Chapter 1

Use of Echinoderm Gametes and Early Embryos for Studying Meiosis and Mitosis

Debadrita Pal, Florencia Visconti, Silvia P. Sepúlveda-Ramírez, S. Zachary Swartz, and Charles B. Shuster

Abstract

The early embryos of sea urchins and other echinoderms have served as experimental models for the study of cell division since the nineteenth century. Their rapid development, optical clarity, and ease of manipulation continue to offer advantages for studying spindle assembly and cytokinesis. In the absence of transgenic lines, alternative strategies must be employed to visualize microtubules and actin. Here, we describe methods to visualize actin and microtubule using either purified, recombinant proteins, or probes in in vitro-transcribed mRNAs.

Key words Sea urchin, Sea star, Mitosis, Meiosis, Cytokinesis actin, Microtubule, Confocal microscopy

1 Introduction

Echinoderm embryos have a long and storied history in the field of cell division, dating back to Hertwig's observations regarding spindle orientation [1] and Boveri's postulates regarding aneuploidy and cancer [2]. Our understanding of the dynamic nature of the mitotic spindle, determination of the cleavage plane, centrosome duplication, and the spindle assembly checkpoint is based on studies by Dan, Inoue, Mazia, Hiramoto, Rappaport, Salmon, Sluder, and others who took advantage of the optical clarity of echinoderm embryos as well as their amenability to physical manipulation of cellular geometry. The capacity to generate large-scale, synchronous cultures also afforded investigators the ability to biochemically analyze the mitotic spindle in isolation [3] and purify critical spindle factors such as cytoplasmic dynein [4] and kinesin-like proteins [5–10]. Thus, the eggs and early embryos of sea urchins played foundational roles in the study of mitosis and cytokinesis.

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Green fluorescent protein (GFP) affords the capacity to study the localization and dynamics of individual components of the mitotic apparatus, and echinoderm oocytes and embryonic blastomeres have been used to great effect to study astral microtubule dynamics [11], actin dynamics during oocyte maturation [12–16], cleavage plane specification [17–20], and Rho GTPase regulation of the actin cortex [21]. One challenge to live cell fluorescence microscopy in echinoderms is that the fluorescently tagged proteins must be expressed exogenously, as the methodologies for developing transgenic lines are still under development. In vitro-transcribed mRNA's may be microinjected at the egg stage, but because GFP and its variants fold comparatively slowly, it is often difficult to get sufficient expression to image prior to the third division, although expression at the zygote or two-cell stage has been reported in several cases [22, 23].

The female gametes of sea urchins and sand dollars (echinoids) are spawned as post-meiotic eggs arrested in a translationally quiescent, G1-like state. The acidified cytoplasm in eggs suppresses translation until fertilization (or parthenogenetic activation) at which time the rise in cytoplasmic pH triggers new protein synthesis and resumption of the cell cycle [24, 25]. Thus, translation of exogenous mRNA in time for the first division can be a challenge, but GFP-tagged proteins purified from bacteria or other expression systems have been successfully used in zygotes [11, 26]. In contrast, germinal vesicle-stage oocytes isolated from sea stars are translationally active, and mRNAs may be injected and oocytes cultured for up to 3 days, allowing for high level expression for imaging of meiosis or mitosis. In the paragraphs below, we will describe methods for purification and injection of recombinant protein or mRNAs for live cell imaging of the actin and microtubule cytoskeletons.

2 Materials

2.1 Expression and Purification of Recombinant Lifeact

1. LB-Miller broth and agar plates (supplemented with 100 mg/ml ampicillin).
2. BL21(DE3) STAR competent cells (Thermofisher).
3. SOC medium.
4. D-Glucose.
5. Isopropyl-β-D-thiogalactopyranoside (IPTG).
6. Ni-NTA Fast Start Kit (Qiagen).
7. Column wash buffer: 20 mM NaH_2PO_4, 500 mM NaCl, 30 mM Imidazole, pH 7.4.

8. Dialysis buffer: 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 10% glycerol, 0. 1 mM DTT, pH 7.0.
9. Pierce BCA Protein Assay kit (Thermofisher).

2.2 In Vitro Transcription

1. SP6 mMessage mMachine Kit (Thermofisher).
2. Poly A Tailing Kit (Thermofisher).
3. NucleoSpin Gel and PCR Clean-Up Kit (Takara Bio).
4. RNaseZap (Thermofisher).
5. Plasmids: Histone H2B-mCherry (Addgene # 108880), pCS2-EMTB-3xGFP (Addgene # 26741), pCS2-Lifeact-mCherry-2A-EGFP-tubulin [27].

2.3 Microinjection and Imaging

1. FluoroDish 35 mm cell culture dish (World Precision Instruments).
2. 1.0 mm diameter borosilicate glass capillary micropipettes with internal filament (World Precision Instruments).
3. Dual-Stage Glass Micropipette Puller (Narishige).
4. 35 mm MatTek dish (20 mm glass diameter).
5. 1-Methyladenine (Fisher).
6. Antibiotics: sulfamethoxazole, trimethoprim, penicillin/streptomycin.

