CHAPTER

Methods for collection, handling, and analysis of sea urchin coelomocytes

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CHAPTER OUTLINE

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Abstract

Sea urchin coelomocytes can be collected in large numbers from adult sea urchins of the species, *Strongylocentrotus purpuratus*, which typically has 12–40 mL of coelomic fluid. Coelomocytes are used for analysis of immune reactions and immune gene expression in addition to basic functions of cells, in particular for understanding structure and modifications of the cytoskeleton in phagocytes. The methods described here include coelomocyte isolation, blocking the clotting reaction, establishing and maintaining primary cultures, separation of different types of coelomocytes into fractions, processing live coelomocytes for light microscopy,

fixation and staining for light and electron microscopy, analysis of coelomocyte populations by flow cytometry, and sorting single cells for more detailed follow-up analyses including transcriptomics or genomic characteristics. These methods are provided to make working with coelomocytes accessible to researchers who are unfamiliar with these cells and perhaps to aid others who have worked extensively with invertebrate cells.

1 COELOMOCYTES IN THE PURPLE SEA URCHIN, *Strongylocentrotus purpuratus*

The echinoid class of the echinoderm phylum, which includes sea urchins, has a coelomic cavity filled with coelomic fluid (CF) that bathes the internal organs. Within the CF of the adult purple sea urchin, *Strongylocentrotus purpuratus*, is a wide variety of coelomocytes that function in the innate immune system (Smith et al., 2018, 2010). The phagocyte class makes up ~70% of the cells and includes the large phagocytes that are categorized as discoidal and polygonal cells based on their cytoskeletal structure (Edds, 1979; Henson et al., 1999), and small phagocytes that are named based on their size (Brockton et al., 2008; Gross, Clow, & Smith, 2000) (Fig. 1A). The vibratile cells have spherical cell bodies with many vesicles and a single long flagellum. Speculations have suggested a wide variety of functions for this cell type, but none have been verified. The two types of spherule cells (also called amoebocytes) are filled with large vesicles that are either colorless or red. The vesicles in the red spherule cells are filled with echinochrome A, an anti-bacterial naphthaquinone (Gerardi, Lassegues, & Canicatti, 1990; Messer & Wardlaw, 1980; Perry & Epel, 1981; Matttew Service & Wardlaw, 1984) that may act through iron binding and sequestration from invading microbes (Coates, McCulloch, Betts, & Whalley, 2018). The colorless spherule cells are similar in morphology, do not express echinochrome A, and have cytotoxic activity when mixed with phagocytes (Arizza, Giaramita, Parrinello, Cammarata, & Parrinello, 2007). Coelomocytes mediate many aspects of the immune response including their involvement in allograft rejection (Coffaro, 1980; Coffaro & Hinegardner, 1977), their cytotoxic and phagocytic activities as demonstrated in vitro (Arizza et al., 2007; Chou, Lun, & Smith, 2018), their response to microbes in vitro (Johnson, 1969), clearance of foreign particles and pathogens in vivo (Coffaro, 1978; Plytycz & Seljelid, 1993; Yui & Bayne, 1983), and the production of a range of anti-pathogen proteins (Barca et al., 2017; Gross, Al-Sharif, Clow, & Smith, 1999; Li, Blencke, Haug, & Stensvag, 2015; Pancer, 2000; Schillaci et al., 2014; reviewed in Smith et al., 2018, 2010). Coelomocytes have been employed in short-term primary cultures to evaluate aggregation and encapsulation responses (Majeske, Bayne, & Smith, 2013), dynamic changes to the cytoskeletal morphology of lamellipodia and the biology of

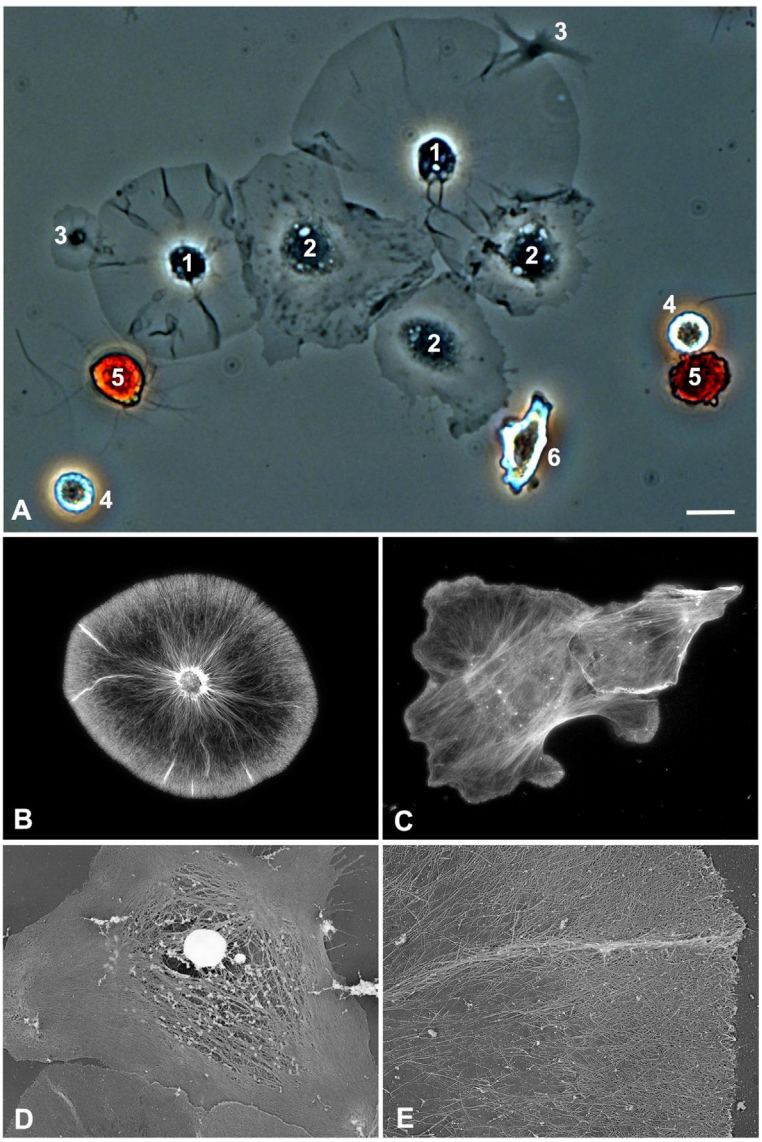

FIG. 1

Coelomocytes from the purple sea urchin, *Strongylocentrotus purpuratus*. (A) A mixed cell population of live coelomocytes by phase contrast microscopy includes discoidal (labeled as (1)), polygonal (2), and small (3) phagocytes, as well as flagellated vibratile cells (4), red spherule cells (5), and colorless spherule cells (6). (B and C) Fluorescent phalloidin staining elaborates the two actin cytoskeletal morphologies in a discoidal phagocyte (B) and a polygonal phagocyte (C). (D and E) Platinum replica TEM of coelomocyte cytoskeletons shows the dense and extensive actin filaments in a polygonal phagocyte (D) and a portion of the lamellipodium of a discoidal coelomocyte (E). Bar = 10 μm.

cell shape (Edds, 1985; Henson et al., 2014), and have been employed as sentinels of environmental stressors and environmental toxins (reviewed in Pinsino & Matranga, 2015). Immune activation in response to *Vibrio diazotrophicus* and lipopolysaccharide has been characterized using immunoquiescent sea urchins with down-regulated immune responsiveness that occurs after long-term housing in artificial sea water and away from the organisms in wild sea water (Gross et al., 1999). Immunoquiescent sea urchins are used to identify immune activators and genes that are induced in coelomocytes during responses to the inducers (Nair, Del Valle, Gross, Terwilliger, & Smith, 2005; Smith, Chang, Britten, & Davidson, 1996). Examples of immune response genes include complement homologues (Clow, Gross, Shih, & Smith, 2000; Gross et al., 2000; Smith, Shih, & Dachenhausen, 1998), a homologue of the Tie1/2 a growth factor receptor (Stevens et al., 2010), and the *SpTransformer* gene family (formerly *Sp185/333*; reviewed in Smith & Lun, 2017). The collection of coelomocytes from adult sea urchins requires blocking the strong clotting reaction of the CF so that the cells can be employed in gene expression analysis, observed in short-term cultures, or can be fixed and stained to evaluate and characterize their structures and protein content. Here we describe some basic protocols for collecting and handling sea urchin coelomocytes for a variety of analyses.

2 COELOMOCYTE COLLECTION AND BLOCKING CELLULAR CLOTTING

A major benefit of working with sea urchin coelomocytes is that they are present in the CF in concentrations of up to 5×10^7 cells per mL of CF and up to 1 mL of CF can be collected from a large animal without killing it. However, the major challenge of handling coelomocytes is that they readily undergo clotting reactions and lysis of cell subsets (D'andrea-Winslow, Radke, Utecht, Kaneko, & Akasaka, 2012; Hillier & Vacquier, 2003). To block this process, CF collected from adult sea urchins must be diluted into an anticoagulant solution. Changes in the chemical composition of the CF that result from bleeding and/or other unknown signals transform the morphology of the large phagocytes, which undergo a significant change in the cytoskeletal morphology and switch from lamellipodial, or bladder-like, to filopodial (Edds, 1977, 1985, 1993; Henson & Schatten, 1983; Henson et al., 1999). The filopodia intertwine among the cells resulting in a cellular clot. Blocking cellular clot formation is necessary prior to evaluation of coelomocytes.

