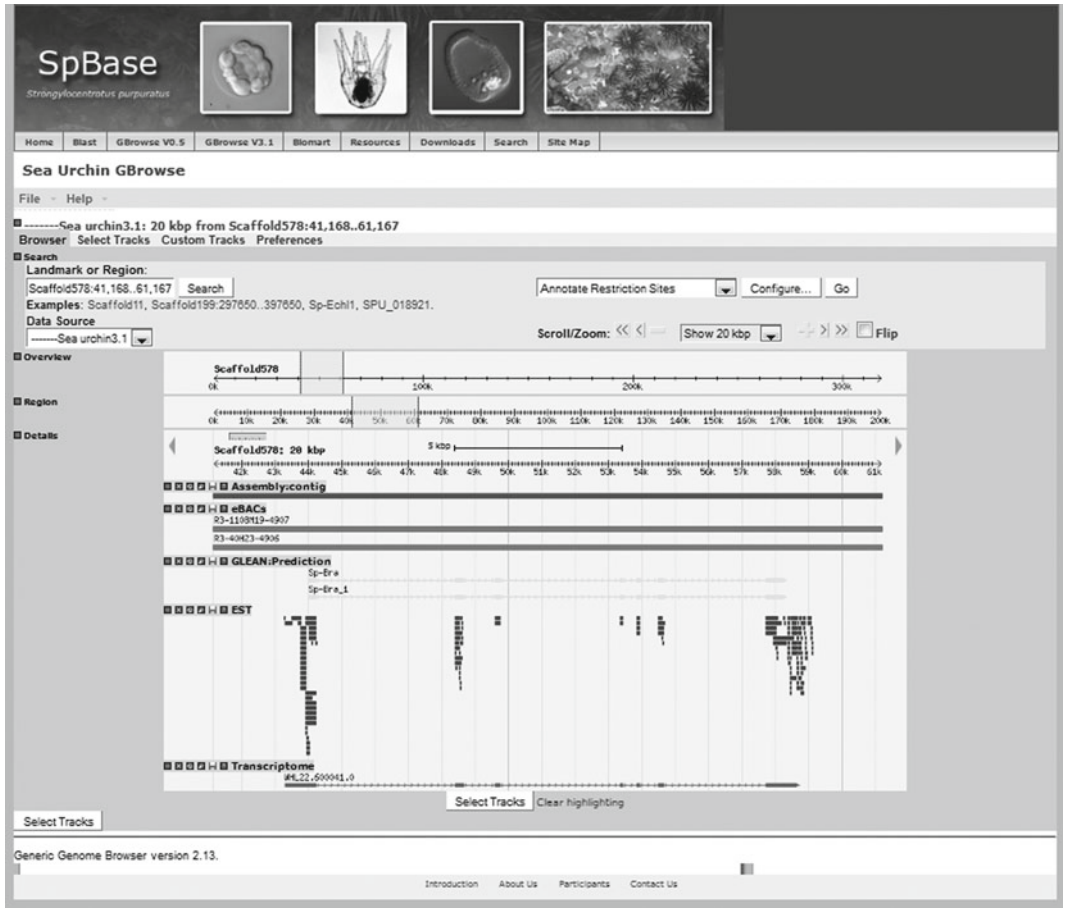

Fig. 4 A typical Gbrowse display showing the sequence features around the chyury gene. In this case, the contig from the genome assembly, the coding exons of two different transcript sets, matching ESTs and BACs used in the assembly are each displayed as separate tracks

genome, but if there are one or a few changes, the resultant PCR primer would not work.

3.4 Finding Noncoding Regulatory Regions

Although rules and methods for identifying sequences that serve as protein-coding templates are reasonably well established, the ones for identifying other functional features like gene regulatory regions are not. Transcription factor core binding sites are often degenerate and of such small size (6–8 bp) as to be present in seemingly random sequence. The strongest method to find regions that are functional outside of protein-coding gene sequences relies on the conservation of base order at an evolutionary distance wherein genetic drift can alter all but the functionally conserved patch of sequence ([22]; *see* [23] for review). Additional rules can be formulated once conserved regions are identified.

In the case of the purple sea urchin, empirical results have shown that a divergence time between genomes of 50 million years reveals functionally conserved regions. The comparison

species that has been used most successfully is *L. variegatus*. The first proof of concept described the modules controlling expression of Sp-Otx [24]. Over 100 of these comparisons with *S. purpuratus* have been made using *L. variegatus* sequences from BAC clones [2, 25].

1. Cartwheel and FamilyRelations are tools to support the analysis, annotation, and comparison of BAC-sized genomic sequences (50–150 kb). They were developed in the Davidson laboratory to support studies of cis-regulation of transcription and the creation of the endomesoderm gene regulatory network [25]. It is the core of an automatic annotation system for regulatory sequences [3].
2. Cartwheel is a Web-based system with a user-friendly interface that allows users to upload sequences and analyze them with a variety of tools, including BLAST and several gene finders. This system is currently maintained by Dr. Titus Brown, Michigan State University (http://woodward.ged.msu.edu/canal/).
3. The unique feature of the system is an un-gapped sequence comparison tool, Paircomp [26]. It uses a window of 10–50 bp to scan one sequence against the other, recording matches at a user-defined percent similarity. The XML output is stored on the Web site for future download and viewing using the FamilyRelations software.
4. FamilyRelations is a graphical user interface written in Java that lets users explore the results of analyses done with Cartwheel. FamilyRelations can display analyses of a single sequence as well as pairwise comparisons and allows interactive viewing of the analyses as well as sequence extraction and motif search.
5. FamilyRelations is available through the Cartwheel site or at http://family.caltech.edu/. There is also an online tutorial to help users get started at http://family.caltech.edu/tutorial/.
6. The FamilyRelations graphical interface displays the sequence similarity between two sequences as either a dot plot or a linear display (Pair View; Fig. 5). In addition to managing the display, the interface also provides the capability to choose and copy sequence elements to the clipboard and search the sequence chosen for motifs specified by the user.
7. Multiple analyses can be associated with a sequence by the Cartwheel program and displayed. An example of this capability is seen in the SUGAR analyses ("Sea Urchin Genome Annotation Resource") produced for many pairs of BAC sequences and posted on the SpBase (http://www.spbase.org/SpBase/resources/bac_sequences.php).

3.5 Data Mining

1. SpBase also provides a tool to create lists of genomic information. Called BioMart (http://www.biomart.org/index.html), it is a fast genome information search and retrieval program

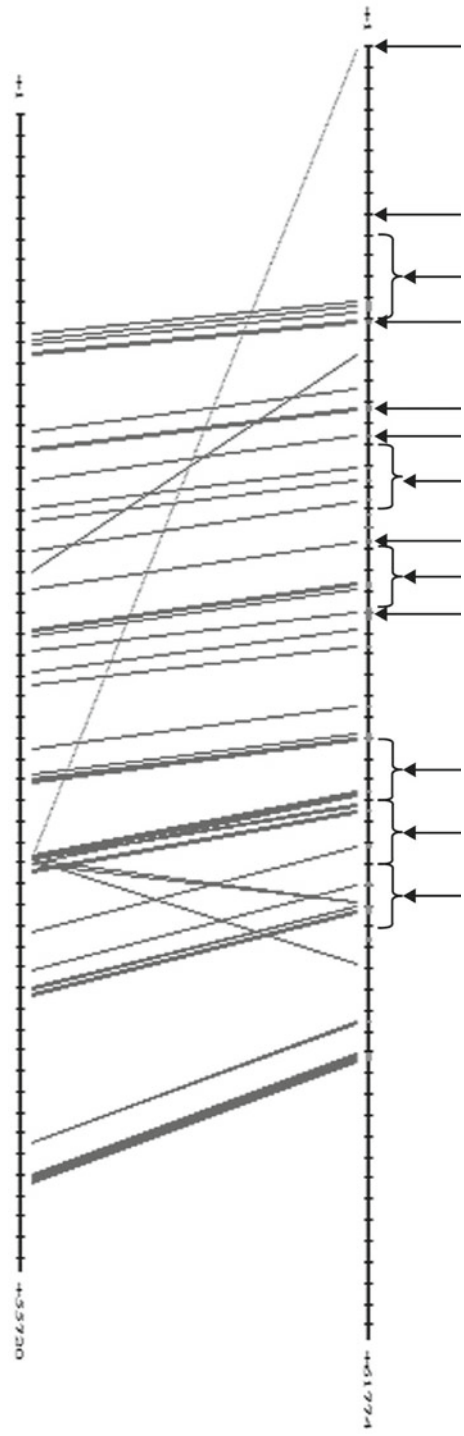

Fig. 5 A typical linear plot of the paircomp sequence comparison in the vicinity of the brachyury gene. The *S. purpuratus* sequence is to the *right* and the *L. variegatus* sequence to the *left*. The *arrows* on the *right* indicate the position of exons and similarly, the brackets mark putative control regions conserved between the two species near the exons

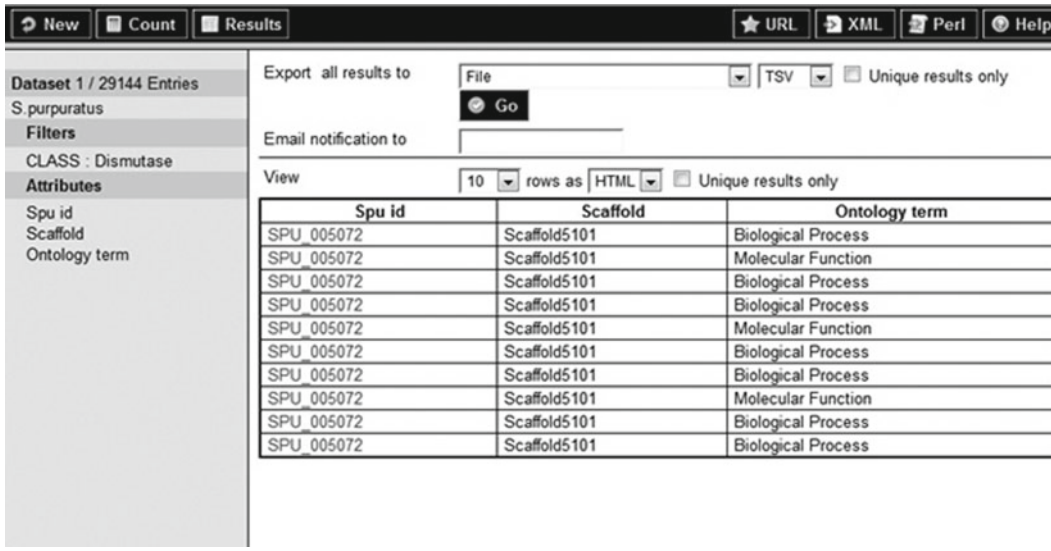

Fig. 6 BioMart. A screen capture of the results' page from a BioMart search for "dismutase" showing the user-specified attribute fields

whose query-building operations allow one to select data by diverse categories, flexibly filter the results, and output data in a tabulated form. This package, a component of GMOD, was developed jointly by the Ontario Institute for Cancer Research (OICR) and the European Bioinformatics Institute (EBI). It is used at SpBase as a fairly simple way to generate large lists of search results. An example of an output page is shown in Fig. 6.

