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Resolving Heterogeneity: Fluorescence-Activated Cell Sorting of Dynamic Cell Populations from Feeder-Free Mouse Embryonic Stem Cell Culture

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Abstract

Embryonic stem cell (ESC) culture comprises a mixture of cells that are primed to differentiate into different lineages. In conditions where ESCs self-renew, these primed populations continuously interconvert and consequently show highly dynamic coordinated changes in their expression of different sets of pluripotency and differentiation markers. It has become increasingly apparent that this transcriptional heterogeneity is an important characteristic of ESC culture. By sorting for specific populations of ESCs it is possible to enrich for cells with a capacity to colonize the embryo proper or the extra-embryonic lineages such as the descendents of the primitive endoderm or trophoblast. Here, we describe a method of isolating specific sub-sets of ESCs from the pluripotent cells present in in vitro ESC culture using SSEA1 antibody staining in combination with reporter lines and fluorescence activated cell sorting (FACS).

Keywords: Embryonic stem cells, Lineage priming, Self renewal, Pluripotency, Endoderm, Transcription

1 Introduction

Embryonic stem cells (ESCs) are characterized by their ability to form any cell type in the adult body (pluripotency) and their capacity to expand indefinitely in culture (self-renewal). These cells are derived from the blastocyst stage of mammalian embryonic development at a point where the first lineage decisions are being made. During early development, the lineage potential of individual cells becomes progressively restricted such that by the late blastocyst stage, three lineage committed progenitor cell types are established: trophectoderm (TE), which will form the placenta, primitive endoderm (PrEn), which will develop into the visceral yolk sac, and epiblast (Epi), which goes on to form the embryo proper (1, 2), including the germ line. The process of specifying these lineages occurs progressively over time, with the TE forming first, at the 8–16 cell stage as an outer epithelial layer that surrounds the inner cell mass (ICM) (3). The specification of Epi and PrEn then becomes apparent at peri-implantation blastocyst stages, from

which most ESC lines are derived. At this stage, the ICM consists of a salt and pepper mix of PrEn and Epi precursors (4–7). These two cell types are marked by the expression of lineage specific transcription factors, including Nanog in the Epi precursors and Gata6 in the PrEn precursors (8). These transcription factors are initially co-expressed in late morula, but become mutually exclusive at E3.5. By E4.5 the Gata6 positive cells form a monolayer of PrEn adjacent to the blastocoel cavity, and the Nanog positive cells form the Epi, which is restricted to the interior of the ICM (8–10). At E3.5, when Nanog and Gata6 become mutually exclusive, individual ICM cells appear committed as lineage tracing experiments show that individual ICM cells at this stage form either Epi or PrEn, but rarely both (9, 11). However, cells from these stages are able to generate both lineages in heterotopic grafting experiments and do not become fully restricted to either Epi or PrEn fate until they have completely segregated and are morphologically distinct at E4.5 (6, 8, 12).

Like the blastocyst, ESC culture is composed of functionally distinct, lineage-primed cell populations that represent precursors of the pluripotent Epi, marked by high Nanog expression (13–15), and the extra-embryonic PrEn, marked by low-level expression of the PrEn marker Hhex in Nanog-low cells (16–18). While the PrEn-primed ESCs don't express Gata6 protein, they do exhibit elevated levels of PrEn RNA expression and have a functional PrEn bias in both in vivo and in vitro differentiation. Thus, ESC culture conditions appear to "trap" cells in a state that is comparable to that in the early blastocyst, as cells are beginning to make their choice between Epi and PrEn. The key to identifying these states is the combination of a marker for PrEn differentiation and a second marker for undifferentiated ESCs. Using this combination of markers, it is possible to sort cells that are both efficiently self-renewing as well as primed for either Epi or PrEn differentiation. In addition to Hhex and Nanog, a number of other markers are also heterogeneously expressed in ESCs (19), including Rex1 (20), Esrrb (21), Dppa3 (Stella) (22), Klf4 and Tbx3 (23), Zscan4 (24), and a two-cell stage specific endogenous retroviral long terminal repeat (25), suggesting that ESC culture contains additional primed populations, although the extent of overlap between these different early lineage markers has yet to be explored.

