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# Imaging Sea Urchin Fertilization

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## 1. Introduction

Imaging animal fertilization presents a number of unique challenges that arise from the unusual nature of the cells involved. Eggs are among the largest cells produced by animals, and sperm are frequently the smallest. Observation of the initial steps of fertilization, including sperm–egg binding and fusion, can be challenging owing to the size discrepancy between these two cell types and, in many species, the rapid time course over which they occur. Subsequent events in fertilization, including the remarkable activities of the microtubular cytoskeleton during the first cell cycle, are usually obscured by the mass of the egg cytoplasm, and only the most obvious features, such as pronuclear formation, are apparent by routine microscopic methods. The application of confocal microscopy methods to the demands of imaging studies in fertilization has proven to be an extremely valuable approach, as the morphological features of most types of animal eggs are extremely well suited to the specific advantages this technology offers. Confocal microscopy is a powerful and flexible tool, and has been skillfully used and developed by a number of researchers not only to study the structural features of fertilization at high resolution, but also to examine dynamic events in living gametes, including imaging signaling events that occur during and after gamete fusion.

Fertilization and subsequent developmental events have now been studied in a wide variety of species by confocal microscopy. The gametes and embryos of each species have their own special characteristics, but three features detrimental to microscopic studies are exhibited by most types of animal eggs: their large size, the presence of extensive yolk or other type of cytoplasmic inclusions, and the presence of specialized extracellular coats of material that not only can obscure cytoplasmic detail, but can significantly interfere with fixation and immunolabeling efforts. A brief summary of a number of studies

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**Table 1**
**Examples of Fertilization-Related Confocal Microscope Studies**

| Species | Study focus | References |
|---|---|---|
| Human | Chromatin organization | ***(1)*** |
| | Sperm penetration | ***(2)*** |
| | Cytoskeletal organization | ***(3,4)*** |
| Rhesus monkey | Cytoskeletal organization | ***(5)*** |
| Pig | Cortical granule exocytosis | ***(6)*** |
| Cow | Centrosome activity | ***(7)*** |
| | Immunocontraception | ***(8)*** |
| Rodents | ER organization | ***(9,10)*** |
| | Calcium dynamics | ***(11)*** |
| | pH measurements | ***(12)*** |
| | Cytoskeletal dynamics | ***(13)*** |
| | Cortical granules | ***(14)*** |
| | Fertilin expression | ***(15)*** |
| *Xenopus* | Cytoskeletal organization | ***(16–25)*** |
| | Yolk platelet pH | ***(26)*** |
| Sea urchin | Protein kinase C activation | ***(27)*** |
| | Cortical granule exocytosis | ***(28)*** |
| | Calcium dynamics | ***(29)*** |
| | ER organization | ***(30)*** |
| | Cytoskeletal organization | ***(31,32)*** |
| Starfish | Calcium dynamics | ***(33)*** |
| | ER organization | ***(34)*** |
| Ascidian | ER organization | ***(35)*** |
| *Drosophila* | Sperm nonequivalence | ***(36)*** |
| | Cytoskeletal organization | ***(37)*** |
| Silkmoth | Cytoskeletal organization | ***(38)*** |

employing a variety of animal species is listed in **Table 1** to illustrate the types of fertilization studies that have been undertaken using confocal microscopy, and as a launching point toward obtaining further information regarding specimen preparation approaches for specific species. In this chapter, methods to prepare sea urchin eggs and embryos for immunochemical confocal studies of cytoskeletal organization are described.

## 2. Materials

### *2.1. Equipment and Supplies*

1. A dozen 50-mL beakers, a half dozen 300- and 600-mL glass beakers, and a few 1- and 2-L beakers.

2. Disposable pasteur pipets and bulbs.
3. Microscope slides; 22 $mm^2$, 1.5 thickness coverslips.
4. Plastic six-well flat-bottom culture dishes (Falcon No. 3046 or equivalent).
5. Half dozen 60-mm to 90-mm diameter bacteriological plastic disposable Petri plates.
6. Two large shallow pans.
7. Inexpensive inverted or upright microscope with 10× or 20× objective lens.
8. 80 µm and 65 µm mesh Nitex nylon cloths.

