Murine intestinal cell dissociation suitable for multi-omics single-cell assays

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ABSTRACT

Here we present an optimized protocol for the single-cell suspension of murine intestines (here we used ileum) optimized for contemporary multi-omics single-cell assays including high-dimensional flow cytometry and single-cell RNA-seq. This protocol provides a rapid and efficient method of digesting mouse ileum into a single-cell suspension with a short processing time, high viable cell yield, and it preserves cell-surface markers throughout the process. Notably, the protocol utilizes a Collagenase-I/Benzonase-based 37°C water bath dissociation method and collects already digested cells at several steps during the process to prevent over-digestion (fractioning technique). Tissues were normalized by length (2.5 cm) and weight (averaged 79.5 mg) and measured for cell yield and viability. We obtained an average total cell yield of ~4.4E6 +/- 1.4E6 cells, and average viability of ~56 +/- 13% (n=7) (Figure 1). By flow cytometry, ~30%-50% of cells were CD45+ immune cells, with the remainder of the cell population being CD45- non-immune cells (i.e., epithelial and stromal cells).

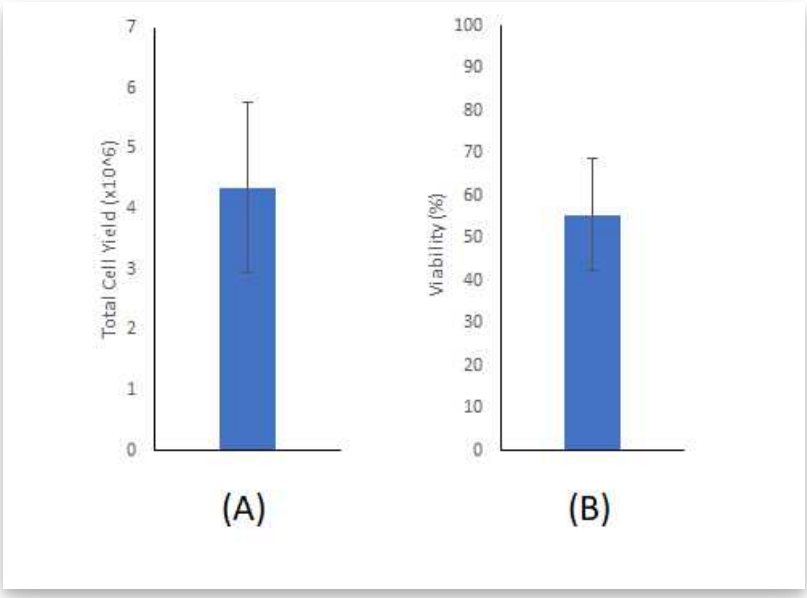

Figure 1: Average yields obtained by using the optimized protocol. Data shown represents 7 independent experiments. A) Total cell yield (Mean +/- SD). C) Percent viability (Mean +/- SD).

To optimize this protocol, several other conditions were tested, including different digestive enzymes, mechanical digestion techniques, the inclusion of an intraepithelial layer (IEL) digestion, and an antioxidant cocktail addition. We summarize our main findings about the optimization of this protocol below:

- Collagenase-I provided the highest number of total yield and viable cells when compared to Collagenase-IV and Liberase.
- Enzymatic digestion in a 37°C waterbath and vortexing the cell solutions proved more successful in cell yield and viability than different combinations of the gentleMACS Tissue Dissociator (Myltenyi) intestine digestion programs, including its m_ intestine _1 and 37_m_LPDK programs and variations in their run times.

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- We have also validated a protocol to separate the IEL before the rest of the ileum, and have found it compatible and therefore able to be adopted with the recommended protocol while maintaining a similar yield of viable cells. An IEL separation protocol has been included in a separate section at the end.
- By using our "fractioning technique", in which cells that are digested early on are removed before digesting the rest of the tissue separately, total cell yield and viability are increased.
- We tested several antioxidant combinations (Gluthatione, Vitamin C, and Vitamin E) and found higher live cell yield when excluding these antioxidants from the Collagenase-I digestion step.

Based on these findings, this murine intestinal digestion protocol is rapid and efficient and can be used for multi-omics single-cell assays such as high-dimensional flow cytometry and single-cell RNA-sequencing.

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GUIDELINES

- Keep all solutions on ice unless otherwise mentioned.
- Note that cells will be isolated into 2-3 different fractions based on the amount of tissue being processed.
- If digesting the IEL, begin immediately after tissues have been obtained and follow with ileum processing as described above.