2.1 PROTOCOL 1. ASPIRATION OF SMALL VOLUMES OF CF FROM AN ADULT SEA URCHIN

1. Use a 1 mL syringe with a 23 gauge needle (3/4 in.) and fill the syringe, including the needle, with ice-cold calcium and magnesium free sea water that includes EDTA as the divalent ion chelator and HEPES as the buffering agent

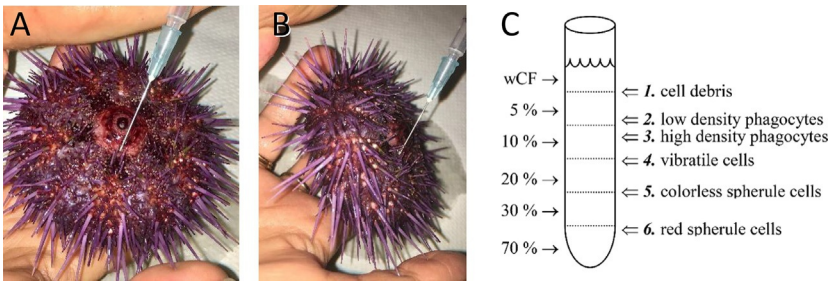

FIG. 2

Collection of coelomic fluid from a sea urchin and separation of coelomocytes into fractions on an Iodixanol step gradient. (A and B) Coelomic fluid is withdrawn from the coelomic cavity by needle aspiration. The needle is inserted through the peristomial membrane into the coelomic cavity at about a 45° angle to withdraw coelomic fluid and to avoid damaging the gut, gonads and other organs. The authors are grateful to Megan Barela Hudgell for assistance with photography. (C) Density centrifugation of coelomocyte subpopulations. Whole coelomic fluid (wCF; cells plus fluid) diluted in CMFSW-EH is centrifuged and the cells separate onto different step concentrations of Iodixanol.

Figure (C) is reprinted by permission from Springer Nature: Gross, P. S., Clow, L. A., & Smith, L. C. (2000). SpC3, the complement homologue from the purple sea urchin, Strongylocentrotus purpuratus*, is expressed in two subpopulations of the phagocytic coelomocytes.* Immunogenetics, 51*, 1034–1044.*

(CMFSW-EH, Recipe 1). Remove all bubbles from the syringe and needle and adjust the volume to be equal to the volume of CF that will be withdrawn from an adult sea urchin.

2. Insert the needle through the peristomial membrane and into the coelomic cavity of an adult sea urchin (Fig. 2A and B). Avoid damaging the gut and gonad by inserting the needle at an angle of about 45° away from the oral/anal axis, aiming toward the body wall.
3. Withdraw the CF into the syringe slowly, which will partially mix with the CMFSW-EH in the syringe. Up to 0.5 mL can be withdrawn from a large adult; however, removing 0.2–0.3 mL is optimal because the sea urchin (*S. purpuratus*) will be more likely to survive. The total number of coelomocytes per mL can range from about $0.5–1 \times 10^6$/mL (Matranga, Toia, Bonaventura, & Muller, 2000) and up to 5×10^7/mL (Brockton et al., 2008) depending on whether the animal is immunoquiescent or immune activated.
4. Remove the needle from the sea urchin and return the animal to the seawater. Withdraw additional ice-cold CMFSW-EH into the syringe to a total of 1 mL. Remove the needle from the syringe and expel the contents into a cold microcentrifuge tube on ice that may contain up to 1 mL of cold CMFSW-EH. Mix by gentle inversion. Removing the needle from the syringe avoids shear forces on the cells being forced through the needle when the syringe is emptied. This can improve cell survival.

5. The final minimum dilution is 0.5 mL CF diluted into a total of 1.5 mL CMFSW-EH or 1:4.
6. The cells are stored on ice and are ready for further use.

2.2 PROTOCOL 2. COLLECTION OF LARGE VOLUMES OF CF AFTER SEA URCHIN SACRIFICE

1. Insert the sharp point of a pair of dissecting scissors through the peristomial membrane.
2. Quickly cut the peristomium around the mouth, which will separate Aristotle's Lantern (echinoid mouthparts) from the body and will sever the radial nerves that run along the five sides of the body wall from the nerve ring.
3. Insert the sharp point of the scissors blade between two teeth and pull Aristotle's Lantern out of the body cavity. This will break the stone canal and the esophagus.
4. Pour the CF from the coelomic cavity through the test opening left by Aristotle's Lantern and into a 50 mL tube containing ice-cold CMFSW-EH (Recipe 1). A glass funnel holding sterile gauze or cheese cloth soaked in CMFSW-EH should be placed in the 50 mL tube so that pieces of tissue, body wall, or spines are caught and do not contaminate the CF. The volume of CMFSW-EH that is pre-loaded into the 50 mL tube should be at least half the estimated volume of the CF for the given sea urchin. CF volume can be estimated from the weight of the sea urchin in grams or by the dimensions of the spheroid test.
   a. CF volume in mL = (weight of the sea urchin in grams) × (0.22) (Smith, Britten, & Davidson, 1995).
   b. Body volume within the test in mL = (4/3) × (π) × (1/2 test diameter in mm)^2 × (1/2 test height in mm) (Elliot, Russell, & Hernandez, 2012).
5. CF diluted in CMFSW-EH is kept on ice. Cells are ready for further processing and evaluation.

2.2.1 Recipe 1. Calcium and magnesium free sea water with EDTA and HEPES (CMFSW-EH)

CMFSW-EH is modified from Humphreys (1963) as reported in Chou et al. (2018). Imidazole can be used as the buffering agent according to Gross et al. (2000). HEPES has been used to avoid interference with the activity of proteins that contain multiple histidines and bind nickel.

460 mM NaCl
10.7 mM KCl
7 mM Na_2SO_4
2.4 mM $NaHCO_3$
70 mM EDTA
20 mM HEPES (or 20 mM Imidazole), pH 7.4

2.2.2 Recipe 2. Artificial coelomic fluid (aCF)

In addition to the requirement for divalent cations, echinoid clotting reactions are also mediated by disulfide bond formation among olfactomedin proteins to generate a protein net that can entangle coelomocytes leading to aggregation (Hillier & Vacquier, 2003). An alternative approach to block clotting by olfactomedin cross-linking is the addition of a reducing agent into the cell suspension. Artificial coelomic fluid (aCF) (Terwilliger, Buckley, Brockton, Ritter, & Smith, 2007) can be modified to an anticoagulant to block olfactomedin crosslinking by the addition of dithiothreitol (DTT). This can be done under circumstances when the addition of EDTA cannot be used and/or if the correct molarity of divalent cations is necessary depending on the subsequent analysis of the coelomocytes. For example, EDTA blocks phagocytosis and amoeboid activity (H-.Y. Chou, Personal Communication) and therefore DTT in aCF is preferred as an anticoagulant for those types of assays.

10 mM $CaCl_2$
14 mM KCl
50 mM $MgCl_2$
398 mM NaCl
1.7 mM Na2HCO3
25 mM Na_2SO_4
pH 7.4
20–30 mM DTT if used as an anticoagulant

3 PRIMARY CULTURES OF SEA URCHIN COELOMOCYTES

The challenges for working with sea urchin coelomocytes are to remove them from the animal, seed them onto plastic or glass culture surfaces, and either block or promote/allow their tendency toward immune activation and cellular clot formation when in contact with foreign surfaces. The parameter that can be used to assess the level of activation is the morphology of the large phagocytes. Non-activated phagocytes have lamellipodia with broad, smooth edges, which is equivalent to their bladder morphology or their three-dimensional morphology in newly collected CF. Activated phagocytes, perhaps in response to a buffer that is not consistent with the chemical make-up of the CF, transform to filopodial, in which the actin cytoskeleton undergoes a major morphological change. This was demonstrated exquisitely by Kenneth Edds who showed that coelomocytes change their cytoskeletal morphology in response to changes in buffer osmolarity (Edds, 1977, 1979, 1980a, 1980b, 1985). Hence, the choice of buffers into which coelomocytes are collected and the buffers that are used for primary cultures is essential for generating reliable results for a wide range of analyses of both coelomocyte morphology and their activities. Short-term primary coelomocyte cultures can be maintained at 14–16 °C for up to 8 h (Brockton et al., 2008) or they can be cultured for a 7 day period although viability decreases from 91.6% to 57% (Majeske et al., 2013).

Longer culture periods of up to 2 weeks have been reported; however, viability estimates were not reported (Bertheussen, 1981a, 1981b; Bertheussen & Seijelid, 1978).

