Chapter 2

Investigating the Interaction Between Hematopoietic Stem Cells and Their Niche During Embryonic Development: Optimizing the Isolation of Fetal and Newborn Stem Cells From Liver, Spleen, and Bone Marrow

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Abstract

Hematopoietic stem cells (HSCs) are maintained in a particular microenvironment termed a "niche." Within the niche, a number of critical molecules and supportive cell types have been identified to play key roles in modulating adult HSC quiescence, proliferation, differentiation, and reconstitution. However, unlike in the adult bone marrow (BM), the components of stem cell niches, as well as their interactions with fetal HSC during different stages of embryonic development, are poorly understood. During embryogenesis, hematopoietic development migrates through multiple organs, each with different cellular and molecular components and hence each with a potentially unique HSC niche. As a consequence, isolating fetal HSC from each organ at the time of hematopoietic colonization is fundamental for assessing and understanding both HSC function and their interactions with specific microenvironments. Herein, we describe methodologies for harvesting cells as well as the purification of stem and progenitors from fetal and newborn liver, spleen, and BM at various developmental stages following the expansion of hematopoiesis in the fetal liver at E14.5.

Key words Hematopoietic stem cells, Hematopoietic development, Liver, Spleen, Bone marrow

1 Introduction

In the past 30 years since the term "niche" was coined by Schofield [1] for the adult BM microenvironment in which hematopoietic stem cells (HSCs) reside, adult HSCs and their niche have been extensively investigated. To date, this has resulted in a huge body of literature identifying a number of cell types and extracellular matrix molecules as components of the niche that play a role in HSC regulation.

Hematopoiesis develops embryonically in an age-dependent and microenvironment-controlled process, with fetal HSC

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migrating through multiple sites. Initially, in the mouse, primitive hematopoiesis starts in the yolk sac at E7.5 [2] and gives rise to erythrocytes. Hematopoiesis then becomes definitive, demonstrating multi-lineage reconstitution capacity, with evidence suggesting a contribution from the aorta-gonad-mesonephros (AGM) region, yolk sac, and placenta [3–5]. HSCs are first detected in the fetal liver at E11.5 [6], where they expand at E14.5 [7] prior to migrating to the BM at E16.5, the permanent location for hematopoiesis throughout adulthood [6]. In addition, the spleen is a temporary fetal hematopoietic organ from E12 shortly after birth [8]. Besides inherent intrinsic properties, fetal hematopoiesis receives extrinsic cues whilst residing within specific microenvironments [9]. However, unlike adult BM, the interaction between fetal HSCs and their niche during hematopoietic development has not been thoroughly investigated and hence remains poorly understood.

Herein, we describe methodologies for harvesting cells as well as the purification of stem and progenitors from fetal and newborn liver, spleen, and BM at various developmental stages following the expansion of hematopoiesis in the fetal liver at E14.5. These methodologies have been utilized to identify key interactions between HSC and specific developmental niches and assess their roles in HSC regulation.

2 Materials

2.1 Isolation of Liver, Spleen, and BM

1. Timed mated embryos or newborn pups.
2. Sterile #11 surgical blade and #3 handle.
3. Sterile straight surgical scissors (16 cm Kelly).
4. 30½G needle attached to a 1 ml syringe.
5. Sterile micro-dissecting knife (12 cm knife).
6. Sterile micro-dissecting scissors (100 mm).
7. Sterile fine tweezers.
8. Sterile beveled forceps.
9. Phosphate-buffered saline (PBS): pH 7.2, 310 mOsm (*see* **Note 1**) supplemented with 2 % serum.
10. Sterile 100 mm tissue culture petri dish.
11. Sterile 35 mm tissue culture petri dish.
12. Dissecting microscope.

2.2 Disaggregation of Liver, Spleen, and BM

1. PBS (pH 7.2), 310 mOsm.
2. PBS (pH 7.2), 310 mOsm supplemented with 2 % serum.
3. 50 ml Conical polypropylene centrifuge tube.
4. 3 ml Syringe plunger.

5. 40 μm Nylon cell strainer.
6. Sterile straight surgical scissors.
7. 3 mg/ml Collagenase I (we use *Clostridium histolyticum*) in PBS made fresh on the day of use.
8. 3 mg/ml Collagenase I/4 mg/ml Dispase II (*Bacillus polymyxa*, neutral protease) in PBS made fresh on the day of use.
9. 37 °C Orbital shaker (Eppendorf Thermomixer comfort model #5355 000.011).
10. 18- and 21-gauge needles attached to 1 ml syringes.
11. Sterile mortar and pestle.
12. Hemocytometer and microscope equipped with phase-contrast or an automated cell counter (Sysmex model KX-21N).