2. The entire contents of SpBase are available for download if one wishes to conduct large-scale data manipulation locally. Various portions of sequence and even the programming code that generates the Web system can be obtained (http://www.spbase.org/SpBase/downloads.php).

4 Notes

1. *Gene naming conventions.* This note describes the scheme for gene names and gene symbols that were used in the annotation of the purple sea urchin genome. The gene symbol uses the specification of the mouse nomenclature whenever possible and adds a sea urchin identifier, e.g., *Sp-Otx*. The specification is described on the mouse Mouse Genome Informatics Web site, The Jackson Laboratory, Bar Harbor, Maine [27]. The salient information is on the nomenclature page "Quick Guide to Nomenclature for Genes" (http://www.informatics.jax.org/mgihome/nomen/short_gene.shtml).

2. *Search Category Definitions.* The search categories are as follows:
   (a) *Gene Official ID* (e.g., SPU_000001). This query uses the SpBase form of the official gene identifier unique in the database. The form of the identifier is three letters "SPU" followed by an underscore and six numbers. The SpBase identifier is derived from and continues on the Official Gene Set identifiers generated at Baylor, the GLEAN numbers. The relationship between SPU numbers and GLEAN number is as follows:

      GLEAN3_#####=SPU_0#####

      SPU numbers higher than the original 28,944 GLEAN3 numbers denote annotated genes for which no GLEAN prediction existed. These additional genes came from individually sequenced cDNAs or other evidence.
   (b) *Scaffold* (e.g., Scaffold100, Scaffold3148). The scaffold identifier refers to the assembly scaffold from the indicated version of the genome assembly. This query uses the number of the scaffold after the identifier "Scaffold" with no space between as in "Scaffold343." It will return a list of the annotated genes predicted from the scaffold sequence. To see all the genes predicted from that scaffold, view the scaffold in Gbrowse with the GLEAN:Prediction track displayed.
   (c) *Official gene name* (e.g., Sp-SFK1, Sp-Exotose, Sp-chordin). The official gene names of the annotated sea urchin genes start with the Sp-gene symbol ("Sp-"). Extensions are usually three to five characters, but additional characters may be added as necessary up to ten in order to distinguish similar symbols. Symbols should begin with an uppercase letter followed by all lowercase letters, unless extensive previous usage dictates otherwise. Punctuation is only used to separate two adjacent numbers (e.g., Lamb1-2) or for designating related (e.g., Es10-rs1) sequences and pseudogenes (e.g., Adh5-ps1). The query can be a text fragment and does not need to include the general prefix (Sp-). A list of the genes whose names match the text fragment will be returned.
   (d) *Synonym* (e.g., Src,EXT,chrd). In order to account for variable usage of gene names, we have tried to collect any synonyms known for annotated genes. For example, Sp-Bra has been called brachyury in many publications. A mouse name that has been for brachyury is "T" or small T locus. Our synonym category can be searched with text fragments as above. A list of genes whose synonyms match the query will be returned.

Table 3
The source and NCBI reference numbers for the EST datasets at Genbank. The clone location and library can be derived from the information on the NCBI page for that clone

Source	Reference	Accession numbers
Coelomocyte cDNAs	[28]	CK828301.1–CK829214.1 CV652690.1–CV652795.1 R61894.1–R62134.1
7 h EST (1000)	[29]	AF122056–AF122818
PMC EST (8293)	[30]	BG780044–BG789442
Clustered ESTs	[32]	CD289359–CD297607 CD303636–CD324638 CD330178–CD342180
Full-length cDNAs (36522)	Coffman, unpub	
HGSC-BCM ESTs (111,497 items)	NCBI: (txid7668[orgn] AND gbdiv_est[prop])	
SMC (H. pulcherrimus)	[31]	AU272543–AU275011

(e) *PubMed ID* PubMed is the bibliographic component of the NCBI's Entrez retrieval system. It accesses a database designed to provide access to citations from biomedical journals. It resides at the National Center for Biotechnology Information (NCBI) at the National Library of Medicine (NLM), one of the institutes of the National Institutes of Health (NIH). PubMed links also lead to full-text journal articles at Web sites of participating publishers as well as to other related Web resources. The PubMed ID (PMID) takes the form of an 8-digit number (e.g., 9628328). Wild card characters cannot be used here. The search returns a list of the genes referred to in the publication.

3. *Clone naming conventions.* The EST sequences derived from purple sea urchin cDNa and deposited in Genbank are from several sources [4, 28–32]. These high-throughput datasets are each derived from one or more cDNA libraries (Table 3). Some of the libraries are still available for clone requests if one should need the actual cDNA.

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Chapter 21

Antibody Inhibition of Protein Activity in Starfish Oocytes

Eiichi Okumura, Masatoshi Hara, and Takeo Kishimoto

Abstract

Antibodies are widely utilized in cell and molecule biology for immunoblots, immunostaining, immunoprecipitation, immunoaffinity purification, and immunoassay. Some antibodies can be used for in vivo inhibition experiments. These antibodies bind to their target molecules and neutralize their functions, providing functional information in the study of their biological role. Here, we describe our methods for obtaining inhibitory antibodies against desired proteins. We then describe in the starfish oocyte system how to inhibit a target protein, even in the nucleus, by injection of antibody into the cytoplasm, and how to evaluate antibody inhibition of cell cycle regulators in small numbers of oocytes.

Key words Antibody inhibition, Neutralizing antibody, Starfish oocyte, Microinjection, Nuclear targeting, H1 kinase, Immunoblot

1 Introduction

Inhibitory antibodies provide valuable tools for studying the biological role of a protein of interest in vivo [1]. These antibodies block the biological function of a target protein through binding to various functional domains such as the catalytic domain, regulatory domain, or binding domain of the target. It is by chance whether an inhibitory antibody is available. Most antibodies are not suitable for in vivo inhibition studies. In order to improve the probability of producing an inhibitory antibody, the immunizing peptide antigen sequence is selected from the functional domain of the desired protein. Mouse or rabbit is typically used for antibody production [2].

The method of antibody inhibition has advantages and disadvantages. Antibody inhibition is highly specific to the antigen protein. The timing of inhibition is controllable, because inhibitory antibodies are usually introduced directly into cells by microinjection. However, the number of cells to be examined is limited. Production of high-titer antibody is required, because the injection volume is limited. Furthermore, injected immunoglobulins

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may interfere further analysis by immunoblots with the primary antibody raised in the same species of animals. If available, another experimental method for inhibition such as chemical inhibitor or dominant-negative competitor should be used in order to complement the results obtained by antibody inhibition.

Here, we describe our methods to prepare and utilize inhibitory antibodies against cell cycle regulators in starfish oocytes: (1) designing the antigen domain of the desired protein and production of its recombinant protein, (2) immunization against rabbit, (3) affinity purification of antibodies from serum, (4) immunoprecipitation to confirm recognition of native protein, (5) microinjection of antibody into starfish oocytes, and (6) evaluation of inhibitory effect in the antibody-injected oocyte. In antibody injection, we especially describe our most recent method to inhibit nuclear protein activity. IgG is too large to pass through nuclear pores and the volume which can be injected directly into the nucleus (called the germinal vesicle (GV) in oocyte) is limited. To overcome these problems, we have developed a method that allows nuclear accumulation of the antibody which was injected into the cytoplasm. For evaluation of the inhibitory effect of the antibody, we usually measure histone H1 kinase activity as a cell cycle marker that represents Cdk1 activity [3] and examine the behavior of cell cycle regulators with immunoblots, in addition to microscopic inspection of the oocyte and nucleus. Because the numbers of the antibody-injected oocytes are very limited, we describe a high sensitive method for histone H1 kinase assay and immunoblots in a small number of oocytes.

Regular methods are based on some books of laboratory manuals [2, 4] and this series of Method in Molecular Biology. Here, we describe in detail some modifications of these methods.

2 Materials

2.1 Peptide Antigen and Peptide Beads Preparation

1. Maleimide-activated KLH (PIERCE; 77605).
2. SulfoLink coupling gel (PIERCE; 20401) for preparation of affinity beads which are used for purification of anti-peptide antibody.

2.2 Vector Construction for Antigen Protein Expression

1. cDNA of the desired protein.
2. Vectors: pET-21 (Novagen) or pTrcHis (Invitrogen) for His-tagged protein expression and pGEX-4T vector (GE Healthcare) for GST-tagged protein expression.
3. Thermal cycler, restriction enzymes, DNA ligation kit ver. 2.1 (Takara; 6021), and other materials for molecular work [4].
4. *E. coli*: DH5α competent cells (Invitrogen), stored at −80 °C.

5. LB medium: 10 g/L NaCl, 10 g/L Bacto Tryptone (BD), 5 g/L yeast extract (BD), pH 7.5 in deionized water at 3 MΩ.
6. Antibiotics: Carbenicillin.

2.3 His-Tagged Antigen Protein Preparation

1. *E. coli*: BL21 (DE3) competent cells (Invitrogen), stored at −80 °C.
2. LB medium: 10 g/L NaCl, 10 g/L Bacto Tryptone (BD), 5 g/L yeast extract (BD), pH 7.5 in deionized water at 3 MΩ.
3. IPTG (isopropyl β-D-1-thiogalactopyranoside).
4. Antibiotics: Carbenicillin.
5. Extraction buffer A: 50 mM Tris–HCl pH 7.9, 0.5 M NaCl, 1 mM DTT, complete EDTA-free protease inhibitor cocktail (Roche; 11873580001).
6. Ni-NTA Agarose (Qiagen; 30210) and 10 mL Poly-Prep Chromatography column (Bio-Rad).
7. Washing buffer A: 20 mM Tris–HCl pH 7.9, 0.5 M NaCl, 20 mM imidazole.
8. Elution buffer A: 20 mM Tris–HCl pH 7.9, 0.5 M NaCl, 250 mM imidazole.
9. Vivaspin column (Sartorius) for concentration of antigen protein.
10. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4.
11. Coomassie Protein Assay Reagent Kit (PIERCE).

2.4 Immunization

1. Adjuvant: AbISCO-300 adjuvant (ISCONOVA), TiterMax Gold (CytRx Corp. GA).
2. 1 mL tuberculin syringes (NORM-JECT 1 mL, HENKE SASS WOLF) and needles of 23 G (TERUMO).
3. 70 % ethanol cotton for disinfection.
4. Electric animal hair clipper (Model 9000, THRIVE) and razors (FEATHER).
5. Two healthy rabbits, New Zealand white (~1.5 kg), per antigen.

2.5 Purification of GST-Tagged Antigen and Preparation of Antigen-Transferred Membrane Pieces

1. Glutathione-Sepharose CL4B (GE Healthcare) and reduced glutathione.
2. Lysis buffer A: PBS containing complete protease inhibitor cocktail (Roche; 11697498001).
3. Elution buffer B: 50 mM Tris–HCl pH 8.0, 10 mM reduced glutathione.
4. 2×-sample buffer: 0.125 M Tris–HCl, pH 6.8, 20 % glycerol, 10 % β-mercaptoethanol, 4 % SDS, and 0.002 % Bromophenol blue.