## 2.2. Reagents and Solutions

1. Instant Ocean AFSW (artificial sea water); make up following manufacturer's instructions.
2. CaFSW (calcium-free artificial sea water): 16 mM Tris-HCl, 2.5 mM EGTA, 488 mM NaCl, 10 mM KCl, 29 mM $MgSO_4$, 27 mM $MgCl_2$. Add 0.8 g of $NaHCO_3$/4 L, pH to 8.1–8.3 with NaOH.
3. 500 mL of 0.5*M* KCl.
4. PBST (phosphate-buffered saline with Tween-20): 140 m*M* NaCl, 13 m*M* KCl, 2 m*M* $KH_2PO_4$, 8 m*M* $Na_2HPO_4$, 5 mL/L of a 20% stock solution of Tween-20 in distilled $H_2O$.
5. 1 mg/mL poly-L-lysine (mol wt >300,000) in water, aliquoted in 1-mL volumes and stored in freezer (*see* **Note 1**).
6. Anti-β-tubulin monoclonal antibody E7 (Developmental Studies Hybridoma Bank, Iowa City, IA). This antibody reacts with tubulin from a wide variety of species from yeast to humans, and works extremely well with fertilized sea urchin eggs and sea urchin sperm. Cells or culture supernatant can be purchased from DSHB. From a flask of cells grown to exhaustion, centrifuge the culture medium (2000*g* for 10 min) to remove cells, add sodium azide to 0.05%, and aliquot in desired volumes and store at –80C. For immunofluorescence microscopy, culture supernatant can usually be used at a 1:10 dilution in PBST.
7. Goat anti-mouse polyvalent (anti-IgG and -IgM) antibody conjugated to fluorescein isothiocyanate (FITC; Jackson Immunoresearch, Malvern, PA). Antibody is reconstituted according to manufacturer's directions, and aliquoted into 25-µL volumes in 200-µL microcentrifuge tubes and stored at –80°C.
8. Hoechst 33258 (Sigma Chemical Co., St. Louis, MO). A stock solution of 5 mg/mL is made in water and stored in the dark at 4°C. Hoechst strongly binds DNA and is a suspected mutagen; wear gloves when handling.
9. Mounting medium: in a 15-mL disposable centrifuge tube, combine 8 mL of glycerol, 2 mL of 0.5*M* CAPS buffer (pH 9.0), and 0.02 g of DABCO (1,4-diazabicyclo[2.2.2]octane) and 0.01 g *p*-phenylenediamine (all from Sigma; phenylenediamine is a suspected carcinogen and it is best to wear gloves when handling). Wrap in aluminum foil and rotate end-over-end for 1–2 h at room temperature, or until phenylenediamine is dissolved. Solution should be a faint tan color. Aliquot into 1-mL microcentrifuge tubes and store at –80°C (this medium will darken with exposure to air, which is accelerated by temperatures above –10°C; it usable for a number of weeks if stored at –10°C, but is usable for

many months when stored at –80°C. It can be used until quite a bit of discoloring has occurred). PBS or PBST may be used instead of CAPS, but make sure pH is not below 7.4; FITC fluorescence is quenched at an acidic pH.

### 2.3. Animals

Sea urchins used for these studies were obtained from Marinus, Inc. (Long Beach, CA). Two species were used: *Lytechinus pictus*, which are gravid from approximately April through September, and *Strongylocentrotus purpuratus*, which are gravid from approx. November through March. Gravid *L. pictus* females usually yield a few (1 to 10) mL of eggs when induced to spawn, whereas over 50 mL of eggs can be obtained from ripe *S. purpuratus* females. *L. pictus* usually ship well, but in peak season, *S. purpuratus* may begin to spawn during shipment; therefore, one should be ready to begin a number of experiments on the day of *S. purpuratus* arrival.

