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Isolating specific embryonic cells of the sea urchin by FACS

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Summary

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

Keywords

Sea Urchin; FACS; embryonic cells; antibody; Vasa

1. Introduction

Lineage-specific gene expression is a major theme of developmental biology. However, the dynamics of differential gene expression may be obscured by experimentation in which the whole embryo is tested. Techniques that fractionate the embryo into its constituent lineages are therefore desirable for performing focused analyses. In the sea urchin embryo, protocols are available for isolating several cell types. For example, micromeres, the skeletogenic cells produced at the 4th embryonic cleavage, can be collected en masse by sucrose gradients due to their small size relative to other blastomeres (Harkey and Whiteley, 1985; Okazaki, 1975). McClay and colleagues devised a procedure for separating the ectoderm from the endoderm based on differential adhesion, which was then adopted for applications of cell adhesion studies and mRNA comparisons (Bruskin et al., 1981; McClay and Marchase, 1979; Wessel et al., 1989). Primary mesenchyme cells, the derivatives of micromeres could also be isolated by differential affinity to lectins which enabled their isolation en masse

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following ingression into the blastocoel (Ettensohn and McClay, 1987). However, all of these methods target a limited number of cell types at particular developmental time points.

Fluorescence activated cell sorting (FACS) affords great flexibility in isolating cells of interest. Traditional FACS uses antibodies against cell surface proteins to label specific live cells for isolation from a suspension, and more recently, of fluorescent protein labeling of cells by genetic means. Such approaches yield viable populations of isolated cells; however, they require antibodies against surface antigens or a model organism in which genetic manipulation is possible (Stoeckius et al., 2009; Yeung and So, 2009). Alternatively, cells may be fixed, permeabilized, and labeled with antibodies against cytoplasmic antigens. However, this approach has typically precluded further downstream analysis of the cell isolates, such as gene expression profiling.

We present a method that combines the flexibility of FACS using antibodies to antigens inside of cells with fixation conditions that allow downstream extraction of RNA for qPCR. In brief, cultured blastula stage embryos are disaggregated into a suspension of single cells, methanol fixed, immunolabeled, and FACS isolated (Figure 1). Fixed, dissociated cells maintain their morphology and can be effectively immunolabeled (Figure 2). By titrating different amounts of YP30-labeled labeled cells (a ubiquitous cytoplasmic protein) into unlabeled cells, we find that positive cells representing as little as 1% of the total population can be effectively FACS isolated (Figure 3). We go on to show isolation of the small micromeres of the sea urchin embryo, a rare (approximately 4 cells within an embryo of 1000 cells) multipotent cell population with germ line competency (Figure 4) (Juliano et al., 2010; Yajima and Wessel). With antibodies specific to small micromere proteins Vasa and Nanos2, the small micromeres can be distinguished and FACS isolated from other cells in the suspension (Figures 2, 4).

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

2. Materials

2.1 Dissociation of embryos into single cells

Reagents

- 4L glass beaker
- Stirring paddle
- 50mL and 15mL conical tubes
- Plastic transfer pipettes
- 45μM and 20μM Nitex® mesh

Solutions

Artificial Sea Water: Coral Life Scientific Grade Marine Salt (Carson, CA), follow manufacturer's instructions

Calcium-free Sea Water

ddH2O	900 mL
NaCl	29.3 gms (0.5 M)
KCl	0.75 gms (0.01 M)
NaHCO3	0.21 gms (1.5 mM)
NaOH	2.5 gms (0.06 M)
EGTA	7.6 gms (0.02 M)

Adjust pH to 8.0 with HCl and then fill to 1 liter with ddH2O

Dissociation Buffer: 1M Glycine

25mM EDTA

pH 8.0

Store at 4°C

2.2 MeOH Fixation of Single Cells

Reagents

- 15mL conical tubes
- 100mm×15mm Petri dishes
- 100% MeOH chilled at −20°C

Solutions

1L 1XPBS-Tween: Dissolve in 800mL of RNase-free Water:

8 g NaCl

0.2 g KCl

1.44 g Na2HPO4

0.24 g KH2PO

Adjust pH to 7.4 with 1M HCl

Add 1mL Tween-20 (0.1% final concentration)

Add RNAse-free water to 1L

Filter sterilize through 0.22μM filter

2.3 Immunolabeling of single cells

Reagents

- 15mL conical tubes
- PBS-Tween (see above for recipe)
- Primary and secondary antibodies of choice