MATERIALS TEXT

- Cell counter (Nexcelom Cellometer K2)
- Cooled large tabletop centrifuge
- 37°C water bath

- 6 cm & 10 cm Petri-dish
- 15 mL/50 mL Falcon tubes
- 70 uM nylon filters (pluriStrainer for 15 mL #43-10070-40; for 50 mL #43-50070-51)
- 9 inch Glass Pasteur pipettes & squeeze bulb

- def-RPMI* (or RPMI-1640)
- NBCS (or FBS)
- Collagenase-I (0.25%) (Stem cell Technologies #07902)
- Benzonase (Sigma #E1014)
- 0.5 M EDTA pH 8.0

Note: *def-RPMI is a custom media prepared in-house and composed of the same formulation of commercially available RPMI-1640, but deficient in the following compounds: biotin, L-glutamine, phenol red, riboflavin, and sodium bicarbonate. These compounds were removed to avoid interference with standard flow-cytometry assays.

Solution preparation: Keep all solutions on ice unless otherwise mentioned.

- **def-RPMI/3%NBCS**: def-RPMI + 3% NBCS (or RPMI-1640 + 3% FBS)
- **def-RPMI/3%NBCS/EDTA**: EDTA @ 5 mM EDTA final concentration in def-RPMI/3%NBCS
- **def-RPMI/3%NBCS/Benzonase**: Benzonase @ 1 uL per 10 mL def-RPMI/3%NBCS
- **Collagenase-I/Benzonase**: Benzonase @ 1 uL per 5 mL Collagenase-I (0.25%)

Additional materials for optional IEL Step:

- DTT

Solution preparation: Keep all solutions on ice unless otherwise mentioned.

- **IEL Buffer**: 35.4 mL def-RPMI + 4 mL NBCS + 400 uL 0.5M EDTA
- **DTT solution**: 6 mg DTT per 400 uL def-RPMI

Obtain Tissues

1 Euthanize mouse, open peritoneal cavity. Take out intestines from the stomach to rectum, and place directly into 10 cm petri-dish containing 10 mL def-RPMI/3%NBCS on ice.

2 Remove connective tissue from intestinal tissue. Untangle as necessary.

3 Separate/cut the ileum from the cecum, and the duodenum from the stomach.

4 Pipette fresh def-RPMI/3%NBCS onto a line of paper towels and lay the full intestine on down. Rinse with more def-RPMI/3%NBCS to prevent from getting dry at all times.

5 Measure and record total length and take pictures.

Return the tissue into def-RPMI/3%NBCS.

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7 Dissect Peyer's patches from ileum (save and process separately if needed).

8 Gently squeeze out feces/bile from the intestine (at least from the lower half) using cotton-tips soaked in def-RPMI/3%NBCS.

9 Cut off the bottom 10 cm of the ileum and transfer to 10 cm petri-dish with fresh def-RPMI/3%NBCS.

10 Separate 10 cm ileal tissue into 4 equal pieces (~2.5 cm each).

11 Cut each piece open longitudinally. Gently rinse in def-RPMI/3%NBCS to clean and scrape the inner wall as necessary to clean. Transfer each piece to a new 6 cm petri-dish with fresh def-RPMI/3%NBCS when complete.

12 Weigh tissues (weigh as little liquid as possible), ensuring that each are similar in weight.

13 Proceed to optional IEL Processing (Step 38) or Ileum Processing (Step 14).

Ileum Processing

14 Using scissors or a razor blade, cut/chop each ileum segment into the smallest pieces possible within its def-RPMI/3%NBCS media.

15 Transfer each cut ileum segment with the media in into a 15 mL conical. Wash the petri dishes with 2 mL of fresh def-RPMI/3%NBCS media and add those to the conical.

16 Centrifuge tubes for 5 min at 450 g and 4°C. **450 x g, 4°C, 00:05:00**

17 Aspirate supernatant.

18 Add 1.5 mL Collagenase-I/Benzonase per sample into each tube.

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19 Incubate tubes in a 37°C waterbath for 10 min, vortexing for 15 sec before, every 2 min during, and after incubation.

20 Place tubes back on ice and fill each one with def-RPMI/3%NBCS/EDTA to 15 mL.

21 Vortex tubes for 10 sec.

22 Pass solutions and tissues through 70 uM filter into new conicals, also rinsing both the original tubes and filters with def-RPMI/3%NBCS/EDTA media into the new conicals. Designate the new conicals which contain the flow-through as 'Fraction 1' and set them aside on ice for later processing (see Step 31). Small tissue chunks are expected to remain and should not be pushed through the filter.

23 Scrape remaining tissues off the filter and add them back to the original tubes. Rinse filter into the original tubes with def-RPMI/3%NBCS/EDTA media as necessary.