2.4 Isolation and Culturing of Echinoderm Gametes and Embryos

1. Echinoderms: *Lytechinus pictus*, *Dendraster excentricus*, *Patiria miniata* (Marinus Scientific, Pt. Loma Marine Invertebrate Lab, or South Coast Bio-Marine, LLC).
2. Calcium-free seawater (CaFASW): 379 mM NaCl, 106 mM $MgCl_2$, 30 mM Na_2SO_4, 10 mM KCl, 2 mM $NaHCO_3$, 2.5 mM ethylene glycol-bis-N,N,N′,N′-tetraacetic acid (EGTA), pH 8.0.
3. Artificial seawater (ASW), pH 8 (Coral Pro Salt, Red Sea).
4. Acetylcholine.
5. Nitex mesh: 150 μm for *L. pictus* and *D. excentricus*; 180 μm for *Patiria miniata*.
6. ST antibiotic cocktail: 10 mg/ml trimethoprim, 50 mg/ml sulfamethoxazole in DMSO. Aliquot and store at −20 °C.

3 Methods

3.1 Expression and Purification of Recombinant Lifeact

1. Lifeact is a low affinity actin probe that binds both monomeric and filamentous actin [28] and in our hands, may be expressed at high levels with no effect on cell division or development (*see* **Note 1**). The probe itself consists of 17 residues of yeast

ABP140 at the N-terminus of a fluorescent protein such as EGFP or mCherry, and for bacterial expression, Lifeact is expressed from pET101 with a C-terminal polyhistidine tag. We typically use either Bl21(DE3) STAR or Rosetta derivatives of BL21 cells and begin starter cultures within 2 days of the initial transformation.

2. Using a sterile inoculation loop, transfer a single colony from the plate into a fresh 100 ml LB media culture supplemented with 100 μg/ml ampicillin and 2% glucose. Culture overnight in a shaking incubator at 30 °C. Note: Lifeact proteins do not appear to be particularly toxic to *E. coli*, and thus we typically do not use BL21(DE3) pLysS cells.
3. Transfer 50 ml of the overnight culture to a fresh 2 l flask containing 500 ml of LB media supplemented with 100 μg/ml ampicillin and 2% glucose, and incubate with vigorous shaking at 30 °C. Monitor cell growth by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer. Once the OD_{600} reaches between 0.6 and 0.8, then add IPTG to 1 mM to induce Lifeact expression 6–8 h, although 3–5 h is usually sufficient. Harvest the bacterial pellet by centrifugation for 15 min at 4000 × *g*. Discard the supernatant and either lyse the pellet immediately or freeze pellet at −20 °C.
4. Recombinant Lifeact may be easily purified by one-step affinity purification, generating protein that is sufficiently clean for microinjection into echinoderm embryos. A single 500 ml culture provides enough protein for thousands of individual injections, and for affinity purification we typically use the QIAGEN Ni-NTA kit, where polyhistidine-tagged proteins are affinity-purified using a pre-packed, 1 ml Ni-NTA column.
5. Allow bacterial pellet to thaw on ice, and while the pellet is thawing, prepare the lysis buffer by supplementing the kit's lysis buffer with 100 μl of prepared lysozyme and 10 μl of benzonase per 10 ml of lysis buffer according to the manufacturer's instructions. Once bacterial pellet has thawed, resuspend it in 10 ml of prepared lysis buffer. Triturate until no bacterial clumps remain.
6. Incubate in ice for 30 min, mixing occasionally. Although not essential, we typically sonicate cells 2–3 times for 45 s to shear chromosomal DNA and ensure that all cells are disrupted.
7. Clarify the lysate at 14,000 × *g* for 30 min at 4 °C. The resulting lysate should be deep red or green depending on the fluorescent protein. Break the seal at the tip of the column and unscrew the cap, and allow the storage buffer to drain. Once the storage buffer has drained, add the clarified lysate to the column and collect the flow-through fraction.

8. The column should appear deep red or green, and while the capacity of the column is above what is needed, but if there is still Lifeact in the flow-through fraction, it may be passed over the column a second time. Wash the column with at least 30 ml of cold wash buffer, running about 10 ml at a time. Allow the buffer to drain out completely each time. Note: the kit recommends using much less wash buffer, but we find that washing with 20–30 volumes results in a much cleaner product.
9. Elute the column 2–5 ml elution buffer, collecting 1 ml fractions and storing them on ice.
10. Dialyze the fractions in a minimum of 200 volumes of dialysis buffer overnight at 4 °C (*see* **Note 2**).
11. Clarify the fractions by centrifugation at 14,000 × *g* to remove any aggregated protein. Determine the protein concentration of the fractions using a Bradford assay and analyze the purity of fractions by SDS-PAGE (*see* **Note 3**).
12. Prepare 10 μl aliquots of your dialyzed protein into 0.5 ml Eppendorf tubes, snap freeze in liquid nitrogen, and store at −80 °C. In our hands, Lifeact is very stable and once thawed, may be stored at 4 °C for at least a week.