2.3 Density Gradient Separation

1. 300 ml Density media: Nycoprep Universal: 60 % (w/v) in water, 1.310 g/cm^3, 580 mOsm mixed with 300 ml 20 mM tricine-NaOH (pH 7.2) and 676.6 ml of 0.65 % NaCl. Confirm that osmolarity is 265 mOsm and density is 1.077 g/ml at room temperature. 20 mM Tricine-NaOH is made by diluting 3.584 g of tricine in 900 ml milliQ water and adjusting the volume to 1 l and pH 7.2. 0.65 % of NaCl is made by adding 6.5 g of NaCl to 900 ml milliQ water and adjusting the volume to 1 l. Both the 20 mM tricine-NaOH and the 0.65 % NaCl should be sterile filtered before use.
2. Cannulas attached to 10 or 20 ml syringes.

2.4 Lineage Depletion

1. Lineage depletion antibody cocktail: A mixture of optimally titrated purified rat anti-mouse antibodies recognizing the cell surface antigens: B220, Gr-1, and Ter119 (*see* **Note 2**) in PBS (pH 7.2), 310 mOsm supplemented with 2 % serum (antibody concentrations are all ≤1 μg/ml) (*see* **Note 3**).
2. Polypropylene 5 ml round-bottom tubes.
3. Magnetic Dynal beads working buffer: PBS, 310 mOsm supplemented with 2 mM EDTA, and 0.1 % (w/v) fraction V bovine serum albumen (BSA; pH 7.4).
4. 4.5 μm Diameter sheep anti-rat IgG Dynabeads (Dynal Biotech ASA, Oslo, Norway).
5. Microtubes.
6. Dynal MPC-S magnet for 2–20 ml samples or MPC-L for 1–8 ml samples.
7. Tube rotator or similar suspension mixer, allowing both tilting and rotation at 4 °C (we use a MACSmix Tube Rotator placed in a fridge).

2.5 Hematopoietic Stem Cell Fluorescence-Activated Cell Sorting

1. HSC antibody cocktail: A mixture of optimally titrated allophycocyanin-cyanine 7 (APCCy7)-conjugated rat anti-mouse B220, Gr-1, CD3, and Ter119 (*see* **Note 4**); Pacific Blue™ (PB)-conjugated rat anti-mouse stem cell antigen 1 (Sca-1); AlexaFluor®647 (AF647)-conjugated rat anti-mouse c-Kit; fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD48; and phycoerythrin (PE)-conjugated rat anti-mouse CD150 in PBS (pH 7.2) and 310 mOsm supplemented with 2 % serum (antibody concentrations are all ≤1 μg/ml).
2. Sterile polypropylene 5 ml round-bottom tubes.
3. Polystyrene, round-bottom tubes: 5 ml with 40 μm cell strainer cap. The cap is swapped to a 5 ml polypropylene tube for filtering cells.
4. Flow cytometer with sorting capability equipped with five solid-state lasers (355, 405, 488, 561, and 635 nm). Band-pass filter settings for the detection of fluorescence for FITC, PE, AF647, PB, and APCCy7 are 528 ± 19, 605 ± 20, 660 ± 10, 460 ± 25, and 780 ± 30, respectively. We use a 70 mm nozzle, sort at 30 psi, and drop delay frequency of 61 kHz for HSC sorting.

3 Methods

3.1 Timed Mating

1. House five sexually mature female mice in one cage (*see* **Note 5**) with mouse chow and acidified water ad libitum.
2. Tease females with bedding from the male for 3 days prior to mating (*see* **Note 6**).
3. Time mate by placing the females into the male's cage late in the afternoon for 12 h (*see* **Note 7**).
4. Separate the mice, and check each female for the presence of a vaginal plug. This is designated as 0.5 days (E0.5).
5. Confirm the pregnancy between E12.5 and E14.5.
6. Check pups' birth twice a day from E18.5. The day when pups are born is designated as day 0 (D0) and then sequentially as D1, D2, D3, etc.