In this chapter, we provide a detailed description of the techniques involved in separating heterogeneous, but self-renewing, ESC populations. We describe the purification of PrEn- and Epi-primed cells from ESC culture by employing a highly sensitive transcriptional reporter for the PrEn marker Hhex (Hhex-IRES-Venus; HV) (16) and the ESC marker SSEA1 (or PECAM1). An overview of the FACS strategy employed to achieve a clear separation of these populations is shown in Fig. 1.

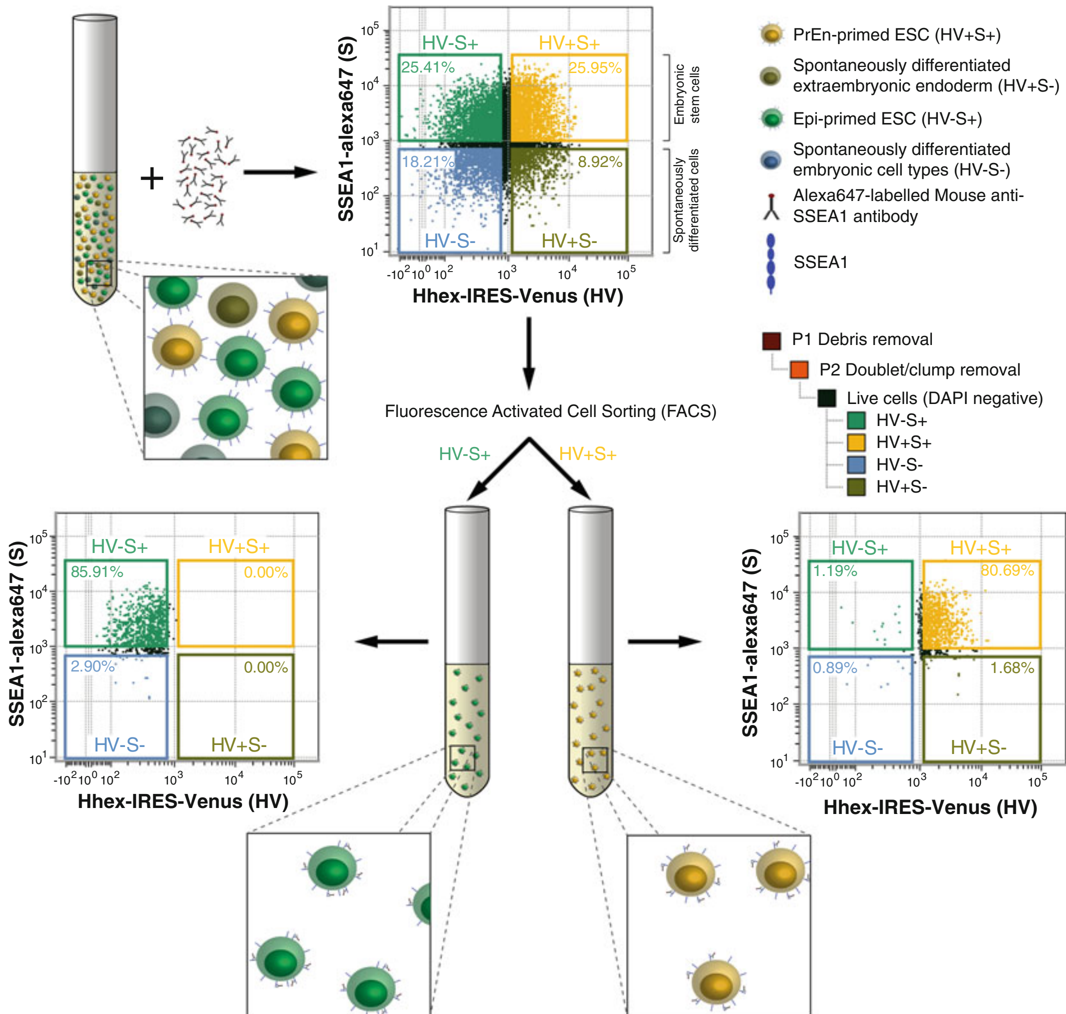

Fig. 1 Schematic overview of the FACS strategy used to isolate primed ESC populations. A single-cell suspension generated from cultured ESCs (*top left*) contains populations of SSEA1$^+$ primed Epi (*green*) and primed PrEn (*yellow*) as well as spontaneously differentiated SSEA1$^-$ embryonic cell types (*light blue*) and extraembryonic endoderm (*dark yellow*). When these cells are stained using a directly conjugated SSEA1 antibody, they can be readily separated in a FACS plot (as shown in the FACS plot at the *top*). Reanalysis of the cells after FACS shows that a large percentage (typically >80 %) of the sorted cells falls within the gate that was used for the respective sorted populations (*bottom half* of the figure). A gating hierarchy (*bottom right*) is used to ensure that the majority of events within the sort gates represent living cells (for details of these gates, *see* Fig. 2)