3.1 PROTOCOL 3. SHORT-TERM (UP TO 8 H) PRIMARY COELOMOCYTE CULTURE

1. Collect CF in ice-cold CMFSW-EH (Protocol 1), count cells and store on ice.
2. Pipette 1×10^5 cells into the center of a Poly-L-lysine coated microscope slide (Polysciences).
3. Place slides in a cold chamber at 14 °C for 1 h to allow settling and adherence of phagocytes to the slide. The other types of coelomocytes will not adhere. Alternatively, cells can be spun onto slides using a cytospin centrifuge at $1000 \times g$ for 5 min at 4 °C.
4. Gently tip the CMFSW-EH off the slide and replace with ice-cold aCF (Recipe 2).
5. Cells can be incubated at 14 °C–16 °C for up to 8 h in aCF.

3.2 PROTOCOL 4. LONG-TERM (1–7 DAYS) PRIMARY COELOMOCYTE CULTURE

1. Autoclave the CMFSW-EH (Recipe 1), cool and add antibiotics.
   a. penicillin G sodium salt (200 U/mL)
   b. streptomycin sulfate (200 mg/mL)
   c. ampicillin sodium salt (25 mg/mL)
   d. adjust to pH 7.4
2. Collect CF (Protocol 1) and dilute at least 1:1 (vol:vol) with ice-cold CMFSW-EH plus antibiotics. Store samples on ice.
3. Work in a laminar flow hood that has been sterilized with UV and/or cleaned with ethanol. Use sterile or cleaned pipetters and tips for manipulating the cells.
4. Place a circular coverslip (18 mm diameter) on the bottom of each well of a six-well cell culture plate (Corning).
5. Pipette 1×10^5 cells onto the center of each circular cover slip.
6. Cover the culture plate with the lid and incubate at 14 °C for 1 h to allow settling and adherence of cells to the cover slips.
7. Return the plate to the sterile hood, remove the lid, gently tip the plate, and aspirate the CMFSW-EH.
8. Wash the cells twice by gently by adding 75–150 µL of ice-cold echinoid coelomocyte culture medium (ECCM; Recipe 3) to each well to cover the cells and incubate at 14 °C.
9. Replace with fresh, cold ECCM daily, working in the sterile laminar flow hood.
10. The coelomocytes can be incubated at 14 °C for up to 7 days in ECCM.

3.2.1 Recipe 3. Echinoid coelomocyte culture medium (ECCM)

1. Prepare separate solutions of commercially available Leibovitz's L-15 Medium, Dulbecco's Modified Eagle Medium and F-12 Nutrient Mixture in autoclaved sterilized deionized water according to the manufacturer's instructions.
2. Add the following components to autoclaved sterilized aCF (Recipe 2). All components are shown in final concentrations.
   0.6% Leibovitz's L-15 Medium
   0.87% Dulbecco's Modified Eagle Medium
   0.26% F-12 Nutrient Mixture
   15 mM HEPES pH 7.4
   14.8 mM $NaHCO_3$
   3% heat inactivated fetal bovine serum
   10 mg/mL insulin from bovine pancreas
   20 mg/mL catalase from bovine liver
   55 mM 2-mercaptoethanol
   200 mM L-glutamine
   10% freshly extracted cell free CF without CMFSW-EH (see step 3 below)
   antibiotics (see above)
   adjust to 950 mOsm and pH 7.4
3. CF is collected from a sea urchin without dilution into CMFSW-EH or other anticoagulant. Clotting is allowed to proceed and the clots and cells are pelleted. The supernatant is collected, aliquoted, frozen, and is used as a component of ECCM.
4. Sterile filter the ECCM through a 0.2 μm filter and store at 4 °C in a tightly sealed sterile container. ECCM will last for a few days at 4 °C prior to experimental application.

4 ISOLATION, EVALUATION, FIXATION, AND IMAGING OF SEA URCHIN PHAGOCYTES

The biological characteristics of sea urchin coelomocytes have been studied since the early part of the 20th century (for reviews see Smith et al., 2018, 2010). Although the original work in identifying and characterizing cell types was done using bright field light microscopy with multiple cytological staining techniques, more recent reports have employed more sophisticated microscopy of live cells, fluorescence microscopy to differentiate coelomocyte subpopulations by cellular cytoskeletal structure and protein localization, as well as super-resolution light microscopy and electron microscopy to identify ultrastructural characteristics (Edds, 1993; Henson et al., 2009, 2014, 2003, 1999, 2015; Henson, Nesbitt, Wright, & Scholey, 1992). Recent work has tended to concentrate on the large phagocyte subset of coelomocytes, which has developed this cell type as a model for understanding actin cytoskeletal structure, function, and dynamics (Edds, 1977, 1979, 1980b, 1993; Henson et al., 2009, 2014, 2003, 1999, 2015). Furthermore, phagocytes have been evaluated for their phagocytosis ability (Chou et al., 2018), contribution to the clotting process

(D'andrea-Winslow et al., 2012; Hillier, Moy, & Vacquier, 2007; Hillier & Vacquier, 2003), and expression of immune response-related proteins (reviewed in Smith et al., 2018, 2010). One key to the use of phagocytes in these studies has been the development of culturing and fixation methods that maximize the structural preservation of these fragile cells.

4.1 PROTOCOL 5. PURIFICATION OF PHAGOCYTES BY A SINGLE STEP SUCROSE GRADIENT

Coelomocytes isolated from sea urchins contain a mixture of three major cell types (Fig. 1A). The majority of the cells are phagocytes, which are excellent for analyses of cytoskeletal structure. An enrichment of phagocytes takes advantage of their relatively high surface area (see (Edds, 1977, 1979; Henson et al., 1992) that allows easy separation from the non-phagocytes by sucrose cushion centrifugation. This method is simpler and faster than the discontinuous density step gradients described in Protocols 9 and 10 that separates all coelomocyte types into fractions. The choice of protocol will depend on subsequent use and evaluation of the cells.

1. Collect the CF from a sea urchin after sacrifice (Protocol 2) and dilute immediately 1:1 (vol:vol) into freshly made ice-cold anticoagulant (Recipe 4) that is supplemented with additional EGTA and Tris for final concentrations of 150 mM EGTA and 100 mM Tris pH 7.4. Mix the CF with the anticoagulant in a beaker on ice by swirling vigorously.
2. Pour 10–13 mL of CF in anticoagulant into a cold 15 mL conical centrifuge tube on ice.
3. Use a 9 in. glass Pasteur pipette to under layer carefully a cushion of 1–2 mL of ice-cold 0.8 M sucrose in anticoagulant.
4. Centrifuge the cells at ~600 × *g* for 5 min at 4 °C in a swinging bucket rotor with a slow start and no brake. A "buffy coat" will appear on the interface between the sucrose cushion layer and the CF diluted in the anticoagulant. This is the phagocyte fraction of coelomocytes.
5. Remove the buffy coat layer with a Pasteur pipette and transfer this enriched phagocyte population to a 15 mL conical tube containing Coelomocyte Culture Media (CCM; Recipe 5) (Henson et al., 1999).
6. Phagocytes can be maintained at low cell density for 24–48 h in CCM in 15 mL centrifuge tubes by placing the tubes on their sides and buried in ice within a cooler. For overnight storage, place the cooler containing the cells within a 4 °C refrigerator.
7. The phagocytes are ready for subsequent evaluation.

4.1.1 Recipe 4. Anticoagulant

500 mM NaCl
26 mM KCl
50 mM EGTA
10 mM Tris-HCl, pH 7.4

4.1.2 Recipe 5. Coelomocyte culture medium (CCM)

0.5 M NaCl
5 mM $MgCl_2$
1 mM EGTA
20 mM HEPES, pH 7.2

4.2 PROTOCOL 6. PREPARATION OF LIVE PHAGOCYTES FOR IMAGING BY LIGHT MICROSCOPY

1. Allow phagocytes in CCM (Protocol 5) to settle for 20–30 min onto ethanol washed 22 × 22 mm coverslips placed on a "pedestal" created by the cross supports of a 60 mm plastic Petri dish that is separated into quadrats (e.g., item 2931 from Kord-Valmark Labware Products) (Fig. 3A). Alternatively, the lid of a 1.5 mL microcentrifuge tube that is placed in a regular 60 or 90 mm plastic Petri dish can be used as a pedestal for a coverslip. The lid can be glued to the Petri dish or affixed using double-stick tape. Small puddles of deionized water are added to the Petri dish to increase the humidity and reduce evaporative loss of the solutions covering the cells. Note that quad Petri dishes serve as coverslip incubation chambers for treating live cells with drugs as well as for fixing and staining cells with antibodies, fluorescent probes, etc., with longer incubation periods often performed when the dishes are kept at 4 °C in a standard refrigerator.
2. For light microscopic observation, blot the CCM (Recipe 5) from the coverslip and invert it cell side down onto a drop of CCM placed on a microscope slide that has spacers to suspend the coverslip above the slide surface. These spacers can be narrow strips of double stick tape (Fig. 3B), parafilm, or strips of glass scored and cut from coverslips. Blot excess CCM from the edges of the coverslip and seal it in place using heated Valap (a 1:1:1 mix of Vaseline or petroleum jelly, lanolin, and parafilm) applied with a small spatula (Fig. 3B) that is heated using a Bunsen burner or an alcohol lamp.
   a. The coverslip can be sealed on all sides.
   b. A crude perfusion chamber can be created by sealing the two sides that are associated with the spacers with Valap and leaving the other two sides open (Fig. 3B). Live cells can be imaged while solutions and/or drugs are "perfused" through the chamber by placing drops on one side of the coverslip and drawing the fluid to the opposite side using filter paper or a Kimwipe (Henson et al., 2009, 2003, 2002, 1999). This approach makes it feasible to image live cells that are subsequently fixed, washed, and stained using the perfusion chamber followed by imaging of staining patterns in the fixed cells by fluorescence microscopy (see Brockton et al., 2008).
3. The cells should be observed initially by phase contrast (Fig. 1A) or differential/relief contrast microscopy because the thin, flattened nature of phagocytes makes them difficult to image using bright-field optics. Note that cells should be

observed in their coverslip chambers within 60 min of creating the chambers. Cells in chambers can be kept on ice or at 4 °C prior to observation or the slides can be placed on a cooling stage at 4–14 °C. A mixed population of living coelomocytes viewed in this type of slide chamber shows discoidal, polygonal, and small phagocytes, in addition to vibratile cells, and red and colorless spherule cells (Fig. 1A).