3.2 In Vitro Transcription of mRNAs for Expression in Echinoderm Oocytes and Embryos

1. We commonly use a pCS2-derived plasmid that encodes Lifeact-EGFP/mCherry with a viral 2A peptide on its C-terminus that allows for expression of Lifeact and a second protein from a single transcript. An EcoRV cloning site is used for subcloning the second protein [27, 29]. To generate mRNA for microinjection, pCS2-derived plasmids are linearized with NotI (located 3′ to the SV40 polyadenylation site) purified using NucleoSpin PCR Clean-Up Kit to obtain in vitro transcription-ready, linearized plasmid. Alternatively, linearized plasmids can be purified for in vitro transcription through ethanol precipitation by adding to the linearization reaction 1/20th volume of 0.5 M EDTA, 1/10th volume of 3 M sodium acetate, and 2 volumes of ethanol. For higher DNA yield, we incubate the reactions overnight at −80 °C, and recover DNA by centrifugation.

2× NTP/CAP	10 μl
10× reaction buffer	2 μl
Linearized template DNA	0.5–1.5 μg
SP6 enzyme mix	2 μl
Nuclease-free water	To 20 μl

2. Assemble the in vitro transcription reaction using SP6 mMessage mMachine Kit according to manufacturer's specification. All reagents from the kit are thawed, centrifuged before

opening, and kept on ice, except for the 10× reaction buffer, which is kept at room temperature after thawing to avoid formation of a precipitate. Assemble the reagents in a PCR tube, and after thoroughly mixing the reagents, the tube is incubated for 2 h at 37 °C. For longer transcripts, it is advisable to add an additional 1 μl of the GTP solution included in the kit.

3. Following the in vitro transcription reaction, add 1 μl of turbo DNase and incubate for an additional 15 min at 37 °C to degrade the template DNA. Polyadenylate the transcribed RNA using a Poly A Tailing Kit according to manufacturer's instructions.

Transcription reaction	20 μl
5× E-PAP buffer	20 μl
25 mM $MnCl_2$	10 μl
10 mM ATP solution	10 μl
Nuclease-free water	36 μl

Assemble the reaction, add 4 μl of E-PAP enzyme, and incubate at 37 °C for 1 h.

4. Add 60 μl of the lithium chloride precipitation solution (7.5 M lithium chloride, 50 mM EDTA) from the mMessage mMachine Kit to the 100 μl polyadenylation reaction and incubate overnight at −20 °C to −80 °C. RNA may be recovered using a number of protocols, including spin columns and phenol-chloroform extraction. However, lithium chloride precipitation affords a simple procedure that offers excellent recovery of injection-ready RNA. If lithium toxicity is a concern, good RNA recoveries can be obtained using half as much of the lithium chloride.
5. Centrifuge at 14,000 × *g* for 30 min at 4 °C in a refrigerated microfuge. Wash 1× with 80% EtOH and centrifuge an additional 5 min at 14,000 × *g*. Carefully remove as much of the supernatant as possible and air-dry the pellet 15–20 min.
6. Resuspend the pellet in 10–20 μl of nuclease-free water and quantify using a spectrophotometer. Store at −80 °C. Yields are typically in the 10–20 μg range.

Note: Be sure to use barrier tips for all steps and treat bench tops, hand gloves, and pipettors with RNaseZap prior to use.

3.3 Isolation and Culturing of Sea Urchin Eggs

A number of species of sea urchins and sand dollar embryos may be used for live cell imaging, and each has its distinct egg size and optimal temperature for development [30]. However, *Lytechinus*

variegatus, *Lytechinus pictus*, and the sand dollar *Dendraster excentricus* are among the most optically clear species for imaging. Here we describe methods optimized for *L. pictus.*