2 Materials

1. ESCs:
   (a) E14 Tg2a (26).
   (b) HV5.1 (16).

2. ESC medium: GMEM (Sigma G5154), 10 % Fetal Calf Serum (FCS), 1× MEM non-essential amino acids, 0.1 μM 2-Mercaptoethanol, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, and 1,000 U/ml of Lif. Store at 4 °C. Equilibrate to room temperature (RT) or 37 °C before use.
3. Dulbecco's phosphate buffered saline (PBS): 1.34 mM KCl, 0.735 mM KH_2PO_4, 68.4 mM NaCl, 4.05 mM NaH_2PO_4, pH 7.4. Store at RT.
4. Attenuated trypsin: 0.025 % trypsin (Invitrogen 15090-046), 1.27 mM EDTA, 1 % Chicken serum (Sigma C5405) in PBS (suitable for cell culture; without CaCl or MgCl). Store at 4 °C.
5. FACS buffer: 10 % Fetal Calf Serum, PBS (suitable for cell culture; without CaCl or MgCl). Keep on ice when in use and store at 4 °C.
6. 5 ml FACS tubes (Corning catalog nr. 352054).
7. 5 ml FACS tubes with cell strainer cap (Corning catalog nr. 352235).
8. Mouse anti-SSEA1 IgM, κ antibody conjugated to Alexa Fluor® 647 (BD Pharmingen catalog nr. 560120), also *see* **Note 1**.
9. Mouse IgM, κ isotype control antibody conjugated to Alexa Fluor® 647 (0.2 mg/ml; BD Biosciences catalog nr. 560806), also *see* **Note 1**.
10. 30 ml universal tubes (VWR 216-1290).

3 Methods

3.1 Cell Culture

All steps are performed in a laminar flow hood.

Prior to starting work in the laminar flow hood, decontaminate it using 20 min UV exposure (usually a built-in UV lamp) and spray the inside of the hood with 70 % ethanol so that all surfaces inside the hood are decontaminated.

Anything that is transferred into the laminar flow hood is sprayed extensively with 70 % ethanol, including (gloved) hands.

Brightfield microscopy on ES cells is done at either 40 or 100× magnification (generally, a 4× or 10× objective on top of 10× internal magnification is used to obtain this level of magnification).

3.1.1 Thawing Cells

1. Check the number of cells in the vial you intend to thaw from the liquid nitrogen and prepare an appropriately sized plate or flask. Normal plating density (5×10^4 cells/cm^2) does not apply as the level of cell death is much higher for cells frozen in liquid nitrogen.
   (a) 0.5–1.5 × 10^6 cells/vial → thaw in one well of a 6-well plate.

   (b) 1.5–4.5 × 10^6 cells/vial → thaw in a 25 cm^2 flask (T25).
   (c) >4.5 × 10^6 cells/vial → thaw in 75 cm^2 flask (T75).

   Note: if the vial to be thawed is very old or has been stored at −80 °C for a few months, increase cell number/cm^2 to compensate for increased cell death.