3.2 Mice Harvesting

3.2.1 Embryos

1. Euthanize pregnant mouse by cervical dislocation. Remove uterus by opening the abdominal cavity and cutting away the connective tissue.
2. Isolate single embryos from the uterus by gently cutting and dissociating the outer muscular uterine layer and yolk sac. Remove the placenta, and euthanize each pup by decapitation (individual institutional animal ethics requirements may vary).

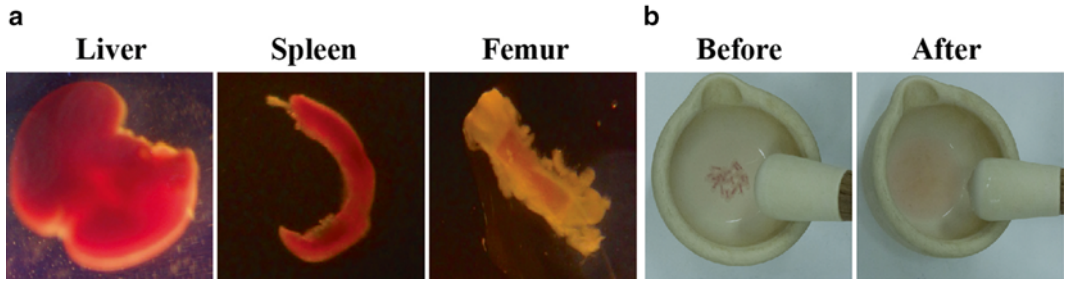

Fig. 1 Fetal organs and bone harvest. (**a**) Images show fetal liver, spleen, and femur. (**b**) Comparison of bones before and after grinding with a mortar and pestle

3.2.2 Newborn Pups

1. Euthanize pups by decapitation with a sharp pair of scissors.

3.3 Isolation of Liver, Spleen, and Long bones

3.3.1 Liver and Spleen

1. In a 100 mm petri dish separate the abdominal and hind portions using a surgical blade.
2. Using 30½G needles attached to 1 ml syringes (for embryos) or fine tweezers (for newborn pups) remove other organs from the posterior end of abdominal portion to expose the liver and spleen.
3. Under a dissecting microscope, use a pair of 30½G needles to remove any extra tissue attached to the liver and spleen (*see* **Note 8**) and place in a 35 mm petri dish containing PBS–2 % serum (Fig. 1a).

3.3.2 Long Bones

1. Lay the hind portion belly down in a 100 mm petri dish, and remove the skin with scissors to expose the transparently red long bones (femur and tibia).
2. Under a dissecting microscope, carefully remove muscles from the bones using a micro knife and/or a pair of micro scissors.
3. Dislocate the femur from the hip and knee, and cut the foot off the tibia at the ankle. Keep bones in individual groups (*see* **Note 8**) in separate 35 mm petri dishes containing PBS–2 % serum (*see* **Notes 9** and **10**) (Fig. 1a).

3.4 Single-Cell Suspensions of Liver, Spleen, and BM

3.4.1 Embryonic Livers

1. Gently push embryonic livers through a 40 μm strainer on top of a 50 ml centrifuge tube with the plunger of a 3 ml syringe.
2. Wash cells in PBS–2 % serum by centrifuging at 400 × *g* for 5 min at 4 °C.
3. Decant supernatant, and resuspend the cell pellet in 10 ml PBS–2 % serum.
4. Refilter the cell suspension through a 40 μm strainer into a new 50 ml conical tube.
5. Perform a cell count.

3.4.2 Newborn Livers

1. Transfer newborn livers into a 50 ml conical tube and chop finely with a pair of long surgical scissors.
2. Add 1 ml Collagenase I for each minced liver, and agitate for 5 min at 37 °C in an orbital shaker, 750 rpm.
3. Repeatedly flush livers through an 18-gauge needle and then a 21-gauge needle until a single-cell suspension.
4. Add 40 ml PBS–2 % serum, and filter through a 40 μm strainer into a new 50 ml conical tube.
5. Wash cells twice in PBS–2 % serum by centrifuging cells at 400 × *g* for 5 min at 4 °C.
6. Decant supernatant, and resuspend the cell pellet in 10 ml PBS–2 % serum.
7. Perform a cell count.

3.4.3 Spleens

Process embryonic and newborn spleens as for embryonic livers (*see* Subheading 3.4.1, **step 1**), except resuspend the cells in 5 ml PBS–2 % serum.