1. Unlike other urchins, the sexes of *L. pictus* may be differentiated by the larger, darker gonopores on females. To induce spawning, inject 0.5 M KCl into the coelomic cavity to induce spawning. Invert female urchins over a 10 ml beaker of artificial seawater (ASW) to collect eggs. Collect sperm "dry" off of the surface of the male urchins using a 1.5 ml tube, and store at 4 °C when not being used (for up to 5 days). Although fertilization rates are best with sperm used within 24 h, sperm collected and stored in this manner may be good for up to 3–4 days, depending on the species.
2. Wash eggs by transferring them to a 50 ml beaker with ASW. Let the eggs settle at the bottom, discard water, and then add fresh ASW. Eggs are best used soon after spawning. Store in a 14 °C incubator for short-term storage (*see* **Note 4**).
3. Once ready to fertilize, resuspend eggs at a concentration of $\leq$1% (v/v) in 10 ml artificial seawater in a 15 ml falcon tube. Dilute sperm 1:1000 in a 1.5 ml Eppendorf tube, mix well, add one or two drops to the egg suspension, and start a timer.
4. Gently invert the falcon tube a few times to disperse and mix the gametes. Add a drop of egg suspension to a slide and confirm fertilization efficiency on a compound microscope. Within 30–45 s, the fertilization envelope should begin to elevate off the egg surface, indicating a successful fertilization.
5. Using either a clinical centrifuge set to the lowest speed or a hand centrifuge, spin down the eggs within 60 s of fertilization. Discard the seawater and re-fill with fresh calcium-free seawater (CaFASW) and gently resuspend the eggs and transfer to a 50 ml beaker with about 25 ml of artificial seawater.
6. Fertilization envelopes must be removed before proceeding with microinjection. To achieve this, pass the egg suspension through a 150 μm Nitex mesh secured on the cap of a 50 ml falcon tube that has been cut in half, allowing the eggs to pass through the Nitex into the new beaker. Repeat this step two to three times to ensure removal of fertilization envelopes. Inclusion of 2 mM 3-amino-1,2,4-triazole in the seawater during fertilization will also prevent hardening of the envelope and aid in mechanical removal of the envelope. The eggs are now ready to be used for microinjections. In general, it is advisable to inject eggs within 30 min of fertilization, since the extracellular hyaline layer will harden over time, complicating injection.

3.4 Isolating and Culturing Sea Star (Patiria miniata) Oocytes

Obtain a small amount of sea star ovary by making a small incision on one of their radial arms using a sharp and clean razor blade (Fig. 1a). Using curved forceps, obtain a small section of the ovary tissue from the incision (Fig. 1a, b). Intact ovary fragments may be kept in petri dishes of ASW supplemented with pen strep solution for up to 5 days. When maintained inside the ovary fragment, these oocytes maintain competency for meiosis and fertilization more robustly than if denuded. When needed, a portion of the oocytes can be subsequently released from the ovary fragments as described below. Oocytes that dissociate from the ovary can be used up to 48 h if stored at 14 °C.

1. To remove oocytes from ovary tissue, place fragments into 10 μM acetylcholine-artificial seawater for 8–10 min (Fig. 1c) to stimulate extrusion of oocytes from the ovary tissue. Alternatively, the ovary tissue may be gently chopped with fine dissection scissors to release oocyte-follicle cell complexes (*see* **Note 5**). After extraction, wash oocytes in ASW. If the oocytes are to be used within 24 h, they may be kept in normal ASW supplemented with antibiotics (100 mg/ml gentamycin or 1× Pen-Strep, 1:100 dilution from stock solution).

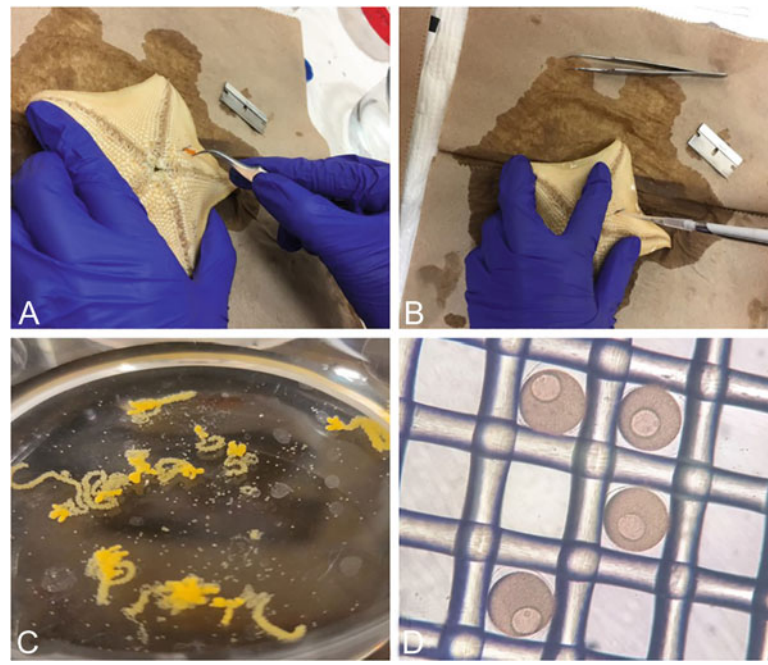

Fig. 1 Isolation of sea star oocytes. A small incision is made on the ventral side of a *P. miniata* sea star, and a small amount of ovary tissue is removed using curved forceps (**a**). Simultaneously, coelomic fluid is taken from the incision with the help of a pipette and saved for long-term oocyte culture (**b**). Oocytes may be isolated from the ovary tissue either by mincing or short-term exposure to acetylcholine, which induces contraction of the tissue and extrusion of the oocytes (**c**). Oocytes trapped within Nitex mesh (**d**)

