Mouse fetal liver erythroid population separation using FACS.

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Method Article

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Abstract

Through-out erythropoiesis cells undergo several morphological and regulatory changes. In mouse, these changes can be characterized through isolation of specific intermediate populations based on the surface levels of Ter119 (Glycophorin A-associated protein/Ly-76) and CD71 (Transferrin receptor 1)[1-2]. This method takes roughly one day and can be used to generate sufficient cells for most high-throughput sequencing techniques with low input adaptations.

Introduction

Reagents

- PBS (Invitrogen: 10010031)

- Bovine Serum Albumin, BSA (Sigma: A4503)

- Glucose (Sigma: G7021)

- EDTA (Invitrogen: 15575-038)

- RPMI (ThermoFisher Scientific: 21875034)

- Fetal Calf Serum, FCS (Sigma: F7524)

- DNaseI (VWR, A3778.0050)

- $MgCl_2$ (Ambion: AM9530G)

- e13.5 Mouse fetal livers 12-20 embryos

- Streptavidin MicroBeads (Miltenyi: 130-048-101)

- Hoechst 33258 (ThermoFisher Scientific: H3569)

Antibodies

- ChromePure Rabbit IgG, 20mg/ml (Jackson, 015-000-003)

- CD41-FITC (BD Bioscience, Clone: MWReg30, Cat: 553848)

- CD45R/B220-FITC (BDBioscience, Clone: RA3-6B2, Cat: 553087)

- CD3e-FITC (BDBioscience, Clone: 145-2C11 553061, Cat: 6053673)

- CD11b/Mac-1-FITC (BDBioscience, Clone: M1/70, Cat: 557396)

- Ly-6G and Ly-6C (Gr-1)-FITC (BDBioscience, Clone: RB6-8C5, Cat: 553126)

- Ter119-Biotin (BD Bioscience, Cat: 553672)

- Streptavidin-APC (Biolegend, Cat:405207)

- CD71-PE-Cy7 (Biolegend Clone: RI7217, Cat: 113811)

DNase stock solution:

- 1x PBS

- 5 mg/mL DNase I

- 100mM $MgCl_2$

Staining solution

- 1x PBS

- 0.2% BSA

- 5 mM glucose

* For liver dissociation add 1 mL DNase stock solution to 50 mL.

* For sorting include 5mM EDTA.

Separation buffer

- PBS (pH 7.2)

- 0.5% BSA

- 2 mM EDTA

Collection buffer

- RPMI

- 10% FCS

Equipment

- Partec CellTrics 30 µm cell filters (Sysmex: 04-004-2326)

- NucleoCounter NC-3000 cell counter, or equivalent (Chemometec)

- LS separator columns (Miltenyi: 130-042-401)

- Pre-sepration filter (30 µM) (Miltenyi:130-041-407)

- MACS MultiStand, or equivalent (Miltenyi: 130-042-303)

- QuadroMACS Separator, or equivalent (Miltenyi: 130-090-976)

- BD FACSAria™ Fusion Sorter, with 100 µM nozzle, or equivalent (BD Bioscience)

Procedure

Dissociation of mouse fetal livers.

*** Chill centrifuge to 4ºC ***

*** Add 1 mL DNaseI stock solution to 50mL of ice-cold Staining Solution. ***

1. Collect livers from e13.5 mouse embryos (12-20 required).

2. Dissociate fetal liver tissue into 3 mL ice-cold staining solution by gently pipetting up and down with a P1000 pipette.

3. Transfer cells to a 12x75 mm tube.

4. Pellet cells by centrifugation at 200 rcf for 5 min (4ºC).

5. Discard supernatant and gently resuspend in 1 mL of ice-cold staining solution to wash cells.

6. Pass resuspended cells through a 30 µm filter.

7. Rinse filter with 2 mL ice cold staining solution ot dislodge cells stuck to the membrane.

8. Pellet cells by centrifugation at 200 rcf for 5 min (4ºC).

9. Discard supernatant and gently resuspend in 1 mL of ice-cold staining solution.

10. Count cells (for NC-3000 cell counter use Solution 13).

Mature erythoid cell depletion.

