Note: this protocol is adapted from Ivanov et al, 2006.

Keywords: gut, lamina propria, immune cell, small intestine

1. Cut out the entire small intestine – from stomach to caecum
    a. As you are cutting it out, remove residual fat and connective tissue
2. Place in ice cold PBS, move on to the next mouse
3. Continue removing all fat and connective tissue
4. Cut out the Peyer's patches
    a. There should be 9-12 PPs in total
5. Cut open the intestine longitudinally
6. Place on a wet paper towel, use rounded forceps to scrape along the mucosa, removing mucous, bacteria, etc
7. Place in a 50 mL conical tube with PBS, invert tube a few times
8. Pour of the supernatant, refill with fresh PBS
9. Repeat the PBS washes 5-6 more times until there is no visible debris
10. Cut the intestine in large fragments
11. Place the fragments in a 15 mL conical tube with 5-10 mL cell dissociation solution
    a. To make 120 mL of cell dissociation solution (adjust as necessary):
        i. 114.5 mL HBSS
        ii. 1.2 mL 0.5 M EDTA
        iii. 1.2 mL 1M HEPES
        iv. 3.12 mL FBS
12. Incubate for 10 minutes at 37C, 100 rpm on the rotator
13. **Vortex** well for 25 seconds, take out the supernatant with a metal strainer, keep the pieces, discard the supernatant
14. Repeat steps 10-12
15. Collect the fragments, rinse in HBSS in a small petri dish, cut using a razor blade (~1mm$^2$)
16. Digest for 20 minutes at 37C with slow rotation in **5mL** digestion mix
    a. To make 180 mL of digestion mix (adjust as necessary):
        i. 126.6 mL RPMI
        ii. 9 mL FBS
        iii. 720 ul DNase I
        iv. 18 mL Dispase (5U/mL)
        v. 360 ul Collagenase D (Roche)
            1. From 500mg/mL stock solution, to make working conc of 1mg/mL
17. Vortex well for 30 seconds
18. Collect the supernatant by filtering through 100um strainer in 50 mL falcon tube at room temperature to avoid cold temperature shock. Keep it on ice afterwards.

19. Put remaining tissue fragment back into the same tube with 5 mL of new digestion mix
20. Repeat steps 16-18 two more times
21. Combine all appropriate supernatants
22. Centrifuge at 3000 rpm at 4C for 10 minutes
23. Prepare Percoll (adjust amounts as necessary)
    a. 100% Percoll = 45 mL stock Percoll + 5 mL 10X PBS (50 mL total)
    b. 80% Percoll = 24 mL 100% Percoll + 6 mL 10% RPMI-FBS (30 mL total)
    c. 40% Percoll = 20 mL 100% Percoll + 30 mL 10% RPMI-FBS (50 mL total)
24. Add 5 mL of 80% Percoll to the bottom of a 15 mL conical tube
25. Resuspend with LPL cells in 1 mL of 40% Percoll to get homogenous solution, then add the remaining 9 mL
26. Gently add 10 mL of the 40% Percoll cell solution on top of the 80% Percoll in the tube
27. Centrifuge at 2500 rpm for 20 minutes, without brake (accel 1, decel 0)
28. Collect and discard the top layer of epithelium and debris
29. Collect white cell layer at the interface between the 40% and 80% Percoll
30. Dilute with 10% RPMI-FBS, invert a few times to mix well
31. Pellet by centrifugation for 7 minutes at 2000 rpm 4C
32. Aspirate SN and resuspend cells FACS buffer, then start FACS stain

Ivanov, II, McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.