2. If desired, the associated somatic follicle cells may be removed by placing the oocytes in cold CaFASW for 15 min (*see* **Note 6**). Replace with fresh CaFASW for an additional 15 min before washing with ASW. CaFASW will remove the follicle cells, and while this can assist with microinjection and their removal can facilitate imaging, it is not absolutely necessary.
3. If long-term culture is desired following microinjection (e.g., to deplete a protein by morpholino injection or to track gradual dynamics of a protein [31]), culturing oocytes (that have not been denuded of their follicle cells) in the presence of coelomic fluid help preserve viability. In a separate 1.5 ml centrifuge tube, extract coelomic fluid from the body cavity of the starfish using a P1000 pipette. The coelomic fluid should appear slightly turbid due to the abundance of coelomocytes. Supplement the coelomic fluid with 1:1000 ST. Sterile filter the coelomic fluid + ST solution through a 0.22 μm syringe filter. Culturing oocytes in the presence of coelomic fluid can maintain viability for up to 2 weeks when the media is changed every 2–3 days [31].
4. To stimulate meiotic maturation, add 1–2 μM 1-methyladenine. When cultured at 14 °C, germinal vesicle breakdown occurs between 30 and 40 min after hormone addition. When cultured at 14 °C, first polar body extrusion in *P. miniata* occurs roughly at about 90 min post hormone addition and the second polar body extrusion occurs at about 120 min post hormone addition. In absence of fertilization, the cell arrests at the egg pronuclear stage.
5. To obtain sperm, make a similar incision on one of the radial arms of a male sea star, and collect a small amount of testes into an Eppendorf tube (this can be stored at 4 °C for up to 5 days). Make a 1:1000 dilution of sperm and add 5 μl of this dilution to a 1 ml suspension of oocytes shortly after GVBD. Although the fertilization envelope will not elevate as dramatically as the sea urchin, it will be visibly separated from the plasma membrane. While removing the fertilization envelope is a more involved process than with sea urchins, it is still possible to microinject after fertilization, and removal of the envelope may not be necessary for many applications.

3.5 Microinjection

The following protocol can be followed regardless of whether you are working with sea urchin eggs or starfish oocytes. The methods described here utilize micromanipulators mounted on an inverted microscope stand (Zeiss Axiovert 200M), but horizontal microinjection systems are equally popular for microinjection and micromanipulation of echinoderm embryos [32].

1. Capillary micropipettes (World Precision Instruments) sufficient for injecting echinoderm gametes may be generated with a simple gravity-based pipette puller such as a Narishige PC-12. For this purpose, 3 in. borosilicate glass capillary micropipettes with an internal filament are used. Heating conditions will need to be empirically determined, but a sharp taper is generally preferable.
2. Clarify RNA or protein at 14,000 × *g* for 15 min at 4 °C in a microcentrifuge to pellet any aggregates and avoid clogging of the micropipettes. Place 0.5 μl of RNA or protein (depending on your experiment) onto the back of the micropipette, and the fluid will rapidly migrate to the tip by capillary action. It is advisable to keep pipettes at 4 °C or on ice until use.
3. This loaded micropipette is then mounted on an instrument holder attached to a Picospritzer pressure injection system pressurized to 60 PSI. The instrument holder is then positioned using either a Leica M or a Narishige NAI-3Z micromanipulator mounted on a Zeiss Axiovert 200M inverted microscope with a Brook temperature stage.

3.6 Methods for Sea Urchin Eggs

1. Generally, we do not need to immobilize *L. pictus* eggs if they are injected soon after fertilization, and we use a fresh 35 mm glass-bottomed dish (*see* **Note 7**).
2. When microinjecting on an inverted microscope, it is necessary to position the instrument holder at a steep angle to avoid the condenser. Position the tip of the micropipette such that it is within the plane of view (that can be easily determined by closing the light aperture), and then slowly lower the pipette using the *z* axis control. Bring the needle down until it is just above the top of the eggs, keeping the needle in focus at all times.
3. Unless you are using micropipettes that have been beveled, it will be necessary to break the tip prior to microinjection. Using the fine control on the micromanipulator, bring the micropipette down until it just touches the glass bottom of the dish. Immediately withdraw the needle from the glass, and inject into the seawater to see if fluid is moving through the tip. Note that tips with large openings may injure the egg.
4. Gently lower needle onto the surface of the egg, and once the needle punctures the membrane, inject the protein or mRNA into the egg. The injected fluid should displace yolk granules in the area immediately adjacent to the tip. The formation of a membrane-enclose vesicle at the injection site indicates that either seawater entered the egg at the injection site or that there was seawater in the pipette tip. Injection systems like the Parker-Hannifin Picospritzer displace fluid from the

micropipette through timed pulses of nitrogen gas, with the longer pulses displacing larger volumes. Generally, we recommend injecting volumes less than 1% of total cell volume, which for *L. pictus* will be ≤73 pl. While each pipette will be slightly different, the system may be calibrated by injecting into vegetable oil and measuring the diameter of the displaced droplet. With the system pressurized to 60 psi, we generally set the pulse timing to 5–8 ms with our needles in the case of sea urchin eggs.

