MEC preparation

by Noriyuki Fujikado

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Mice

C57BL/6J Background

Adult (5 weeks)

Neonate (1-3 days)

Solutions needed

RPMI-1640

FCS

25mM HEPES

20 mg/ml Collagenase (Sigma C6885)

20 mg/ml Collagenase/Dispase (Roche 10269638001)

20 mg/ml DNase (Sigma DN25)

0.5M EDTA

TRIzol Reagent (15596-018)

PBS

Basic medium

- 2% FCS
- 25mM HEPES
- RPMI-1640

Collagenase medium

- 0.5 mg/ml Collagenase
- 0.2 mg/ml DNase
- Basic medium

Collagenase/Dispase medium

- 0.5 mg/ml Collagenase/Dispase
- 0.2 mg/ml DNase
- Basic medium

Protocol

Enrichment of MECs

1. Put thymi in a culture dish on ice.
2. With forceps, remove as much connective tissue as possible.
3. Wash the thymi with PBS.
4. Mince the thymi with scissors in the basic medium.
5. Transfer the minced thymi to 15 ml centrifuge tube.
6. Pipette up and down with a large orifice P1000 tip to break up clumps.
7. Leave to stand for a few minutes to sink the minced thymi to bottom of the tube.
8. Discard the supernatant (including thymocytes).
9. Repeat 6-8 (usually 1 or 2 more times)
10. Add Collagenase medium.
11. Incubate at 37℃ for 15-20 minutes with stirring, pipetting up and down sometimes.
12. Pipette up and down with a large orifice P1000 tip to break up clumps.
13. Leave to stand for a few minutes to sink the cells to bottom of the tube.
14. Discard the supernatant (including thymocytes and cortical cells).

    *Control collagenase fraction is needed to keep for compensation.
15. Repeat 12-14 (usually 1 or 2 more times)
16. Add Collagenase/Dispase medium.
17. Incubate at 37℃ for20-30 minutes with stirring, pipetting up and down sometimes.
18. Pipette up and down gently (trying not to make babbles) with P1000 tip or Pasteur pipette until you have a single-cell suspension.
19. Add 10mM EDTA and pipette up and down gently to break up rosettes.
20. Wash the cells with FACS Buffer (2%FCS/PBS).
21. Filtration the cells with mesh.
22. Spin down and resuspend in FACS Buffer.

Staining for sorting

1. Add CD45-PE-Cy5 (1:100), Ly51-PE (1:800), I-A/I-E (1:1200) and incubate 20 min on ice. For compensation, following single staining will be needed; Unstain, CD4-FITC (1:100), CD45-PE-Cy5 (1:100), Ly51-PE (1:800), I-A/I-E (1:1200), DAPI (1:100). DAPI sample are needed to kill at 60℃, 5 min before staining.
2. Wash the cells with FACS Buffer.
3. Resuspend in 0.2-0.5 ml FACS Buffer.

Sorting

1. Filtration the cells with mesh and stain with DAPI just before sorting.
2. View the cells on a FSC vs. SSC plot and gate on the wide cells. After remove the doublet cells on a FSC vs. Pulse width plot and dead cells on a DAPI vs. SSC plot, view the cells on a CD45 vs. SSC plot and gate on the CD45 low to negative cells.
3. View the cells on a Ly51 vs. I-A/I-E plot. The MEC^{hi} cells are the population of $Ly51^{-/lo}$ I-A/I-E^{hi} cells. ($Ly51^{-/lo}$ I-A/I-E^{lo} ; MEC^{lo}, $Ly51^{+}$ I-A/I-E^{+}; CECs, $Ly51^{-}$ I-A/I-E^{-}; Fibroblasts) By using control cells, set the gates of GFP^{+} and GFP^{-} on a GFP vs. I-A/I-E plot. And then, sort GFP^{+}.MEC^{hi} and $GFP^{-}MEC^{hi}$ cells to 0.5 ml of Torizol Reagent on ice.

DNase (1:100) _____ µl (100 µl)

Collagenase (1:40) _____ µl (100 µl)

Basic medium _____ ml (10 ml)

DNase (1:100) _____ µl (100 µl)

Collagenase/Dispase (1:40) _____ µl (100 µl)

Basic medium _____ ml (10 ml)

2x Ab mix

CD45-PE-Cy5 (1:100) _____ µl (20 µl)

Ly51-PE (1:800) _____ µl (2.5 µl)

I-A/I-E (1:1200) _____ µl (1.7 µl)

FACS Buffer _____ ml (1 ml)