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Rapid Isolation and Flow Cytometry Analysis of Murine Intestinal Immune Cells after Chemically Induced Colitis

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[Abstract] Chemically induced murine colitis models are widely used to understand intestinal homeostasis and inflammatory responses during acute and chronic gut inflammation such as Inflammatory Bowel Disease (IBD). Resident populations of immune cells together with those recruited during an inflammatory response maintain intestinal immunity by mounting an effective immune response to enteropathogenic microbes while at the same time maintaining tolerance against commensals. To better understand the disease mechanism, the study of different immune cell populations and their dynamic changes during infection and inflammation is essential. However, isolating healthy and viable immune populations, particularly hyperactivated neutrophils and macrophages from the inflamed gut (*i.e.*, active disease site), is very challenging as tissues are usually subjected to rigorous enzymatic digestion for an extended period. Here, we describe a method that uses a cell dissociator (Medimachine II from Syntec International) to separate intestinal tissue after short enzymatic digestion to obtain a single-cell suspension. This technique facilitates the isolation of immune cells from mouse intestinal tissues at high quantity and with superior viability in a very short time frame. This protocol delivers 80 to 90% cell viability that is 1.5 to 2-fold higher when compared to conventional methods of isolating cells from inflamed mouse colons. The composition, phenotype, activation state, and gene expression profile of cells isolated using this protocol can be assessed by using multiple methods including, but not limited, to flow cytometry, quantitative PCR, and functional readouts such as reactive oxygen species (ROS) production.

Keywords: Immune cells, Medimachine II, Lamina propria, Colitis, Cell viability, Flow cytometry

[Background] An intricate network of various immune cells and secreted cytokines and chemokines function in cooperative ways to protect the host from adverse environmental conditions and microbial infections to maintain or restore homeostasis (Hooper and Macpherson, 2010). The GI tract is constantly exposed to commensal, opportunistic, and pathogenic microbes, creating perturbations that can lead to life-threatening infections and chronic inflammation. The host defense system in the gastrointestinal (GI) tract is comprised of a single layer epithelium fortified with adjoining cells and lymphoid tissues that operate efficiently to maintain barrier homeostasis. This barrier strikes a balance of remaining tolerant to commensal microbes and dietary antigens while preserving the capacity to mount an inflammatory response against pathogens.

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IBD, a general term for chronic inflammatory conditions of the GI tract, is comprised of two main clinical entities: Crohn's disease and ulcerative colitis. A broadly compromised or dysregulated immune system due to environmental changes, genetic background, or a qualitative and quantitative abnormal gut microbiota is a hallmark of IBD pathogenesis (de Souza and Fiocchi, 2016). Despite several decades of research, our understanding of onset, recovery, and recurrent flares in IBD remains tenuous. Recruitment and activation of immune cells at inductive sites [Peyer's patches (PP); isolated lymphoid follicles (IELs); and mesenteric lymph nodes (MLN)] and initiation of effector functions in the lamina propria and epithelium is a sequential and complex process. In chronic disease conditions, constant activity of immune cells (*i.e.*, ROS generation, chemokine, and cytokine secretion) in conjunction with architectural damage of tissues further induces immune cell recruitment, hence forming a perpetual destructive loop (Brandtzaeg *et al.*, 2008). To fully comprehend the spatiotemporal dynamics of transmigrated cells and their crosstalk with other cell types, efficient isolation and characterization of distinct immune cell populations is essential. A relatively simple and rapid protocol for isolating immune cells from intestinal tissue will be highly enabling as intestinal immunity is at the center stage in IBD, colorectal cancer (CRC), or host-microbe interactions. Isolating healthy and viable cells from inflamed gut, including hyperactivated neutrophils and macrophages in early acute inflammation or various T lymphocytes at later stages of the disease, is challenging but crucial for immunophenotyping and functional assays of selected cell populations. The majority of the existing protocols describes isolation of cells from normal (non-inflamed) tissues during homeostasis (Weigmann *et al.*, 2007; Geem *et al.*, 2012; Reißig *et al.*, 2014); however, those that isolate from inflamed tissues have extended digestion period and often lack in viability data (Drakes *et al.*, 2004; Bowcutt *et al.*, 2015; Li *et al.*, 2016, Gui *et al.*, 2018).