5. SDS-PAGE and Western blot equipment.
6. PVDF membrane: Immobilon-P Transfer Membrane (Millipore; IPVH00010).
7. Ponceau-S staining solution: 0.1 % ponceau-S, 0.05 % acetic acid.
8. Ponceau-S destaining solution: 0.05 % acetic acid.
9. Blocking solution: 5 % skim milk in TBS, freshly prepared.

2.6 Antibody Purification with Peptide Beads or Antigen-Transferred Membrane Pieces

1. TBS: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl.
2. TBS-T: TBS with 0.1 % Tween-20.
3. Wash buffer: 50 mM Tris–HCl, pH 7.5, 500 mM NaCl.
4. 0.15 M NaCl.
5. Elution buffer: 0.2 M glycine-HCl pH 2.0.
6. 1 M Tris-base for neutralization.
7. pH testing paper or drop pH meter.
8. Vivaspin column (Sartorius) for concentration of purified antibodies.
9. Coomassie Protein Assay Reagent Kit (PIERCE, 23200).

2.7 Starfish and Oocyte Extracts

1. Starfish, *Asterina pectinifera* (renamed *Patiria pectinifera*), are collected during the breeding season and kept in laboratory aquaria supplied with circulating commercial artificial seawater at 14 °C.
2. Commercial artificial seawater: Sealife (Marine-tech), Marin art (Osaka Yakken).
3. Artificial seawater (ASW): 462 mM NaCl, 10 mM KCl, 9.2 mM $CaCl_2$, 36 mM $MgCl_2$, 18 mM $MgSO_4$, 20 mM H_3BO_3, pH 8.2 [5] (*see* **Note 1**).
4. Ca-free ASW: 476 mM NaCl, 10 mM KCl, 36 mM $MgCl_2$, 18 mM $MgSO_4$, 20 mM H_3BO_3, pH 8.2 [5].
5. Tweezers and 35-mm dishes.
6. Microscope and stereomicroscope.
7. Extraction buffer B: 160 mM Na-β-glycerophosphate, 40 mM EGTA, 30 mM $MgCl_2$, 200 mM KCl, 200 mM sucrose, pH 7.3 for stock solution stored at 4 °C and for working solution, add final conc. 2 mM DTT, 1 mM Na-orthovanadate, 0.1 % Nonidet P-40, 50 μM benzamidine, 50 μM PMSF, 50 μg/mL leupeptin, 50 μg/mL soybean trypsin inhibitor [6].

2.8 Immunoprecipitation

1. Protein A-Sepharose 4B (Sigma; P9424-5ML) or Protein G Sepharose 4B (Sigma; P3296-5ML).
2. Immunoprecipitation buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 % Triton X-100, 0.1 % SDS, 50 mM NaF,

10 mM Na pyrophosphate, 0.1 mM Na-orthovanadate, 0.1 mM Na *p*-nitrophenyl phosphate, and 0.1 mM $ZnCl_2$, stored at 4 °C for a few months.

2.9 Microinjection

1. Glass injection needles formed from 50 μL microcap (Drummond Scientific; 1-000-0500) using a glass needle puller (NARISHIGE).
2. 1 mL syringe (TERUMO; SS-01T) with long needle (Hamilton; 90122).
3. Silicone oil (Shin-Etsu Chemical; KF-96-500CS).
4. Glass chamber for holding oocytes [7].
5. Microscope with injector and micromanipulator (Leitz) [8].

2.10 Expression and Purification of ZZ-IBB

1. pET21-ZZ-IBB: ZZ-domain [9] was amplified by PCR from pEZZ18 (GE Healthcare; 27-4810-01) and cloned into pET21a (Novagen; 69740-3) along with the coding region of IBB [10] (for ZZ-IBB, *see* Subheading 3.12).
2. Rosetta 2 (DE3) Competent Cells (Novagen; 71397-3).
3. Plusgrow II (Nacalai Tesque; 08202-75).
4. Antibiotics: Carbenicillin and chloramphenicol.
5. IPTG (isopropyl-β-D-thiogalactopyranoside).
6. Binding buffer: 20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 5 mM imidazole and complete EDTA-free protease inhibitor cocktail (Roche; 11873580001).
7. Nonidet P-40 (NP-40).
8. Ni-NTA Agarose (Qiagen; 30210).
9. Wash buffer B: 20 mM Tris–HCl, pH 7.5, 0.5 M NaCl and 20 mM imidazole.
10. Elute buffer C: 20 mM Tris–HCl, pH 7.5, 0.5 M NaCl and 200 mM imidazole.
11. Coomassie Brilliant Blue (CBB) staining solution: 0.05 % CBB, 25 % methanol, and 7 % acetic acid.
12. CBB destaining solution: 25 % methanol and 7 % acetic acid.
13. PBS.
14. Dialysis tube: Spectra/Por2 (Spectrum Labs; 132678).
15. Vivaspin 500 10,000 MWCO PES (Sartorius; VS0102).

2.11 Small-Scale Oocyte Extracts for High Sensitive Histone H1 Kinase Assay and Immunoblots

1. Lysis buffer B: 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 100 mM KCl, 100 mM Sucrose, 1 mM DTT, 0.5 mM Na-orthovanadate, 25 mM NaF, 0.5 % NP-40, and complete EDTA-free protease inhibitor cocktail (Roche; 11873580001), pH 7.3.

2.12 High Sensitive Histone H1 Kinase Assay

1. Kinase buffer: 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, and complete EDTA-free protease inhibitor cocktail (Roche; 11873580001), pH 7.3.
2. H1 reaction mixture: 0.6 mg/mL histone H1 and 14.8 MBq/mL [γ-^{32}P]-ATP in kinase buffer.
3. 4×-sample buffer: 0.25 M Tris–HCl, pH 6.8, 40 % glycerol, 20 % β-mercaptoethanol, 8 % SDS, and 0.004 % Bromophenol blue.
4. Coomassie Brilliant Blue (CBB) staining solution: 0.05 % CBB, 25 % methanol, and 7 % acetic acid.
5. CBB destaining solution: 25 % methanol and 7 % acetic acid.
6. Bio-imaging analyzer: BAS 2000 (Fuji Film).

2.13 High Sensitive Immunoblots

1. Transfer buffer: 100 mM Tris–HCl, 192 mM glycine, 0.05 % SDS, and 20 % methanol.
2. Semidry blotter: HorizBlot (ATTO; AE-6677).
3. PVDF membrane: Immobilon-P Transfer Membrane (Millipore; IPVH00010).
4. Filter paper (Advantec; 526).
5. TBS-T: 0.05 % Tween 20 in TBS.
6. Can Get Signal Immunoreact Enhancer (Can Get Signal) Solution 1 and 2 (Nacalai Tesque; NKB-101).
7. Secondary antibody: Anti-rabbit IgG HRP-linked F(ab′)$_2$ fragment donkey HRP-conjugated anti-rabbit IgG (GE Healthcare; NA9340-1ML) or polyclonal rabbit anti-mouse immunoglobulins-HRP (Dako; P0260).
8. ECL Plus Western Blotting Detection System (GE Healthcare; RPN2132).
9. Imaging system: Image Quant LAS 4000 (GE Healthcare).

3 Methods

3.1 Designing the Antigen for Inhibitory Antibodies

1. Search databases for homologous sequences to the desired protein.
2. Generate sequence alignments on a program such as DNASIS (Hitachi Solutions. Ltd.).
3. Search a homologous domain of the desired protein to the conserved catalytic domain such as a kinase or a phosphatase domain. Design antigen including a catalytic domain. If there is some information about motifs such as the binding domain to the target and the regulatory domain of the activity, these domains are also available for antigen (Fig. 1) (*see* **Note 2**).

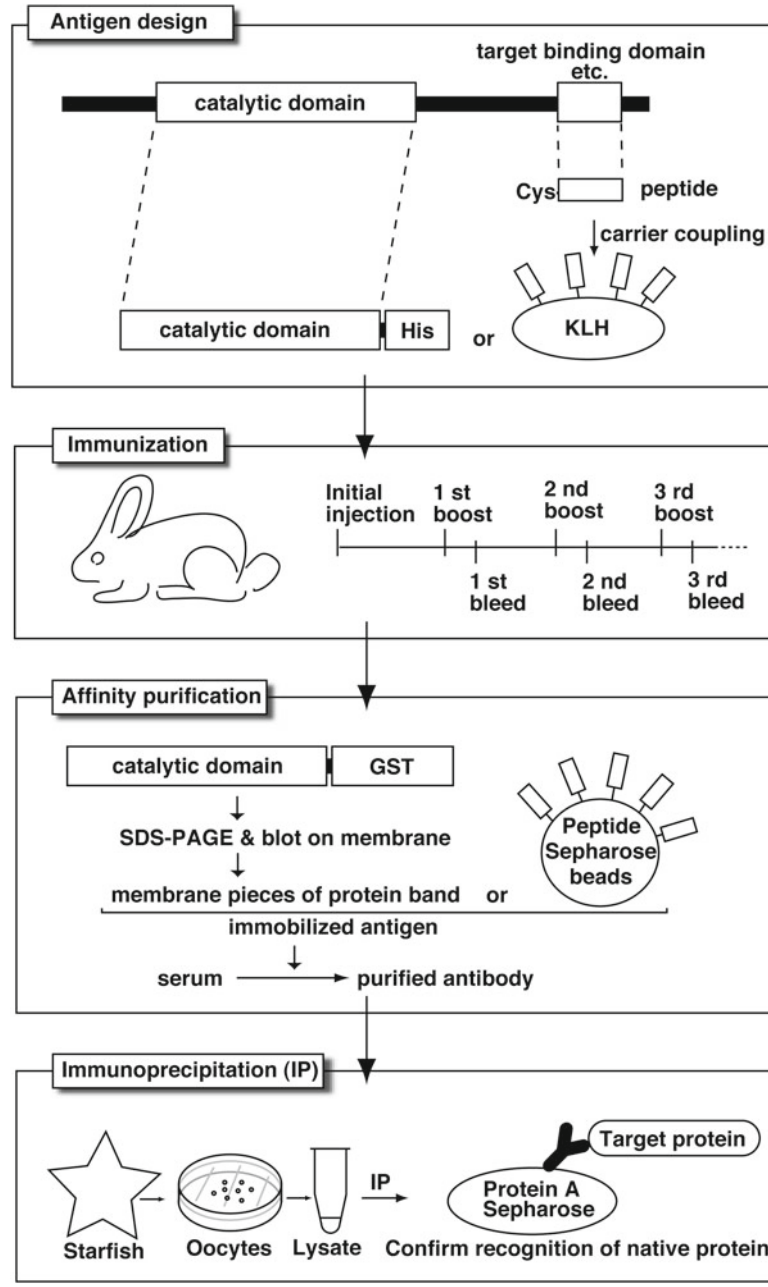

Fig. 1 Flow chart of antibody preparation

4. Full-length recombinant protein and N- and C-terminal 10–20 peptides are designed as control antigens for non-inhibitory antibodies, which can be utilized in activity assay in immunoprecipitates, immunostaining, and immunoblots.
5. In case of short peptide antigens, cysteine is added at the end for chemical coupling with maleimide-activated carrier proteins such as KLH (keyhole limpet hemocyanin).