3.4.4 BM

1. Transfer bones into a sterile mortar.
2. Grind bones with a pestle (*see* **Note 11**).
3. Remove the supernatant and filter through a 40 μm strainer into a 50 ml conical tube.
4. Further grind bones if not dissociated into small fragments (Fig. 1b). Rinse the crushed bone fragments with PBS–2 % serum, and filter the supernatant as in **step 3**, until all bone fragments become white. Top up the 50 ml tube to 50 ml with PBS–2 % serum and set aside on ice until **step 9**.
5. Transfer the crushed bone fragments into a new 50 ml conical tube containing Collagenase I and Dispase II (1 ml per 12–18 bones) and place at 37 °C in an orbital shaker, 750 rpm, for 5 min (*see* **Note 12**).
6. Add 20 ml straight PBS to the digested bone fragments, and shake vigorously for 20 s.
7. Filter the cell suspension through a 40 μm strainer into a new 50 ml conical tube.
8. Repeat **steps 6** and **7**, and filter cells into the same 50 ml conical tube. Top the tube with 10 ml PBS–2 % serum.
9. Centrifuge the cell suspension tubes (from **steps 4** and **8**) at 400 × *g* for 5 min at 4 °C.
10. Decant supernatant, resuspend pellets, pool cells in 10 ml PBS–2 % serum, and perform a cell count.

3.5 Density Separation for BM, Liver, and Spleen

1. Top up each cell suspension to 20 ml with PBS–2 % serum (*see* **Note 13**).
2. Underlay 10 ml Nycoprep using a cannula attached to a 10 or a 20 ml syringe.
3. Centrifuge the gradients at 600 × *g* for 20 min at room temperature with no deceleration.
4. Using a 10 ml pipette, collect mononuclear cells from the interface between the PBS–2 % serum and the Nycoprep solution and place in a new 50 ml conical tube.
5. Fill the tube with PBS–2 % serum and centrifuge at 400 × *g* for 5 min at 4 °C.
6. Decant supernatant, resuspend cells in 10 ml PBS–2 % serum, and perform a cell count.

3.6 Lineage Depletion

1. Pellet cells by centrifuging at 400 × *g* for 5 min at 4 °C.
2. Stain cells at 1×10^7 cells/ml in the cocktail of lineage markers on ice for 20 min.
3. Wash cells with PBS–2 % serum by centrifuging at 400 × *g* for 5 min at 4 °C to remove unbound antibodies.
4. Resuspend cells in 2 ml PBS supplemented with 2 mM EDTA and 0.1 % BSA and transfer into 5 ml sterile polypropylene tube. Perform a cell count (*see* **Note 14**) and set aside on ice until **step 10**.
5. Resuspend Dynabeads.
6. Place the required volume of Dynabead suspension, to give half a bead per cell, into two individual 1.7 ml microtubes. The optimal number of Dynabeads per cell has previously been established as half a bead per cell with the depletion repeated with a second half a bead per cell [10].
7. Remove the azide from the Dynabeads by adding 1 ml of 2 mM EDTA with 0.1 % BSA into each aliquot and mixing well. Place the tubes in the magnet for 1 min prior to decanting the supernatant and move from the magnet.
8. Repeat **step 7**.
9. Resuspend the Dynabeads in 500 μl 2 mM EDTA with 0.1 % BSA.
10. Add the first aliquot of washed Dynabeads to the cells, and mix well.
11. Incubate the mixture with gentle tilting and rotation for 5 min at 4 °C.
12. Place the cells in the magnet for 2 min.
13. Transfer the supernatant containing the unbound cells to a new 5 ml sterile polypropylene tube.

14. Rinse the rosetted bead–cell complexes with 1 ml 2 mM EDTA with 0.1 % BSA and place in the magnet for 1 min prior to collecting any residual unbound cells to the collection tube used in **step 13**.
15. Add the second aliquot of washed Dynabeads into the unbounded cell suspension.
16. Incubate the mixture with gentle tilting and rotation for 10 min at 4 °C.
17. Place the cells in the magnet for 2 min.
18. Transfer the supernatant containing the unbound cells to a new 5 ml sterile polypropylene tube.
19. Rinse the rosetted bead–cell complexes with 1 ml 2 mM EDTA with 0.1 % BSA and place in the magnet for 1 min prior to collecting any residual unbound cells to the collection tube used in **step 18**.
20. Measure the total volume of the unbound lineage-negative cell suspension, and perform a cell count.