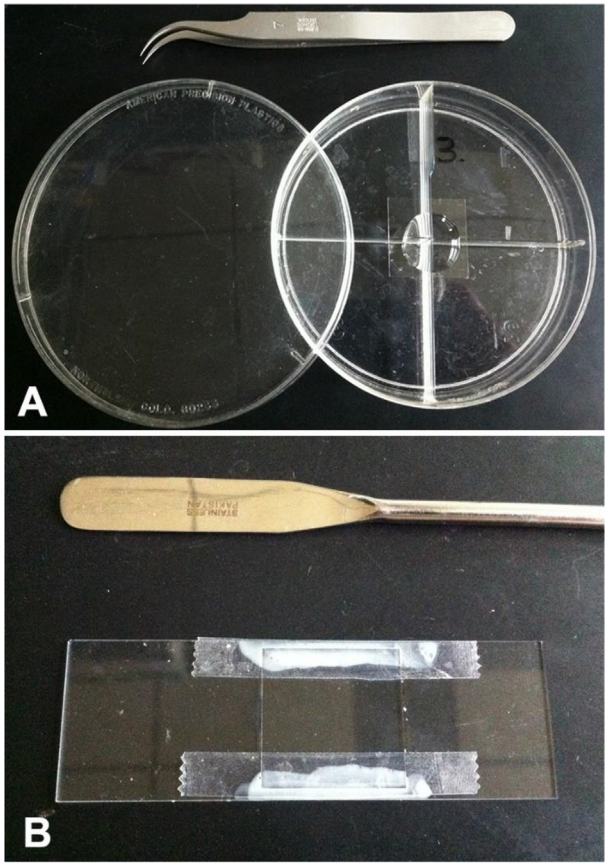

FIG. 3

A quad Petri dish for incubating coelomocytes on coverslips and a slide-based cell perfusion chamber. (A) A 90 mm quad Petri dish is used as an incubation chamber for a coverslip onto which coelomocytes are settling. The coverslip rests on the cross supports in the center of the dish. The fine, curved, #7 forceps are used to manipulate the coverslip (top). Petri dishes are numbered on the bottom to keep track of multiple samples. (B) A slide-based perfusion chamber is constructed using double stick tape as spacers that supports a coverslip over the slide. The coverslip is shown sealed on two sides with Valap. The spatula used for applying the melted Valap is at the top of the image.

4.3 PROTOCOL 7. PHAGOCYTE FIXATION FOR LIGHT MICROSCOPY

For optimal preservation of the fragile phagocytes, formaldehyde or organic solvent-based fixatives should not be used directly, but instead the cells should be treated with prefix at a very low percentage of glutaraldehyde (Recipe 6) (Henson et al., 1999; Hyatt, Shure, & Begg, 1984). Examples of fluorescently stained filamentous actin in phagocytes (Fig. 1B and C) demonstrate the remarkable preservation that is afforded by a multipart fixation protocol that is described below.

1. Blot CCM from the coverslip onto which cells have settled and attached (Protocol 6), and replace with 200–300 µL of prefix for light microscopy (Recipe 6) to cover the surface of the coverslip. Incubate for 3–5 min using the quad Petri dish chambers (Protocol 6, step 1). Once the cells are fixed, all subsequent steps can be carried out at room temperature.
2. Blot the prefix from the coverslip, do not wash the coverslip to remove residual prefix, and cover the cells with fix for light microscopy (Recipe 8) for 5 min.
   a. If the antibodies to be used for staining the cells bind to antigens that are exposed by treatment with organic solvents, blot off the fix and immerse the coverslips in either 100% methanol or acetone for 5 min at −20 °C. The solvents can be chilled in glass coverslip Coplin jars in a −20 °C freezer.
3. Blot off the fix (Recipe 8) or the organic solvent and transfer coverslips to a six-well plate for three to four rinses with standard PBS. Care must be taken so that the coverslips do not dry by ensuring that a thin film of PBS is always present to avoid disrupting the cells from dehydration.
4. For immunofluorescent staining, transfer cells on coverslips from the six-well plate to the quad Petri dish, blot off the PBS and replace with Blocking Buffer (Recipe 9) for 20–30 min.
5. Blot off the Blocking Buffer (do not rinse) and replace with 100–200 µL of primary antibody at the appropriate dilution in Blocking Buffer. Incubate the coverslip in a quad Petri dish for 30–60 min.
6. Blot off the primary antibody and rinse the coverslip two–three times with PBS. Replace the solution with the secondary antibody labeled with a fluorochrome using the same approach as with the primary antibody followed by washes in PBS.
7. After completing the antibody staining steps, mount the coverslip on a slide using mounting media.
   a. For conventional wide field or confocal microscopy, use 10–15 µL of ProLong Gold mounting media with or without a nuclear stain such as 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific).
   b. For super-resolution light microscopy, use non-hardening Vectashield (Vector Laboratories) because it is less subject to shrinkage artifacts that have been evident with ProLong media once it has gone through the curing and hardening process. Although DAPI can be used to stain nuclei for super-resolution imaging, it should not be included in the mounting media if cells are to be imaged by Structured Illumination Microscopy (SIM) or Stimulated Emission Depletion (STED). In addition, for STED imaging, the intensity of the depletion laser warrants the use of ProLong Diamond mounting medium.

4.3.1 Recipe 6. Prefix for light microscopy

0.000025% Glutaraldehyde in CCM (Recipe 5)
Dilute 25% EM grade glutaraldehyde 1:10,000 in CCM (Recipe 5)

4.3.2 Recipe 7. AC320 fixation buffer

75 mM KCl
2 mM $MgCl_2$
320 mM sucrose
20 mM EGTA
20 mM PIPES pH 7.0.

4.3.3 Recipe 8. Fix for light microscopy

1–3% formaldehyde
0.1–0.5% Triton X-100
in AC320 fixation buffer (Recipe 7)

4.3.4 Recipe 9. Blocking buffer

2% normal goat serum
1% bovine serum albumin
in standard PBS

4.4 PROTOCOL 8. PHAGOCYTE FIXATION FOR ELECTRON MICROSCOPY

1. Settle phagocytes in anticoagulant onto coverslips (Protocol 5). Fix cells with prefix for electron microscopy (Recipe 10) for 5 min in a quad Petri dish incubation chamber (Protocol 6).
2. For thin section transmission electron microscopy (TEM) or whole mount scanning electron microscopy (SEM) (Henson et al., 2014), follow protocols described in 2a–c.
   - **2a.** Blot the coverslip to remove the prefix, cover the cells with fix for electron microscopy (Recipe 11) in either 100 mM sodium cacodylate pH 7.3, or in AC320 Fixation Buffer (Recipe 7), and incubate for 30 min. At this stage, cells can be held in fixative for several weeks at 4 °C prior to further processing.
   - **2b.** Transfer the coverslips to a six-well plate and rinse five times in PBS. Move the plate on a bed of ice to a chemical fume hood and postfix with 1% osmium tetroxide in PBS for 30 min.
   - **2c.** Following extensive washes of the coverslips with deionized water, dehydrate cells through a graded ethanol series followed by further processing required for thin section TEM, or whole mount SEM. Removal of the glass coverslip from flat embedded cells prior to sectioning can be accomplished with digestion in hydrofluoric acid (Moore, 1975) or by freezing and popping the coverslip off the embedded cells (Howatson & Almeida, 1958).

3. For platinum replica TEM of cytoskeletons (Henson et al., 1999;Svitkina, 2007, 2009), follow the protocols in 3a–c.
   - **3a.** Blot coverslips to remove the prefix for electron microscopy (Recipe 10) and incubate in 0.5% Triton X-100 in AC320 Fixation Buffer (Recipe 7) for 3–5 min. Blot to remove the Triton/AC320 and incubate the cells in fix for electron microscopy (Recipe 11) as in step 2a.
   - **3b.** Rinse the coverslips in deionized water to remove the fix, incubate the cells in 0.2% aqueous tannic acid followed by 0.2% aqueous uranyl acetate (30 min in each).
   - **3c.** Dehydrate the cells in a graded ethanol series, critical point dry, and rotary shadow with platinum and carbon following published methods (Svitkina, 2007, 2009).