3.2 Preparation of Short Peptide Antigen

1. Order synthesis of the designed peptide at 10 mg scale (from a supplier such as Sigma-Aldrich Co. or Operon Biotechnologies). Or synthesize antigen peptide by solid-phase peptide synthesis utilizing Fmoc amino-protecting group and further purify them by reverse phase chromatography with FPLC, if available.
2. Conjugate synthesized peptide with maleimide-activated KLH following the instruction manual of Imject Maleimide-Activated mcKLH (PIERCE; 0838w). Use 4 mg of the peptide and 4 mg of the carrier protein.
3. Dialyze the conjugated mixture against over 100× volume of PBS for 5 h, with buffer exchange at 1 and 3 h.
4. Recover the antigen conjugate and divide the solution into eight aliquots of 1 mg protein in each tube. Freeze and stock them at −80 °C for further use.

3.3 Vector Construction for His-Tagged and GST-Tagged Antigen Protein Expression

His-tagged protein is used for immunization and GST-tagged protein is used for affinity purification of antibody to eliminate the antibody against the His-tagged domain.

1. cDNA of the desired protein was amplified by PCR with forward and reverse primers which contain restriction enzyme sequences to insert multiple cloning sites of vectors.
2. Cut 5′ and 3′ end of the PCR product with restriction enzymes.
3. Cut vectors with same restriction enzymes. Use pET21 or pTrcHis for His-tagged protein expression. Use pGEX-4T vector for GST-tagged protein expression.
4. Ligate DNA fragments of **steps 2** and **3** with Takara DNA ligation kit.
5. Transform DH5α *E. coli* cells with the ligated DNA, plate cells on the LB plate containing carbenicillin, and incubate overnight at 37 °C.
6. Pick six single colonies and incubate them overnight at 37 °C in 1.6 mL LB medium containing 100 μL/mL carbenicillin.
7. Purify plasmid DNA with plasmid mini prep kit.
8. Confirm DNA sequences of insert DNA by sequencer.

3.4 Preparation of His-Tagged Antigen Protein

1. Transform BL21 (DE3) *E. coli* cells with the His-tagged protein expression vector according to the manufacturer's instructions, plate cells on the LB plate containing carbenicillin, and incubate overnight at 37 °C.
2. Pick a single colony and incubate it overnight at 37 °C in 10 mL LB medium containing 100 μL/mL carbenicillin.
3. Inoculate 1,000 mL LB medium containing 100 μL/mL carbenicillin with the 10 mL overnight culture and incubate for 3 h at 37 °C.

4. Add IPTG at final conc. of 1 mM and incubate for 4 h at 37 °C to induce the protein expression (*see* **Note 3**).
5. Harvest the cells by centrifugation at 4,000 × *g* for 15 min at 4 °C.
6. Add PBS to the cell pellet and resuspend cells with a pipette until the pellet is completely dissociated.
7. Pellet the cells again and freeze in liquid nitrogen.
8. Thaw the cells expressing His-tagged protein and suspend them in 10 mL extraction buffer A.
9. Sonicate the cell suspension on ice with a microtip for an interval of 15 s sonication and 30 s cooling. Repeat this for several rounds until the sample becomes no longer viscous.
10. Centrifuge lysate at 12,000 × *g* for 20 min at 4 °C.
11. Recover clear supernatant (*see* **Note 4**).
12. Add 2 mL of 50 % of Ni-NTA slurry to 10 mL cleared lysate and mix gently with rotator in a cold room at 4 °C for 1 h.
13. Load the mixture into a Bio-Rad 10 mL column and collect flow-through.
14. Wash the column twice with 5 mL of extraction buffer A.
15. Wash the column with 6 mL of wash buffer A.
16. Elute the His-tagged protein with 6 mL elution buffer A and collect 1 mL aliquots of the eluate.
17. Analyze by SDS-PAGE and CBB staining and then pool the eluted protein fractions.
18. Concentrate the fraction pool with Vivaspin (*see* **Note 5**).
19. For buffer exchange, repeat several rounds of dilution with PBS and reconcentration with Vivaspin.
20. Assay the protein concentration with Coomassie Protein Assay Reagent Kit (PIERCE). The protein concentration at 2 mg/mL is preferable.
21. Freeze and stock at −80 °C in the aliquots of 1 mg protein in each tube.

3.5 Immunization and Serum Preparation

At immunization, we handle rabbits following the guideline of the center for experimental animals in our Institute.

1. For initial immunization, mix 1 mg of antigen protein solution (2 mg/mL, 0.5 mL) with 45 μg of AbISCO-300 adjuvant (3 mg/mL, 15 μL) per rabbit by a vortex mixer shortly (*see* **Note 6**).
2. Before initial injection, collect 5–10 mL pre-immune blood by insertion of needle into the ear vein of a rabbit. Prepare pre-immune serum by leaving the blood overnight in cold room and collect the supernatant after centrifugation at 2,000 × *g*

for 15 min at 4 °C. Add sodium azide at final conc. 0.05 %. Store the serum at 4 °C (or at −30 °C for over several months storage) for use as the negative control.

3. Cut the hair on the back of rabbit with an electric animal hair clipper.
4. Disinfect the back skin with 70 % ethanol cotton.
5. Inject the antigen mixture into about five subcutaneous administration sites on a rabbit back.
6. For boost 4 weeks after the initial injection, inject the same amount of AbISCO-300 adjuvant (3 mg/mL, 15 μL) and a half amount of antigen protein solution (2 mg/mL, 0.25 mL) per rabbit into about five subcutaneous administration sites on a rabbit back.
7. Test bleed 5–10 mL per rabbit 1 week after the first boost injection and prepare serum. Test serum whether they can be used for immunoblots with antigen and endogenous protein from starfish oocytes. Pre-immune serum is used as a negative control (*see* **Note 7**).
8. Repeat boost injection and test bleeding in a 4-week cycle until final bleeding.

3.6 Purification of Antibody Using Peptide Affinity Column

1. Cross-link synthesized antigen peptides to SulfoLink coupling gel according to its instruction (PIERCE; 0527). Use 1 mg peptides per 1 mL gel.
2. Pour 2 mL of 50 % slurry of gel suspension into 10 mL Poly-Prep Chromatography column.
3. Wash the column with 6 mL of 0.2 M glycine-HCl (pH 2.0).
4. Wash the column with 10 mL of TBS.
5. Add 1 mL of TBS and 1 mL of serum.
6. Incubate the column for overnight in cold room with gentle mixing by a rotator.
7. Recover flow-through fraction and further test its immunoreactivity by immunoblots for confirmation of antibody absorption to the column.
8. Wash the column with 10 mL TBST.
9. Wash the column with 3 mL of 0.15 M NaCl.
10. Elute the antibodies with 1 mL of 0.2 M glycine-HCl (pH 2.0) for six times.
11. Neutralize the eluted fractions immediately by Tris-base, along with pH monitoring by the pH test paper or a drop pH meter.
12. Confirm by protein assay reagent whether each of the eluted fractions contains antibody.
13. Pool fractions containing antibodies and concentrate them to approx. 50 μl with Vivaspin.

14. For buffer exchange, repeat several rounds of dilution with PBS and reconcentration with Vivaspin.
15. Measure protein concentrations of purified antibodies (*see* **Note 8**).

3.7 Purification of GST-Tagged Antigen Protein for Antibody Purification

1. Thaw the cells expressing GST-tagged protein (prepared in Subheading 3.3) and suspend them in 10 mL of lysis buffer A.
2. Sonicate the cell suspension on ice with a microtip for an interval of 15 s sonication and 30 s cooling. Repeat this for several rounds until the sample becomes no longer viscous.
3. Centrifuge lysate at 12,000 × *g* for 20 min at 4 °C.
4. Recover clear supernatant (*see* **Note 9**).
5. Add 2 mL of 50 % of Glutathione-Sepharose slurry to 10 mL of cleared lysate and mix them gently with rotator in a cold room at 4 °C for 1 h.
6. Load the mixture into a Bio-Rad 10 mL column and recover flow-through.
7. Wash the column twice with 5 mL of lysis buffer A.
8. Wash the column with 5 mL of PBS.
9. Elute the GST-tagged protein with 6 mL elution buffer B and collect 1 mL aliquots of the eluate.
10. Analyze by SDS-PAGE and pool the eluted protein fractions.
11. Concentrate the pooled fraction with Vivaspin (*see* **Note 5**).
12. Add SDS-PAGE sample buffer to a part of concentrated GST-tagged protein solution. Another part is used for the immunoprecipitation test.

3.8 Purification of Antibody Using Antigen-Transferred Membrane Pieces

1. Cast the gel for SDS-PAGE without comb. The concentration of acrylamide is selected so that the GST-tagged protein runs around middle of the gel.
2. Load about 200 μg of GST-tagged protein per mini slab gel on the flat stacking gel (*see* **Note 10**).
3. After gel electrophoresis, transfer to PVDF membrane and stain it with ponceau-S. Cut out the stained band of the GST-tagged protein. Collect the stained band from 4 to 5 mini gels.
4. Destain the membranes with 0.05 % acetic acid solution, then cut them into small pieces (3 × 10 mm), and put them into 2-mL tube.
5. Block membrane pieces with 1.5 mL of 5 % skim milk in TBS for 30 min at RT with gentle mixing by rotator.
6. Wash membrane pieces five times with TBS.
7. Wash membrane pieces five times with 1.5 mL of 0.2 M glycine-HCl pH 2.0.

8. Wash membrane pieces five times with 1.5 mL of TBS.
9. Add 1 mL of 2× diluted serum with TBS.
10. Incubate overnight in cold room with gentle mixing by rotator.
11. Wash membrane pieces five times with 1.5 mL of TBS-T.
12. Wash membrane pieces two times with 1.5 mL of 0.15 M NaCl.
13. Elute antibody by adding 0.5 mL of 0.2 M glycine-HCl pH 2.0 to membrane pieces and incubating for 3 min. The eluate is recovered to a new tube. Repeat this five rounds.
14. Neutralize the eluted fractions by Tris-base immediately, along with pH monitoring by the pH test paper or a drop pH meter.
15. Confirm by protein assay reagent whether each of the eluted fractions contains antibody.
16. Pool fractions containing antibodies and concentrate them to approx. 50 μL with Vivaspin.
17. Measure protein concentrations of purified antibodies (*see* **Note 8**).

3.9 Oocyte Isolation and Preparation of Oocyte Extracts

1. Dissect a piece of ovary from female starfish with a tweezers and put it into Ca-free ASW in 35-mm dishes.
2. Hold a closed end of ovary with a tweezers and squeeze out oocytes gently from open end with another tweezers under a stereomicroscope.
3. Wash oocytes several times with Ca-free ASW until follicles are removed.
4. Wash isolated oocytes three times with ASW. These are used for microinjection or preparation of oocyte extracts.
5. To prepare oocyte extracts, put isolated oocytes into a microtube and centrifuge at 5,000 × *g* for 10 s at 4 °C.
6. Remove the supernatant seawater by aspiration.
7. Add the oocytes pellet with 5 volumes of ice-cold extraction buffer B and freeze the mixture in liquid nitrogen.
8. Thaw the oocyte suspension, followed by homogenization with a microtip sonicator for 3 s on ice.
9. Centrifuge at 12,000 × *g* for 20 min at 2 °C.
10. Recover the supernatant to a fresh tube as an oocyte extract.