3.7 Methods for Sea Star Oocytes

1. Because the extracellular coat surrounding oocytes can sometimes make microinjection challenging, we remove the jelly coat using pH 5 ASW. Oocytes are gently stirred in pH 5 ASW for 45 s and then collected in 85 μm mesh. The oocytes collected in the mesh are then washed in ASW (pH 8.2) twice before use or storage.
2. Immature oocytes may be immobilized by settling them in a dish that has 180 μM Nitex mesh attached to the glass-bottomed dish using vacuum grease (Fig. 1d) [33].
3. Microinjection proceeds much the same as for sea urchin eggs, except that the injection volumes may be larger (~240 pl).
4. After microinjection, the oocytes are stored in this same dish overnight at 14 °C incubator. The RNA takes about 4–7 h to translate into fluorescent protein inside the oocyte.

3.8 Confocal Imaging

1. We typically image embryos on an Olympus IX83 inverted microscope equipped with an Andor Dragonfly 550 confocal imaging system, using either an Andor iXon 888 Electron Multiplying CCD (EMCCD) camera or an Andor Zyla 4.2 scientific cMOS camera. For rapid 4D imaging, we typically use the more sensitive EMCCD camera to reduce exposure time and illumination of the sample (*see* **Note 8**). The system may be driven either by Andor Fusion or IQ imaging acquisition programs.
2. For either urchin embryos or sea star oocytes, we use a 60× Apochromat silicone oil objective (NA 1.30).
3. For any imaging longer than 10–15 min, it is highly advisable to use a temperature-controlled stage. For confocal microscopy, we use the Oko Touch stage incubator set to 14 °C. For wide-field fluorescence, DIC, or polarizing microscopy, we use Brook Industries cooling stage inserts.
4. To prepare embryos for imaging, Remove fluorescent oocytes and embryos from the injection dish using a P10 pipette tip under a fluorescent stereo microscope, and carefully place them in the center of the MatTek 35 mm dish containing 200 μl of ASW. Cover the droplet with a 22 mm^2 coverslip (Fig. 2).

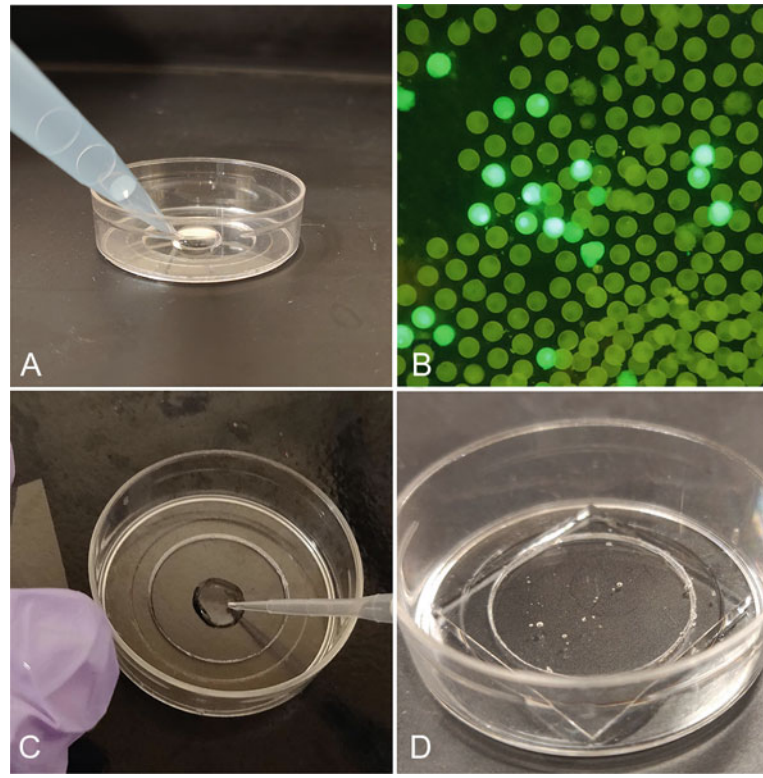

Fig. 2 Preparing the dish for imaging. Place 200 μl of ASW on to a MatTek dish (**a**), and under a fluorescent stereo microscope, pick out the fluorescent oocytes or embryos using a 0.5–10 μl pipettor (**b**). Carefully place the oocytes/embryos in the ASW (**c**). Place a 22 × 22 mm coverslip onto the drop of ASW to prevent any drifting during imaging (**d**)

5. Mount the dish in the stage incubator and locate the fluorescent embryos by wide field fluorescence.
6. When setting the parameters for imaging, it is important to consider the kinetics of cell division in your species. Although the timing of cell division in echinoderms will vary depending on temperature and species, for *L. pictus* cultured at 14 °C, nuclear division (nuclear envelope breakdown through reformation) lasts approximately 26 min for the first cleavage. Prometaphase is quite rapid, with chromosome congression to the metaphase plate achieved within 5 min [34], and upon anaphase onset, poleward chromosome segregation progresses at 1.2 μm/min [35]. The lag between anaphase onset and furrow initiation lasts ~7.5 min [35], and once initiated, the rate of furrow ingression is fairly invariant across species, lasting approximately 8 min. As mentioned above, these numbers may vary from species to species, but they provide a general ruler for acquisition settings.

