

Chapter 2

A Unique FACS Method to Isolate Stem Cells in Planarian

Tetsutaro Hayashi and Kiyokazu Agata

Abstract

Fluorescence-activated cell sorting (FACS) is a useful method for stem cell biology, which enables us to isolate the living stem cells of interest from mixture of a variety of cells. In general, the target cells for FACS need to be labeled for various cell surface markers. However, in non-model organisms, we usually do not have specific labels for such cell surface markers. Here, we describe a method for isolating stem cells from non-model organisms, such as planarians, based on physiological and morphological properties of the stem cells. This method may also be applicable to other non-model animals.

Key words: Planarian, Non-model organisms, Fluorescence-activated cell sorting, Stem cells, Neoblasts

1. Introduction

Fluorescence-activated cell sorting (FACS) has been applied to isolate near-pure populations of hematopoietic stem cells (HSC) from mammals by combinatory use of cell surface markers (1). However, it has been considered that FACS is not applicable to purify stem cells from non-model organisms since no cell surface markers useful for FACS have been isolated yet for such organisms. Therefore, other strategies should be developed to purify living stem cells from these organisms. Previously, a subset of mouse HSC was isolated from bone marrow based on their physiological characteristics, that is, their ability to rapidly expel the fluorescent DNA-binding dye Hoechst 33342 (2). The HSC-rich cell fraction isolated by this method is called the side population (SP) fraction. However, this method was not applicable to isolate stem cells from non-model animals since their whole bodies contained a variety of cells expelling the Hoechst nuclear dye.

Therefore, we tried to develop a new method to isolate stem cells from non-model animals, such as planarians, based on their physiological properties. Hoechst 33342 can stain live cell nuclei and is useful to measure the DNA content of the nuclei. Therefore, using this dye, we could separate S/G2/M phase cells from G0/G1 phase cells based on their staining intensity. We also stained the dissociated cells with calcein AM, a fluorescent dye that stains active cytoplasm. We found that combinatory staining using these two different fluorescent dyes provides a powerful method to separate stem cells from differentiated cells since stem cells have scant cytoplasm, proliferative ability, and high sensitivity to γ -ray irradiation.

Planarians are known to have high regenerative capability, which is supported by adult pluripotent stem cells called “neoblasts” (3, 4). The neoblasts have been defined by their morphological characteristics, including their minimal cytoplasm. Moreover, these cells are specifically eliminated by γ -ray irradiation, and irradiated planarians completely lose the capability to regenerate (5). Based on these features, we have developed the FACS-based approach we describe here consisting of simple staining methods for the isolation and purification of γ -ray-sensitive stem cell populations from adult planarians (6). We also propose that this method could be applied to the isolation of stem cells from almost all organisms even if they lack known stem cell surface markers.

2. Materials

Prepare all solutions using distilled water (unless otherwise specified) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1. Dissociating and Staining Reagents

1. Saline solution and sheath buffer for planarians: Holtfreter's solution diluted 5/8 in distilled water (5/8 Holtfreter; 21.88 g NaCl, 0.63 g CaCl_2 , 0.31 g KCL, 1.25 g NaHCO_3 in 10 L distilled water, pH 7.4). Store at room temperature.
2. Trypsin stock solution ($\times 50$): 5% (w/v) trypsin (DIFCO #215240). Store at -20°C .
3. Hoechst stock solution: 1 mg/mL Hoechst 33342 (Sigma) in distilled water. Store at -20°C .
4. Calcein AM stock solution: 1 mg/mL calcein AM (Dojindo) in DMSO. Store at -20°C .
5. Propidium iodide (PI) stock solution: 1 mg/mL PI (Dojindo) in distilled water. Store at -20°C .

2.2. Prepare Worms Appropriate for FACS

1. Planarians: A clonal strain of the planarian *Dugesia japonica* (ssp.) is used in this experiment. Ten adults approximately 8 mm in body length are employed for each cell sorting fractionation (see Note 1). To reduce contamination from gut content debris, the animals are starved for more than 1 week before each experiment.
2. γ -ray-irradiated planarians: 10 animals (in same condition as in Subheading 2.2, item 1) are irradiated with 15 gray of γ -ray with a cesium source (Gammacell 40 Exactor, Best Theratronics). At least 4 days after irradiation, animals are used for this experiment.