3.10 Immunoprecipitation and In Vitro Activity Assay of the Protein

It is essential for the inhibitory antibody to bind to its target protein. Immunoprecipitation is performed to confirm whether the prepared antibody can recognize and bind to the native form of desired endogenous protein.

1. Add 0.2 μL of purified antibody to 10 μL of oocyte extracts and briefly mix them by a vortex mixer (*see* **Note 11**). His-tagged or GST-tagged protein solution is also used for precipitation in a control experiment.
2. Incubate the mixture on ice for 1 h.
3. Add 10 μL of 50 % slurry of protein A-Sepharose 4B suspended in immunoprecipitation buffer.
4. Incubate the mixture on ice for 1 h with gentle and brief mixing by a vortex mixer in 10–15 min intervals.
5. Wash the beads three times with 1 mL of the immunoprecipitation buffer.
6. Recover beads, add 2× SDS-PAGE sample buffer to them, and analyze by immunoblots to confirm that the antibody binds to the endogenous antigen protein (*see* **Note 12**).
7. For in vitro enzyme assay to test whether the antibody directly inhibits the activity of the protein of interest, wash the beads of **step 5** three times with an assay buffer appropriate for measuring enzyme activity. Then, use beads for enzyme assay of the protein of interest. For a positive control of the activity assay, prepare immunoprecipitates using other non-inhibitory antibodies such as anti-N-terminal or anti-C-terminal peptide antibodies (*see* **Note 13**).

3.11 Microinjection of Purified Antibodies into Starfish Oocytes

Microinjection is performed according to the methods previously described by Kishimoto [8]. Only the holding chamber of oocytes is modified [7]. If the target protein localizes in the nucleus, antibodies are injected into the cytoplasm along with the ZZ-IBB protein described in Subheadings 3.12 and 3.13 [11] (Figs. 2 and 3).

1. Inject the purified and concentrated antibodies into the cytoplasm of an isolated oocyte. The injection volumes are less than 1/10 volume of an oocyte.

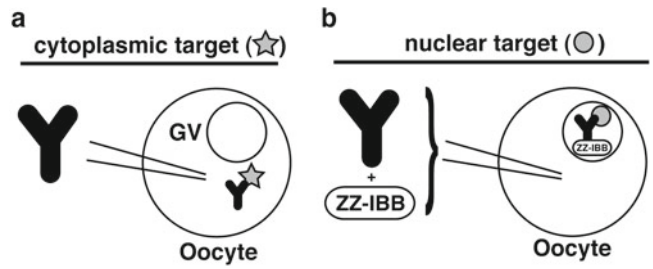

Fig. 2 Antibody targeting to cytoplasm or nucleus. (**a**) For cytoplasmic target protein, antibody (Ig, indicated by *bold Y*) is microinjected into the cytoplasm of a starfish oocyte. (**b**) For nuclear target protein, Ig is co-injected with recombinant ZZ-IBB protein which has both binding domain with Ig and nuclear localization activity derived from importin α

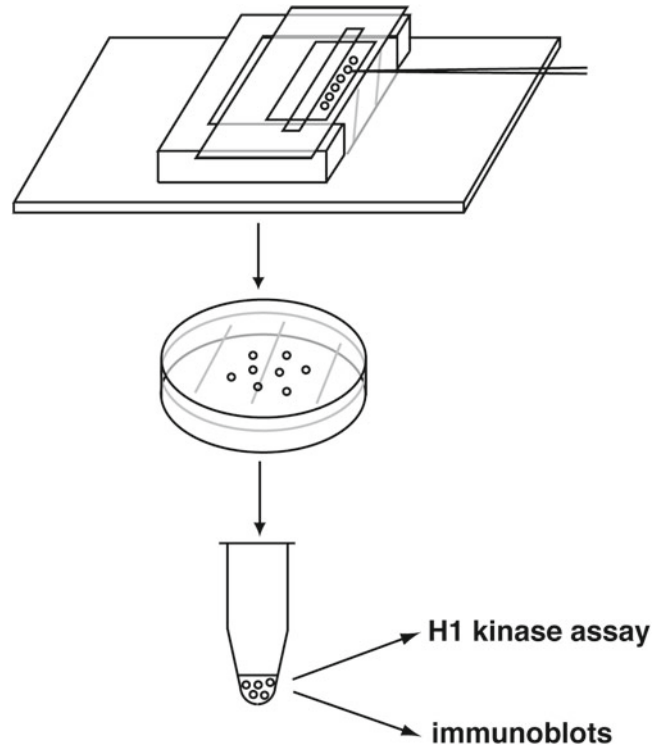

Fig. 3 Microinjection, incubation, and sampling. Antibody is injected under microscope by a glass needle into a starfish oocyte held by the glass chamber which is filled with artificial seawater (ASW) (refs. [7, 8]). Injected oocytes are taken out by the injection needle and transferred into a 35-mm dish filled with ASW. After treatment with 1-methyladenine (maturation-inducing hormone), oocytes are transferred into tube for the H1 kinase assay or immunoblots

2. Incubate the injected oocyte for 30 min at 20–21 °C. Then, evaluate the effect as Subheadings 3.14 and 3.15.
3. If the antibodies show inhibitory effect, prepare a series of serial dilution of the purified and concentrated antibodies.
4. Inject a series of purified antibodies into the cytoplasm of an isolated oocyte. The control IgG is also injected at the highest concentration as a control.

3.12 Expression and Purification of ZZ-IBB

ZZ-IBB consists of two synthetic "Z" domains derived from IgG-binding domain of protein A [9] and IBB (importin β-binding domain of importin α), a nuclear localization signal [10]. ZZ-IBB allows the antibody which is injected into the cytoplasm to accumulate into the nucleus, resulting in inhibition of the activity of the nuclear protein [11].

1. Transform Rosetta 2 (DE3) *E. coli* cells with pET21-ZZ-IBB according to the manufacturer's instructions, plate cells on the LB plate containing carbenicillin and chloramphenicol, and incubate overnight at 37 °C.

2. Pick a single colony and incubate it overnight at 37 °C in 10 mL Plusgrow II medium containing 100 μL/mL carbenicillin and 20 μL/mL chloramphenicol.
3. Inoculate 100 mL Plusgrow II medium containing 100 μL/mL carbenicillin and 20 μL/mL chloramphenicol with the 10 mL overnight culture and incubate for 1 h at 37 °C.
4. Add IPTG at a final concentration of 1 mM and incubate for 3 h at 37 °C to induce the protein expression.
5. Harvest the cells by centrifugation at 4,000 × *g* for 15 min at 4 °C.
6. Resuspend the cells in 10 mL binding buffer and lyse the cells by sonication.
7. Centrifuge the lysate at 26,000 × *g* for 15 min at 4 °C.
8. Recover the supernatant, mix it with 1 mL (bed volume) Ni-NTA Agarose resin, and incubate and rotate the mixture for 30 min at room temperature.
9. After removing the supernatant, wash the resin three times with 10 mL binding buffer and wash buffer B.
10. Elute the ZZ-IBB protein from the resin four times with 1 mL elution buffer C and check the purity by SDS-PAGE and CBB staining.
11. Dialysis against PBS using dialysis tube.
12. Concentrate using Vivaspin.
13. Divide into aliquot, freeze in liquid nitrogen, and store at –80 °C.

3.13 Transport the Neutralizing Antibody into the GV

1. Thaw the neutralizing antibody and ZZ-IBB on ice (*see* **Note 14**).
2. Mix the neutralizing antibody with ZZ-IBB and add NP-40 into the mixture to a final concentration of 0.05 % (Fig. 2) (*see* **Note 15**).
3. After incubation for 30 min on ice, centrifuge the mixture at 12,000 × *g* for 15 min at 4 °C.
4. Inject supernatant into the cytoplasm of immature starfish oocytes (*see* **Note 16**).
5. Incubate the injected oocytes for 30 min and proceed to further experimental process.

3.14 Small-Scale Evaluation of Antibody Inhibition by Measuring H1 Kinase Activity for the Cell Cycle Progression

1. Recover five starfish oocytes with 3 μL sea water into a 0.5 mL micro test tube and freeze in liquid nitrogen (*see* **Note 17**).
2. Stand the tube on ice, add 7 μL lysis buffer B, and incubate for 30 min on ice.
3. Lyse the oocytes by vortexing at 4 °C and then centrifuge the lysate at 12,000 × *g* for 15 min at 4 °C.
4. Recover 0.5 μL supernatant and transfer into 4.5 μL ice-cold kinase buffer in a new 0.5-mL micro test tube. The following

procedures are performed in a room for radioisotope experiments.

5. Add 5 μL H1 reaction mixture and incubate for 15 min at 25 °C.
6. Add 10 μL 2× sample buffer and heat for 5 min at 96 °C to terminate the kinase reaction.
7. Run samples on 12.5 % SDS-PAGE and stain the gel with CBB staining solution.
8. After destaining the gel with CBB destaining solution, detect the radioactivity incorporated into histone H1 using BAS 2000 image analyzer (Fuji Film). Alternatively, excise the histone H1 bands from the gel and quantify the radioactivity incorporated into histone H1 using a liquid scintillation counter.

3.15 Small-Scale Evaluation of Antibody Inhibition by Immunoblots for Modification of the Cell Cycle Regulators

Unless otherwise indicated, the procedure below is performed at room temperature.

1. Add 4 μL 4× sample buffer into the tube from **step 3** in Subheading 3.14 after recovering sample for histone H1 kinase assay at **step 1** in Subheading 3.14 and heat for 5 min at 96 °C.
2. Separate proteins by appropriate percentage of SDS-PAGE.
3. Equilibrate filter paper and PVDF membrane in transfer buffer with gentle rocking.
4. After electrophoresis, equilibrate the gel in transfer buffer with gentle rocking for 10 min.
5. Transfer the proteins from the gel to the membrane using semidry blotter. Briefly, place on the anode plate of the blotter in following order: three sheets of the filter paper, the membrane, the gel, and three sheets of the filter paper. Set the cathode of the blotter and transfer the protein to the membrane at 2 mA/cm^2 constant current for 1 h.
6. Recover and wash the membrane three times with TBS-T for 5 min. It should be noted that skipping a blocking step here is the essence of high sensitive immunoblotting (*see* **Note 18**).
7. Incubate the membrane with primary antibody at appropriate dilution with Can Get Signal Solution 1 for overnight at 4 °C (*see* **Note 19**).
8. Wash the membrane three times with TBS-T for 5 min.
9. Incubate the membrane with HRP-conjugated secondary antibody diluted 1:10,000 in TBS-T for 1 h (*see* **Note 20**).
10. Wash the membrane three times with TBS-T for 5 min.
11. For detection of the protein, incubate the membrane with ECL Plus Western Blotting Detection System and visualize with LAS 4000.

