

Procedure	Purpose	Procedure standards	Readouts	Detailed Procedure
<b>1. Euthanize mouse</b>		Use 6 to 10 week old, male, C57BL6 mouse	Log time of death and age of mouse (Log: Lines 1 and 2)	Drop anesthetize mouse in isoflurane for two minutes (or until mouse stops moving, besides breathing). Euthanize by cervical dislocation.
<b>2. Weigh mouse</b>	Comparison purposes		Weight of mouse (Log:Line 3)	
<b>3. Dissect out jejunum</b>		-Remove 2 cm of duodenum -Collect first 10 cm of jejunum		Cut intestine at pyloric sphincter and caecum. Measure and remove first 2 cm of duodenum. Collect first 10 cm of jejunum. Be careful not to stretch.
<b>4. Flush intestine</b>	Clear out fecal matter			Flush with ice cold PBS from proximal end using a pipette tip attached to a 10 mL slip tip syringe
<b>5. Cut open intestine, and lightly swirl in ice cold PBS in dish with tweezers</b>	Remove any remaining fecal matter			Mark proximal end of intestine by cutting a small slit at the end. Fillet open the intestine by cutting down the length of the intestine through the lumen.
<b>6. Place intestine in dissociation reagent #1 and place on ice</b>	Remove calcium ions from epithelium and prime crypt cells for release from intestinal tissue	-On ice for 20 minutes -10 mL of dis reagent #1 (PBS, 30mMEDTA, and 1.5 mM DTT)		
<b>7. Weigh conical tube #2 alone, and conical tube with dissociation reagent #2</b>	For later weight recordings and calculations	Exactly 5 mL of dis. reag. #2	Weight of conical tube #2 alone, and with dissociation reagent #2 (Log:Lines 5 and 6)	
<b>8. Place in dissociation reagent #2 and incubate</b>	Release cells from intestinal tissue	-Incubate at 37° C for 8 min. -dis reag. #2 (PBS and 30 mM EDTA)		

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<b>9. Shake conical tube for 30 seconds</b>	Remove crypts from the rest of the intestinal tissue	Use accelerometer to standardize shaking intensity -Shake until solution becomes cloudy with cells, and tissue begins to sink toward middle of the solution	Log frequency of shake (See Figure 1, Log:Line 4)	Shake conical tube for 30 seconds at the proper acceleration and frequency (view video and practice with iPhone and Context Logger).
<b>10. Remove intestinal remnant tissue and place in separate conical tube of dis. reagent #2</b>	To determine the amount of tissue dissociated from muscularis.			Ensure that all of the solution remains in the tube, and not on the remnant tissue. Excess fluid clinging to tissue will skew the measurements.
<b>11. Weigh original conical tube with dis. reagent #2 and dissociated tissue</b>	Ensure an adequate amount of cells have been removed. Should be about 0.3-0.5g.		Weigh cells and dissociation reagent #2 (Log:Line 7)	
<b>12. If necessary, place intestinal remnant tissue back in original conical tube and shake for 15 seconds.</b>	Remove additional epithelial cells from intestinal basement membrane			Shake conical tube for 15 seconds at the proper acceleration and frequency
<b>13. Fix intestinal remnant tissue (muscularis) in PFA</b>	Verify the amount of crypts removed from intestinal tissue. Although optional, assessing the amount of epithelium removed can be valuable for troubleshooting.		Optional: Determine Percent of crypts removed (See Figure 2)	Fix in 4% PFA (in PBS) overnight, then 30% sucrose (in water) for 1-2 days. Swiss role intestine and embed in OCT Compound. Freeze. Cut sections, stain (On 2 slides: (1) H&E and (2) EpCAM/DAPI), photograph entire section and analyze percent of crypts removed by measuring distances in Adobe Photoshop or Image J. See Figure *** for protocol.
<b>14. Centrifuge solution</b>		1280g at 4° C for 5 minutes		
<b>15. Remove supernatant</b>	Stop EDTA and			

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and rinse cells with 10 mL of 1X PBS at 4° C and 1mL FBS	DTT reaction process			
16. Centrifuge solution		1280g at 4° C for 5 minutes		
17. Remove supernatant, and centrifuge solution again	Ensure accurate pellet amount	1280g at 4° C for 1 minute		
18. Remove supernatant and weigh pellet			Weigh pellet (See Figure 3, Log:Line 9)	Turn open conical tube upside-down and dab on a paper towel to ensure all supernatant has been removed
19. Add 10 mL PBS at 4° C, 1mL FBS, and 50 uL DNaseI (2000 U/mg, 10 mg/mL)	Prevent clumping of cells			Mix well to ensure DNase is properly distributed
20. Aliquot 5 drops of 10 uL of solution	Crypt counting		Count # of crypts in individual drops (See Figure 4) Record #; average and std. dev. (Log:Lines 11 through 17)	Create a 1:5 dilution of cells to have a proper density of cells to count. Invert tube twice each time before pipetting 10 uL from center of solution to ensure sample is homogenous and representative samples are taken.
21. Filter crypt solution through 100 um and 70 um filters	Removes villi and large clumps of cells			
22. Aliquot 5 drops of 10 uL of solution	Crypt counting		Count # of crypts in individual drops (See Figure 4) Record #; average and std. dev. (Log:Lines 18 through 24)	Create a 1:5 dilution of cells to have a proper density of cells to count. Invert tube twice each time before pipetting 10 uL from center of solution to ensure sample is homogenous and representative samples are taken.
23. Centrifuge crypt solution		1280g at 4° C for 5 minutes		