2. Coat the flask or plate with 0.1 % gelatin in PBS (~200 μl/cm^2) for at least 5 min at RT.
3. Put the cryovial containing, the cells in ES medium with 10 % DMSO, in a hot water bath at 37 °C until ~ 50 % of the vial has liquefied, then transfer the cryovial into a laminar flow hood.
4. Using a 1 ml pipette, transfer all liquid from the cryovial into a universal tube containing 9 ml of ES medium (at a temperature between RT and 37 °C).
5. Transfer ~500 μl warm ES medium to the cryovial to liquefy the remaining solidified DMSO and then transfer all liquid into the same universal, repeat this step until the entire content of the cryovial has been liquefied and transferred into the universal tube.
6. Pipette up and down with ~50 % volume ~15–20× using a 10 ml pipette to ensure the cells are in single-cell suspension.
7. Centrifuge at 330 × *g* for 3 min at RT.
8. Remove the supernatant (preferably using a vacuum pump to prevent cell loss as the pellet is quite delicate).
9. Resuspend in 400 μl/cm^2 by pipetting up and down with ~50 % volume ≥20× using a 10 ml pipette to ensure the cells are in single-cell suspension.
10. Transfer this single-cell suspension into the appropriate culture vessel.
11. Directionally shake the culture vessel to evenly distribute the cells across the surface:
    (a) Quickly move the culture vessel forwards and backwards five times in a row and then immediately switch to quickly moving the culture vessel from left to right and back again five times in a row.
    (b) Repeat the previous step at least three times.
    (c) Check the culture vessel under a microscope to make sure that the cells are distributed evenly across the surface and if not, repeat the directional shaking until the distribution of cells is even.
12. Transfer the culture vessel to a 37 °C incubator (5 % CO_2, 95–98 % humidity).
13. Tap the culture vessel and change the culture medium after 1 day to remove dead cells, then change the culture medium every other day.

14. Grow the cells until the culture vessel is 60–95 % confluent (this should take ~1–3 days using the densities indicated above).
15. Proceed with passaging as described in the next section.
16. Passage the cells at least three times before starting any experiments to ensure that the cells have recovered from the stress of thawing and have adjusted to the culture conditions.

3.1.2 Passaging Cells

1. Check the culture vessel under the microscope and proceed with passaging if the confluency is 60–95 %.
2. Wash the culture vessel with 400 μl/cm^2 PBS at RT.
3. Aspirate PBS and add 40 μl/cm^2 attenuated trypsin.
4. Shake the culture vessel to spread the trypsin across the entire surface area.
5. Incubate at 37 °C for 2–4 min.
6. Vigorously tap the flask 20–30× to release the cells from the plate and reduce cell clump size.
7. Inactivate the attenuated trypsin by adding 9 volumes of ES medium.
8. Transfer the cell suspension to a universal tube and pipette up and down with ~50 % volume ≥20× using a 10 ml pipette to ensure that the cells are in single-cell suspension (if in doubt, check the level of dissociation under the microscope).
9. Centrifuge at 330 × g for 3 min at RT.
10. Dilute the cells at a ratio of 1:2 up to 1:10 into the final plating volume (400 μl/cm^2 (=10 ml/T25)) depending on the size of the pellet.

    OR

    Count the cells using your favorite cell counting method (e.g., using a hæmocytometer) and resuspend the cells at a concentration of 1.25×10^6 cells/ml, then dilute the resuspended cells 1:10 into the appropriate volume for plating (400 μl/cm^2; final cell concentration should be 1.25×10^5 cells/ml (=5×10^4 cells/cm^2))
11. Transfer the appropriate plating volume into the prepared culture vessel(s) (Table 1).
12. Directionally shake the culture vessel to evenly distribute the cells across the surface:
    (a) Quickly move the culture vessel forwards and backwards five times in a row and then immediately switch to quickly moving the culture vessel from left to right and back again five times in a row.
    (b) Repeat the previous step at least three times.

Table 1
Area, volume, and cell amounts for seeding and harvesting mouse ESCs on various types of cell culture plastics

Flasks	*Growth area* (cm^2)	*Flask volume* (ml)	*Cell amount for plating*	*Low confluence* (~60 %)	*High confluence* (~95 %)
25 cm^2	25	10	1.25E + 06	2.50E + 06	7.50E + 06
75 cm^2	75	30	3.75E + 06	7.50E + 06	2.25E + 07
150 cm^2	150	60	7.50E + 06	1.50E + 07	4.50E + 07
Dishes	*Growth area* (cm^2)	*Dish volume* (ml)	*Cell amount for plating*	*Low confluence*	*High confluence*
35 mm	9	3.6	4.50E + 05	9.00E + 05	2.70E + 06
60 mm	21	8.4	1.05E + 06	2.10E + 06	6.30E + 06
100 mm	55	22	2.75E + 06	5.50E + 06	1.65E + 07
150 mm	152	60.8	7.60E + 06	1.52E + 07	4.56E + 07
	For each individual well			*Approximate cell yield*	
Multiwell plates	*Growth area* (cm^2)	*Well volume* (μl)	*Cell amount for plating*	*Low confluence*	*High confluence*
96 well (V bottom)	0.38	152	1.90E + 04	3.80E + 04	1.14E + 05
96 well (flat bottom)	0.32	128	1.60E + 04	3.20E + 04	9.60E + 04
48 well	0.95	380	4.75E + 04	9.50E + 04	2.85E + 05
24 well	1.9	760	9.50E + 04	1.90E + 05	5.70E + 05
12 well	3.8	1,520	1.90E + 05	3.80E + 05	1.14E + 06
6 well	9.5	3,800	4.75E + 05	9.50E + 05	2.85E + 06