3. Methods

Carry out all procedures at room temperature unless otherwise specified. Pipetting is performed using wide-orifice Cell Saver tips.

3.1. Cell Dissociation

1. Prepare ice in a 10-cm Petri dish using distilled water for cell dissociation (see Note 2). Two filter papers are superimposed on the ice and soaked moderately with distilled water. Then, stack a piece of Saran wrap (smaller or same size as the dish) on the filter papers as a top layer (see Note 3).
2. Gently place ten planarians on the ice stage (see Subheading 3.1, step 1) by using a pipette and sufficiently remove the water surrounding them by using Kimwipes twisted into string-like shapes. Cut planarians into smaller pieces using a surgical knife (No. 14) (see Note 4).
3. Immediately put the planarian homogenate in 980 μ L 5/8 Holtfreter in a 1.5-mL conical tube and add 20 μ L trypsin solution ($\times 50$).
4. Suspend the dissociating cell solution at once and incubate for 5 min at 20°C.
5. After incubation, gently pipette this dissociating cell solution at least 60 times and completely dissociate into single cells (see Note 5).
6. Centrifuge the dissociated cell solution at $1,500 \times g$ for 5 min and then wash two times.
7. Resuspend with 1 mL 5/8 Holtfreter and then filter through a 35- μ m pore size cell strainer (Becton Dickinson) and a 20- μ m pore size nylon net filter (Millipore) to remove tissue fragments (see Note 6).

3.2. Cell Staining

1. Add Hoechst solution and calcein AM solution, 18 and 0.5 $\mu\text{L}/\text{mL}$, respectively, to the filtered sample.
2. Invert the sample tube about three times and incubate for 120 min at 20°C (see Note 7).
3. Centrifuge and remove the supernatant. Resuspend in 2 mL 5/8 Holtfreter (or resuspend in two times the volume before centrifugation).
4. Finally, add PI solution 1 $\mu\text{L}/\text{mL}$ and incubate at least 5 min on ice.
5. Analyze by FACS or microscopy.

3.3. FACS Sorting of Cells, General Notes

5/8 Holtfreter is used as a planarian sheath buffer for FACS. Metabolic activation of live cells will begin to affect Hoechst intensity when cells are warmed above 10–15°C, so the sample should be cooled at 4°C during FACS analysis. Dissociated planarian cells are characterized using each fluorescent dye as follows: Hoechst 33342 is used for DNA staining; calcein AM is used to assess cell size; and propidium iodide (PI) is used to detect and eliminate dead cells from the sample. Flow cytometry analysis and cell sorting are performed using high-speed, multiparameter cell sorters. Machines must be optimized for flow rate to achieve maximal cell purity and viability. We use a BD FACSVantage SE (three laser system: 488 nm, 633 nm, and UV wavelengths; Becton Dickinson) and BD FACStation software (Becton Dickinson, version 5.1.1). At present, BD FACS Aria is not useful because it cannot use the “index sorting option” for single-cell PCR analysis (7). Moreover, when the sample includes the mucin-producing cells, like planarian cells, a flow cell system (e.g., FACS Aria) is also not useful compared to the jet-in-air system (e.g., FACS Vantage) because a flow cell system easily gets fouled with the mucus and is more difficult to clean than the jet-in-air system. Flow cytometry data can be analyzed offline using FlowJo software (Tree Star, Inc., Macintosh version 8.1.1).

3.4. Instrument Setting for FACS

1. Set the nozzle (pore size: 70 μm). This size is adequate to analyze and sort dissociated planarian cells.
2. Set optical detector as follows: FL1 (the log setting): band-pass filter (BP) 530/30 for calcein AM, FL3 (log setting): BP 630/22 for PI, FL4 (linear settings): BP 424/44 for Hoechst 33342 (Blue) and FL5 (linear settings): BP 670/42 for Hoechst 33342 Red (see Note 8).
3. The coefficient of variation (CV) in FL4 must be less than 2.0.

3.5. Gating the Stem Cell Population

1. Carry out adjusting fluorescent compensation and positive/negative gating in each fluorescent channel using a fluorescence-minus control and single staining ones.