## 3. Methods

### 3.1. Spawning

1. Prepare for spawning by setting up a workstation containing disposable glass pasteur pipets and bulbs; a number of 50-mL, 300-mL, and 600-mL glass beakers; a small scissors; a blunt forceps, an ice bucket filled with crushed ice; 0.5*M* KCl; and two large shallow waterproof trays (large photographic developing trays work well). Place 1–2 inches of water in the first tray, and add small amounts of ice until the desired temperature is reached (about 12°C for *S. purpuratus*, and 18°C for *L. pictus*; small amounts of ice should be added periodically to maintain the temperature in the desired range. A piece of styrofoam can be placed under the tray of water to reduce the warming rate of the water bath). Cover the bottom of the second tray with 1–2 inches of crushed ice. Fill a number of 50-mL beakers to the brim with AFSW and place in the first water bath tray. Place a few opened 60 to 90-mm diameter plastic petri dishes on the ice in the second tray.
2. Transport urchins in a small container filled with chilled AFSW (the same temperature as the holding aquarium) to the workstation. Open the coelomic cavity by cutting the mouth around the peristomial membrane with a scissors, and discard the mouthparts. Examine gonads to determine the sex of the animal and to assess the reproductive status; ripe ovaries are tan-to-yellow to orange in *L. pictus*, and a brighter orange in *S. purpuratus*. The testes in both species are more tan, and exude white semen when nicked. Ripe gonads should fill a large percentage of the coelomic cavity, whereas poorly developed gonads resemble thin shreds of brown-to-tan material lying along the wall of the coelomic cavity.
3. Gently deliver a few milliliters of 0.5*M* KCl into the coelomic cavity (a wash bottle is convenient), and immediately place females upside down (opened coelomic cavity facing up) on the top of the 50-mL beakers filled with AFSW. Place males upside down on petri plates over ice. Allow spawning to continue for approximately 10 min (until the flow of eggs becomes slow), then remove females

from the beakers of AFSW. If pigment has been released into the AFSW (frequently a problem with *S. purpuratus*), immediately aspirate to just above the level of the eggs and replace with fresh AFSW. Check for thick puddles of sperm underneath males; collect and transfer the thick semen with a pasteur pipette to 1-mL centrifuge tubes, and place on ice (*see* **Note 2**).

### 3.2. Fertilization

1. Strip jelly coats from eggs by passing eggs over a Nitex screen (80 µ*M* diameter for *L. pictus*, and 65 µ*M* for *S. purpuratus*). To do this, gently transfer eggs to pour this suspension over the Nitex cloth into a second empty 600-mL beaker; repeat this step two to three times, collecting the filtrate from the last nitex passage into a clean 600-mL beaker. Let eggs settle (about 10 min), remove AFSW by aspiration, and resuspend in fresh AFSW. Stripping the jelly coats allows for a greater degree of developmental synchrony among a population of eggs by allowing sperm rapid access to the egg surfaces at the time of addition.
2. While the eggs are settling in the final AFSW wash in step 1, prepare 100 mL of a 2× fertilization envelope stripping solution consisting of 0.01% trypsin and either 12 or 20 m*M* dithiothreitol (DTT) (12 m*M* for *L. pictus*, and 20 m*M* for *S. purpuratus*) in CaFSW at the desired temperature (*see* **Note 3**). This solution should be used within approx. 30 min or so of preparation. Also at this time, make a 1:1000 dilution of sperm by adding about 10 µL of semen to 10 mL of AFSW. Mix to obtain a homogeneous suspension.
3. Gently pull up 4 to 5 mL (about two Pasteur pipetfuls) of settled dejellied and washed eggs (collected as they rest in a layer on the bottom of the 600-mL beaker) to 100 mL of fresh AFSW in a 300-mL beaker. Add one half Pasteur pipetful (about 1 mL) of diluted sperm, immediately gently mix by passing eggs back and forth once or twice between another beaker, and start a timer. Remove small aliquots of eggs to check under a microscope at low magnification. The fertilization envelope should begin to rise from one point on each egg within about 1 min. At 2 min from the addition of sperm, fertilization envelopes should be rising from 95% or more of the eggs. At no later than the 2-min mark, add an equal volume of 2× stripping solution and gently mix by passing the egg suspension back and forth between two beakers once or twice. Let the egg beaker rest in the ice bath without disturbance for 5–10 min.
4. At no later than the 10-min mark (earlier, if the eggs settle more quickly) aspirate off as much of the sea water as possible (at least 70–80%), and add fresh CaFSW to fill the beaker. Exposure of eggs to the full-strength stripping solution for longer than 10 minutes frequently results in the formation of small membrane "blebs" as the first cell cycle progresses. Let eggs settle for another 5–10 min, and repeat the wash with fresh CaFSW. At this point, fertilized eggs can be cultured by one of two methods. Relatively low numbers of eggs can be cultured in Petri dishes in a water bath or refrigerated incubator at the appropriate temperature (adjust concentration so that only about 50% of the surface of the dish is covered with eggs), or larger numbers can be cultured in 1-L beakers with stir-