(c) Check the culture vessel under a microscope to make sure that the cells are distributed evenly across the surface and if not, repeat the directional shaking until the distribution of cells is even.

13. Transfer the culture vessel to a 37 °C incubator (5 % CO2, 95–98 % humidity).

    At a plating cell density of 5 × 10^4 cells/cm^2, ES cells will need to be passaged approximately every other day.

3.2 Preparing Cells for FACS

FACS buffer is kept on ice as much as possible to try and keep the cells at around 4 °C during the entire procedure.

Every experiment should include a sample of unstained cells that resemble the stained (reporter) cells, such as E14Tg2a ESCs (for reporter lines the parental is usually the best choice).

Before starting, check the minimum volume required to analyze a sample on the flow cytometer used for sorting and adjust the volume of the controls accordingly.

1. Wash the culture vessel with 400 μl/cm^2 PBS at RT.
2. Aspirate PBS and add 40 μl/cm^2 attenuated trypsin.
3. Shake the culture vessel to spread the trypsin across the entire surface area.
4. Incubate at 37 °C for 2–4 min.
5. Vigorously tap the flask 20–30× to release the cells from the plate and reduce cell clump size.
6. Inactivate the attenuated trypsin by adding 9 volumes of ice-cold FACS buffer and transfer the cell suspension to a universal tube.
7. Pipette up and down with ~50 % volume ≥20× using a 10 ml pipette to ensure the cells are in single-cell suspension (if in doubt, check the level of dissociation under the microscope).
8. Count the cells using an aliquot of this cell suspension and leave the rest of the cells on ice.
9. Preparation of antibody solutions used for staining: For every one million cells that are to be stained, prepare 100 μl of 125 ng/ml Alexa Fluor® 647-conjugated Mouse IgM, κ anti-SSEA1 antibody (concentration varies per lot, so check the concentration and calculate the dilution before starting the protocol; the concentration given here is based on the stock concentration of lot # 2237758, which was 50 μg/ml and was diluted at 1:400) and for control cells, prepare 100 μl per million cells of 125 ng/ml Alexa Fluor® 647-conjugated Mouse IgM, κ isotype control antibody (stock = 0.2 mg/ml → 1:1,600 dilution); centrifuge the prepared antibody solutions at max speed (>10,000 × *g*) for 15 min at RT to remove antibody aggregates, then store the solutions on ice in the dark until use (*see* **Note 1**).
10. Centrifuge the cells at 330 × *g* for 3 min.
11. Remove the supernatant and wash the cells by resuspending in ice-cold FACS buffer (~1 ml per million cells), then split the cells into control and stained cell fractions (*see* **Note 2**).
12. Centrifuge the cells at 330 × *g* for 3 min.
13. Remove the supernatant and resuspend in 100 μl per million cells of isotype control or antibody solution (as prepared in **step 9**).
14. Incubate for 15 min on ice.
15. Centrifuge the cells at 330 × *g* for 3 min.

16. Remove the supernatant and wash the cells by resuspending in ice-cold FACS buffer (~1 ml per million cells).
17. Repeat steps 15 and 16 once.
18. Centrifuge the cells at 330 × *g* for 3 min.
19. Resuspend the cells that are to be sorted by repeated gentle pipetting using a 1 ml pipette at 1–1.5 × 10^7 cells/ml in FACS buffer containing 1 μg/ml DAPI and run them through a cell strainer. Resuspend the control cells in FACS buffer containing 1 μg/ml DAPI at 1 × 10^6 cells/ml in 5 ml FACS tubes (to ensure sufficient volume is available for analysis).