This protocol describes a straightforward method for rapid isolation of highly viable immune cells from murine intestinal tissues such as colonic and ileal lamina propria, PP, and MLN using Medimachine II (Syntec International, Ireland). This protocol has also been used to isolate viable cells from the murine spleen and can easily be adapted to phenotype diverse cell populations from human tissues. Here, we used chemically induced models of acute colitis in mice, such as 2,4,6-trinitrobenzene sulfonic acid (TNBS) and dextran sodium sulfate (DSS) treatment, for isolating cells from colonic lamina propria at the peak of disease.

Materials and Reagents

1. 1- and 10-ml syringes (sterile)
2. 100 µm, 70 µm, and 40 µm cell strainers (sterile)
3. 15- and 50-ml tubes (sterile, without skirts)
4. 6-well sterile tissue culture plates (Fisher Scientific, catalog number: 10578911)
5. C57BL/6J mice (The Jackson Laboratory, USA)
6. 70% industrial methylated spirit (70% IMS)

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7. Anti-mouse CD45, Brilliant Violet 510 antibody (BioLegend, clone 30F11, catalog number:103137)
8. Anti-mouse CD11b, APC (BioLegend, clone M1/70, catalog number: 101211)
9. Anti-mouse Ly6G, PE antibody (BioLegend, clone 1A8, catalog number: 127607)
10. Collagenase D (Sigma-Aldrich, catalog number: 11088866001), stock (500 mg/10 ml HBSS)
11. Dispase (Corning, catalog number: 354235), stock (5,000 U/100 ml HBSS)
12. DNase I (Sigma-Aldrich, catalog number: 10104159001), stock (100 mg/10 ml HBSS)
13. DPBS, calcium and magnesium-free (Thermo Fisher Scientific, catalog number: 14190144)
14. EDTA, 500 mM in H_2O, sterile filtered (Sigma-Aldrich, catalog number: E4884-500G)
15. FBS, heat-inactivated (Sigma-Aldrich, catalog number: F7524-500ML)
16. HBSS, calcium and magnesium-free (Thermo Fisher Scientific, catalog number: 14175095)
17. HEPES solution (Sigma-Aldrich, catalog number: H0887-100ML, 1 M)
18. Liberase TM (Sigma-Aldrich, catalog number: 5401119001), Stock (5 mg/2 ml HBSS)
19. Non-essential amino acids (Sigma-Aldrich, catalog number: M7145-100ML, 100×)
20. Sodium Azide (NaN_3), 100 mM solution in sterile water (Sigma-Aldrich, catalog number: S2002-25G)
21. Penicillin-streptomycin (Thermo Fisher Scientific, catalog number: 15140122, 10000 U/ml)
22. Red blood cell (RBC) lysis buffer (Thermo Fisher Scientific, catalog number: 00-4333-57)
23. RPMI-1640 with L-glutamate, without phenol red (Gibco, catalog number: 11835-063)
24. Sodium pyruvate (Sigma-Aldrich, catalog number: S8636-100ML, 100 mM)
25. Trypan Blue solution (Sigma-Aldrich, catalog number: T8154-20 ML)
26. ViaKrome 808 Fixable Viable Dye (Beckman Coulter, catalog number: C36628)
27. Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific, catalog number: 65-0865-14)
28. Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) (BD Biosciences, catalog number: 553142)
29. eBioscience™ flow cytometry staining buffer (Thermo Fisher, catalog number: 00-4222-26)
30. eBioscience™ intracellular (IC) fixation buffer (Thermo Fisher, catalog number: 00-8222-49)
31. Epithelial removal solution (500 ml) (see Recipes)
32. Washing solution (500 ml) (see Recipes)
33. 2x Digestion solution (250 ml) (see Recipes)
34. Complete RPMI with FBS/cRPMI (500 ml) (see Recipes)
35. Digestion solution (10 ml) (see Recipes)
36. Flow Cytometry Staining Buffer/FACS buffer (see Recipes)

Equipment

1. Beckman Coulter CytoFLEX LS flow cytometer (or any other compatible cytometer)
2. Light microscope
3. Medimachine II® (Syntec International)
4. Swing-bucket Eppendorf® cooling centrifuge

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5. Tissue culture incubator set to 37°C
6. Water bath set to 37°C
7. Vortex mixer
8. Hemacytometer (Sigma-Aldrich, catalog number: Z359629-1EA)
9. Mouse dissecting tools
10. Medicons and Medicons Max (Syntec International, catalog number: 79500 S)

Procedure

1. Euthanize C57BL/6 mice either on day 8 of oral DSS treatment or on day 2 of intrarectal TNBS instillation.
2. Spray 70% IMS on the mouse abdomen and dissect out the small or large intestine. Remove surrounding connective tissue quickly and keep the intestinal tissue in 2 ml ice-cold **cRPMI** in a 6-well plate with a lid. Move to Tissue Culture Room to use Biosafety cabinet Category 2.
3. Cut the intestine open longitudinally to wash out feces with Washing solution (room temperature, RT). Remove excessive liquid by using blotting paper (or Kim wipes), cut tissues into approximately 1 cm pieces, and immerse them in pre-warmed Epithelial removal solution (30 ml/ colon in a conical tube). Place horizontally in the incubator on a shaker at 37°C, 200 rpm for 10 min.