TEM imaging shows the elaborate actin cytoskeleton in platinum replicas of phagocytes fixed according to methods described here (Fig. 1D and E).

4.4.1 Recipe 10. Prefix for electron microscopy

0.001% glutaraldehyde in CCM (Recipe 5)

4.4.2 Recipe 11. Fix for electron microscopy

2.5% glutaraldehyde
in 0.1 mM sodium cacodylate, pH 7.3
or AC320 Fixation Buffer (Recipe 7)

5 FRACTIONATION OF COELOMOCYTES

The diversity of coelomocyte types in the purple sea urchin and their range in sizes, shapes, and inclusions enables enrichment of subpopulations by density centrifugation, which can be combined with subsequent processing and evaluation of individual cell types. Density centrifugation has been used successfully with two different media, Percoll and Iodixanol (Gross et al., 2000; Smith, Britten, & Davidson, 1992). Percoll (Sigma-Aldrich) is available in a buffer that is isosmotic with mammalian cells; hence, it must be altered so that it will not promote coelomocyte clotting and lysis, and so that the addition of buffer or anticoagulant will not dilute the Percoll concentration below the highest concentration required for the gradient. This is most easily accomplished by dialysis of Percoll into CMFSW-EH or other anticoagulant (Smith et al., 1992). After dialysis, different concentrations of Percoll are generated by dilution with an anticoagulant. All steps must be carried out at 4 °C to maximize cell viability and to reduce phagocytosis of the Percoll silica particles by the large phagocytes.

5.1 PROTOCOL 9. DISCONTINUOUS PERCOLL DENSITY GRADIENT

1. Dialyze the Percoll overnight against a large volume (2–4 L) of CMFSW-EH (Recipe 1) or other anticoagulant at 4 °C with stirring. Use dialysis tubing with the largest molecular weight cut-off to increase the speed of the exchange to remove divalent cations that drive coelomocyte clotting. Store the dialyzed Percoll at 4 °C in sealed tubes.
   a. In some cases, blocking cell clotting may also require the addition of 20 mM DTT (Majeske, Oren, Sacchi, & Smith, 2014).
2. Dilute the dialyzed Percoll with anticoagulant to the following percentages.
   a. 2.5%; cell debris float on this layer and it separates CF proteins and ions from the cells. It can be discarded after centrifugation.
   b. 20%; a mixture of phagocytes enriched with polygonal phagocytes float on this layer.
   c. 30%; a mixture of phagocytes enriched with discoidal phagocytes float on this layer.
      i. Phagocytes can also be collected as a single fraction, which will float on 40% Percoll.
   d. 50%; a mixture of vibratile and colorless spherule cells float on this layer.
   e. 70%; red spherule cells float on this layer, although some may pellet through this layer and can be collected from the bottom of the tube.
3. To reduce cells sticking to the walls of tube in which the gradient is formed and in which cell fractions are washed, use glass centrifuge tubes that have been silanized. This is done by coating the internal surfaces of a clean dry test tube with commercially available Rain-X® or Sigmacote® (Sigma-Aldrich) followed by extensive washing in deionized or distilled water. Silanization may be omitted.
4. Formation of a discontinuous gradient is generated most easily by under layering as described above for the sucrose cushion (Protocol 5). The volume of each step will depend on the size of the test tube in which the gradient is created and is based on the number of cells to be separated. Typically for a 12 mL tube, the gradient steps are about 2 mL, although the 2.5% Percoll layer at the top can be 0.5 mL.
5. Work on ice with the series of diluted Percoll.
6. Load the 2.5% Percoll into the bottom of the tube.
   a. For under layering, bubbles must be avoided because they will disrupt the interfaces of the layers above.
7. Place a 9 in. glass Pasteur pipette into the test tube so that it rests on the bottom and below the 2.5% Percoll layer.
8. Use a 5 mL syringe attached to a long 16 gauge needle to withdraw 20% Percoll, remove the bubbles from the syringe, and adjust the volume to 2 mL. Expel the Percoll into the glass pipette so that it flows below the 2.5% Percoll. This first under layer will generate bubbles, but this will also

partially fill the glass pipette with Percoll and subsequent bubbles will be avoided.

9. Repeat with higher and higher concentrations of Percoll until the gradient is complete. Higher concentrations of Percoll will under layer smoothly.
10. Remove the glass pipette when the gradient is complete.
    a. The gradient can also be generated by over layering less dense layers of Percoll on top of more dense layers. This is done with a glass Pasteur pipette or other pipetting mechanism, but must be done slowly and with constant flow to avoid mixing at the interface.
11. Very slowly over layer the coelomocytes in CMFSW-EH (Recipe 1) onto the 2.5% Percoll layer. Add the cells using a glass Pasteur pipette with a continuous motion moving around the top edge of the 2.5% Percoll meniscus. This approach may reduce mixing between the 2.5% Percoll layer and the cell solution.
12. Centrifuge the gradient in a pre-chilled centrifuge in a swinging bucket rotor at 250 × *g* at 4 °C for 15 min with a slow start and no brake.
13. Unload the layers of cells from the top using Pasteur pipettes and place the cells in silanized glass tubes on ice.
14. Dilute the cell fractions with 5–15 mL of ice cold CMFSW-EH (Recipe 1) depending on the size of the tube. Pellet the cells at 400–500 × *g* and resuspend in a small volume of 200–500 µL CMFSW-EH.
15. Count the cells and determine viability by trypan blue exclusion with a light microscope or by 1.5 mM propidium iodide (PI) exclusion with a fluorescent microscope or by flow cytometry.

Coelomocytes can also be separated into fractions using a continuous Percoll density gradient of 40–60% that is generated by centrifugation in a fixed angle rotor at 30,000 × *g* for 30 min at 4 °C (Coates et al., 2018). After loading coelomocytes in CMFSW-EH, the gradients are subsequently centrifuged in a swinging bucket rotor at 400 × *g* for 15 min at 4 °C. Fractions are unloaded as described above (Protocol 9, step 13).

5.2 PROTOCOL 10. DISCONTINUOUS IODIXANOL DENSITY GRADIENT

Fractionation by discontinuous Percoll density gradients does not separate the vibratile and colorless spherule cells, and the phagocytes may phagocytose the Percoll particles. To avoid these problems, separation can be accomplished by using Iodixanol (available as Optiprep (Sigma-Aldrich)) as the gradient medium. Iodixanol is not supplied in a buffer, which deletes the necessity for a dialysis step required for Percoll (Protocol 9). Iodixanol has been employed for separating sea urchin coelomocytes into separate fraction by several groups using very similar step gradients (Arizza et al., 2007; Barca et al., 2017; Gross et al., 2000; Liao & Fugmann, 2017) (Fig. 2B).

1. Dilute the Iodixanol to the range of percentages to be used in forming the gradient. The diluent is CMFSW-EH (Recipe 1) according to Gross et al. (2000) or ISO-EDTA (Recipe 12) according to Barca et al. (2017).

   a. 5%; cell debris floats on this layer and separates fragments and proteins in the CF from the underlying cell fractions. This layer can be removed discarded.
   b. 10%; low density phagocytes float on this layer. High density phagocytes may penetrate the layer and be located just below the top interface surface (Fig. 2C).
   c. 20%; vibratile cells float on this layer.
   d. 30%; colorless spherule cells float on this layer.
   e. 70%; red spherule cells float on this layer, but may also pellet through this step to the bottom of the gradient.
2. The gradient is formed by under layering in a silanized glass tube of about 15 mL (or larger) as described above for Percoll. However, the delivery of Iodixanol must be done very slowly and constantly to form optimal interfaces between layers.
3. Centrifuge the gradient at 800–1500 × *g* at 4 °C for 30 min in a swinging bucket rotor with a slow start and no brake. Variations in rotor speed are based on Barca et al. (2017) and Gross et al. (2000).
4. Fractions of cells that layer on top of the gradient or steps within a gradient are removed from the top with a silanized glass pipette.
5. Cells are diluted in anticoagulant, pelleted, and resuspended as described in Protocol 9, step 14 for fractions obtained from Percoll gradients.

5.2.1 Recipe 12. ISO-EDTA

20 mM Tris, pH 7.5
0.5 M NaCl
70 mM EDTA

6 FLOW CYTOMETRY AND SORTING TO ANALYZE SUBPOPULATIONS OF COELOMOCYTES

There are six morphotypes of coelomocytes in the purple sea urchin, *S. purpuratus* (see Section 1), which are differentiated based on size, inclusions, cytoskeletal morphology, presence of flagellae, and the production of echinochrome A. Flow cytometry is an efficient means to evaluate subpopulations of coelomocytes and can be employed in combination with a variety of perturbations to sea urchins followed by subsequent analyses of the cells.

6.1 PROTOCOL 11. FLOW CYTOMETRY AND SORTING OF LIVE COELOMOCYTE SUBPOPULATIONS

1. Cells in CMFSW-EH (Recipe 1) can be analyzed using any flow cytometer; however, the descriptions below apply to the FACS Aria II and gates for sorting have been defined using the FACSDiva™ software (BD Biosciences).