3.3 Fluorescence-Activated Cell Sorting Parameters

The parameters given here are based on sorting with a BD FACS Aria III (for laser/filter combinations, *see* Table 2). Ensure that the sorter is set up so that it collects the following parameters: Linear scale—FSC-A, FSC-W, FSC-H, SSC-A, SSC-W, SSC-H; Biexponential scale—DAPI, Venus (or other reporter fluorescent protein, depending on the cell line used and the populations to be collected), APC/Alexa-647 (or other fluorophore conjugated antibody, depending on the (reporter) cell line used and the populations to be collected).

Use a nozzle of at least 100 μm diameter (*see* **Note 3**).

For further details on cell sorting by flow cytometry, *see* ref. (27).

Table 2
Laser/filter/mirror combinations used to detect the indicated dyes

UV dyes	Application	Laser	Filters
DAPI	Identify dead cells	375 nm	450/40
Green/Yellow Dyes			
GFP	Reporter gene expression	488 nm	530/30
FITC	Antibody staining	488 nm	530/30
Venus	Reporter gene expression	488 nm	530/30
Orange/Red Dyes			
RFP	Reporter gene expression	561 nm	582/15
PE	Reporter gene expression	561 nm	582/15
Alexa-568	Antibody staining	561 nm	582/15
mCherry	Antibody staining	561 nm	610/20
Far-Red Dyes			
APC	Antibody staining	633 nm	660/20
Alexa-647	Antibody staining	633 nm	660/20

Gate name	% of Parent	% of Total
P1	70.02	70.02
P2	95.50	66.88
Live Cells	96.39	64.46
HV-S+	0.01	0.01
HV+S+	0.00	0.00

Gate name	% of Parent	% of Total
P1	64.98	64.98
P2	94.84	61.63
Live Cells	96.25	59.32
HV-S+	65.47	38.84
HV+S+	0.00	0.00

Gate name	% of Parent	% of Total
P1	70.85	70.85
P2	94.32	66.83
Live Cells	96.54	64.51
HV-S+	0.00	0.00
HV+S+	0.03	0.02

Gate name	% of Parent	% of Total
P1	66.91	66.91
P2	94.54	63.26
Live Cells	97.88	61.91
HV-S+	21.81	13.50
HV+S+	22.93	14.20

Gating Hierarchy

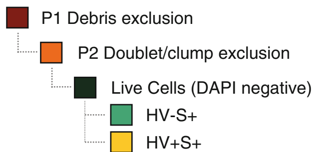

Fig. 2 Gating strategy and requisite control samples. Samples for FACS should always include a negative control (E14 Tg2a negative control) and single positive controls (E14 Tg2a 1:400 SSEA1-alexa647 and HV5.1 negative control) to ensure the gates for the sorted sample (HV5.1 1:400 SSEA1-alexa647) are positioned correctly. Gating forward scatter area (FSC-A) against side scatter area (SSC-A) allows exclusion of cell debris and particulates (the *black dots* to the *left* of P1 in the *dot plots* at the *top*) in the sort tube. Cell clumps can then be removed by gating cells from P1 on forward scatter width (FSC-W) against forward scatter height

3.3.1 Plots

Create the following plots (*see* Fig. 2):

1. Dot plot of SSC-A vs FSC-A.
2. Contour plot with outliers of FSC-H vs FSC-W.
3. Contour plot with outliers of DAPI vs FSC-A.
4. Contour plot with outliers of reporter (Venus) vs. cell surface marker staining (SSEA1-Alexa 647).

3.3.2 Gating

The positioning of gates is determined by including negative control cells (In this case, E14Tg2a stained with Alexa 647-conjugated isotype control IgM at the same concentration as Alexa 647-conjugated SSEA1 (Fig. 2, left column)) and single positive cells (In this case, E14Tg2a stained with Alexa 647-conjugated SSEA1 (Fig. 2, middle left column) and HV5.1 stained with Alexa 647-conjugated isotype control IgM at the same concentration as Alexa 647-conjugated SSEA1 (Fig. 2, middle right column).) to distinguish aspecific staining or autofluorescence from weakly stained cells.

Create the following gates on the plots (numbered as in the Subheading 3.3.1, *see* Fig. 2):

1. Gate on SSC-A vs FSC-A to exclude cell debris and the majority of dead cells (P1 in Fig. 2); select the main population with medium to high FSC-A levels.