   Notes:

   a. *The duration of the incubation should be optimized for the selected tissue. Examples are 15 min for untreated mouse ileum and 5-10 min for inflamed and damaged colon (after chemical colitis treatment with DSS or TNBS).*
   b. *If working with Peyer's patches or spleen, skip steps 2 and 3 and directly proceed to step 5. No digestion is required for MLNs (as cells are inside the sac); hence, directly proceed to step 6 (i.e., by placing MLNs directly in Medicons).*
4. Collect tissues and wash with 30 ml Washing solution (RT) to remove EDTA. Collect the tissue in a sterile petri dish for the next step.
5. Cut intestinal tissues into smaller pieces on a sterile plastic petri dish using a sterile scalpel. Mix minced tissue with 5 ml of Digestion solution (freshly prepared within 30 min and pre-warmed at 37°C in the water bath) in 50 ml tubes. Place tubes vertically in tissue culture incubator (37°C, no shaking) for 10 min. Peyer's Patches can directly be placed in the 1-2 ml Digestion solution without any mincing.

   Note: For intestinal lamina propria cell isolation use Medicon MAX with 16 ml volume capacity. For other tissues such as Peyer's patches or smaller organs use Medicons with 1.6 ml capacity (up to 5 Peyer's Patches and all MLNs per one Medicon).
6. Vortex the tubes at maximum speed for 5-10 s and collect the supernatant (containing some single cells) in a fresh 50 ml collecting tube using a 100 µm cell strainer. Move the tissue from the cell strainer into Medicons MAX by carefully placing them on the sieve using sterile tweezers

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and add 15-16 ml c-RPMI (8-10°C) through the collecting port using a syringe. Place the Medicon MAX into the corresponding slot of the Medimachine II and dissociate cells for 10 s at medium speed (10,000 rpm). Collect the liquid from the Medicons using a sterile syringe and combine it into the collecting tube above by using a 100 µm cell strainer. Add cRPMI (8-10°C) to a final volume of 45 ml and keep the tube on the ice until the next step.

Note: Move Peyer's Patches directly into the Medicons from the digestion solution without vortexing, add 1.6 ml c-RPMI (8-10°C) and proceed for cell dissociation as described above in step 5. As mentioned MLNs do not need digestion and thus move all MLNs directly into the Medicons. Collect cells through 70 µm cell strainer into 15 ml tubes and add c-RPMI to a final volume of 10 ml.

7. Centrifuge the collecting tubes containing cells at 450 × *g* for 15 min at 10°C. Discard the supernatant and proceed with the remaining cells to red blood cell lysis using RBC lysis buffer if required (see note below). Resuspend the pellet carefully with 35 ml cRPMI (8-10°C) and filter through a 40 µm cell strainer into a new tube.

   Note: If the pellet has reddish coloration, it usually requires RBC lysis. This is often observed in inflamed colonic tissues. Gently resuspend the cell pellet in 500 µl of ice-cold RBC lysis buffer and allow lysis for 3-4 min at room temperature. Add 10 ml cRPMI (8-10°C) and centrifuge at 450 × g for 15 min at 10°C. Wash once more with 10 ml cRPMI (8-10°C) before proceeding.
8. Centrifuge tube at 450 × *g* 10°C for 15 min, discard the supernatant, and resuspend the pellet in the desired volume of c-RPMI (for example, 2 ml for colonic or ileal lamina propria and 1 ml for Peyer's Patches or MLNs).
9. Viability can be immediately assessed by the Trypan Blue exclusion test. Mix cell suspension and Trypan blue in 1:1 ratio, load mixture to haematocytometer, and count cells under a light microscope. Viable cells are impermeable to Trypan Blue, whereas the dye permeates into dead cells, which appear blue under a light microscope.