2. Cells are first separated from debris by gating based on Forward Scatter (FSC) and Side Scatter (SSC; Fig. 4A) similar to protocols established for other invertebrate species (Corey et al., 2016; Rosental, Koshekbaeva, Fernhoff, Tasi, & Traylor-Knowles, 2017).
3. Live cells are gated based on exclusion of propidium iodide (PI) (1 μg/mL) (Fig. 4B).
4. After gating, the live cell population is analyzed for different subpopulations of cells based on their intrinsic size as assessed by FSC and granularity as assessed by SSC using a log scale. Depending on cell characteristics, gates are defined to encompass different clusters of cell populations (Fig. 4C).
5. The gated populations are sorted with a nozzle of 100 μm and a flow rate of 1500 cells/s. Cells are sorted directly into 5 mL tubes containing 3 mL of staining medium (Recipe 13) to minimize cellular stress. The number of cells needed per population will depend on the downstream process and may range from 10,000 to 40,000 cells for each population (Fig. 4C).
6. Additional analysis and sorting can take advantage of the natural fluorescence of the red spherule cells that are detected in the far red channel with an

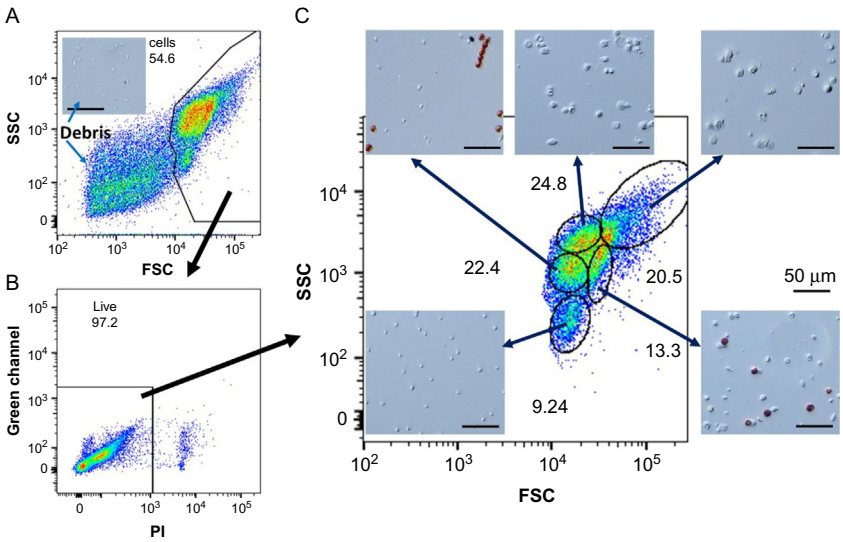

FIG. 4

Coelomocytes from the purple sea urchin, Strongylocentrotus purpuratus, are analyzed by fluorescence-activated cell sorting (FACS). (A) The gate excludes debris from cells and uses side scatter (SSC) to evaluate granularity and forward scatter (FSC) to evaluate intrinsic size. The inset shows cell debris. B. Live cells are gated based on exclusion of propidium iodide (PI). C. Coelomocyte morphotypes can be isolated according to their FSC and SSC properties on a log scale. Gates are indicated as black ovals and numbers within each gate indicate the percentage of cells in the population for that morphotype. Representative images of the sorted cell populations are shown. The red spherule cells are evident based on the red color of echinochrome A. Scale bar = 50 μm.

excitation of 633 nm that is used to excite fluorochromes such as allophycocyanin (APC) or Alexa Fluor 647. This gating should be done in combination with a second channel that does not excite the auto-fluorescence of the red spherule cells such as the excitation for Alexa Fluor 488 (green) or FL1 (vs FL4), which will depend on the sorting instrument. Because the auto-fluorescence in the far red channel for the red spherule cells is not very strong, adding a second channel for analysis (even without a specific stain) helps to gate optimally for the red spherule cells.

7. Data collected by flow cytometry are analyzed after the run and the sort using the FlowJo program (Flowjo, LLC) (Fig. 4).

6.2 PROTOCOL 12. SORTING LIVE COELOMOCYTES FOR ANALYSIS BY MICROSCOPY

1. When sorted cells will be imaged and analyzed by microscopy, first pellet the sorted cell fractions by centrifugation at 500 × *g* for 5 min at 4 °C.
2. Resuspend the pelleted cells in staining medium (Recipe 13) at 1.66×10^5 to 3.33×10^5 cells/mL and pipette 30 µL into each well of an 18-well flat µ-Slide coated with poly-L-Lysine (Ibidi, GmbH). This is about 5 to 10×10^3 cells in each well, which is enough for observation by microscopy.
3. Incubate the cells for 1 h to overnight at 4 °C to allow the cells to settle to the bottom of the well and to adhere to the slide.
4. Image the cells by inverted light microscopy (Fig. 4C) or by fluorescence or confocal microscopy if they have been stained with fluorescent antibodies or other cell markers (see Protocol 6 or Brockton et al., 2008).

6.2.1 Recipe 13. Staining Medium in 3× PBS

20 mM HEPES pH 7.4
1.5% heat-inactivated fetal calf serum
3× PBS
- 452.1 mM NaCl
- 8.91 mM KCl
- 33 mM Na_2HPO_4
- 5.94 mM KH_2PO_4

6.3 PROTOCOL 13. SURFACE LABELING COELOMOCYTES WITH ANTIBODIES IN SOLUTION

Evaluating coelomocytes by sorting can take advantage of identifying surface proteins with antibodies, and has also been employed after incubating coelomocytes with lectins labeled with fluorochromes (Liao & Fugmann, 2017). The protocol described below can be used to label live *S. purpuratus* coelomocytes, and takes advantage of the surface expression of SpTrf proteins on small phagocytes (Brockton et al., 2008). Surface labeling of this phagocyte subset is used prior to analysis by fluorescence activated cell sorting (FACS) (Protocol 14).

1. Collect 200–500 µL of coelomic fluid and dilute into an equal volume of ice cold CMFSW-EH (Recipe 1). All steps should be carried out with cold reagents on ice.
2. Filter debris and cell clots from single cells by slowly pipetting the cell suspension through a standard medicinal gauze pad or a 35 µm nylon mesh cell strainer tube cap into a 12 × 75 mm flow cytometry test tube (Falcon) on ice.
3. Count cells and adjust the concentration to 1×10^6 cells/mL.
4. Pellet two 1 mL samples of the cells by centrifugation at 400–500 × *g* for 5 min at 4 °C.
5. Resuspend the pellet in 100 µL staining medium (Recipe 13) by gently flicking the tube rather than pipetting the cells, which can cause damage from shear forces.
   a. Resuspend one sample in staining medium containing primary antibody at the appropriate dilution. For this example, coelomocytes are incubated with a mix of three polyclonal rabbit antibodies against the SpTrf proteins (formerly called Sp185/333; Brockton et al., 2008) at a dilution of 1:100 in staining medium.
   b. Resuspend the second sample in staining medium with the same dilution of normal rabbit serum. This sample will serve as the background control.
   c. Subsequent steps are identical for both cell samples.
6. Incubate cells for 30 min on ice.
7. Pellet the cells as in step 4, discard the supernatant, and resuspend the pellet in 500 µL staining medium.
8. Pellet the cells and resuspend the pellet in 30 µL in staining medium containing goat anti-rabbit immunoglobulins conjugated to Alexa Fluor 488 (1:250 dilution) (Thermo Fisher Pierce).
9. Incubate on ice for 30 min.
10. Pellet the cells and resuspend in 500 µL staining medium.
11. Pellet the cells, resuspend in 100 µL staining medium containing 1 µg/mL propidium iodide (PI) and incubate on ice for 5 min. Nuclear staining by PI is used to identify dead cells that are excluded from the analysis.
12. Evaluate 10 µL of each cell suspension with a fluorescent microscope (40 × objective) to verify cell surface labeling for the secondary antibody (green) and nuclear staining for the PI (red) to determine whether the antibody binding protocol was successful and to estimate the level of background and the percentage of live cells.
13. These samples are prepared for evaluation by FACS (Protocol 14).

7 SINGLE CELL SORTING OF ANTIBODY-LABELED SEA URCHIN COELOMOCYTES

Differences in gene expression among single cells that are identical morphologically suggest that cells have a range of gene expression parameters, or that a given cellular morphotype is actually mixture of different cell types. For example, naïve lymphocytes

are morphologically similar but are a mixture of very different types of cells that have different functions when terminally differentiated (Spitzer et al., 2015). Similarly, there are three morphotypes of sea urchin phagocytes, polygonal, discoidal, and small; however, whether each type is functionally uniform has been under speculation. For example, subsets of polygonal phagocytes express the C3 complement homologue, SpC3 (Gross et al., 2000), subsets of all three types of phagocytes express a single *SpTrf* gene that can differ among cells (Majeske et al., 2014), and different coelomocyte morphotypes or subsets of morphotypes can be identified by specific lectins that bind to surface sugars (Liao & Fugmann, 2017). FACS can be used to characterize coelomocytes based on the gating approach that optimizes separation according to the staining method that is employed and/or the characteristics of one or more cell types.