   FSC-A measures diffracted light and gives an approximation of cell size while SSC-A measures refracted and reflected light and is indicative of granularity or internal complexity of the detected particle. Cell debris is usually low in FSC-A and dead cells tend to have lower FSC-A and higher SSC-A values than live cells. Applying an initial crude selection based on these parameters simplifies the next gating steps considerably as the position of the gates outlined below is much easier to determine when the majority of unwanted events have been excluded already.
2. In a plot showing only events included in P1, gate on FSC-H vs FSC-W to exclude cell clumps (P2 in Fig. 2); select the main population as identified in a contour plot with outliers.

Fig. 2 (continued) (FSC-H) and excluding events that are larger (i.e., have a bigger FSC-W value) than the main population as defined by the contours in the contour plot with outliers in the *second row* of plots. Finally, dead or dying cells are excluded by including only those events in P2 that are DAPI negative (Live cells gate in the contour plots with outliers shown in the *third row from the top*). This sequential gating strategy ensures that only living single cells are included in the dot plots used to set the sorting gates (*bottom row* of plots). The percentages relative to the parent gate and to the total number of events for each of the gates are shown in the tables at the *bottom*. A schematic overview of the gating hierarchy is shown below the tables

In particular the pulse width (FSC-W) is useful to remove cell clumps as it relates to particle size (28).

3. In a plot showing only events included in P1, gate on DAPI vs FSC-A to exclude dead/dying cells (Live cells in Fig. 2); select DAPI negative cells only.

   Cell impermeant DNA dyes, including DAPI, can enter the cell when membrane integrity is compromised upon necrosis, thus labeling the DNA of dead cells. Note that cells in early phases of apoptosis are not excluded from flow cytometry analysis using this method (29–32).
4. In a plot showing only events included in the Live cell gate, gate on reporter vs. cell surface marker to select the populations of interest (in this case HV^{-} S^{+} and HV^{+} S^{+}, gated to include 20–25 % of the total number of events in the plot).

   See the gating hierarchy given in Figs. 1 and 2 for an overview of all the gates and how they are related to eachother.

3.4 Collecting Cells After Sorting

1. Collect cells in 5 ml FACS tubes containing 1 ml FACS buffer: 10 % FCS in ice-cold PBS (*see* **Note 4**).
2. Sort up to 750 k cells per tube (when using a 100 μm nozzle; *see* **Note 3**).
3. Keep on ice after sort.
4. Spin FACS tubes at 500 × *g* for 3 min at 4 °C.
5. Coat 1.5 ml Eppendorf tubes with BSA by pipetting 1 ml of 7.5 % BSA (sterile) into the tube, let it sit for 10 s and then wash the tube with 1 ml PBS (the same 1 ml of BSA can be used to coat all tubes needed).
6. Check the tube to see if the cells have pelleted.
7. Partially remove supernatant until ~200 μl is left.
8. Resuspend the cells in the remaining 200 μl and transfer to 1.5 ml BSA-coated Eppendorf tube.
9. Spin at 500 × *g* for 3 min at 4 °C.
10. Check the tube to see if the cells have pelleted.
11. Carefully remove sup (keep an eye on the pellet!) using a P1000 until ~10–20 μl is left, add 1 ml of ice-cold PBS and resuspend pellet by repeated pipetting.
12. Spin at 500 × *g* for 3 min at 4 °C.
13. Check the tube to see if the cells have pelleted.
14. Carefully remove sup (keep an eye on the pellet!) using a P1000 until ~10–20 μl is left, then remove the rest of the PBS using a P200.
15. Resuspend pellet by flicking the tube.

3.4.1 RNA Isolation

1. For RNA add the appropriate lysis and RNA extraction reagent (such as 350 μl buffer RLT (without β-mercaptoethanol) when using Qiagen Rneasy columns or 400 μl TRIzol (Ambion) or TRI reagent (MRC) when using Guanidinium thiocyanate-phenol-chloroform extraction) and ensure cell lysis by repeated pipetting. Leave at room temperature for at least 2 mins and then store at −20 °C or −80 °C.
2. Proceed with RNA isolation according to the manufacturer's protocol.