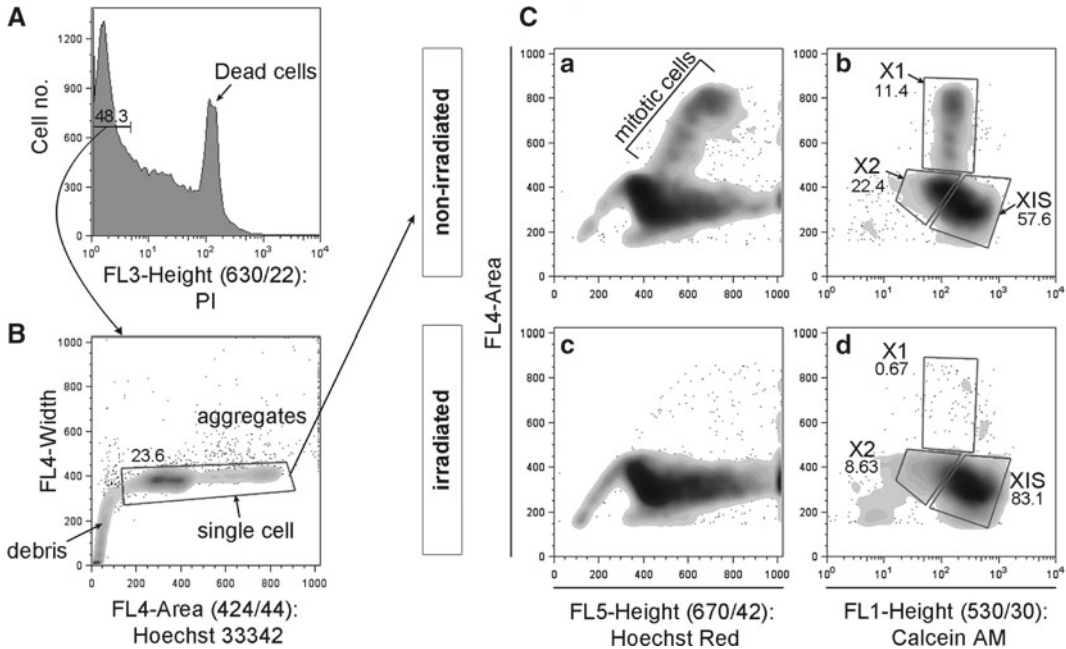


Fig. 1. Sorting profile for the purification of planarian stem cells. (A) Histogram of PI intensity. Negative staining region is gated as a live cell-containing population. (B) Density plots showing outliers in FL4-Area vs. FL4-Width. Single cell population is gated to divide from the aggregates and the debris. (C) Density plots showing outliers in Hoechst Red (a, c)/calcein AM (b, c) vs. Hoechst 33342. (a, b) Non-irradiated animal. (c, d) γ -ray-irradiated animal. (a, c) The mitotic cells are observed in non-irradiated animals, but not in irradiated animals. However, no difference of these animals is observed in the non-mitotic cell region. (b, d) Three populations are identified, X1, X2, and XIS, by comparison of non-irradiated with irradiated animals. Numbers in each graph indicate percentage of events. Data are collected for samples containing 10,000 cells in the single cell population.

2. Negative cells for PI staining are gated to exclude the dead cells (Fig. 1A).
3. The single cell population is identified by using FL4-Area vs. FL4-Width plot (Fig. 1B). This step is important to eliminate two-cell aggregates (see Notes 9–11).
4. Comparison of the profiles of non-irradiated and γ -ray-irradiated samples shows one region of high Hoechst 33342 staining cells eliminated by γ -ray irradiation in the FL5 Hoechst Red vs. FL4 Hoechst Blue plot (Fig. 1C, a, c). These cells seem to be mitotic planarian stem cells. Thus, mitotic stem cells can be sorted simply by staining with only Hoechst 33342 and PI.
5. Identify the mitotic stem cell population using comparison of the profiles of non-irradiated and γ -ray-irradiated samples in the calcein AM vs. Hoechst plot (Fig. 1C, b, d). This population shows high Hoechst 33342/weak calcein AM staining and is eliminated by γ -ray irradiation: this population corresponds to the population of cells designated X1 (see Note 12).