2.4 FACS sorting and RNA extraction for RT-qPCR

Reagents

- FACS collection tubes
- Trizol® LS, Catalog #10296028 (Invitrogen; Carlsbad, CA)
- Glycogen, Catalog #10901393001 (Roche; Indianapolis, IN)
- RQ1 DNase, Catalog #M6101 (Promega; Madison, WI)
- Acid Phenol:Chloroform, Catalog # AM9720 (Ambion; Austin, TX)
- 5M ammonium acetate pH5.2
- 100% EtOH
- High Capacity cDNA Reverse Transcription Kit, Catalog # 4368814 (Applied Biosystems; Foster City, CA)

3. Methods

3.1 Dissociation of embryos into single cells

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

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

3.2 MeOH fixation of single cells

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

3.3 Immunolabeling of single cells

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

10. Spin single cells for 5 minutes at ~255 × g and remove supernatant.
11. Add up to 15mL of PBS-Tween
12. Repeat steps 10–11 twice.
13. Check labeling under fluorescent microscope (Figure 2).

3.4 FACS labeling and RNA extraction for RT-qPCR

1. Bring labeled cells immediately to FACS machine for sorting (Figures 3,4A).
2. Sort cells directly into Trizol® LS. Fill an appropriately sized tube (depending on the instrument) with 750μL of Trizol® LS and sort cells into tube until the volume reaches 1mL. To collect more cells, use multiple tubes. Both the positive and negative populations can be collected if this is desired for downstream applications. If necessary, after sorting adjust the final sample volume with nuclease free or Trizol LS water such that the final ratio is 3 parts Trizol LS to 1 part aqueous sample.
3. Isolate RNA from sorted cells following the Trizol® LS manufacturer's protocol. During isopropanol precipitation, add 0.5μL of glycogen to help visualize the pellet.
4. Re-suspend the pellet in 10μL of DNase solution (8 μL nuclease-free water + 1 μL10× buffer +1 μL DNase) (See Note 8)
5. Incubate at 37°C for 30 minutes
6. Bring volume up to 300μL with nuclease-free water
7. Extract RNA with acid phenol:chloroform following manufacturer's instructions
8. EtOH precipitate RNA overnight at −20°C (0.1 volumes of ammonium acetate and 2.5 volumes of 100% ethanol)
9. Re-suspend pellet in 10μL of nuclease-free water
10. Use entire volume for cDNA synthesis
11. Proceed with qPCR to test the relative abundance of candidate genes between distinct cell populations (See Note 9).

4. Notes

4. 1 Protocol Notes

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

3. Although single cells can be fixed using other methods, such as 4% paraformaldehyde in seawater, we found that the RNA is better preserved using MeOH fixation. Furthermore, the quantity of RNA obtainable is much higher from MeOH fixed cells versus paraformaldehyde. Be aware that different fixing conditions will likely have a direct impact on the effectiveness of your antibody labeling. We suggest testing your antibody labeling with different fixes on whole embryos before choosing a condition to use for single-cell labeling.

4. Ideal concentrations for labeling differ greatly between different antibodies. The concentration used for a specific antibody should be determined empirically.

5. The volume used for antibody labeling should be as high as possible to reduce the possibility of cell clumping. To conserve antibody, consider saving the solution and re-using for future experiments.

6. We find that using blocking reagents such as Bovine Serum Albumin (BSA), including those certified as RNAse free, will cause severe RNA degradation and should thus be avoided if possible. This could affect the quality of the antibody labeling, and these conditions should be tested in whole embryo labeling before single-cell labeling is done. In general, all incubation times and centrifugations should be minimized, and whenever possible, performed at 4°C to minimize RNA degradation. RNAse-free reagents should be selected whenever available.

7. If it does not decrease the quality of the antibody labeling, consider decreasing the labeling time, which may increase the quality of the RNA recovered from the sorted cells.

8. Alternatively, the pellet can be resuspended in 10μL of nuclease-free water and can be used directly for cDNA synthesis (step 10). In this case though, there will likely be contaminating genomic DNA in the sample. If primer pairs are designed to span an intron for use in RT-qPCR, contaminating genomic DNA should not be a problem.

9. In our hands, the RNA collected from this method is not of high enough quality for microarray or deep sequencing. Therefore this method cannot be used for gene discovery, but is better suited for testing if candidate genes are enriched in your cell population by RT-qPCR. This would be particularly useful for testing genes of low abundance that cannot be detected by whole-mount *in situ* RNA hybridization. However, it is possible that further improvements to this protocol could yield sufficiently high quality RNA for gene discovery.