ring. A simple stirring device can be constructed from a clock motor attached to a Plexiglas plate, a glass rod to act as a shaft, and a Teflon paddle attached to the glass rod.

### 3.3. Fixation and Immunolabeling

1. Calculate the number of samples required, and fill a corresponding number of wells in six-well dishes with freezer temperature (–10°C) absolute methanol. Keep dishes in the freezer until used.
2. Two minutes before each desired timepoint, remove zygotes from the culture with a Pasteur pipet and puddle on polylysine-coated glass coverslips (22 $mm^2$, 1.5 thickness). After 2 min, pick up each coverslip with forceps and lower straight down into a well filled with cold methanol. Tip the coverslip during this process as little as possible, i.e., do not let the puddle run off coverslip, and do not "slide" the coverslip into methanol, which can result in mechanical damage to the fragile zygotes. Replace the dish in the freezer, and fix cells for a minimum of 5–10 min (*see* **Note 4**). Cells can be stored in methanol in the freezer for extended periods of time (sea urchin embryos stored for 4 years in absolute methanol at –80°C have been successfully labeled with anti-tubulin and anti-lamin antibodies).
3. To rehydrate cells, remove coverslips from the methanol dishes, briefly drain, and place in similar six-well dishes filled with PBST. After 5 min, transfer coverslips to wells containing 0.5% Carnation nonfat powdered milk in PBST ("blocking" solution), and incubate for 30–60 min at 37°C.
4. Remove coverslips from blocking solution, briefly drain, and place coverslips cell-side-up on a flat sheet of parafilm in a humid container. Apply 100–200 µL of primary antibody at an appropriate dilution and incubate 4–5 h at room temperature (*see* **Note 5**). The parafilm can be labeled with a "Sharpie" indelible marker to identify coverslips.
5. Remove and save antibody, if desired, and wash coverslips in three changes of PBST over 15–20 min. Serially transferring the coverslips through PBST-filled wells in six-well dishes works well for this purpose. During this washing step, rinse residual antibody off of the parafilm and dry with Kimwipes.
6. Replace coverslips cell-side-up on parafilm and apply 100–200 µL secondary antibody at a 1:50 dilution in PBST. If 5 mL or less of secondary antibody is prepared, clear the solution before use by centrifuging in a microcentrifuge (10,000*g* for 3 min), and avoid using solution from the bottom of the microcentrifuge tube. If larger volumes are used, it is usually more convenient to clear the solution by Millipore filtration (0.22 µm pore size) with a disposable syringe filter unit. Failure to clear the secondary antibody frequently results in contamination of coverslips with finely particulate fluorescent background material. Label cells for 1 h at 37°C.
7. Remove secondary antibody from the surface of the coverslip and save, if desired (with the addition of sodium azide to 0.05%, diluted secondary antibody is usually good for 3–4 wk when stored at 4°C), and apply 100–200 µL of PBST containing 5 µg/mL Hoechst 33258. Incubate 5–10 min at room temperature.