4 Notes

1. The salinity of seawater is not the same among various oceans in the world. The artificial seawater used in our lab is the modified version of van't Hoff's artificial seawater which is buffered by 20 mM boric acid at pH 8.2.
2. In our previous studies, we obtained four types of neutralizing antibodies. One is anticatalytic domain of Cdc25 phosphatase antibody [12], which inhibits its phosphatase activity directly. Second is the anti-C-terminal domain of Myt1 antibody [13], which inhibits Myt1 kinase activity toward Cdc2 but not autophosphorylation, consistent with a report [14] that the C-terminal domain binds to Cdc2. Third is anti-Plk1 antibody [6], which inhibits activation of Plk1 through blocking T-loop phosphorylation but not the activity itself of Plk1. Fourth is anti-C-terminal catalytic domain of Greatwall kinase antibody [11], which inhibits its kinase activity in immunoprecipitates and presumably also suppresses its kinase activation when the antibody is injected together with ZZ-IBB into the cytoplasm of immature oocytes.
3. To increase the yield of soluble His-tagged proteins, the condition of IPTG induction is sometimes changed: decrease the ITPG concentration to 0.1 mM and/or lower the culture temperature to 18 °C.
4. If the His-tagged proteins are mostly insoluble, one may try to purify them under denaturing conditions. AbISCO adjuvant is applicable to soluble antigen and can be used with solution containing 3 M Urea. When most of protein becomes precipitated during dialysis against 3 M Urea/PBS, suspension of the precipitated protein in PBS is injected into rabbit along with another adjuvant TiterMax Gold, which is applicable to insoluble antigen.
5. Various kinds of proteins are concentrated well by Vivaspin with low rate of protein absorption, but some proteins cannot be concentrated because of their limited solubility. If so, addition of 8 M Urea/PBS to the pellet can solubilize the precipitated proteins. Then, the concentration of Urea should be diluted lower than 3 M for immunization.
6. High specificity and high titer are required for inhibitory antibody. Immunization of large amount of antigen (0.5–1 mg per rabbit) increases the likelihood of obtaining high-titer antibodies.
7. Titer and specificity of the antibody are tested by immunoblots. The method is similar to that described in Subheading 3.15, except for addition of blocking with 5 % skim milk to reduce the nonspecific signals. Oocyte samples are usually loaded alongside of His-tagged and GST-tagged proteins as controls.
8. The final concentration for inhibition of protein activity varies from 10 μg/mL to 5 mg/mL in our experiments. The inhibitory antibody against synthetic peptide usually exhibits its effect

at lower concentration than that against bacterially expressed protein, although the yield after antibody purification is relatively low in the case of synthetic peptide antigen.

9. Glutathione beads are applicable to soluble proteins in native condition. If the protein amount is insufficient, alternative culture conditions described in **Note 3** are suggested. If the protein is still insoluble and forms inclusion bodies, their pellet may be washed with lysis buffer A, followed by several rounds of sonication. The suspension added with sample buffer mainly contains GST-tagged protein and can be used for purification.
10. 200 μg of loading proteins is usually moderate. If more amount of proteins are loaded and transferred on a membrane, the proteins are sometimes eluted from the membrane together with antibodies. Pretreatment of the membrane pieces with 0.2 M glycine-HCl (pH 2.0) reduces the coelution. Purified antibodies should be checked whether they contain the GST-tagged protein.
11. If the signal of the desired protein is detected in control beads, oocyte extracts are preincubated with protein A beads to reduce nonspecific binding.
12. In some cases, endogenous proteins cannot be precipitated, while control recombinant proteins can be precipitated. A possible reason would be that endogenous proteins may form complexes with some partner that masks the epitope.
13. One way to confirm neutralizing activity is to assay the activity of purified desired protein in the presence or absence of the antibody. The desired protein can be purified by peptide elution from immunoprecipitates with peptide antibodies.
14. Once ZZ-IBB is thawed, it can be stored on ice for a few days.
15. Because injection of an excess amount of ZZ-IBB causes delay in meiotic progression in starfish oocytes, the injection amount of ZZ-IBB as well as that of the antibody should be optimized. A high-concentration protein solution is viscous and clogs the glass micropipette. To avoid this, NP-40 is added into the injection material at a final concentration of 0.05 %. It should be noted that an injection material which contains more than 0.1 % NP-40 is toxic to starfish oocytes.
16. To check whether ZZ-IBB can transport IgG into the GV, ZZ-IBB is first mixed with a fluorescently labeled IgG (e.g., Alexa488-IgG) and then injected into cytoplasm of an immature oocyte [11]. Thereafter, accumulation of the fluorescent signal into the GV can be examined by fluorescence microscopy.
17. Because histone H1 kinase activity can be measured with oocyte extracts equivalent to less than 0.25 oocytes, it is possible to carry out this procedure with less than five oocytes, if you have

a high-titer antibody against the protein of interest. The frozen oocytes can be stored at –80 °C at least a few weeks.

18. In general, the blocking is believed to be an essential step in immunoblotting to reduce background noise by preventing nonspecific interaction between the antibodies and the blotting membrane or nonspecific proteins. However, the blocking reduces the signal of the target protein as well as the background. In our immunoblotting procedure, we found that omission of the blocking step does not so increase the background noise. Instead, this enhances the signal of the target protein, resulting in increase of signal/noise ratio. It is recommended that an optimal condition is determined in each antibody (i.e., dilution factor of antibody). It should be noted that immunoblotting without the blocking step may not be successful for some antibodies, because of their titer and specificity.
19. Can Get Signal Solution 1 increases the sensitivity and specificity of antibody and reduces the background signal, resulting in a high signal/background ratio. Can Get Signal Solution 1 is optimized for dilution of primary antibody. It is recommended that an optimal dilution factor is determined for each antibody. In most cases, the antibody dilution factor can be increased by using Can Get Signal Solution 1, compared to conventional diluents such as PBS or TBS.
20. To enhance the signal of the target protein, the secondary antibody may be diluted with Can Get Signal Solution 2 which is optimized for the secondary antibody dilution.

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Appendix on Starfish Genomes

Sequencing of the genome and EST of the starfish *Asterina pectinifera* (renamed *Patiria pectinifera* in the 2007 NCBI Taxonomy Browser) is now ongoing in the Japanese team. For details, contact Takeo Kishimoto at kishimoto.takeo@ocha.ac.jp. Some *A. pectinifera* EST sequences are already available in DDBJ/EMBL/GenBank. EST data from another species of the starfish, *Patiria miniata*, are also available on the website of Baylor College of Medicine, http://blast.hgsc.bcm.tmc.edu/blast.hgsc?organism=Pminiata.

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Chapter 22

Immunophotoaffinity Labeling of the Binding Proteins for 1-Methyladenine, an Oocyte Maturation-Inducing Hormone of Starfish

Tetsuo Kida, Shinjiro Matsuda, Atsushi Kuyama, and Tetsuo Toraya

Abstract

Starfish oocytes are naturally arrested at the prophase stage of the first meiotic division and resume meiosis in response to 1-methyladenine (1-MeAde), the oocyte maturation-inducing hormone of starfish. Putative receptors for 1-MeAde have not yet been characterized biochemically, although the specific binding of 1-MeAde to the isolated cortices of starfish oocytes was reported so far. Based on the structure-activity relationship of 1-MeAde analogs, we have designed a photoaffinity labeling reagent. The photoaffinity labeling of oocyte membrane fractions, followed by immunoblotting analysis with anti-1-MeAde antibody, results in the detection of an almost single protein band. This 1-MeAde-binding protein might be a possible candidate of the maturation-inducing hormone receptor of starfish.

Key words Immunophotoaffinity label, Photoaffinity label, Immunoblot, Oocyte maturation, Maturation-inducing hormone, Starfish, *Asterina pectinifera*, 1-Methyladenine, 1-Methyladenine-binding proteins, Receptor

1 Introduction

Fully grown oocytes of starfish are arrested at the prophase stage of the first meiotic division. Reinitiation of meiosis is triggered by 1-methyladenine (1-MeAde), the oocyte maturation-inducing hormone (MIH) of starfish [1], which is produced and released by the ovarian follicle cells in response to the gonad-stimulating substance (GSS), a peptide hormone from the radial nerve [1]. Recently, it has been reported that GSS is closely related to the vertebrate relaxin [2].

Upon exposure of oocytes to 1-MeAde, the maturation-promoting factor (MPF) becomes activated in the cytoplasm [3] and induces oocyte maturation. A MPF has been identified as a complex of *cdc2* kinase (*cdk1*) with cyclin B [4, 5], but the hormonal signal transduction pathway has not yet been fully elucidated. Putative receptors for 1-MeAde have also not yet been

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characterized biochemically, although the specific binding of 1-MeAde to the isolated cortices of starfish oocytes was reported independently by Yoshikuni et al. [6, 7], by Tadenuma et al. [8], and by ourselves [9]. 1-MeAde-induced maturation of oocytes was inhibited by microinjection of pertussis toxin, suggesting the involvement of pertussis toxin-sensitive G-protein in the signal transduction pathway [10]. A starfish G-protein serving as pertussis toxin substrate was purified from the plasma membranes of oocytes [11]. The cDNA encoding its α subunit was cloned, and the deduced amino acid sequence was reported [12]. The βγ subunits of starfish G-protein were shown by Chiba et al. to induce oocyte maturation when injected into cytoplasm [13] and to coexist with cytokeratin filaments in starfish oocytes [14].

Isolation and characterization of 1-MeAde receptors would help us to understand the entire signal transduction pathway from the receptors to MPF. Many analogs of 1-MeAde were synthesized and tested for oocyte maturation-inducing activity [15–18]. Relationship between biological activities as 1-MeAde agonists and antagonists and their competitive binding for 1-MeAde receptors was also investigated with several analogs of 1-MeAde [6, 9]. Based on these structure-binding studies of 1-MeAde analogs, we have designed a photoaffinity labeling reagent for 1-MeAde receptors [19].

In this chapter, we report the methods of photoaffinity labeling of 1-MeAde-binding proteins of starfish oocytes and of detection of the labeled proteins by immunoblotting analysis with rabbit anti-1-MeAde antibody. Figure 1 outlines the principle of immunophotoaffinity labeling method described here.