4.2 Other potential uses for FACS-sorted cells

Many new experimental applications would be possible if an investigator were capable of isolating specific cell populations. The most direct application of the defined protocol herein is in mRNA analysis though improvement of the mRNA fidelity will be important in order to take full advantage of the protocol.

Alternatively, if antibodies are available that recognize a cell surface component, they can be used in live-cell immunolabeling and traditional FACS protocols. In this situation, the cells need not be fixed, and the mRNA will be protected as long as the cell remains intact.

Chromatin Immunoprecipitation (ChIP) for the analysis of epigenetic modifications and for promoter occupancy tests would benefit from this FACS protocol. In this case, the DNA is sheared to small (500bp) fragments following cell isolation and EDTA can be added to the fixed cells to minimize nucleases that may chemically cleave the DNA. In this application the isolated cells present a more homogeneous population of chromatin that may be more effective than use of the whole embryo and may enable a more refined condition for analysis of DNA or protein modifications, and for trans-factor analysis of promoter occupancy.

With new proteomic technologies enabling whole cell analysis, this FACS protocol will enable analysis of protein products. In many cases, cells will accumulate an mRNA but not translate the protein, so such analysis will inform what proteome the cell is working with at the time of isolation. This approach will also enable more effective identification of post-translational modifications of specific proteins including phosphorylation, glycosylation, ubiquitylation and sumoylation. Since the protein samples are trypsinized prior to analysis, fixation with methanol or even formaldehyde will not prevent protein identification, and for phosphorylation and glycosylation, a pre-purification column (e.g. Fe^{3+} IMAC enrichment of phosphopeptides) will enable more focused analysis of the biochemical question at hand (Cao et al., 2007).

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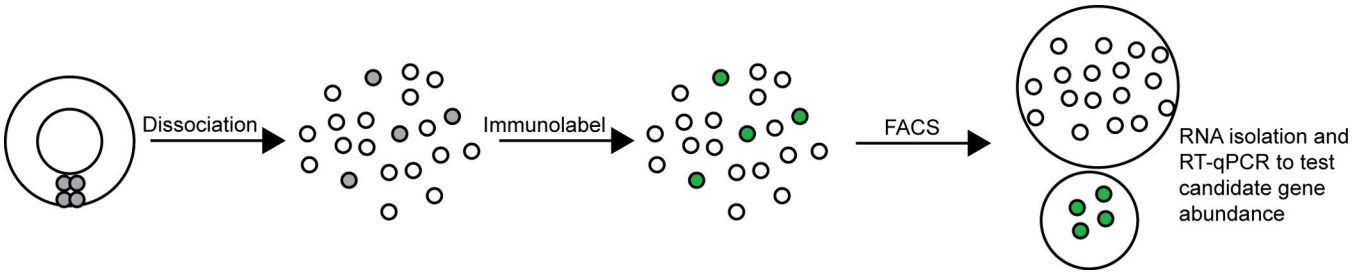

Figure 1. Scheme for isolating specific *S. purpuratus* embryonic cell populations by FACS