8. Briefly drain coverslips and wash in PBST as in step 5.
9. Mount coverslips on glass microscope slides, using phenylenediamine/DABCO/CAPS mounting medium. Remove an aliquot of mounting medium from the freezer and allow it to come to room temperature. Place one drop in the middle of a clean microscope slide, and also place a few small broken pieces of a no. 1.5 coverslip around the mounting medium to support the coverslip and reduce egg deformation. Pick up the coverslip from the last PBST wash with a forceps and carefully wipe the back dry with a Kimwipe. Invert coverslip (cell side down) over the drop of mounting medium and glass pieces. Seal the edges of the coverslip with nail polish, and store slides in a –10°C or –80°C freezer. Slides may be stable for many weeks in the freezer, but should be photographed as soon as possible (immediately is best, but within 1–3 days is usually acceptable) because some redistribution of fluorochrome does occur with time (in particular, nuclear fluorescence frequently appears with extended storage).

### 3.4. Cytoskeletal Experiments

The first cell cycle in fertilized sea urchin eggs takes approximately 1.5 h to complete, depending on the species and culture temperature. During this time, the microtubule cytoskeleton undergoes a series of remarkable structural reorganizations, which attend its functions in bringing the pronuclei together and in forming the mitotic spindle (**Table 2**). The dynamic behavior of microtubules during the first cell cycle is readily amenable to disruption by a wide variety of pharmacological and physical treatments. For an illustration of a typical cytoskeletal disruption experiment, the treatment of *L. pictus* zygotes with the carbamate herbicide CIPC (chlorpropham) is described (for further details, *see* **ref. *39***).

Prepare a 100 m*M* stock solution of CIPC [N-(3-chlorophenyl)carbamate] (Sigma) in dimethyl sulfoxide (DMSO). This solution appears to retain its activity for weeks, but not months, when stored at room temperature. Add 1, 10, or 100 µL of CIPC stock solution/100 mL of egg culture just before adding sperm, to obtain a 1 µ*M*, 10 µ*M*, and 100 µ*M* concentration, respectively. Fertilize and culture the eggs as described previously, with CIPC added to all AFSW and CaFSW solutions. Fix and immunolabel as described, and compare microtubule patterns between CIPC-treated embryos and control embryos (treated with equivalent amounts of DMSO only). A range of cytoskeletal perturbations at each timepoint should be apparent, with little or no effects noticeable at 1 µ*M*, significant alterations occurring at 10 µ*M*, and gross disruption of cytoskeletal organization and cleavage patterns resulting from treatment with 100 µ*M* CIPC (**Fig. 1**).