7. Like most cells, phototoxicity can be an issue with echinoderms, and our choice of camera depends on the nature of the experiment and the brightness of the sample. For single fluorophore, single plane imaging, the 4.2 megapixel Zyla scMOS camera (6.5 μm pixel size, 82% QE, 0.9 e- read noise) provides high resolution images and sufficient dynamic range to capture both the bright perinuclear actin and the fine cytoplasmic actin and single microvilli labeled by recombinant Lifeact-EGFP in dividing *L. pictus* embryos (Fig. 3a). However, for rapid multichannel 4D imaging such as spindle assembly and chromosome segregation in 16 cell embryos (Fig. 3b) or polar body extrusion during meiosis I (Fig. 3c), we rely on the iXon 888 (13 μm pixel size, 97% QE, <1 e- read noise). The higher sensitivity of the EMCCD affords the capacity to rapidly section through the sample while keeping laser powers below 15%.

4 Notes

1. Studies in yeast suggest that apart from direct chemical labeling at the C-terminus, any genetically encoded epitope or fluorescent tags of actin compromise functionality by interfering with formin-based actin polymerization [36, 37]. Thus, researchers have relied on indirect probes for the actin cytoskeleton; the most widely used probes are Lifeact [28], utrophin [38], and F-tractin [39]. Each of these proteins have differential affinities for monomeric and filamentous actin, and potential dose-dependent effects have been described for each [40–42]. However, in our hands, we have not detected a deleterious effect on actin organization or dynamics with Lifeact in echinoderm embryos, either expressed from injected mRNA or injected as a recombinant protein.
2. We typically add glycerol to the dialysis buffer as a stabilizer but find that injection of recombinant protein solutions containing glycerol disrupts meiotic maturation of sea star oocytes. Thus, for any experiments involving recombinant proteins and sea star oocytes, it is advisable to dialyze in the absence of glycerol.
3. It will not be unusual to see some GroEL contamination in the most concentrated fractions, although after dialysis, GroEL often aggregates and may be removed by centrifugation. Persistent chaperone contamination may be removed by including 1 mM ATP in the wash buffer.
4. Sea urchin eggs will lose viability more rapidly if stored at temperatures above their normal culturing conditions. For longer-term storage, eggs for *S. purpuratus* and *L. pictus* may be kept at 4 °C, although fertilization efficiency may drop after 12–24 h. It is generally thought that bacterial contamination