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

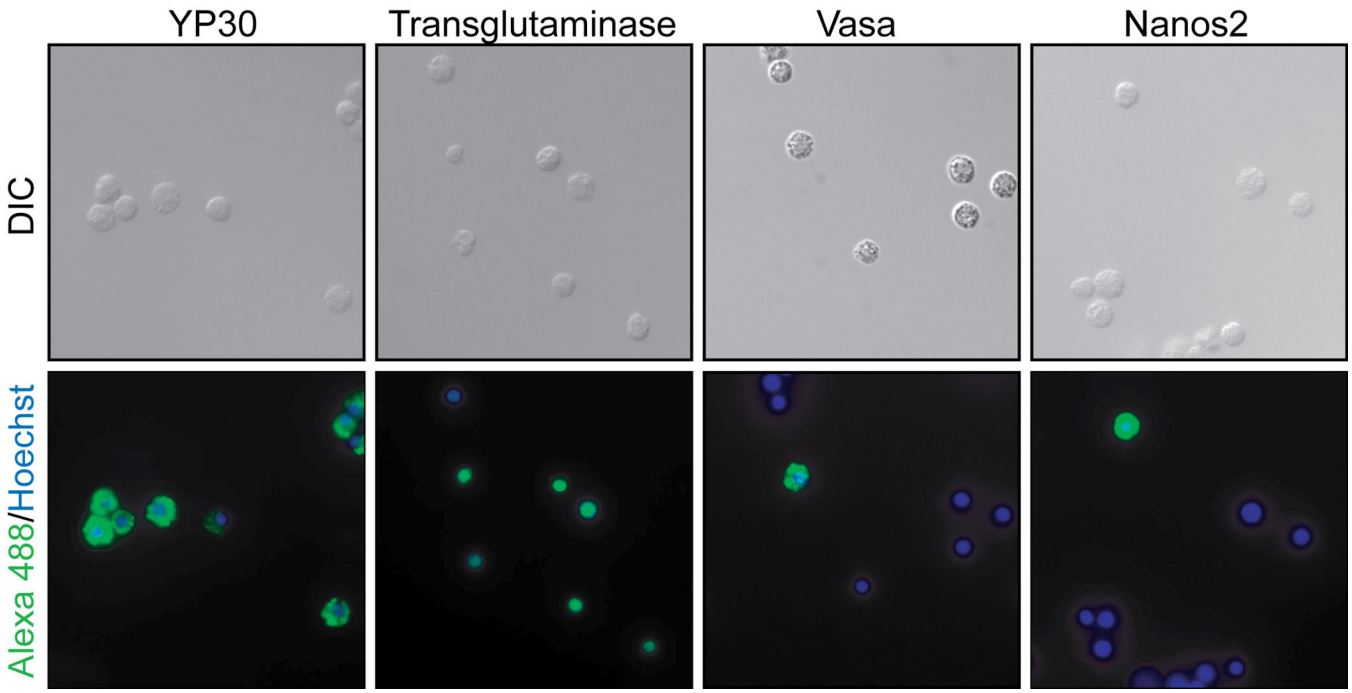

Figure 2. Immunolabeled single cells from mesenchyme blastula embryos

Mesenchyme blastula stage embryos were dissociated, fixed, and immunolabeled with antibodies that recognize YP30 (ubiquitous, cytoplasmic), Transglutaminase (ubiquitous, nuclear), Vasa (small micromere specific, cytoplasmic), or Nanos2 (small micromere specific, cytoplasmic) (Juliano et al., 2010; Voronina et al., 2008; Wessel et al., 2000). Cells were uniformly labeled with antibodies against transglutaminase and YP30. Approximately 1–2% of cells were labeled with the small micromeres-specific antibodies.

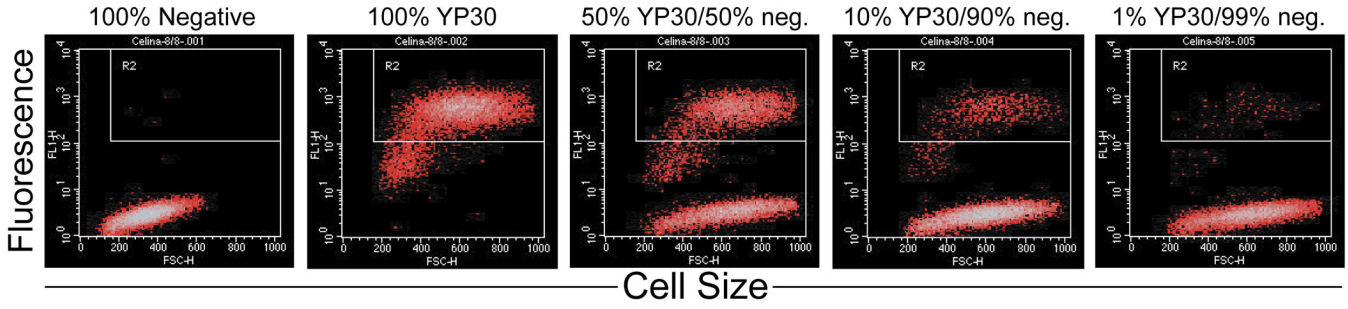

Figure 3. Separation of YP30 immunolabeled from unlabeled single embryonic *S. purpuratus* cells by FACS

FACS plots demonstrate successful separation of dissociated mesenchyme blastula cells based on YP30 immuno-labeling (see Figure 2). First, cells immunolabeled with YP30 or a secondary alone negative control were run separately followed by the successful sorting of mixed populations containing 50%, 10%, or 1% YP30 immunolabeled cells. The R2 population were gated as YP30-positive cells.