   For flow cytometry analysis, cells can be stained with chosen dyes and antibodies, as demonstrated in this study. A brief protocol for staining for flow cytometry is as follows.

   a. Resuspend 10^6 cells in 100 µl 10 mM HEPES buffer containing live dead dye in concentration suggested by the manufacturer. Incubate for 15 min in the fridge followed by washing with 10 mM HEPES and centrifuging at 450 × *g* at 10°C for 6 min.
   b. Prepare 1:100 dilution of Fc-blocking solution in FACS buffer (anti-mouse CD16/CD32 antibodies cocktail). Add 10 µl/tube Fc-blocking solution and mix well. Incubate at room temperature for 5 min and add antibodies directed to cell surface proteins in suitable concentrations (CD45; 1:100, Ly6G; 1:50, CD11b; 1:100). Add flow cytometry staining buffer up to 100 µl. Incubate in the dark at 4°C for 20 min followed by washing twice with FACS buffer and centrifuging at 450 × *g* at 10°C for 6 min.
   c. To fix and store stained cells for analysis later, dilute Intracellular (IC) fixation buffer with FACS buffer in a 1:1 ratio. Resuspend the cell pellet obtained above with 100 µl of diluted IC fixation buffer. Keep in the dark at 4°C until analysis.

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A summary of distinguishing steps and different volumes required for a variety of tissues (Colon, Peyer's patches, Spleen and MLNs) have been shown in Figure 1.

A rapid and improved protocol for isolation of immune cells from inflamed mouse colon, Peyer's patches, spleen, and mesenteric lymph nodes

	Steps	Colon	PP and Spleen	MLN
1- 2	Tissue collection	2 ml cRPMI ice cold	2 ml cRPMI ice cold	2 ml cRPMI ice cold
3	Epithelial removal	30 ml / colon, 200 rpm (horizontal shaker), 10 minutes, 37 °C	Not required	Not required
4	Washing	30 ml/ colon	Not required	Not required
5	Enzymatic digestion	5 ml/ colon (minced), 10 minutes, no shaking, 37 °C	1-2 ml, 10 minutes, no shaking, 37 °C	Not required
6	Load remaining tissue into Medicons	Vortex and collect supernatant, use Medicon MAX (16 ml)	Directly load tissue into Medicon (1.6 ml)	Directly load tissue in Medicon (1.6 ml)
	Dissociate	10 seconds	10 seconds	10 seconds
7	Washing and RBC lysis	450 g, 15 minutes, 10 °C RBC Lysis required	450 g, 15 minutes, 10 °C RBC Lysis required	450 g, 15 minutes, 10 °C RBC Lysis usually not required
8	Resuspend cells	2 ml cRPMI	1 ml cRPMI	1 ml cRPMI
9	Count cells and perform viability assay using Trypan Blue. Proceed to Flow cytometry or assay of choice			

Figure 1. Flow diagram of the isolation procedure (top) and representative images of Medimachine II and Medicons (bottom)

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Data analysis

1. In this study, colon samples were stained with ViaKrome 808 fixable viable dye and other samples (MLN, PP, and spleen) with Fixable viable dye eFlour 780. CD45 was used to detect all leukocytes, as shown in Figures 2 and 3. CD11b (Macrophages and Neutrophils) and Ly6G (Neutrophils) positive cells from colonic tissue (DSS- and TNBS-induced colitis) are shown in Figure 3. Consistent cell viability of 80-90% was observed in cells extracted from colonic Lamina propria after colitis. Distinct CD45, CD11b, and Ly6G positive populations are shown in histograms.