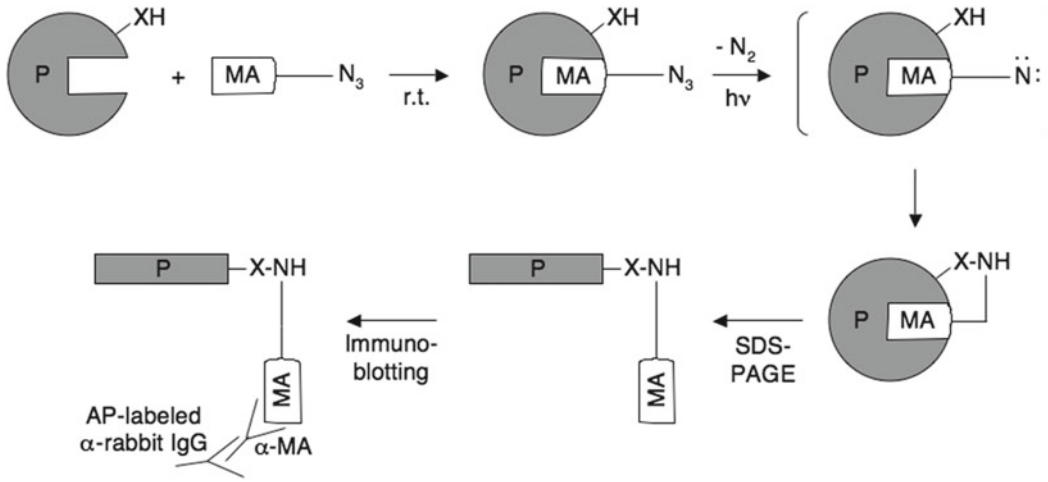

Fig. 1 Principle of immunophotoaffinity labeling. MA—N_3, photoaffinity labeling reagent such as Reagent I (N^6-ANB-AH-CM-1-MeAde); MA, 1-methyladenine; P, 1-methyladenine-binding proteins including receptors; -XH, generic functional group reactive to a nitrene; AP, alkaline phosphatase

2 Materials

Prepare all solutions using pure water and reagent-grade commercial products. Store all reagents in a refrigerator (unless otherwise indicated). Prepare and stock the photoaffinity labeling reagents in the dark (*see* **Note 1**).

2.1 Synthesis of 1-MeAde Analog I

1. Adenosine.
2. Iodoacetic acid.
3. DOWEX-50 (H^+ form) column (bed volume, 300 mL).

2.2 Synthesis of Reagent I

1. *N*-5-azido-2-nitrobenzoyloxysuccinimide.
2. *N*,*N*-dimethylacetamide.

2.3 Preparation and Affinity Purification of Antibody Against 1-MeAde

1. 1-MeAde analog I (Fig. 2a): N^6-(6-aminohexyl)carboxamidomethyl-1-methyladenine (N^6-AH-CM-1-MeAde) synthesized as described below.
2. Dimethyladipimidate · 2HCl in ice-cold water (50 mg/mL).
3. 50 mM sodium phosphate buffer, pH 8.2.
4. Saline: 0.9 % (w/v) NaCl.
5. Tris-buffered saline (TBS): 0.15 M NaCl, 0.01 M Tris–HCl buffer; pH 7.4.

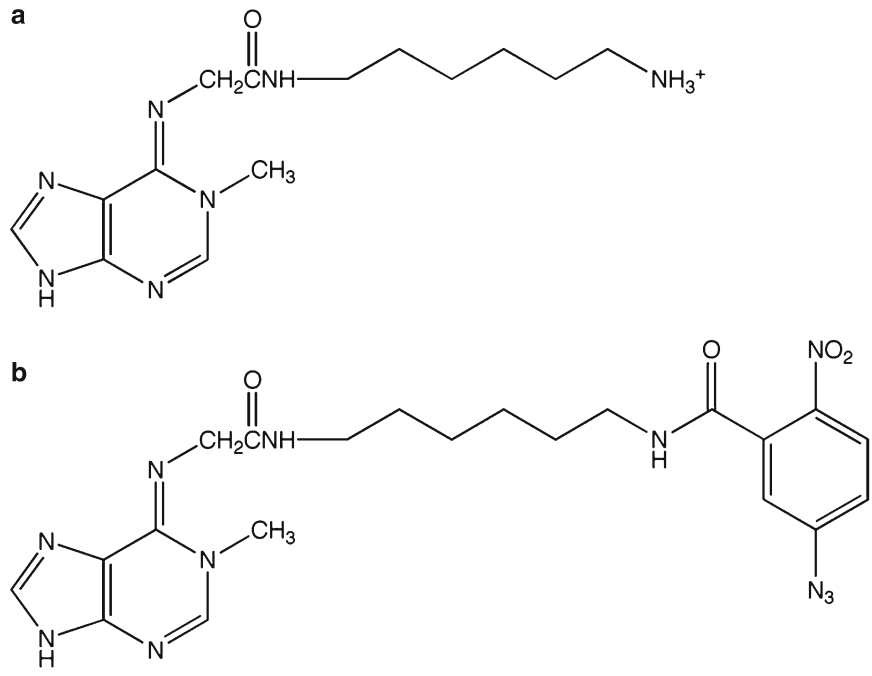

Fig. 2 1-MeAde analog and photoaffinity labeling reagent used in this study. (**a**) 1-MeAde Analog I (N^6-AH-CM-1-MeAde). (**b**) Photoaffinity labeling Reagent I (N^6-ANB-AH-CM-1-MeAde)

6. 1 % (w/v) Triton X-100, 1 M NaCl, 20 mM Tris–HCl; pH 7.5.
7. 0.1 M glycine · HCl buffer, pH 2.5.

2.4 Crude Membrane Preparation

1. Modified van't Hoff's artificial seawater (ASW): 460 mM NaCl, 10.1 mM KCl, 9.2 mM $CaCl_2$, 35.9 mM $MgCl_2$, 17.5 mM $MgSO_4$, 20 mM H_3BO_3. Adjust pH to 8.2 with NaOH.
2. 2 Ca^{2+}-free artificial seawater (CaFASW): The same as ASW, except that 9.2 mM $CaCl_2$ and 460 mM NaCl are replaced by 476 mM NaCl.
3. CaFASW, 2 mM EGTA; pH 6.5.
4. Homogenizing buffer (HB)/1 mM PMSF: 20 mM HEPES buffer (pH 7.4) containing 5 mM EGTA and 5 mM $MgCl_2$. Add 1 % volume of 100 mM phenylmethanesulfonyl fluoride (PMSF) in dimethyl sulfoxide (DMSO).

2.5 Photoaffinity Labeling

1. WSB: Water-saturated 2-butanol.
2. Reagent I (Fig. 2b): 208 mM N^6-(6-(5-azido-2-nitrobenzoyl) aminohexyl)carboxamidomethyl-1-methyladenine (N^6-ANB-AH-CM-1-MeAde). Keep this reagent in the dark.

2.6 SDS-PAGE and Transfer to a PVDF Membrane

1. Solution A: Weigh 29.2 g of acrylamide monomer and 0.8 g of *N,N*-methylenebisacrylamide and make up to 100 mL with water. Store at 4 °C.
2. Solution B: Dissolve 18.2 g of Tris and 0.4 g of sodium dodecyl sulfate (SDS) in 80 mL of water. After adjusting pH to 8.8 with HCl, make up to 100 mL with water. Store at 4 °C.
3. Solution C: Dissolve 6.06 g of Tris and 0.4 g of SDS in 80 mL of water. After adjusting pH to 6.8 with HCl, make up to 100 mL with water. Store at 4 °C.
4. Solution D: Weigh 0.1 g of ammonium persulfate and make up to 1 mL with water. Prepare this solution fresh each time.
5. *N,N,N,N*-Tetramethylethylenediamine (TEMED): Store at 4 °C.
6. 2× Sample buffer: 125 mM Tris–HCl buffer (pH 6.8), 4 % SDS, 20 % glycerol, 10 mM 2-mercaptoethanol, 0.02 % bromophenol blue (BPB).
7. SDS-PAGE running buffer: 0.3 % Tris, 1.44 % glycine, 0.1 % SDS.
8. Marker proteins: Sigma MW-SDS-70L and MW-SDS-6H.
9. Blotting buffer: 25 mM Tris, 192 mM glycine, 0.1 %(w/v) SDS, 10 %(v/v) methanol; pH 8.3.
10. PVDF membrane (ProBlott; Applied Biosystems).

2.7 Immunoblotting

1. Blocking solution: Bovine serum albumin (BSA) in TBS (20 mg/mL).
2. Affinity-purified anti-1-MeAde antibody: Dilute appropriately in the blocking solution.
3. TBS-tween (TBST): TBS containing 0.05 % Tween-20.
4. Affinity-purified goat anti-rabbit IgG antiserum conjugated with alkaline phosphatase (AAF-012-1; EY LABORATORIES, INC): Dilute to 1/5,000 in the blocking buffer.
5. NBT solution: Dissolve 15.0 mg of nitroblue tetrazolium (NBT) in 200 mL of 70 % dimethylformamide (DMF).
6. BCIP (x-phosphate) solution: Dissolve 10.0 mg of 5-bromo-4-chloro-3′-indolylphosphate (BCIP) (x-phosphate) in 200 mL of 100 % dimethylformamide (DMF).
7. Buffer A: 100 mM Tris–HCl buffer (pH 9.5), 10 mM NaCl, 50 mM $MgCl_2$.
8. 10 mM Tris–HCl buffer (pH 8.0), 1 mM EDTA.

3 Methods

Carry out all the procedures at 4 °C unless otherwise indicated. Perform the photoaffinity labeling experiments in the dark or under a safety light.

3.1 Synthesis of 1-MeAde Analog I

1. Dissolve 2.5 g of adenosine and 8.0 g of iodoacetic acid in 200 mL of water. The pH of the solution was adjusted to and maintained at pH 6.5 with 2 M LiOH. After 22 days at 30 °C, adjust pH to 3.0 with 1 N HCl and make up to 2 L with water. Load this solution on a DOWEX-50 (H^+ form) column (bed volume, 300 mL). Wash the column with 5 bed volumes of water and then with 3 bed volumes of 30 %(v/v) ethanol. Elute 1-carboxymethyladenosine (1-CM-Ado) from the column with 30 % ethanol containing 1 N NH_3.
2. Pool the 1-CM-Ado-containing fractions (about 670 mL), concentrate, and make up to 200 mL with water. Adjust pH to 11.0 with 2 N LiOH and heat the solution at 70 °C for 2 h to convert 1-CM-Ado to N^6-carboxymethyladenosine (N^6-CM-Ado) (Dimroth rearrangement). Neutralize the solution to pH 7.0 and make up to 4 L with water. Load this solution on a diethylaminoethyl (DEAE)-cellulose column (acetate form) (bed volume, 300 mL). Wash the column with 5 bed volumes of water and elute N^6-carboxymethyladenosine (N^6-CM-Ado) from the column with 1 % acetic acid. Pool the fractions that show UV quenching and evaporate it to dryness under reduced pressure.