7.1 PROTOCOL 14. FLOW CYTOMETER SETUP

7.1.1 Hardware setup

1. Turn on the FACSAria flow cytometer (BD Biosciences, San Jose, CA) and fill the sheath tank with sheath fluid. Standard phosphate buffered saline (PBS) that does not contain calcium or magnesium is the most commonly used sheath fluid.
2. Install a 100 µm nozzle underneath the flow cell and initiate the flow of sheath fluid. When a sample is introduced into the flow cell, it is injected into the center of a stream of sheath fluid. Within the flow cell, hydrodynamic focusing aligns particles (beads or cells) into a single file at the interrogation point where each particle intercepts the laser(s). After passing through the flow cell, the sample stream enters the nozzle where the drop drive breaks the stream into highly uniform droplets for sorting.
3. Launch the Cytometer Setup and Tracking (CST) program. Run CST beads (BD Biosciences) for the Performance Check and verify that standard settings do not deviate from established optimal settings for the instrument.
4. Launch the FACSDiva program, which is the acquisition and analysis program on the FACSAria. Run Accudrop beads (BD Biosciences) on the sorter to determine the drop delay setting. When a bead is present at the interrogation point and meets the sorting criteria, an electrical charge is applied to the fluid stream just as the droplet containing that bead breaks off from the stream. Droplets detach from the stream a few millimeters downstream from the nozzle. Drop delay setting is the time between when a bead intercepts the laser(s) at the interrogation point and when it reaches the droplet breakoff point.
5. Install the splash shield below the sort aspirator drawer and activate the Automated Cell Deposition Unit (ACDU).
6. For test sorting, tape a lid to the bottom of a 96-well or 384-well plate to prevent even the slightest shifting of the lid during movement of the ACDU. The edges of the lid must be oriented exactly over the edges of the plate bottom. Secure the plate onto the ACDU with well A1 at the bottom left corner. Test sorting is performed by sorting beads in droplets onto the plate lid to confirm that charged droplets will be deposited at the center of each well of the plate.

7. Use the factory-default setting for a standard 96-well or 384-well plate as a starting point and move the ACDU to the "Home" location, which is the position where the first test sort bead will land on the lid over well A1.
8. Turn on the deflection plates, which are high-voltage plates that deflect the fluid stream to the side during sorting of beads or cells that meet the sorting criteria. An electrical charge is applied to the stream just as the droplet containing that bead breaks off from the stream. When the charged droplet passes by the charged deflection plates the electrostatic attraction and repulsion deflects the droplet to the right or left, depending on the charge polarity induced on the droplet. For sorting with the ACDU, charged droplets will only be deflected to the left.
9. Briefly apply voltage to deliver small test droplets of sheath fluid onto the lid of the plate.
10. Adjust the ACDU iteratively until droplets land on the lid and are centered over the A1 well. Save the *x*- and *y*-coordinates as the new "Home" location.
11. Move the ACDU to the diagonally opposite or the "farthest" location on the plate, which is well H12 for 96-well plate or well P24 for 384-well plate. Repeat the procedure as in steps (9 and 10). Save the *x*- and *y*-coordinates as the new farthest location.
12. Run some beads (e.g., 6–8 μm polystyrene beads; Spherotech) through the sorter and draw a gate around the bead population on a FSC vs SSC plot. Sort the gated population onto the lid of the 96-well or 384-well plate at 30 beads/well. Confirm that the beads hit the lid over the center of each well. If not, repeat steps 9–11 to fine-tune the plate alignment.

7.1.2 FACSAria software set up

1. Three lasers provide excitation wavelengths of 405, 488, and 640 nm and several detection filters are used on the FACSAria instrument (Table 1). These lasers and bandpass (BP) filters are used to sort coelomocytes (Table 2).
2. The most commonly observed autofluorescent molecules are NADPH and flavins. They emit in the violet/blue/green wavelengths. Viewing red fluorescence vs violet fluorescence facilitates identification of the red spherule cells because they form an obvious cluster that can be gated. This cluster of cells is positive for red fluorescence and negative for violet fluorescence. Table 2

Table 1 Lasers and Band Pass Filters.

Excitation Laser (nm)	Band Pass Filter (nm)	Detection
405	450 ± 25	Violet fluorescence
488	488 ± 5 488 ± 5 530 ± 15 610 ± 10	Forward Scatter Side Scatter Alexa Fluor 488 Propidium iodide
640	670 ± 7	Red fluorescence

Table 2 Set-up for Coelomocyte Sorting[a].

Plot	*x*-Axis	*y*-Axis
A and E	488 nm (FSC)	488 nm (SSC)
B and F	530 nm (AF488: secondary Ab)	610 nm (PI)
C and G	610 nm (PI)	488 nm (SSC)
D and H	670 nm (Red: echinochrome A)	450 nm (Violet: autofluorescence)

FSC, forward scatter; SSC, side scatter; AF488, Alexa Fluor 488; Ab, antibody; PI, propidium iodide.
[a]These plot parameters refer to the data shown in Fig. 5.

shows the parameters used to evaluate coelomocytes stained with a primary antibody followed by a secondary antibody conjugated to AF488.

3. Draw four bivariate plots on a global worksheet in the workspace (all parameters are in log scale) (Fig. 5).

7.1.3 Coelomocyte sample analysis

1. Run an aliquot of the control cells (Protocol 13) and record all of the events on the four plots (Fig. 5A–D).
2. Display all of the events on Plot A and draw a polygonal Gate 1 in the upper right region of the plot that contains coelomocytes and excludes debris with low FSC and SSC signals (Fig. 5A).
3. Apply Gate 1 to Plot B to display the coelomocytes (Fig. 5B). Draw a polygonal Gate 2 in the lower right region that encompasses AF488-positive/PI-negative events, which are live coelomocytes. Secondary antibody conjugated to AF488 binding to the cells indicates the background level of non-specific binding to the cells.
4. Apply Gate 1 in Plot A to Plot C (Fig. 5A and C) to display coelomocytes and draw a rectangular Gate 3 (Fig. 5C) in the upper left side that encompasses PI-negative events or live coelomocytes.
5. Apply Gate 3 to Plot D (Fig. 5D) to display live coelomocytes and draw an elliptical Gate 4 in the lower right region encompassing Red-positive/Violet-negative events, which are live, red spherule cells containing echinochrome A.
6. Run through the sorter an aliquot of the coelomocytes labeled with anti-SpTrf antibodies (or other primary antibody to a surface molecule on coelomocytes) and secondary antibody labeled with AF488 (Protocol 13). Record the events on the four plots (Fig. 5E–H) as was done for the control cells. Use the same display and gating scheme as described above for the control cells.
7. Plot E displays "All Events" and Gate 1 contains coelomocytes (Fig. 5E). The percentage of coelomocytes is similar to the percentage control cells (Fig. 5A).
8. Plot F displays live coelomocytes that are PI-negative (Fig. 5F). The cell sample is labeled with primary anti-SpTrf antibodies followed by secondary antibody conjugated to AF488. Gate 2 encompasses live, small phagocytes that bind anti-SpTrf/AF488 and show a significantly higher percentage of cells compared to the control cells (Fig. 5B).