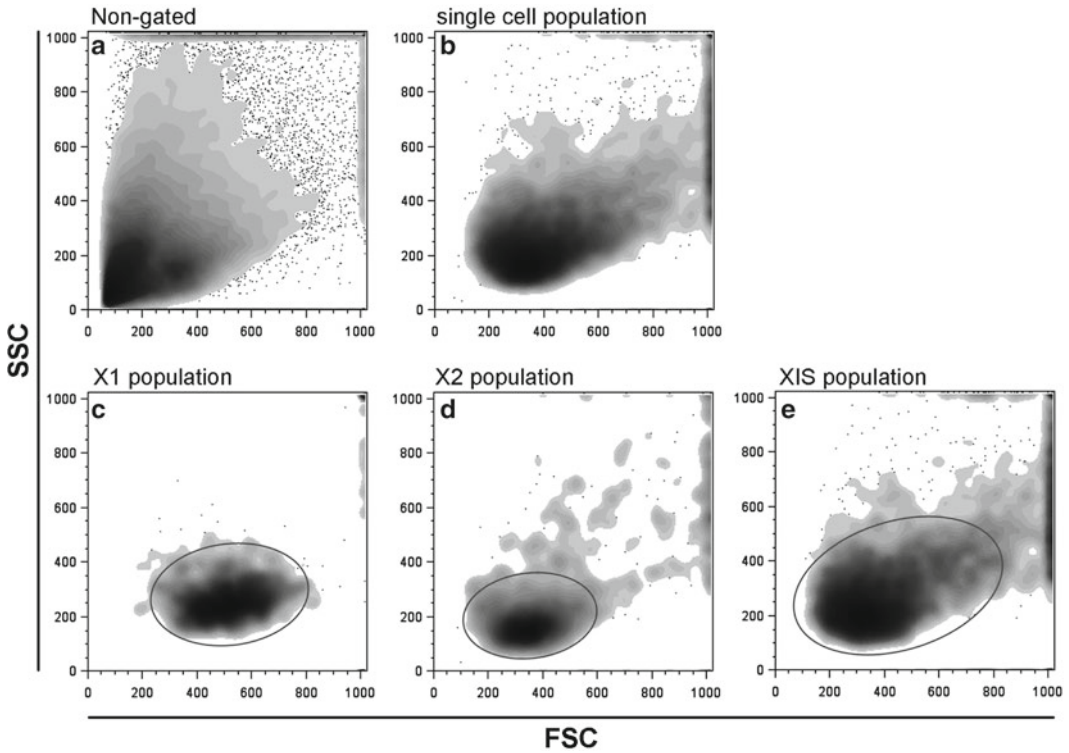


Fig. 2. The profiles of the different populations show differences in cell size and granularity. Density plots showing outliers in FSC vs. SSC. (a) The non-gated population shows a widely spread pattern in both size and granularity. The appropriate cells cannot be isolated in this population. (b) Single cell population shows the pattern appropriate for planarian live cells. (c–d) Each population shows a different pattern. Especially, the X1 and X2 populations show high homogeneity, in contrast to the XIS population.

6. Identify the non-mitotic stem cell population (Fig. 1C, b, d). This population shows weak Hoechst 33342 and calcein AM staining and is absent in the γ -ray-irradiated sample, and corresponds to the cell population designated X2 (see Note 12).
7. Identify the differentiated cell population (Fig. 1C, b, d). This large region separate from X1 and X2 and present on both plots maps to a γ -ray-insensitive cell population designated XIS (see Note 12).
8. Confirm the difference of each population in forward scatter (FSC) vs. side scatter (SSC) plot. Each population shows different features of cell size and granularity, respectively (Fig. 2).
9. Create the proper gate for size and granularity in each population (FSC and SSC, respectively) (Fig. 2c–e, circles).
10. Sort the target population and observe the sorted cells morphologically using fluorescence microscopy.