3.4.2 Protein Isolation

1. For protein add 100 μl/million cells of Laemmli lysis buffer (2 % w/v SDS, 10 % v/v glycerol, 120 mM Tris–HCl pH 6.8).
2. Sonicate on ice (probe):
   (a) 100–250 μl: 10 s at 20 % power.
   (b) 250–500 μl: 15 s at 30 % power.
3. Boil lysates at 95 °C for 5 min.
4. Incubate on ice for 10 min.
5. Spec on Nanodrop using protein 280 setting to get the approximate protein concentration.
6. Store at −20 °C.
7. Just before loading the samples onto a gel, add 10 % v/v 1 M DTT containing bromophenol blue; load 20–60 μg of protein per lane in equal volumes (i.e., all samples in the same volume) of Laemmli (maximum of ~25 μl for standard 10 lane gels).

3.5 Data Presentation

The flow cytometry plots presented in this chapter were generated using FCS Express 4 Flow Research (v. 4.07.0011) and figures were then assembled in Adobe Illustrator CS6 (v. 16.0.3). Plots were copied from FCS express 4 directly into Adobe Illustrator to generate high resolution vector based images. Small size pdfs of the figures were obtained by rasterizing (using "Object > Flatten Transparency. . .") areas within the figure that contained high vector densities.

4 Notes

1. Alternatively, use 500 ng/ml APC-conjugated PECAM1 Rat IgG2a, κ antibody (1:400 from 0.2 mg/ml; BD Pharmingen catalog nr. 551262) and 500 ng/ml APC-conjugated Rat IgG2a, κ isotype control (1:400 from 0.2 mg/ml; BD Pharmingen catalog nr. 554690). These antibodies are also available with alternative conjugates and their equivalent directly conjugated isotype controls (*see* Table 3). For other conjugates and cell lines with different genetic backgrounds (the lines used here were derived from E14Tg2a 129P2/OlaHsd background

Table 3
Antibodies and isotype controls used to detect self-renewing embryonic stem cells (all from BD Pharmingen)

SSEA1 (CD15)	*Clone*	*Host species*	*Ig subtype*	*Conjugate*	*Catalog nr.*
SSEA1-Alexa-647	MC480	Mouse	IgM, κ	Alexa-647	560120
SSEA1-PE	MC480	Mouse	IgM, κ	PE	560142
SSEA1-FITC	MC480	Mouse	IgM, κ	FITC	560127
IgM isotype control-Alexa-647	G155-228	Mouse	IgM, κ	Alexa-647	560806
IgM isotype control-PE	G155-228	Mouse	IgM, κ	PE	555584
IgM isotype control-FITC	G155-228	Mouse	IgM, κ	FITC	553474
PECAM1 (CD31)	***Clone***	***Host species***	***Ig subtype***	***Conjugate***	***Catalog nr.***
PECAM1-APC	MEC13.3	Rat	IgG2a, κ	APC	551262
PECAM1-PE	MEC13.3	Rat	IgG2a, κ	PE	553373
PECAM1-FITC	MEC13.3	Rat	IgG2a, κ	FITC	553372
IgG2a isotype control-APC	R35-95	Rat	IgG2a, κ	APC	554690
IgG2a isotype control-PE	R35-95	Rat	IgG2a, κ	PE	553930
IgG2a isotype control-FITC	R35-95	Rat	IgG2a, κ	FITC	553929

ESCs (26); see http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml) than the ones described here, the optimal concentration should be determined empirically.

2. Usually, the number of cells needed for a quick analysis of the negative control is ~2×10^5, but if you're working with precious cells, this can be reduced to ~5×10^4. Due to the small volume used for staining cell numbers below the standard 2×10^5 (=20 μl of diluted antibody), staining should be done in a 0.5 ml eppendorf tube to ensure proper mixing of the cells with the antibody.
3. When using a nozzle size bigger than 100 μm, less than 750 k cells will fit into a FACS tube containing 1 ml of buffer. The amount of cells that can be collected in one tube will depend on the size of the nozzle.
4. Survival of cells after the sorting procedure is strongly enhanced by the presence of at least 2 % serum. By adding 1 ml of 10 % FCS in PBS to the 5 ml FACS tube prior to sorting the FCS percentage is always above 2 %. Vortexing the sort tube containing 1 ml of 10 % FCS in PBS prior to starting

the sort also ensures that the sides of the tube are coated with FCS to prevent cells sticking to the side of the tube.

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