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<b>24. Remove supernatant and resuspend pellet in 10mL HBSS (minus Ca and Mg) with 0.3U/mL dispase</b>	Dissociates whole crypts to single cells	Incubate crypt/dispase solution in 37°C water bath for 10 min. Remove tube every 2 min. and shake vigorously.		For single cell dissociation with dispase, solution should be shaken as vigorously as possible 7 shakes/second – can measure on iPhone if necessary) for 15 seconds every 2 min. Unlike in step 7, we have not observed any detrimental effects in terms of cell viability associated with vigorous shaking. Rather, an increase of shaking force at this point in the prep appears to yield a more complete dissociation of crypts to single cells.
<b>25. Add 1mL FBS and 50uL DNaseI (10 mg/mL) to single cell solution</b>	Prevent clumping of cells			
<b>26. Centrifuge single cell solution</b>	Removes dispase	1280g at 4° C for 5 minutes		
<b>27. Resuspend pellet in 10mL HBSS (minus Ca and Mg)</b>				
<b>28. Aliquot 5 drops of 10uL of solution</b>	Assess level of dissociation		<b>Count single cells and cell aggregates. Compare ratio of single cells to cell aggregates.</b>	Invert tube twice each time before pipetting 10 uL from center of solution to ensure sample is homogenous and representative samples are taken. If ratio of cell aggregates to single cells is not satisfactory, dispase concentration may have to be optimized per manufacturer to obtain a more fully dissociated prep.
<b>29. Filter single cell solution through 40um filter</b>	Removes cell aggregates and non-dissociated crypts			
<b>30. Centrifuge filtered single cell solution</b>		1280g at 4° C for 5 minutes		

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<b>31. Resuspend single cells in appropriate volume of IESC sorting media for immediate FACS isolation/flow analysis or labeling with primary conjugated antibodies (if not staining, skip to step 38)</b>	Exposes cells to essential nutrients in media and anoikis inhibitor and helps preserve viability during staining and/or sort.	<b>IESC sorting media:</b> (Adv. DMEM/F12 (Gibco # 12634), + B27 w/o Vitamin A (50X; Invitrogen # 12587010), + N2 (100X; Invitrogen # 17502048), +2mM L-glut, +Pen (0.1 mg/ml) /Strep (0.25µg/ml), + 10mM HEPES (Gibco # 15630-106), +10µm Y27632 (Sigma Cat. # Y0503)		When resuspending cells, add enough media so that final [c] is approx. $1.0-2.0 \times 10^7$ cells/mL. Keep cells on ice until immediately before analysis or sort
<b>OPTIONAL</b>  <b>32. If labeling with antibodies, add 10% FBS to single cell suspension and aliquot into individual FACS tubes for staining as called for by labeling protocol</b>	FBS blocks non-specific binding of primary conjugated antibodies			
<b>OPTIONAL</b>  <b>33. Stain cells as per standard operating procedure for antibody labeling for flow cytometry</b>				Keep cells on ice for duration of labeling
<b>OPTIONAL</b>  <b>34. Centrifuge stained</b>		460g at 4° C for 5 minutes		

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single cell solution				
<b>OPTIONAL</b> <b>35. Remove supernatant and rinse in excess volume of IESC sorting media without FBS (approx 3mL)</b>	Remove any unbound antibodies from sample to ensure “clean” analysis by flow			
<b>OPTIONAL</b> <b>36. Centrifuge stained single cell solution</b>		460g at 4° C for 5 minutes		
<b>OPTIONAL</b> <b>37. Resuspend pellet in IESC sorting media without FBS</b>				
<b>38. Prepare sample and stain with Trypan Blue, and count dead cells</b>			% of Trypan + and – cells (Log:Lines 25 and 26)	Make a dilution of 1:50 of cell solution. Combine equal parts Trypan Blue and cell solution. Place 10 uL of the combined solution onto a hemacytometer and count all + and – cells within the grid.
<b>39. Add PI</b>	Determine viability from prep	1ug/mL	Log time (Typically it takes us 3 hours from Death of animal to this point) (Log:Lines 27 and 28)	
<b>40. Analyze and/or sort cells</b>		Follow gating parameters depicted in figures 7-10. Sort for 2 hours. From time of death to analysis of cell viability should be no	Log: 1) Time of death of animal to sort start time. 2) Length of sort. 3) % of PI + and – cells from a	If sorting, cells should be collected into FACS tubes containing 500uL IESC sorting media with 1µg/ml PI. Collect on ice. Collect 50,000 cells.

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		more than 4 hours.	PI/FSC-Height histogram (see Figure 10) 4) total number of cells collected. 5) flowrate of instrument (Log:Lines 29 through 35)	
<b>41. Post-sort analysis</b>	To assess post-sort viability		Collect at least 25,000 cells and log: 1) % PI + and – cells. (Log:Lines 36 and 37)	