This protocol was adapted from An optimized protocol for isolating lymphoid stromal cells from the intestinal lamina propria by Stzepourginski et al 2015.

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# Lamina Propria Stromal Cell Isolation

ActSeq - 37° steps should be changed to room temperature and incubation times increased by 50%

1. Excise intestine and open intestine longitudinally, washing in Ca&Mg-free HBSS until all fecal matter has been removed
   a. ActSeq – Extract all intestines and place in Ca&Mg-free HBSS + **ActD** before moving on
2. Remove smooth muscle using scraping technique and place remaining lamina propria (LP) in Ca&Mg-free HBSS on ice until all mice are finished. Discard detached smooth muscle and epithelial cells.
3. Cut Intestine into 2-5 cm pieces (longer and they'll tie themselves in knots) and transfer to and new 50 ml tube (**CHEL** tube), adding 20 mls of chelation buffer.
   a. I've pooled the LPs of up to 3 mice and treated them as a single biological replicate at this point to increase the cell yield per sample.
4. Incubate for 10 min at 37° on slow rocker.
5. Wash tissue pieces by vortexing and shaking the **CHEL** tube vigorously, then discard the supernatant.
6. Add a fresh 20 mls of Ca&Mg-free HBSS to the **CHEL** tube and repeat until obtaining a clear supernatant devoid of epithelial cells.
   a. If the smooth muscle has been removed, this is usually takes 2 washes, with 30-60 seconds of shaking/vortexing each time.
7. Transfer LP pieces from **CHEL** tube to a new 50 ml tube (**RXN** tube), cutting them into as small of pieces as possible at the same time.
8. Add 10 mls of pre-warmed dissociation buffer.
9. Incubate for 20 min rocking at 37°
   a. To speed up dissociation, you can use a 10 ml serological pipette to triturate the LP fragments as needed.
      i. I generally do 5-10 pulls at the 10- & 20-minute marks of both digestion periods. Try to minimize the number of pulls if possible as they have a great effect on the final viability.
      ii. **DO NOT** keep pulling if a large chunk plugs the pipette; creating a vacuum in the pipette is the fastest way to turn your cells to confetti. Pre-wetting the pipette will increase your yield by preventing the tissue pieces from sticking to the inside of the pipette.
10. Spin the tubes for 60 seconds @ 200-300 RCF to get the larger chunks at the bottom.
    a. Spin time/speed often needs adjustment based on 'chunkiness' and viscosity of your sample.
11. Remove as much of the supernatant as possible without picking up the larger tissue chunks (usually 4-6 mls) and pass it through a 70µm filter into a fresh tube (**QNCH** tube, but use same quench tube for 1st and 2nd rounds).
12. Add 1 volume of quench buffer to the **QNCH** tube through the filter to pick up any cells stuck in mesh, then place the **QNCH** tube on ice.
    a. You can use a serological pipette to propel the quench buffer through the filter, pushing chunks that may be lodged in the holes to maximize recovery. The same can be done with the dissociation buffer in the opposite direction to get larger chunks back into the **RXN** tube
    b. To increase your yield and conserve filters, you can gently rub the filter with the rubber end of a 1ml syringe plunger. This helps break up the larger clumps and get the suspension to pass through the filter more easily.
13. Add a fresh 5 mls of dissociation buffer to the **RXN** tube and Repeat steps 6-8. For the second cycle, transfer all the remaining contents of the **RXN** tube to the **QNCH** tube through the 70µm filter
    a. You should end up with a single tube per biological replicate containing 30mls of filtered cell suspension.
    b. The original protocol calls for 3 rounds at 20 minutes. I feel like this is overhandling the cells and 2 incubations at higher enzyme concentrations seems to work. Plenty of wiggle room here though.
14. Spin down the cellular suspension for 15 minutes @400 RCF, 4°C.

a. I've heard of people going as fast as 800g for 5 min, but the 10x protocol says to never spin faster than 400g. Not sure how imperative this is, but I play it safe for scRNAseq runs and just do what they tell me.

15. Resuspended the pellet in 500 uls of the appropriate staining buffer, transfer to flow tube.
    a. 500µls is suited for 2 small intestines pooled together. I stain my cells at 10e6/ml, but just be sure whatever volume you use is as much as you can add without reducing the concentration below what you intend to stain at, you'll add more later anyway.
16. Stain, wash, and sort as appropriate.
    a. I stain for 30 minutes on ice @ 10e6 cells/ml. Then I wash with staining buffer and spin down at 500G for 5 minutes, resuspending the pellet in staining buffer for sorting.
        i. If using a small tip (70µm or less), just resuspend in pure accumax but try to get the sort done ASAP.
    b. Immediately before sorting, I add one volume of accumax to the flow tubes and filter through a 70µm syringe filter to prevent clumping during the sort.

| Reagent | Composition | [ActD] |
|---|---|---|
| Chelation solution | HBSS without Ca&Mg; 0.5M EDTA | 30 µM |
| Dissociation solution | DMEM; 2-4 Wunsch units Liberase TM; 1-5 units DNaseI | 30 µM |
| Quenching solution | DMEM; 10% FBS | None |
| Staining solution: | HBSS with Ca&Mg; 3% FBS | 3 µM |

**(References)**

Stzepourginski I, Eberl G, Peduto L. An optimized protocol for isolating lymphoid stromal cells from the intestinal lamina propria. J Immunol methods. 2015 Jun;421:14-19. Doi: 10.1016