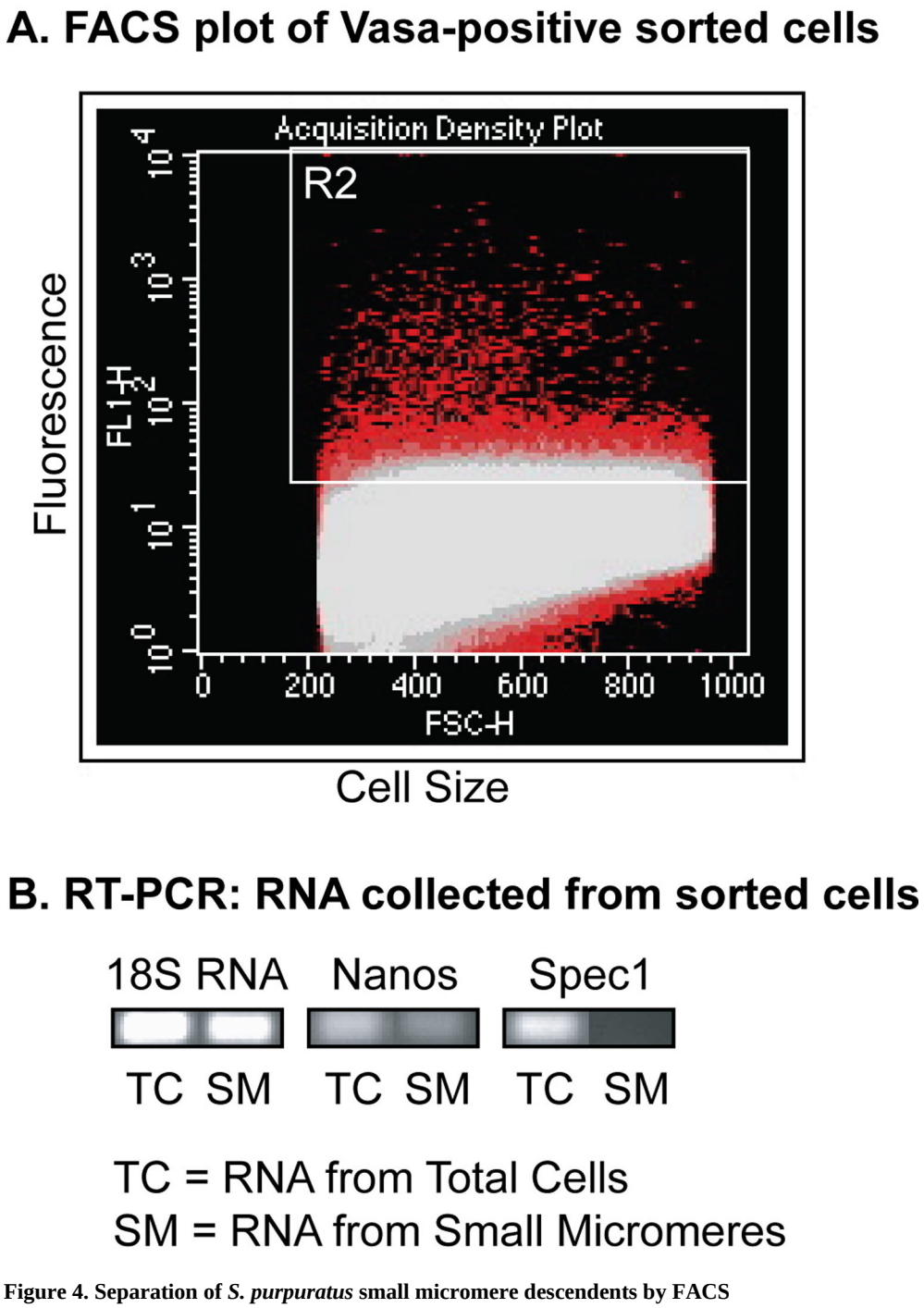

Figure 4. Separation of *S. purpuratus* small micromere descendents by FACS

(A) Single cells dissociated from mesenchyme blastula were immunolabeled with a Vasa antibody (see Figure 2) and separated by FACS on the basis of fluorescence. 270,000 cells were collected from the sort (box denoted by R2), which represented 0.75% of the total cells sorted. **(B)** 50ng of total RNA was isolated from the Vasa-positive small micromere descendents (SM). RT-PCR demonstrates that these cells contain the small micromere-

specific gene *nanos*, but not the ectoderm specific gene *spec1*. As a control for the RT-PCR, total RNA was isolated from the total cell population before FACS (TC).