3.6. Exploitation of Sorted Stem Cell Population

1. Quantitative reverse transcription–PCR analysis: Total RNA from the cells collected by FACS is prepared using an RNeasy Micro Kit (Qiagen). For this, collect at least 1.0×10^5 cells in a 1.5-mL conical tube. First-strand cDNA synthesis is performed using a First-strand cDNA synthesis kit (Amersham Biosciences), and then the cDNA is used for semiquantitative real-time PCR analysis (see ref. (6)).
2. Single-cell PCR analysis: Single-cell sorting is carried out using the index sorting option (refer to the chapter of Index Sorting in the CloneCyt Plus User's Guide: Becton Dickinson Biosciences; see Note 13). A single cell is collected in each well of a 96-well plate and used to synthesize cDNA (see ref. (7) for following steps).
3. Fix for electron microscopic analysis: The sorted cells are centrifuged at $1,800 \times g$ for 10 min using a swinging-bucket rotor (Tomy, TMS-21). Remove the supernatant fluid. The cells are fixed in 1.2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4°C. The cells are then washed for 15 min in 0.1 M sodium cacodylate buffer and postfixed in 2% osmium tetroxide in the same buffer for 1 h at 4°C and then encompassed with 0.1% agarose gel in the same buffer to prevent the cells from being dispersed (see ref. (8) for following steps).
4. Fix for immunostaining or in situ hybridization analysis: The sorted cells are centrifuged at $1,800 \times g$ for 10 min using a swinging-bucket rotor, and the supernatant fluid is removed. Fix the cells in cold 2–4% paraformaldehyde on ice for 5 min. Wash at least once with PBS and follow the appropriate subsequent procedures.

4. Notes

1. It is difficult to count the cell number of dissociated cells from the whole planarian body because the sample contains many types of cells, debris, aggregates, and so on. Therefore, it is necessary to control the animal size and number and the period of starvation, in order to keep the same conditions.
2. Because planarians lose the ability to move when they are cooled, chilling on ice makes it easy to treat them.
3. By using the wrap, the planarian homogenate can be collected easily.
4. Cut planarians until there is absence of pieces and formation of a thick liquid.
5. Pipette until absence of small pieces.

6. This manipulation is critical for Hoechst staining. Especially, without filtering through the 20- μ m pore size nylon net filter, the nucleus is not stained sufficiently.
7. The concentration of Hoechst and staining time are essential for isolation of stem cells. At first, to avoid over/under Hoechst staining, investigation of the optimal conditions for staining, cell concentration, dye concentration, staining time, and temperature should be necessary. The optimal conditions produce the population expected, the G2/M phase, in the region of highest Hoechst intensity (Fig. 1C, a, b).
8. When using FACSVantage SE and BD FACStation software, the setup of pulse processing in FL4 is needed to use FL4-Width and FL4-Area parameters (refer to the chapter of Using Pulse Processing in the Pulse Processor Plus User's Guide: Becton Dickinson Biosciences).
9. To strictly collect single cells and eliminate doublet cells, we conduct FACS based on the following principal. Single dividing cells (G2/M phase cells: 4N/cell) and doublet non-dividing cells (adherent G1/G0 phase cells: 2N/cell) can be distinguished by comparison of the area and width of the Hoechst fluorescence signal, where "area" is equal to the intensity times the width (corresponding to the time of passage of a cell through the laser beam). Although the Hoechst areas of a single dividing cell and doublet non-dividing cells are the same, the width of doublet cells is twice that of a single dividing cell. Thus, by sorting living cells according to the area and width of Hoechst intensity, one cell fraction is defined as a population of singlet cells.
10. The cells showing higher intensity of FL4-Width than the single cell population seem to be aggregates. On the other hand, the negative population of FL4-Area seems to be debris. This analysis needs saturated staining for Hoechst 33342.
11. When using FACSVantage SE and BD FACStation software, instrumental setting is needed (refer to the chapter of DNA analysis in the Pulse Processor Plus User's Guide: Becton Dickinson Biosciences).
12. Results from single-cell PCR and electron microscopic analysis demonstrated that the X1 population mainly contains mitotic stem cells, the X2 population contains non-mitotic stem cells and differentiated cells, and the XIS population contains almost all differentiated cells (7, 8).
13. The order in which the cells are collected in wells and FACS profiling data (FCS file) of the sorted cells are recorded at the time of collection using the index sort function of FACSVantage SE option.

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