1. Adjust volume of cells in staining solution to achieve 0.5-1 x 10^7 cells per 100 µL.

2. Block Fc receptors by adding ChromePure Rabbit IgG (final concentraion 200 µg/mL).

3. Add 10 µL of Ter119-biotin per mL of cells.

4. Incubate on ice for 30 min.

5. Wash the cells by adding 3 mL of cold staining solution.

6. Pellet cells by centrifugation at 200 rcf for 5 min (4ºC).

7. Resuspend cells in 90 µL cold staining buffer per 10^7 cells.

8. Set aside 2 x 10^6 cells (18 µL) for staining controls and make up to 1 mL with staining solution. Keep on ice.

9. Set aside 8 x 10^6 cells (72 µL) for unenriched sort and make up to 100 µL with staining solution. Keep on ice.

10. Add Streptavidin MicroBeads to the remaining cells (10 µL per 10^7 cells).

11. Mix well by pipetting and incubate for 15 min at 4°C.

12. Wash the cells by adding 3 mL of separation buffer.

13. Pellet cells by centrifugation at 200 rcf for 5 min (4ºC).

14. Whilst cells are washing, place LS separator columns (1 for every 1x10^8 cells) into

the magnet and rinse with 2 mL of separation buffer.

15. Discard supernatant and resuspend pellet in 500 µL of separation buffer per 10^8 cells.

16. Place an 30 µm pre-separation filter in each LS column, and a 15 mL collection tube below the column.

17. Apply 500 µL of resuspended cells to each LS column, collecting the flow through which contains Ter119-negative progenitor cells.

18. Wash each column twice with 1 mL of separation buffer, allowing the first mL to flow through before adding the second.

19. Pellet cells by centrifugation at 200 rcf for 5 min (4ºC).

20. Discard supernatant and resuspend pellet in 100 µL of staining buffer.

21. Count Ter119-negative cells (for NC-3000 cell counter use Solution 13).

Cell staining

1. Using staining solution, adjust the volume of Ter119-negative cells in staining solution to achieve 1 x 10^7 cells per 100 µL.

2. Gently resuspend staining control and unenriched cells (set aside earlier) by vortexing.

3. Aliquot staining control cells into 9 tubes of 100 µL each.

4. Prepare the Lineage cocktail by combining 3 µL of each antibody in Figure 1.

5. Add the relevant conjugated antibodies to each tube according to Figure 2.

6. Cover stained cells and incubate on ice for 45 min in the dark.

7. Add 1 mL ice-cold staining solution to wash cells.

8. Pellet cells by centrifugation at 200 rcf for 5 min (4ºC).

9. Discard supernatant and resuspend in 1 mL ice-cold staining solution.

10. Pellet cells by centrifugation at 200 rcf for 5 min (4ºC).

11. Re-suspend full stain in 400 µL ice-cold staining solution (+ 5 mM EDTA).

12. Re-suspend controls in 200 µL ice-cold staining buffer (+ 5 mM EDTA).

13. Make a 1:150 dilution of Hoechst in ice-cold staining buffer.

14. Add 1:100 of diluted Hoechst to the relevant tubes immediately prior to sorting (Figure 2).

Cell sorting.

1. Establish voltages and flow using unstained and single stain samples:

A. Gate cells for forward and side scatter to isolate single cells.

2. Establish population gating using flourescence minus one (FMO) samples:

A. Gate out Hoechst-positive cells to remove dead and dying cells.

B. Gate out FITC-positive cells to remove non-erythroid cells.

C. Gate for specific population on levels of CD71 and Ter119 (Figure 3).

3. Sort mature populations into 500 µL collection buffer using unenriched sample.

4. Sort progenitor populations into 500 µL collection buffer using progenitor full stain sample.

Troubleshooting

Time Taken

Anticipated Results

References

1. Socolovsky M. et al. Negative autoregulation by FAS mediates robust fetal erythropoiesis. PLoS Biol. 5, e252 doi:10.1371/journal.pbio.0050252 (2007)

2. Oudelaar A.M. et al. Dynamics of the 4D genome during in vivo lineage specification and differentiation. *Nat Commun*. 11:2722. doi:10.1038/s41467-020-16598-7 (2020)

Figures

Antibody
CD41
CD45R/B220
CD3e
CD11b/Mac-1
Ly-6G and Ly-6C (Gr-1)

Figure 1

Lineage cocktail

Sample	Source	Strep (APC)	CD71 (PE-Cy7)	Lin (FITC)	Hoechst (1:150)
Unstained	Staining control	–	–	–	–
Ter119 single stain	Staining control	+	–	–	–
CD71 single stain	Staining control	–	+	–	–
Lin single stain	Staining control	–	–	+	–
Hoechst single stain	Staining control	–	–	–	+
Ter119 FMO	Staining control	–	+	+	+
CD71 FMO	Staining control	+	–	+	+
Lin FMO	Staining control	+	+	–	+
Hoechst FMO	Staining control	+	+	+	–
Unenriched	Unenriched cells	+	+	+	+
Progenitor full stain	Ter119-Negative	+	+	+	+
	Volume:	1:300	1:1,000	1:100	1:100