3. Dissolve 0.55 g of N^6-CM-Ado and 2.5 g of hexamethylenediamine (HMDA) in 40 mL of water and adjust pH to 4–5. Add 0.4 g of 1-(3-dimethyaminopropyl)-3-ethylcarbodiimide (water-soluble carbodiimide, WSC) and allow to react at room temperature for 2 h. Then, add WSC six times, 0.1 g each time, with gentle stirring. Add 80 mL of water to terminate the reaction and centrifuge to collect supernatant. Load the supernatant onto the florisil (100–200 mesh) column (bed volume, 103 mL) and wash the column with 5.7 L of water. Elute the N^6-(6-aminohexyl)carboxamidoadenosine (N^6-AH-CM-Ado) with 80 %(v/v) acetone containing 1 %(v/v) acetic acid and evaporate it to dryness under reduced pressure.
4. To N^6-AH-CM-Ado in 4 mL of 50 %(v/v) 1,4-dioxane, add 0.75 g of 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetnitrile (Boc-on) and 531 μL of triethylamine (Et_3N) in 33 mL of 50 %(v/v) 1,4-dioxane three times, 0.25 g of Boc-on and 177 μL of Et_3N each time. Evaporate the reaction mixture to dryness under reduced pressure. After washing with ether, add 2.5 L of water and 300 mL of ether to extract N^6-Boc-AH-CM-Ado. Evaporate the water layer to dryness under reduced pressure. Take up the residue into 20 mL of 10 % 2-butanol in water-saturated 2-butanol (WSB) and load the sample on a silica gel column (bed volume, 120 mL) which has been equilibrated with the same solvent. Evaporate the N^6-Boc-AH-CM-Ado-containing fractions to dryness under reduced pressure.
5. Dissolve N^6-Boc-AH-CM-Ado in 5.5 mL of *N,N*-dimethylacetamide. Add 400 μL, 200 μL, and 100 μL of methyl iodide on days 1, 4, and 5, respectively. On day 6, terminate the reaction by evaporating the mixture to dryness under reduced pressure. After washing with ether, N^6-Boc-AH-CM-1-MeAdo was evaporated to dryness under reduced pressure.
6. Dissolve N^6-Boc-AH-CM-1-MeAdo in 10 mL of 0.5 N HCl and seal the solution in a glass tube. After heating at 95 °C for 45 min, neutralize the reaction mixture with 2 N NaOH and evaporate the hydrolyzate to dryness under reduced pressure to obtain N^6-(6-aminohexyl)carboxamidomethyl-1-methyladenine (N^6-AH-CM-1-MeAde) (1-MeAde analog I in Fig. 2a).

3.2 Synthesis of Reagent I

1. Add 19.4 mg of *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) in 0.5 mL of *N,N*-dimethylacetamide to *N6-AH-CM*-Ado in 4.5 mL of *N,N*-dimethylacetamide and allow to react at room temperature in the dark for 50 h with gentle stirring.
2. Add 400 μL of methyl iodide to this reaction mixture and allow to react at room temperature in the dark for 47 h.

After washing three times with 90 mL of ether, 30 mL each time, evaporate the mixture to dryness under reduced pressure.

3. Dissolve N^6-(6-(5-azido-2-nitrobenzoyl)aminohexyl)carboxamidomethyl-1-methyladenosine (N^6-ANB-AH-CM-1-MeAdo) in 1.5 mL of 0.5 N HCl and seal the solution in a glass tube. After heating at 95 °C for 30 min, neutralize the sample to pH 6–7 by passing through a DEAE-cellulose column (bed volume, 10 mL) twice. Evaporate the effluent under reduced pressure to obtain crude N^6-(6-(5-azido-2-nitrobenzoyl)aminohexyl)carboxamidomethyl-1-methyladenine (N^6-ANB-AH-CM-1-MeAde) (Reagent I in Fig. 2b).
4. Dissolve crude N^6-ANB-AH-CM-1-MeAde in 100 mL of 70 %(v/v) ethanol and purify the desired product by first thin-layer chromatography (TLC) on silica gel 60 F_{254} (20×20 cm) using water-saturated 2-butanol (WSB), second TLC on the same adsorbent using WSB containing 1 % acetic acid, and third TLC on 20 mM borate buffer-impregnated silica gel 60 F_{254} (20×20 cm) using WSB. Photosensitive and oocyte maturation-inducing bands were collected and extracted from silica gel. λ_{max} for N^6-ANB-AH-CM-1-MeAde (Reagent I) in water: 317 nm (peak) and ~270 nm (shoulder).

3.3 Preparation and Affinity Purification of Antibody Against 1-MeAde

1. Dissolve 7.7 mg of keyhole limpet hemocyanin (KLH) into 1.6 mL of 0.1 M $NaHCO_3$ with gentle shaking and then mix with 5 mg of the 1-MeAde analog I (Fig. 2a) in 0.25 mL of 0.1 M $NaHCO_3$. Add 55 μL of dimethyladipimidate·2HCl in ice-cold water (50 mg/mL) and allow to react at room temperature for 3.5 h. Add 200 μL of 50 mM sodium phosphate buffer (pH 8.2), centrifuge, and store the supernatant solution at −80 °C.
2. Add 0.5 mL of saline to 0.4 mL of the above 1-MeAde-KLH solution and then mix with 1.1 mL of Freund complete adjuvant (FCA). Inject 0.1–0.15 mL aliquots to the back of female adult rabbits. Inject the mixture of 0.2 mL of the above 1-MeAde-KLH solution, 0.7 mL of saline, and 1.1 mL of Freund incomplete adjuvant (FIA) for the booster injections on days 21 and 32 after the first immunization.
3. Sacrifice the rabbit 7 days later to collect the whole blood from carotid. After 1 h at room temperature, collect sera by centrifuge and store at −80 °C.
4. Add 1.5 mL of KLH (12 mg/mL in TBS) to 3 mL of the antiserum. After 2 h at room temperature, shake at 4 °C for 2.5 days. Centrifuge at 15,000×*g* to collect the supernatant.
5. Apply the supernatant (4.4 mL) onto a 1-MeAde-Sepharose 4B column (bed volume, 3 mL) and wash the column

successively with 4 bed volumes of TBS, 10 bed volumes of 1 %(w/v) Triton X-100/1 M NaCl/20 mM Tris–HCl (pH 7.5), 10 bed volumes of TBS, and 4 bed volumes of 0.15 M NaCl. Elute the antiserum with 4 mL of 0.1 M glycine·HCl buffer (pH 2.5), pH of the eluate being immediately adjusted to 7.5.

6. Pass the affinity-purified antibody three times through 3 mL of a KLH-Sepharose 4B column, which was preliminarily equilibrated with 5 bed volumes of TBS.

3.4 Oocyte Membrane Fractions of the Starfish Asterina Pectinifera

1. Obtain *Asterina pectinifera* immature oocytes without follicle cells from dissected ovaries by the transfer from Ca^{2+}-free artificial seawater (CaFASW) [6] to modified van't Hoff's artificial seawater (ASW) [20]. Wash three times with CaFASW.
2. Prepare dejellied oocytes from immature oocytes by washing three times with CaFASW/2 mM EGTA (pH 6.5). After washing with ASW, pack the dejellied oocytes by centrifuge at 120×*g* for 30 s.
3. Add 2 packed volumes of HB/1 mM PMSF and homogenize using Teflon homogenizer (5 strokes). Centrifuge at 17,000×*g* for 15 min to remove the supernatant. Add 2 precipitant volumes of HB/1 mM PMSF, resuspend, and centrifuge to remove the supernatant. This operation was repeated twice. Store the oocyte membrane fractions at −80 °C.

3.5 Photoaffinity Labeling of 1-MeAde-Binding Proteins

1. Add 2 μL of 208 mM Reagent I to 10 μL of a crude membrane suspension or its 1 % Triton X-100/1 mM EDTA extract in the dark. After 30 min at room temperature, photo-irradiate the mixture at 0 °C for 2 min with a 250-W tungsten light bulb from the distance of 20 cm.
2. Add 12 μL of 2× Laemmli's sample buffer. After mixing, heat the mixture at 100 °C for 5 min.

3.6 SDS-PAGE and Transfer to a PVDF Membrane

1. For preparing a 7.5 % running gel, mix 2.25 mL of Solution A, 2.25 mL of Solution B, and 4.5 mL of water. Degas with an aspirator. Add 0.01 mL of TEMED and 0.04 mL of Solution D and cast gel in gel cassette. Overlay gently with 50 μL of water.
2. For preparing a stacking gel, mix 0.90 mL of Solution A, 1.5 mL of Solution C, and 3.0 mL of water. Degas with an aspirator. Add 0.01 mL of TEMED and 0.02 mL of Solution D and cast gel in gel cassette. Insert a gel comb immediately without introducing air bubbles.
3. After centrifugation, load supernatants of the heated samples in the sample buffer and marker proteins (MW-SDS-200 kit) on a 7.5 % gel. Electrophorese at 10 mA till the sample has

entered the running gel and then continue at 15 mA till the dye front has reached the bottom of the gel.

4. After electrophoresis, pry the gel plates open, rinse the gel with water, and transfer into a blotting buffer (25 mM Tris/192 mM glycine/0.1 %(w/v) SDS/10 %(v/v) methanol; pH 8.3). Place the gel on a filter paper.
5. Cut a PVDF membrane (ProBlot; Applied Biosystem) to the size of the gel and immerse the membrane in methanol and then in the blotting buffer. Lay a PVDF membrane on the gel and then a filter paper. Place the PVDF membrane-gel-filter paper sandwich to a trans-western blotting apparatus (ATTO AE-3280). Fill up with the blotting buffer and transfer at 10 V for 6 h. Remove the PVDF membrane for immunoblotting.

3.7 Detection of Labeled Proteins by Immunoblotting

1. Block the PVDF membrane with the blocking solution (20 mg/mL BSA in TBS) at 4 °C overnight.
2. Add 10 mL of diluted anti-1-MeAde antibody to the PVDF membrane in a hybridization bag and incubate at room temperature for 12 h.
3. Wash the membrane with TBS-tween three times, 15 min each time, and then with TBS for 60 min with gentle shaking.
4. Add affinity-purified goat anti-rabbit IgG antiserum conjugated with alkaline phosphatase (AAF-012-1; EY LABORATORIES, INC) (1/5,000-diluted with 20 mg/mL BSA in TBS) to the membrane in a hybridization bag and incubate at room temperature for 2 h.
5. Wash the membrane with TBS-tween three times, 15 min each time, and then with TBS for 60 min with gentle shaking.
6. Add 1 mL of the mixture of 4.5 μL of NBT (nitroblue tetrazolium) solution, 3.5 μL of BCIP (5-bromo-4-chloro-3′-indolylphosphate, x-phosphate) solution, and 1 mL of buffer A and let bands develop.
7. Add 10 mM Tris–HCl buffer (pH 8.0)/1 mM EDTA to stop the reaction.
8. Wash with pure water and scan the membrane for bands after being air-dried (Fig. 3).

4 Note

1. To avoid undesired photolysis of a photoaffinity labeling reagent, carry out all the procedures in the dark. Use a safety light in the dark room if necessary.

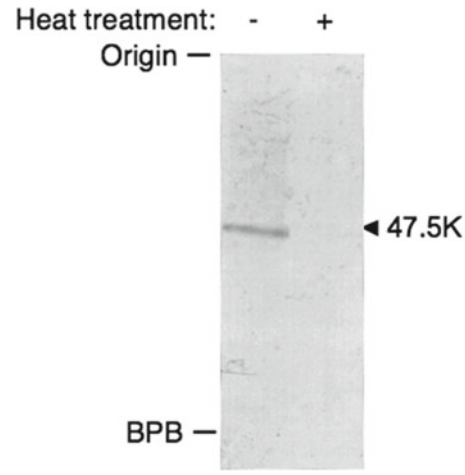

Fig. 3 Immunoblotting analysis of 1-MeAde-binding proteins of starfish oocyte membrane. Crude membrane fraction was extracted with 1 % Triton X-100/1 mM EDTA. The extract was photoaffinity labeled with Reagent I before and after heat treatment at 100 °C for 5 min. The 1-MeAde-binding proteins in the gel were analyzed by immunoblotting with the anti-1-MeAde antibody. *BPB*, bromophenol blue

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