24 Centrifuge tubes for 5 min at 450 g at 4°C. **450 x g, 4°C, 00:05:00**

25 Aspirate supernatant.

26 Add 1 mL Collagenase-I/Benzonase per sample to remaining pellet/tissue chunk in tubes and resuspend the pellet with vortexing.

27 Incubate tubes in a 37°C water bath until tissue pieces are mostly dissociated or for a max of 10 min, vortexing for 15 sec before, every 2 min during, and after incubation.

28 After incubation, fill tubes with 15 mL def-RPMI/3%NBCS/EDTA.

29 Pass solutions and tissues through 70 uM filter into new conicals, also rinsing both the original tubes and filters with def-RPMI/3%NBCS/EDTA media into the new conicals. Designate the new conicals which contain the flow-through as 'Fraction 2.'

29.1 If cells are still not mostly digested after this step, repeat the process from Step 23 until sufficiently digested.

30 Use the back of a 1 mL syringe plunger to push any undigested chunks through the filter into the Fraction 2 conicals. Rinse the back of the syringe plunger over the filter with 2 mL def-RPMI/3%NBCS/EDTA.

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31 Layer tubes from Steps 22 and 29 with 100% NBCS (or FBS). To layer, use a 9 inch Glass Pasteur pipette and squeeze bulb to fill up the pipette with as much 100% NBCS as possible. Place the pipette such that its tip is at the bottom of the sample tubes and carefully take off the bulb. Allow the 100% NBCS to slowly form a layer at the bottom of the tube. Carefully lift the pipette out of the tube and keep the tube upright to maintain the layers through centrifugation.

32 Centrifuge tubes for 5 min at 450 g and 4°C. **450 x g, 4°C, 00:05:00**

33 Aspirate supernatant.

34 Resuspend cell pellets at 250-500 uL defRPMI/3%NBCS/Benzonase depending on the pellet size.

35 Each fraction is ready to be counted.

36 Fractions can be pooled or used separately depending on the research question.

37 Cells are ready for additional purification steps or protocols.

Optional: Intraepithelial Layer (IEL) Processing

38

If digesting the IEL, begin immediately after tissues have been obtained and follow with ileum processing as described above (Steps 1-13).

39 On ice, in new 6 cm petri-dishes (one per sample) add 2 mL IEL buffer.

40 Add 20 uL DTT solution per dish (or 100 uL per 10 mL buffer for larger quantities).

41 Add one 2.5 cm ileal piece to each 6 cm petri-dish.

42 Chop up tissues into small pieces in the dishes using scissors or a razor blade.

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43 Transfer solution and tissue pieces to a 15 mL tube. If you rinse the dishes with additional media, be sure to maintain concentrations of DTT.

44 Incubate for 10 min on ice, vortexing at 5 and 10 min.

45 Pass each sample through a 70 uM filter to a new conical, using additional def-RPMI/3%NBCS to rinse the original tube and pass through the filter as well. Tissue chunks are expected to remain and should not be pushed through the filter.

46 Transfer tissue chunks left on top of the filter to a new 15 mL tube for total ileum processing (refer to Step 18) before continuing with Step 47 with the flow-through.

47 For the flow-through, fill up the 15 mL tube with def-RPMI/3%NBCS/Benzonase to 15 mL and layer with 100% NBCS (or FBS). To layer, use a 9 inch Glass Pasteur pipette and squeeze the bulb to fill up the pipette with as much 100% NBCS as possible. Place the pipette such that its tip is at the bottom of the tube and carefully take off the bulb. Allow the 100% NBCS to slowly form a layer at the bottom of the tube. Carefully lift the pipette out of the tube and keep the tube upright to maintain the layers through centrifugation.

48 Spin for 5 min at 450 g and 4°C. **450 x g, 4°C, 00:05:00**

49 Aspirate supernatant.

50 Resuspend pellet in 200 uL 37°C Collagenase-I/Benzonase.

51 Incubate at room temperature for 5 min.

52 Fill up tube to 10 mL with def-RPMI/3%NBCS containing 5 mM EDTA (def-RPMI/3%NBCS/EDTA).

53 Pass sample through a 70 uM filter into a clean 15 mL conical. Use the back of a syringe plunger (1mL syringe) to push through any undigested chunks. Rinse the back of the syringe plunger over the filter with 2 mL def-RPMI/3%NBCS/EDTA.

54 Layer with 100% NBCS.

55 Spin for 5 min at 450 g and 4°C. **450 x g, 4°C, 00:05:00**

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56 Aspirate supernatant.

57 Resuspend pellet in 250 uL def-RPMI/3%NBCS/Benzonase.

58 Cells are ready to be counted.

59 Cells are ready for additional purification steps or downstream protocols.

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