## 4. Notes

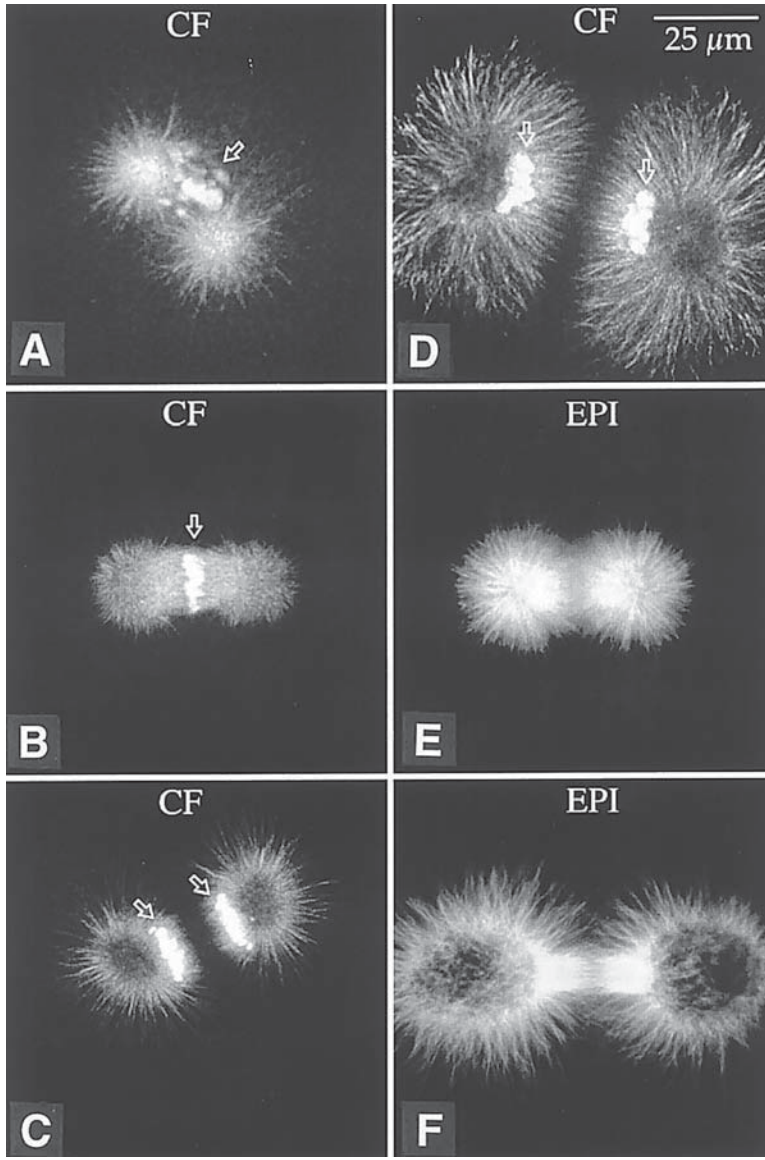
1. The polylysine is used to make polylysine-coated coverslips in the following way. Transfer a few hundred 22 $mm^2$ 1.5 thickness glass coverslips to a beaker

**Table 2**
**Developmental and Cytoskeletal Landmarks, First Cell Cycle, *L. pictus*, 18°C**

| | |
|---|---|
| 0–5 min: | Sperm incorporation |
| 5–10 min: | Beginning of sperm aster formation; sperm-associated centrosomal material only faintly detectable |
| 10–20 min: | Sperm aster greatly increases in size; pronuclei approach each other; maternal centrosomal material recruited around nuclei (especially sperm nucleus) |
| 25–35 min: | Pronuclei fuse; centrosomal material completely surrounds the zygote nucleus; bipolarization of microtubule organizing centers occurs at the end of this period |
| 45–55 min: | Chromatin in zygote nucleus appears more homogeneous; microtubules form "streak"; centrosomal material becomes elongated in plane of "streak" |
| 55–65 min: | Chromosomes begin to condense; microtubules begin to form bipolar spindle apparatus; centrosomal material surrounds nucleus, with concentrations at the spindle poles |
| 65–75 min: | Prophase progresses to prometaphase; centrosomal material completely separated and localized to each of the spindle poles |
| 75–80 min: | Metaphase |
| 80–85 min: | Anaphase |
| 85–95 min: | Telophase and cytokinesis |

containing hot water and standard laboratory detergent, such as Alconox. Let sit for 5–10 min, with periodic agitation. Decant detergent, and rinse extremely well with warm tap water (at least 10 changes), followed by a final rinse with distilled water. Decant distilled water and add 95% ethanol to completely immerse coverslips, and cover with parafilm to store. For coating, remove desired number of coverslips and place on paper towels. Rub the ethanol off of the exposed surface of the coverslip with a Kimwipe until the coverslip "squeaks." Let dry completely, and add a 10- to 15-µL puddle of 1 mg/mL polylysine to one half of the coverslips. Invert the other one half of the coverslips over the polylysine-coated coverslips to form pairs of