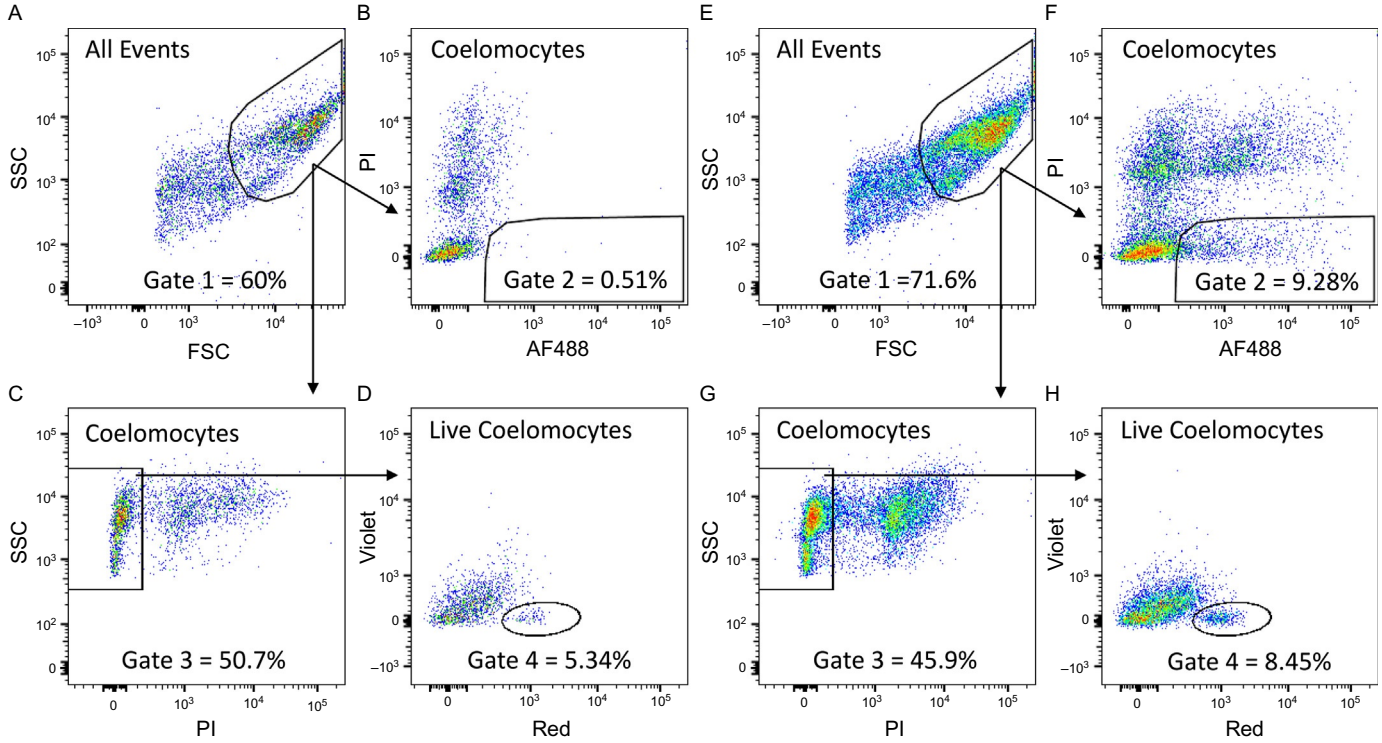

FIG. 5

Single cell sorting of sea urchin coelomocytes labeled with anti-SpTrf antibodies. (A–D) Control coelomocytes processed with the secondary antibody conjugated with AF488 only illustrate background staining. (A) All events are displayed and Gate 1 encompasses all coelomocytes and excludes debris that have low forward scatter (FSC) and side scatter (SSC) signals. (B) All coelomocytes are displayed and live cells exclude propidium iodide (PI). Gate 2 shows non-specific binding of the secondary antibody on some of the live, control cells. (C) All coelomocytes are displayed and Gate 3 shows live coelomocytes that exclude PI. (D) Live coelomocytes are displayed, and Gate 4 shows live, red spherule cells that contain echinochrome A. They form a discreet cluster that is positive for red fluorescence (Red) and is negative for violet fluorescence (Violet). (E–H) Coelomocytes that have been processed for labeling with anti-SpTrf antibodies and secondary antibody conjugated with AF488. (E) All events are displayed and Gate 1 encompasses coelomocytes and excludes cell debris. (F) All coelomocytes are displayed and live cells exclude PI. Gate 2 shows live, small phagocytes that are positive for anti-SpTrf/AF488. (G) All coelomocytes are displayed and Gate 3 shows live coelomocytes that exclude PI. (H) Live coelomocytes are displayed and Gate 4 shows live, red spherule cells that contain echinochrome A.

9. Plot G displays coelomocytes and Gate 3 contains live coelomocytes, which show a similar percentage as the control cells in Plot C.
10. Plot H displays live coelomocytes (Fig. 5H) and Gate 4 shows live, red spherule cells containing echinochrome A. The percentage of red spherule cells is similar to those in the control cells (Fig. 5D).
11. Once the gating has been established for different cell types, the protocol can be used to sort single cells of a known type into single wells for subsequent analysis.

7.1.4 Single cell sorting into 96-well or 384-well plate

1. Prepare an empty 96-well or 384-well plate for testing the sorter, cover it with the same type of seal as used for the plates for single cell sorting, and secure it onto the ACDU.
2. Using Gate 3 (live coelomocytes; Fig. 5C) as the sorting criterion, sort from the control sample and deposit 30 cells/well onto the seal of the plate.
   a. This serves as a final confirmation that the trajectory of sorted cells hits the center of each well accurately. This procedure may not be necessary for single cell sorting into a 96-well plate, but will enhance the success of depositing a single cell into a small target for a 384-well plate.
3. Prepare a 96-well or 384-well plate for sorting. Dispense 7 µL of staining medium (Recipe 13) into each well of A1-A12 and B1-B12 of a 96-well plate, or A1-A24 and B1-B24 of a 384-well plate. Secure the plate onto the ACDU.
4. Using Gate 2 (live, small phagocytes; Fig. 5F) as the sorting criterion, sort from the SpTrf-stained cell sample to deposit a single $SpTrf^{+}$ cell into each well of A1-A12 of a 96-well plate, or A1-A24 of a 384-well plate.
5. Using Gate 4 (live, red spherule cells; Fig. 5H) as the sorting criterion, sort a single red spherule cell from the SpTrf-labeled cell sample, into each well of B1-B12 of a 96-well plate, or B1-B24 of a 384-well plate. Observe the cell under a light or fluorescence microscope to verify the presence of a single cell of the expected cell type in each well.
6. If more than one cell or the incorrect cell type is observed in a well, troubleshoot the procedure before proceeding. Confirm that the drop delay setting is accurate and the gating strategy is correct. Repeat steps 4–5 until a single cell of the expected cell type is observed in each well.
7. Dispense 7 µL of collection buffer from the supplier for whole genome analysis or whole transcriptome analysis kits into each well of D1-D12, F1-F12, and H1-H3 of a 96-well plate, or G1-G24, L1-L24, and P1-P6 of a 384-well plate. Secure the plate onto the ACDU.
8. Using Gate 2 (live, small phagocytes; Fig. 5F) as the sorting criterion, sort from the SpTrf-labeled cell sample to deposit a single small phagocyte into each well of D1-D12 of a 96-well plate, or G1-G24 of a 384-well plate.
9. Using Gate 4 (live, red spherule cells; Fig. 5H) as the sorting criterion, sort a single red spherule cell from the SpTrf-labeled cell sample into each well of F1-F12 of a 96-well plate, or L1-L24 of a 384-well plate. Wells H1-H3 and

P1-P6 serve as negative controls for the 96-well and 384-well plates, respectively.

10. Immediately seal the plate and centrifuge at 400 × *g* at 4 °C for 5 min. Store at −80 °C until further processing.
11. Repeat steps 7–9 for coelomocytes from other sea urchins.

8 LIMITING DILUTIONS OF COELOMOCYTES FOR SINGLE CELL ANALYSIS

If a cell sorter is unavailable or there is no experienced technician familiar with sorting non-mammalian cells in high salt solutions, limiting dilutions of coelomocytes can be used as an alternative and inexpensive approach (Majeske et al., 2014). Limiting dilutions can be combined with an initial step of density gradient centrifugation to enrich for particular cell types (Protocols 9 and 10).

8.1 PROTOCOL 15. LIMITING DILUTIONS FOR SINGLE CELL GENE EXPRESSION ANALYSIS

1. CF collected in CMFSW-EH (Recipe 1) is adjusted to approximately 1×10^4 cells/μL. All steps should be done on ice.
2. Place 10–50 μL of cells in the wells of one row in a 96-well plate.
3. Perform serial 1:10 dilutions into CMFSW-EH from the first row of wells to the next row, and repeat, to generate a dilution series of 1×10^3, 1×10^2, 1×10^1, and 1 cell/μL for each row of wells.
4. To account for variations in cell concentration in the initial sample that may be compounded by pipetting error during the dilution series, additional dilutions of 1 cell/μL should be done. This includes two serial dilutions of 1:2 and a 1:10 dilution, which generate samples of 0.5, 0.25 and 0.1 cell/μL.
5. Pipette 1 μL from dilutions of 1, 0.5, 0.25, and 0.1 cell/μL into cell lysis buffer. The composition and volume of the lysis buffer will depend on whether whole genome amplification or transcriptome analysis will be done and which kit or method has been selected.
6. A positive control to verify the presence of a cell in a well must be included in the analysis. Depending on the subsequent approach, RT-PCR can be used to verify transcripts from housekeeping genes such as *actin*, large ribosomal protein 8 (*SpL8*), and/or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Primers for these genes can also be used to verify the presence of genomic DNA by PCR.

9 CONCLUSION

The methods described here have been used successfully with the coelomocytes from the purple sea urchin, *S. purpuratus*, for a wide range of analyses, including characterization of subclasses of coelomocytes, expression patterns of specific genes and

proteins, and functions of proteins. These approaches can be augmented by separating coelomocyte types into fractions or single cells. We assume that these methods are applicable to other species of echinoids with no or little modifications, but this will also depend on the focus of the research. It is noteworthy, that many echinoids do not produce echinochrome A and are not purple or red, such as *Lytechinus pictus*. Hence, this characteristic of *S. purpuratus* cannot be employed for all other species. It is not known whether these methods can be applied directly to non-echinoid species or whether significant modifications will be required for optimization. The types and characteristics of coelomocytes in members of the other classes of echinoderms can be quite different (see Smith et al., 2018).

The evaluation of coelomocytes from adult purple sea urchins is a species of choice because it is a large invertebrate, has a large body cavity filled with CF particularly for non-gravid animals in which the gonads are small, and is an excellent source of large numbers of coelomocytes. If small volumes of CF are collected from individual animals, experiments can be designed with multiple days as sampling time points. It should be kept in mind when designing experiments, that the genetic variations among individual sea urchins can introduce significant noise into a data set such that it often obscures the signal. Consequently, experimental design should employ many (>10) animals for each experimental group (e.g., pre vs post treatment) and therefore would require easy access to large populations of sea urchins. Alternatively, the benefit of using a large invertebrate is that individual animals can serve as their own control by collecting small volumes of CF and evaluating the coelomocyte before compared to after treatment over time.

CONTRIBUTORS

All co-authors contributed equally to this chapter and are listed in alphabetical order.

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