Linheng Li Lab

Repopulation Assay

Materials

3ml syringes
$22_{1/2}$G needles
70 um filter
6 well plates
50ml and 15ml conical tubes and racks
5ml round bottom flow tubes without the cap and racks
Disposable Pasteur pipettes and rubber bulbs
1X DPBS + 2%FBS
Hemolytic buffer (1X, in H_2O):

NH4Cl	8.3g/L, MW: 53.49, Sigma #A9434
$NaHCO_3$	1.0g/L ($KHCO_3$), MW: 84.01, Sigma #S5761
EDTA	37 mg/L, MW: 292.25

The day before

1. Have the list of your transplanted mice and check on Facility if there is dead one to omit from the list.
2. Give your transplanted mice a consecutive numbers.
3. Label: 5ml round bottom flow tubes for each sample and controls
   50ml tubes for B6 BM (x2) and Ptprc BM (x2)
   6 well plates for B6 and Ptprc
4. Aliquot 3ml of lysis buffer into pre-labeled 5ml round bottom flow tubes and keep them in 4˚C
5. Assemble Disposable Pasteur pipettes and rubber bulbs

Procedure

Control BM:
1. Sacrifice B6 & Ptprc to get bone marrow cells
2. Flush out the BM cells from the leg bones with $22_{1/2}$G needle + 3ml syringe
3. Homogenize cells with $22_{1/2}$G needle + 3ml syringe (passing the needle 10 times),
4. Then filter into 50ml tube with 40 or 70 uM filter.
5. Wash one time with 1xDPBS+2%FBS (Centrifuge at 4˚C, 500g, for 5min)
6. Resuspend the cell pellet into 4ml 1xDPBS+2%FBS. (To make ~ **$5x10^3$**cells/ul)
7. Filter into a fresh 50ml tube.

RBC Lysis:
1. Line up the pre-labeled 5ml flow tubes containing lysis buffer on ice
2. Transfer blood sample into the tube using Pasteur pipettes
3. Keep the samples on ice until you put them in the 37°C water bath.
4. Wipe off condensed water from the top (cover) to avoid water dropping into your tube
5. Incubate in 37°C water bath for 5 minutes- shake every 1:30 min
6. After 5 minutes incubation, stop the lysis reaction by adding 1ml of 1xDPBS+2%FBS

7. Centrifuge the tubes at 4˚C, 1500 rpm, for 5min. After centrifugation,
8. Pour off supernatant and resuspend the pellet in 5ml of 1xDPBS+2%FBS
9. Filter the cell suspension into pre-labeled 5ml round bottom flow tube using disposable pipettes (Save some left over cell suspensions from several tubes into unstained Ptprc blood tube)
10. Spin down and remove supernatant carefully (at 4˚C, 500g, for 5min).
11. Resuspend the pellet in 200ul of 1x DPBS+2%FBS for Antibodies Staining.
12. Aliquot B6BM & Ptprc BM into pre-labeled 5ml round bottom flow tubes.

Antibody treatment

1. Add proper antibodies for each tube, then incubate 30 minutes on ice/tilted rotator
2. Wash one time with 4ml of 1x DPBS+2%FBS, centrifuge at 4˚C, 1500 rpm, for 5min.
3. Resuspend the cell pellet in 200ul 1xDPBS+2%FBS

Antibody cocktails (always keep them cold) 3.5ul antibody mix per sample

0.25ul of CD45.2	FITC	(B6, Donor)
0.25ul of CD45.1	PE-Cy5	(Ptprc, Recipient)
0.25ul of Mac-1	PE-Cy7	(Myeloid/Lymphoid)
1.25ul of Gr-1	APC-Cy7/APC-eF780	(Myeloid/Lymphoid)
0.25ul of B220	PE	(B cells)
1.25ul of CD3	APC	(T cells)

Color Comp Tubes, 10^6 cells/tube, 200ul (Mac-1 or Gr-1 conjugated)

CC 1) FITC	B6	1 ul
CC 2) PE-Cy5	**Ptprc**	1 ul
CC 3) PE-Cy7	B6	1ul
CC 4) APC -Cy7/ APC-eF780	B6	1 ul
CC 5) PE	B6	1 ul
CC 5) APC	B6	1 ul

Unstained B6 BM
Unstained Ptprc BM
Unstained Ptprc Blood

Isotype Tubes, 10^6 cells/tube, 100ul of B6BM + 100ul of PtprcBM

Tube1	Tube2	Tube3	Tube4	Tube5
IsoFITC	CD45.2-FITC	CD45.2-FITC	CD45.2-FITC	CD45.2-FITC
IsoPE-Cy5	CD45.1-PE-Cy5	CD45.1-PE-Cy5	CD45.1-PE-Cy5	CD45.1-PE-Cy5
	Iso-PE-Cy7	Mac1-PE-Cy7	Iso-PE-Cy7	Mac1-PE-Cy7
	Iso-APC-Cy7	Gr1-APC-eF780	Iso-APC-Cy7	Gr1-APC-eF780
	Iso-PE	Iso-PE	B220-PE	B220-PE
	Iso-APC	Iso-APC	CD3-APC	CD3-APC

Sample Tubes

Tube5
CD45.2-FITC
CD45.1-PE-Cy5
Mac1-PE-Cy7
Gr1-APC-eF780
B220-PE
CD3-APC

CyAn analyzer
Throughout the procedure, keep the tubes on ice.