Fig. 1. (*facing page*) First cell cycle mitosis in CIPC-treated zygotes **(A–D)** and Triton X-100/glycerol extracted specimens **(E,F)**. **(A–D)** Confocal microscopy (CF); **(E,F)** routine epifluorescence microscopy (EPI). **(A)** Prophase; the incipient spindle poles have failed to become aligned in a straight line with the main mass of chromatin (i.e., have not become perfectly apposed). **(B)** Metaphase; note the clublike appearance of the spindle, and the truncated astral microtubules. **(C)** Anaphase; astral microtubules are again truncated, and the chromosomes are arrayed over an unusually broad plane. **(D)** Telophase; microtubules are relatively dense, but fail to penetrate the most peripheral areas of the cortex. Cytokinesis in embryos treated with 10 µ*M* or higher CIPC frequently fails to be




completed. **(E,F)** Control (non-CIPC-treated) zygotes subjected to Triton X-100/glycerol extraction prior to fixation in cold methanol. Routine epifluorescence microscopy. **(E)** Metaphase; **(F)** anaphase. Note the distortion of the microtubule cytoskeleton in the anaphase spindle (compare with **Fig. 2F**). The metaphase spindle in this example is very well preserved for extracted specimens; frequently, detectable physical distortion occurs in this stage, as well as all others, following extraction. **(A–D)** Double labeled with E7 anti-β-tubulin antibody and Hoechst; arrows indicate chromatin.

coverslip "sandwiches" (cleaned surface to cleaned surface). Let sit for 5 min, and slide each pair apart, and place wet-side-up on paper towels. Examine the wet surfaces of each coverslip: if the residual polylysine immediately beads up into tight droplets, discard coverslip (this indicates surface was not sufficiently clean, and eggs will not stick well). Save those coverslips on which the polylysine remains largely spread out (some contraction of the puddle is acceptable).

2. Some researchers prefer to use a syringe and inject 0.5*M* KCl into the coelomic cavity through the peristomial membrane. However, removing the mouth allows the status of the gonads to be visually determined; in addition, animals appear to generally release more gametes with this approach. On the other hand, the more traumatic method of mouth removal also seems to result in the release of more pigment along with the eggs (especially in *S. purpuratus*), which can be inhibitory to fertilization if not washed away.
3. There are a number of alternate methods to strip the fertilization membrane from the fertilized egg, including the use of urea or aminotriazole (ATA) instead of trypsin/DTT. The ATA method ***(40)*** is very convenient, and has the advantage that close attention does not have to be paid to the time of addition of stripping solution. However, the quality of fixation and immunolabeling is very sensitive to the presence of hyalin, which is secreted onto the surface of the egg. The trypsin/DTT method, followed by culturing in CaFSW, appears to be the most effective in removing this material, and usually results in the best images after processing is complete.
4. Alternate approaches toward preparing sea urchin embryos for microscopy include the use of formaldehyde-based fixatives, and extracting eggs and embryos prior to fixation. Direct methanol or formaldehyde fixations usually result in excellent morphological preservation, but the large size of the eggs results in a "muddy" image of immunolabeled cytoskeletal elements when viewed by routine epifluorescence microscopy (compare **Fig. 2 A**, **C**, **E** with **B**, **D**, **F**). To circumvent this problem, extraction methods have been developed to visualize the cytoskeleton more clearly by routine epifluorescence microscopy ***(41)***. However, extraction can produce significant artifacts (compare **Figs. 1F** and **2F**), and the most faithful preservation of morphological relationships is obtained by direct fixation in either methanol or paraformaldehyde. Methanol fixation is usually better for immunocytochemistry, and produces excellent images when coupled with confocal microscopy.
5. Primary antibodies are diluted so that a 1-h incubation at 37°C produces acceptable labeling results (strong specific labeling with low background). The following incubation times and temperatures yield roughly comparable labeling intensities: 1 h at 37°C, 3–5 h at room temperature, or overnight (about 16 h) at 4°C. Subjectively, the best results are obtained from a 3- to 5-h incubation at room temperature. The intensity of immunolabeling can be markedly increased by incubating overnight at room temperature. Many solutions have been used prior to the primary antibody to "block" nonspecific protein binding sites, including BSA (bovine serum albumin) and 10–20% normal goat serum. However, a solution of 0.5% nonfat milk is at least as effective for all antibodies so far tested in this laboratory, and is extremely simple and economical to use.

