

Procedure for Mouse Intestinal Mesenchyme Isolation

Li Lab - Stowers Institute; Version1

(This protocol was adapted from Stzepourginski et al. 2015; PMID: 25599879 DOI: 10.1016/j.jim.2014.11.013)

A. Preparation of Mouse Intestine

- 1) Prepare mouse 3 mice (6-8 weeks old) for sterile extraction of intestine by shaving the abdominal area and prepping the skin with Betadine Solution
- 2) Using sterile technique in a laminar flow hood, excise entire intestine.
- 3) Remove Peyer's patches, eliminate majority of bowel contents by squeezing down the length of intestine and discard.
- 4) Remove as much mesenteric fat/connective tissue as possible. Leftover tissue can cause clumping, filter clogging, and generally make your life miserable later.
- 5) Set up 5-6 60mm, sterile disposable petri dishes per mouse on ice for cold, serial washing of the intestine.
- 6) Open intestine longitudinally using a small scissors and forceps, and wash in calcium- and magnesium-free 4°C, HBSS without Phenol Red until all fecal matter and mucus have been removed.
- 7) Cut each intestine into 2 cm pieces, and transfer to pieces from individual intestine into a separate 50mL conical tube incubate for 20 minutes at 37 °C in 30mL of calcium- and magnesium-free HBSS containing 10 mM EDTA under gentle agitation (80 rpm.)

Tips from Darrick: Dissociation of epithelium from mesenchyme. Be sure that it is in 37°C HBSS for the whole 20 minutes. Samples that go straight from the ice to the shaker have a greatly reduced amount of epithelial cell dissociation. I always stop at 20 minutes for reproducibility and viability reasons. The only cause for concern is if the supernatant looks extremely clear after your first or second wash in the next step. However the only real issue is there will be a greater number of epithelial cells during the FACS part of the experiment. But since you will be negatively selecting against them with the EPCAM antibody I've never seen it be an issue)

B. Removal of Epithelial Cells Post Chelation

- 8) After chelation process, remove epithelial cells by vortexing tissue pieces in 30mL of calcium- and magnesium-free HBSS and discard the supernatant by aspirating with a pipette or pouring the through a Moria Spoon.

(Tips from Darrick: We have a Moria Spoon that we pour it through, but we have also used a serological pipettes to aspirate it out. This is really dependent on the tools you have at hand. Leave as little supernatant as possible, and there is no need to spin.)

- 9) Repeat with 30mL of fresh calcium- and magnesium-free HBSS and repeat until obtaining a clear supernatant devoid of epithelial cells (~ 3 times).

(Tips from Darrick: It might be a good idea to do a practice run where you wash at least 5 times to see just how clear it gets at its cleanest.)

C. Isolation of Mesenchymal Cells

- 10) Transfer individual mouse samples from a 50mL conical to a GentleMACS Dissociator tube (see Supply Table for vendor and Catalog#) using a moria spoon or other device to minimize the amount of fluid transferred and add 5 mls of Dissociation Mix.
- 11) Place tubes on the GentleMACS Dissociator mouse ("m") intestine program and run once before the initial incubation.
 - a. Remove tubes from machine and incubate for 10 minutes at 37 °C in 5mLs of Dissociation Mix.

- i. At the 5 minute mark during incubation, place tubes on GentleMACS Dissociator and again run the mouse ("m") intestine program, then return to water bath for the remaining 5 minutes.
- b. After 10 minutes, harvest as much supernatant as you can (without disturbing the larger tissue pieces at the bottom of the tube) and transfer to a new 50mL collection conical, add 5mL of Stop Solution and store on ice.
- c. Top up the Dissociator tube with 5mL of fresh Dissociation Mix.

(Tips from Darrick: We've tried a variety of times and concentrations of Liberase in step 5. The concentration listed is the highest we've gone, however we've used as little as one quarter that amount on some samples. It really depends on the fragility and abundance of the cell type you are trying to isolate. Higher concentrations with longer incubation times typically yield more cells so are better for lowly abundant cell types as long as they aren't particularly fragile. Lower concentrations and shorter times are suitable for abundant cell types and allow for faster tissue processing.)

- d. Repeat steps a. through c. 3 times for a total time of ~30 minutes, replacing the tubes on the GentleMacs Dissociator at each 5 minute mark, collecting the digested supernatant at each 10 minute time point and transferring to the collection conical for that mouse.

(Tips from Darrick: I think it's important to mention here that if you don't have a GentleMacs Dissociator, you can keep the samples in a 50mL conical tube and use a 10mL serological pipette to gently pipette the tissue pieces up and down several times every 5 minutes. If you do use serological pipettes it's a good idea to use scissors to chop the 2cm pieces down to >0.5cm pieces immediately before step 10.)

- 12) After completion of the 3 cycles, filter the dissociated tissue in the collection tube through a 70µm mesh or filter, using the rubber end of a syringe plunger to mechanically dissociate any large tissue pieces that the filter collects. Rinse with Stop Solution and collect into a new 50mL conical.
- 13) Spin down the filtered cellular suspension @600g for 5 minutes at 4°C.
- 14) Discard supernatant and floating debris by decanting, add 5mLs of DMEM or Stop Solution, and resuspend pellet by gently shaking. Then top to 40 ml with additional DMEM or Stop Solution.
- 15) Repeat spin @600g for 5 minutes at 4°C. Decant supernatant and dry off the inside of the tube with a kimwipe to remove as much fluid as possible without disturbing the pellet.