3.7 HSC FACS

1. Pellet cells by centrifuging at $400 \times g$ for 5 min at 4 °C.
2. Stain cells at 1×10^7 cells/ml in the HSC antibody cocktail on ice for 20 min.
3. Add 3 ml PBS 2 % serum, and filter the cell suspension through a cell strainer into a new 5 ml sterile polypropylene tube (*see* **Note 15**). Centrifuge cells at $400 \times g$ for 5 min at 4 °C to remove unbound antibodies.
4. Resuspend cells at $25–30 \times 10^6$ cells/ml in PBS–2 % serum (*see* **Note 16**) and place on ice until sorted.
5. To set up HSC sorting by flow cytometry, the following samples are required for each tissue being sorted (*see* **Note 17**).
   (a) $0.5–1 \times 10^6$ Unstained cells to set voltage for forward scatter, side scatter, APCCy7, PB, AF647, PE, and FITC.
   (b) Individual tubes containing $0.5–1 \times 10^6$ cells stained with APCCy7, PB AF647, PE, and FITC for compensation controls.
   (c) $0.5–1 \times 10^6$ Adult BM cells stained with HSC antibody cocktail as a positive control.
6. Run the cell samples stained with HSC antibody cocktail and sequentially gate through FSC-H versus FSC-A, SSC-A versus FSC-A, SSC-A versus APCCy7, AF647 versus PB, and FITC versus PE (Fig. 2). Fetal HSCs phenotypically are defined as lineage$^+$Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^-$.
7. Sort cells at predetermined optimal input speed and collect into culture medium or PBS–2 % serum depending on the functional assay requirement.

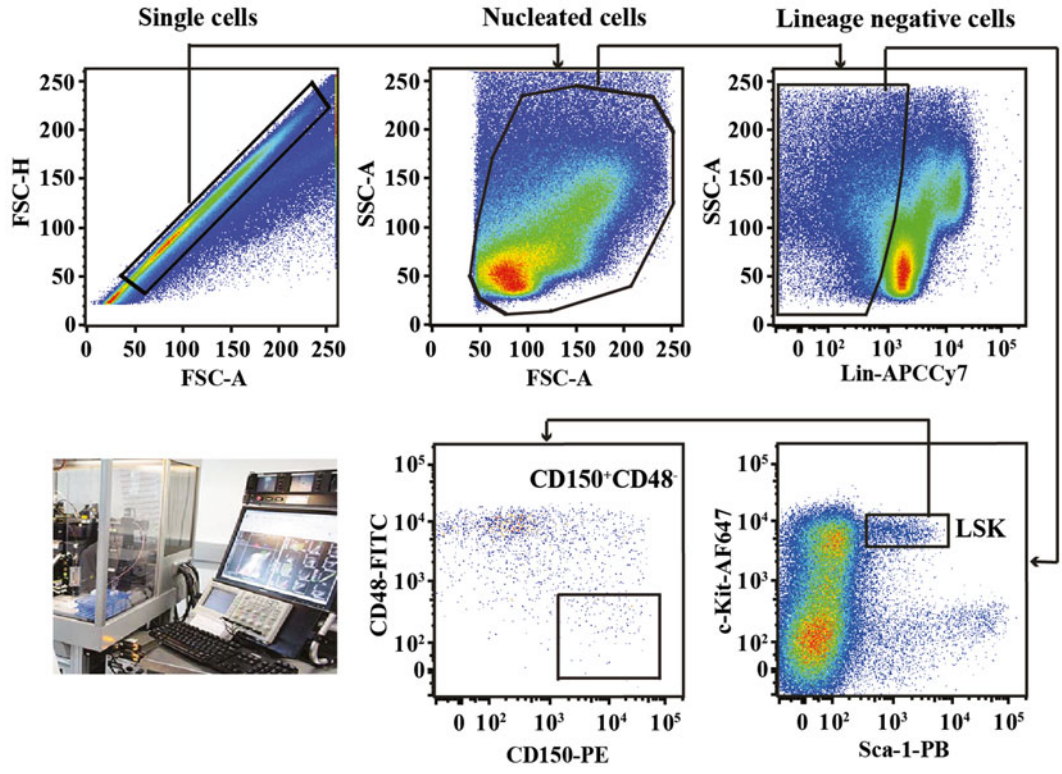

Fig. 2 HSC gating strategies for flow cytometry. HSCs are sequentially gated through single-cell gate, nucleated cell gate, and lineage-negative cell gate and then selected as Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^-$

3.8 Summarized Optimizing Isolation of Fetal and Newborn Liver, Spleen, and BM at a Variety of Developmental Ages

Since fetal hematopoiesis is a developing process, maturation, expansion, and migration occur at different time points and in different organs. Hence, the strategies for isolating fetal HSC are not always the same. The optimized time points and methodologies for tissue harvesting, single-cell preparation, and HSC enrichment are summarized in Table 1.