Figure 2

Antibody staining matrix

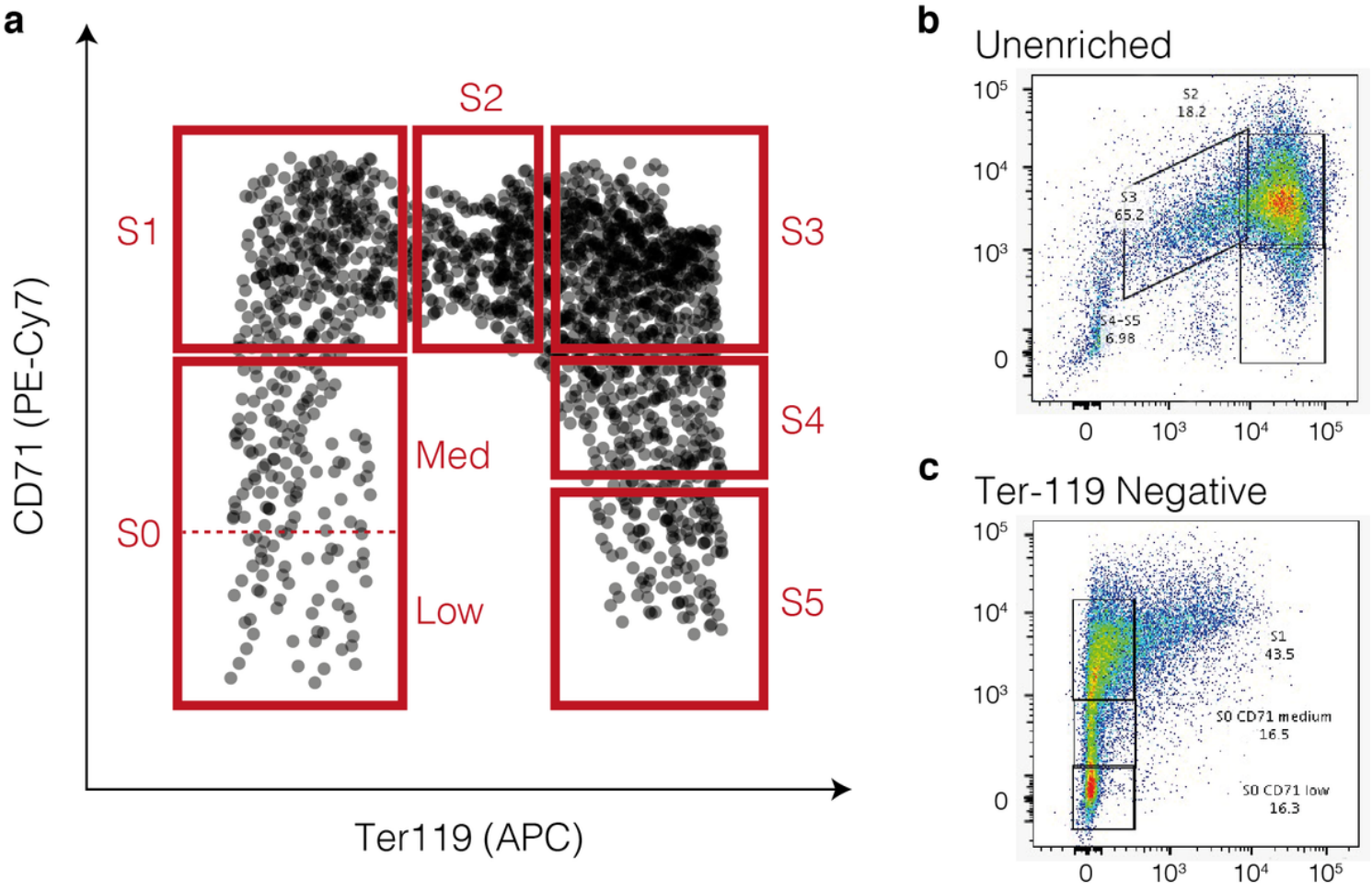

Figure 3

Erythroid populations. a, Schematic of gating for erythroid populations. Haematopoietic Stem and Progenitor cells and Burst-Forming Unit-Erythroid cells (S0-low), early Colony-Forming Unit-Erythroid cells (CFU-E) cells (S0-medium), late CFU-E (S1) and maturing terminal differentiating cells (S2-S5). Example FACS plot showing the distribution of cells in the unenriched (b) and Ter-119 depleted populations (c)