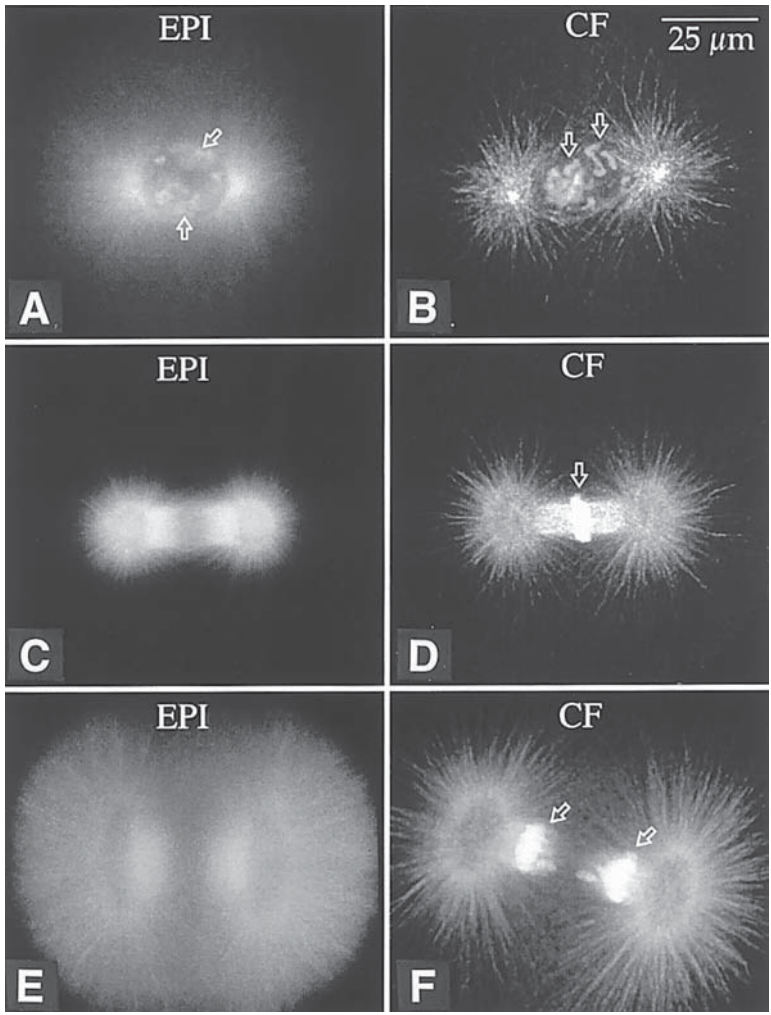



Fig. 2. First cell cycle mitosis by epifluorescence microscopy (EPI) and confocal microscopy (CF). **(A,B)** Prophase. **(C,D)** Metaphase. **(E,F)** Anaphase. All specimens were fixed by immersion in cold methanol and labeled with E7 anti-β-tubulin antibody. All images except for **(C)** and **(E)** are double exposures, showing both tubulin and Hoechst staining; *arrows* indicate Hoechst-stained chromatin. Note the marked increase in clarity and detail in the CF images.

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