4 Notes

1. This osmolarity is appropriate for murine cells and results in better cell recovery.
2. Mac-1 is excluded from the lineage depletion antibody cocktail due to its previously described presence on fetal and newborn HSC.
3. In order to obtain accurate and high-quality fluorescence-activated cell sorting (FACS) profiles, proper titration is required for all antibodies. The optimized working concentration allows the optimal separation of positive cells from negative

Table 1
Optimized methodologies for fetal and newborn HSC isolation

Organ		E14.5–16.5	E17.5	E18.5	D0–D4	D5–D8
Liver	Observation time points	HSC isolation				–
	Methods summary	Mash with plunger through a 40 μm strainer Density separation (*see* **Note 18**)			Mince with scissors Digest with Collagenase I Flush with 18G and 21G needles Filter through a 40 μm strainer	–
Spleen	Observation time points	–		HSC isolation		
	Methods summary	–		Mash with plunger through a 40 μm strainer Density separation Lineage depletion		
BM	Observation time points	–		HSC isolation		
	Methods summary	–		Grind with a mortar and pestle Digest with Collagenase I and Dispase II Density separation Lineage depletion		

cells without causing any shift for isotype control from unstained.

4. B220, CD3, Gr-1, and Ter119-APCCy7 are included to exclude residual lineage-committed cell contamination.
5. Co-housing females results in individual estrous cycles being synchronized.
6. The murine estrous cycle is 4–5 days, with ovulation on the third day. Therefore teasing females 3 days prior to mating will produce the maximum number of pregnancies.
7. Only using female mice that are confirmed to be actively in estrous can increase the success of the timed mating.
8. Due to the size of embryos and newborn pups, a number of organs need to be pooled to provide sufficient cells. Table 2 shows the number of organs required for harvesting 50×10^6 single cells from different organs at a variety of ages.
9. It is very difficult to clean embryonic and newborn bones as they are extremely soft and even gentle squeezing will result in the loss of marrow. As a consequence, holding the muscle around the bone whilst cleaning works best.

Table 2
The number of organs required for harvesting 50×10^6 cells

Mouse age	No. of livers	No. of femurs	No. of spleens
E14.5	6	_	_
E15.5	2	_	_
E16.5	2	_	_
E17.5	2	_	_
D0	3	220[a]	40
D1	5	160[a]	30
D2	5	120[a]	20
D3	4	80[a]	15
D4	4	60	7
D5	_	30	4
D6	_	30	3
D7	_	20	3
D8	_	15	2

[a]Calculated based on observed cellularity

10. As the cellularity of both the femur and tibia is low, the humerus is also often collected. We have experimentally shown that there is no significant difference in the cellularity or the HSC frequency between a femur, tibia, and humerus at E18.5 and D1–8 bones.
11. The processing of fetal and newborn BM is different from adult BM [11] in that due to their tiny size they are not separated into central and endosteal fractions.
12. 5 min is experimentally determined for ideally digesting bones, since after 5 min there is a progressive loss of c-Kit and Sca-1 [12], which are important surface markers for isolating HSC.
13. The maximum number of cells per tube for a density separation is 2×10^8.
14. The maximum number of cells per tube for a Dynal bead separation is 3×10^8.
15. As these cells tend to clump easily additional refiltering may be required immediately prior to sorting.
16. $25–30 \times 10^6$ Cells/ml is the ideal concentration for obtaining optimal yields and purity when sorting cells on our Influx1 cell sorter.

17. For convenience and saving our enriched cell samples, we use un-fractionated single cells for the compensation controls. Furthermore, CD45-conjugated antibodies are used for compensation as CD45 is highly expressed on un-fractionated cells.
18. Fetal liver HSCs are not enriched by lineage depletion, as E14.5 fetal liver cells do not express lineage antigens at high levels. Such a population only begins to appear after E16.5, but still comprises a very low proportion.

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