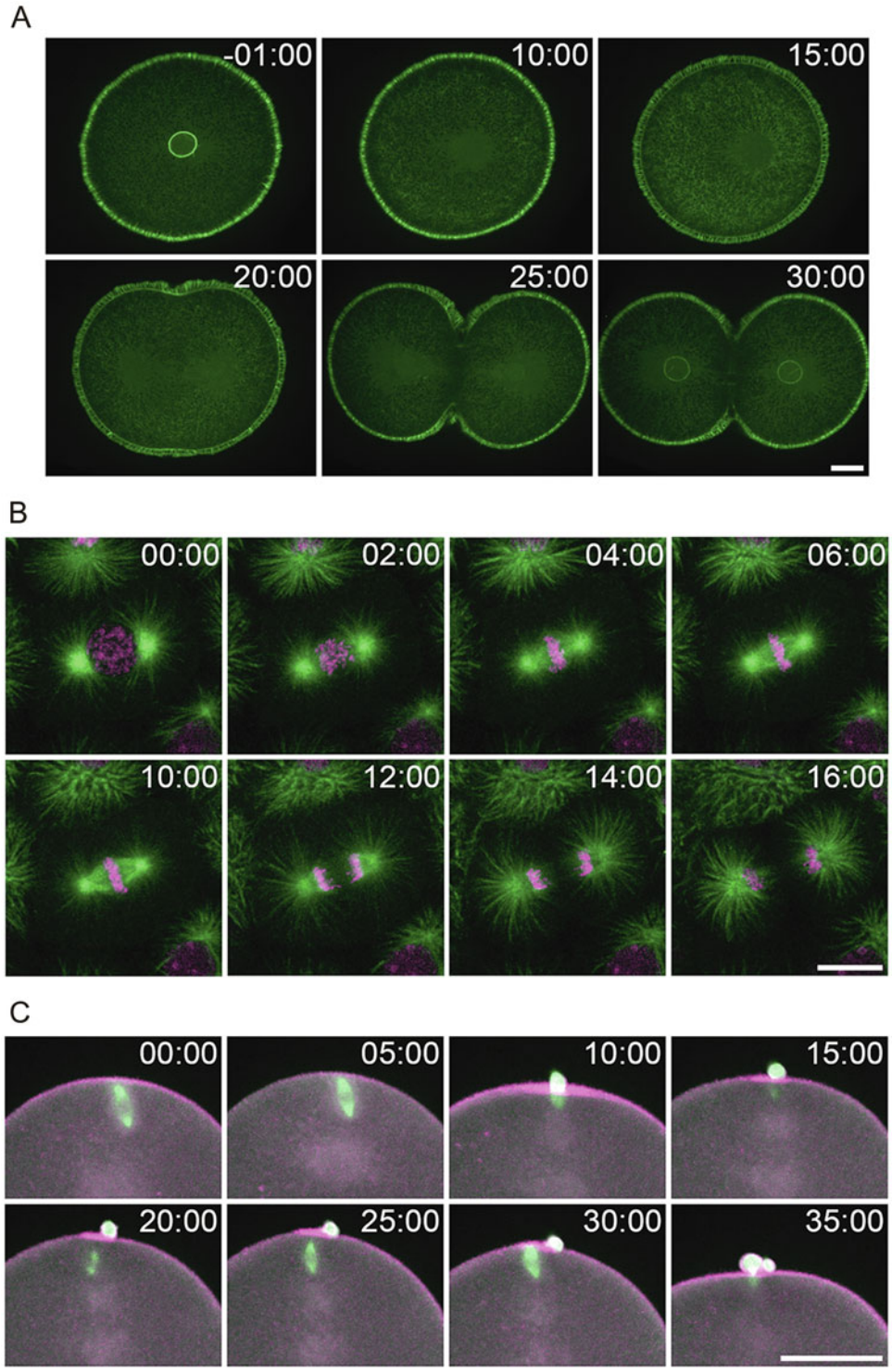

Fig. 3 Live cell imaging of cell division in echinoderm embryos and oocytes. (**a**) *L. pictus* embryos were injected with recombinant Lifeact-EGFP and single plane time-lapse images were acquired by spinning disk confocal microscopy with a Zyla4.2 scMOS camera every 30 s. (**b**) *L. pictus* embryos expressing ETMB-3xEGFP (green) and mCherry-histone H2B (magenta) were cultured until the 16-cell stage and Z stacks

results in breakdown of the jelly coat, and thus addition of antibiotics such as gentamycin may also help preserve egg viability.

5. There are several possible variations in how oocytes may be isolated from ovary tissue. For many of our experiments, it is necessary to remove follicle cells prior to microinjection, culturing, and imaging. In this case, we place the ovary tissue into cold CaFASW for 15 min and then replace with fresh CaFASW for an additional 15 min. This enables the oocytes to extrude better in presence of acetylcholine and strips the follicle cells in the process. After extrusion, acetylcholine/CaFSW is replaced with fresh ASW and then either used immediately or stored at 14 °C as described.
6. Follicle cells are the source of the maturation hormone, 1-methyladenine. For some species such as *Asterias forbesi*, it may be necessary to prevent spontaneous maturation. However, inclusion of 10 mM L-phenylalanine in the seawater has been reported to suppress spontaneous maturation [43].
7. It is common practice with sea urchin embryos to inject eggs prior to fertilization, as the cortical stiffness is very low and there is no secreted hyaline layer that can clog needles or fertilization envelope. However, any extracellular calcium that enters the egg during microinjection can cause spontaneous activation. For imaging during the early cleavages, we typically fertilize and remove the fertilization envelope prior to microinjection. However, if microinjecting following fertilization, it is important to inject soon after microinjection (within 30 min), as any hyaline on the egg surface will harden in the presence of calcium ion in the seawater. This will not only clog the needle, but the embryos will be less adherent to the glass and will tend to roll out from under the needle (if injecting using an inverted microscope).
8. As with any imaging system, there are trade-offs with the different imaging modes when imaging live samples [27]. The scanning speeds and amount of laser power associated with traditional laser scanning confocal microscopes (LSCM) often result in photobleaching and phototoxicity but have been employed successfully for decades with echinoderm embryos. However, LSCM run in resonant scanning mode dramatically increases scanning speed and lowers the laser

Fig. 3 (continued) (50 planes at 0.5 μm intervals) were then acquired by spinning disk confocal microscopy with an iXon 888 EMCCD camera every 60 s. (**c**) *P. miniata* oocytes were injected with a mRNA encoding Lifeact-mCherry-2A-EGFP-tubulin (magenta and green, respectively), and embryos were cultured overnight. Oocytes were activated with 2 μM 1-methyladenine, and Z stacks (40 planes at 0.5 μm intervals) were then acquired by spinning disk confocal microscopy with an iXon 888 EMCCD camera every 30 s. Bars, 20 μm

wattage required to image live samples [29]. Spinning disk confocals are known for reducing both phototoxicity and photobleaching, but in our hands, spinning disk confocals cannot image as deeply into a sample as a resonant scanning LSCM.

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