D. Cell Counting and Preparation for Antibody Staining of Mesenchymal Cells

- 16) Resuspend washed pellet in 500µL of Staining Buffer, pass suspension through a pre-wetted 70µm syringe filter, and collect in flow tubes.

(Tips from Darrick: For a large pellet, increase staining buffer amount to 1000µL. If you had to err on the side of caution I'd go with 500µL, then dilute if necessary after counting. The danger of resuspending in too small of a volume is it will tend to clump and you'll lose more cells during filtration. At too high a volume the staining volume is increased, or you have to spin them down again which stresses the cells even more.)

- 17) In order to count cells, remove 20µL of cell suspension and add to flow tube containing 180µL of staining buffer. Add 2µL of .1 mg/mL of Dapi. Let sit for 10 minutes before counting cells on a suitable cytometer. (Note: Remember to account for the 10 fold dilution.)
 - a. If necessary, dilute sample to 10e6 cells per ml for staining

- 18) Add Antibody for the cell type of interest.

(Tips from Darrick: I've found staining 5e6 cells per sample is sufficient to get the 1000 cells per biological replicate we've needed, and I usually do this in 500µLs. For color compensation controls I use 2.5e5, and for isotype controls I use 5e5, both in 200µL of staining buffer.)

- 19) Allow cells to stain in Antibody solution, on ice for 30 minutes.

- 20) Wash cells by topping up flow tubes with staining buffer (usually 3.5mL), and spinning down at 500g for 5 minutes. Resuspend in 200 μ L for color compensation and isotype controls. For samples, volume will vary based on total number of cells stained.

(Tips from Darrick: To save on cost I've also diluted my staining solution 10x with HBSS w/ca&mg and used that for the wash step as they are only in it for 5 minutes. It can get expensive if you have a lot of samples to wash.)

- 21) Proceed to sorting procedure as soon as possible after staining, holding cells on ice until sort begins.

(Tips from Darrick: Our Sorter can handle about 10 e6 cells per ml, but the person doing the protocol should know what concentration is ideal for the machine and what the flow rate is. I find on our machine as long as I keep it between 5-10 e6 cells per ml and run it at a fairly fast flow rate (e.g. 3000-4000 events a second) it won't clump and I get the necessary number of cells fairly quickly and efficiently.)

Reagents						
Solution	Vendor	Catalog#	Reagent	Amount /Reagent/Mouse	Total/Experiment (3 mice)	
Rinse Solutions (Stock)	Lonza	04-315Q	Hanks Buffered Salt Solution (HBSS) wo Phenol Red; wo Ca & Mg	1L - 2L @4°C; 1L @ 37°C	3L - 6L @4°C; 3L @ 37°C	
Chelation Solution	Lonza	04-315Q	Hanks Buffered Salt Solution (HBSS) wo Phenol Red; wo Ca & Mg	49mL @37°C (from Stock)	147mL@37 °C (from Stock)	10mM - Final Working Solution
	no preference		EDTA – Store Stock @ 0.5 M, pH-8.0	1 mL of EDTA Stock	3 mL of EDTA Stock	
Dissociation Mix	Gibco	11995-065	DMEM	15mL	45mL	
	Roche	5401020001	Liberase TL	1 Wunsch unit /mL	45 Units/45mL	
	Worthington	LS006362	Dnase I	10U/mL	450 U/45mL	
Stop Solution	Gibco		DMEM	9mL	27mL	
	Peak Serum	PS-FB2	Fetal Bovine Serum (FBS)	1mL (10%)	3mL	
Staining Buffer (Total amounts needed vary /antibody panel)	Lonza	10-527F/12	Hanks buffered Salt Solution (HBSS) wo Phenol Red w Mg, Ca	4.8mL	48 mL	For normal antibody panel make a total of 50mL as a Working Solution
	Peak Serum	PS-FB2	Fetal Bovine Serum (FBS)	150µL (3%)	1.5mL	
	Sigma	Y0503	Y-27632 *	50µL	500µL	
Optional Supplies						
Item	Vendor	Catalog#	Description		Cost	
GentleMACS Dissociator Tubes	Miltenyi Biotec	130-096-334	25 C-tubes/single packed; sterile		\$150	
		130-096-334	100 C-tubes/ 4 x 25 packed sterile		\$560	
GentleMACS Dissociator Machine	Miltenyi Biotec	130-093-235 (130-095-937 130-096-427)	Automated, programmable tissue dissociator		Upon request and style	
Moria Perforated Spoon	Fine Science Tolls	10370-17	20mm tip, 5mm depth		\$219	

Antibody Chart				
Cell Type	Antibody	Vendor	Catalog #	Volume/ 1e6 cells
Endothelial	Cd31-APC	ebioscience	17-0311-80	0.5 µL
Epithelial	Epcam-APC	biolegends	118213	3.3 µL
Erythrocytes	Ter119-A7	biolegends	116223	3.3 µL
Hematopoietic	Cd45-A7	biolegends	103153	2.0 µL
PDGFRα +ve	PDGFR-α	biolegends	135905	3.3 µL

*(Tips from Darrick: The first four are our basic panel, all but the PDGFR-α are negative selectors. Though when I have needed to substitute a fluorophore or manufacturer I've never run into a problem or needed to adjust the volumes. I've put my own personal titrations here, but I really feel like people should do their own to be thorough and really know their own patterns due to variations in animals, and the inherent variability of the process. For the positive marker all cells of interest we've used a genetic marker, with the exception of PDGFR-α. * Freeze [Y-27632](#) 1M Stock (320µg/mL=100x) @ 500µL in microfuge tubes)*