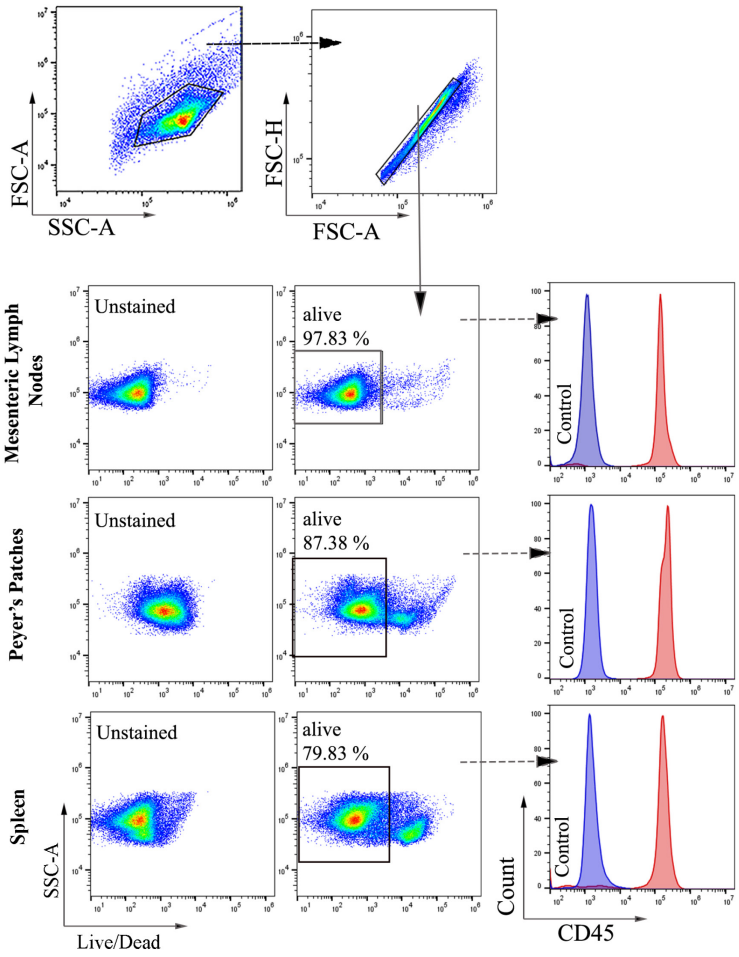

Figure 2. Representative images of flow cytometry analysis and gating strategy of cells extracted from Mesenteric Lymph Nodes (MLNs), Peyer's Patches (PP), and spleen (A) of untreated mice. Total cells were selected based on forward and side scatter, followed by selecting singlets. From singlets, the Fixable Viable Dye eFlour™ 780 negative population (alive) was selected for further gating of CD45 positive cells. Filled blue and red histograms represent unstained and CD45 Brilliant Violet 510 stained cells, respectively (n = 6).

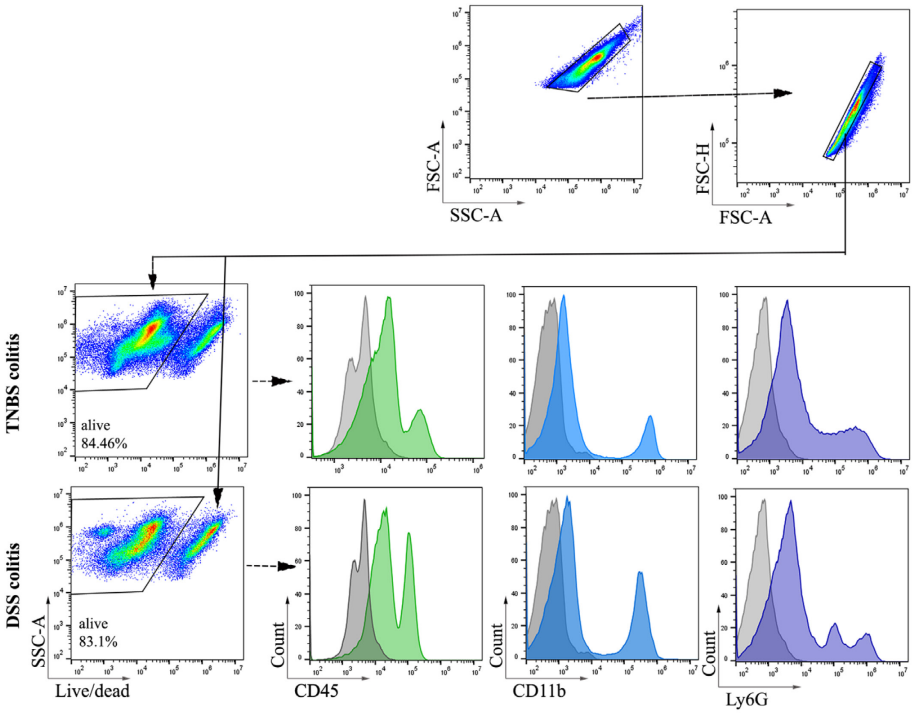

Figure 3. Representative images of flow cytometry analysis and gating strategy of cells extracted from C57BL/6 mice colonic lamina propria after DSS- and TNBS-induced colitis. Colonic tissues were harvested after 48 h (day 2) of 2.5% TNBS enema. For DSS-induced colitis, colonic tissues were collected on day 8 (6 days of oral 3% DSS treatment followed by water). Total cells were selected based on forward and side scatter, followed by selecting singlets. ViaKrome 808 Fixable Viable Dye negative populations (alive) derived from singlets were selected for gating of CD45, CD11b, and Ly6G positive populations. A filled grey histogram represents unstained (FMO) cells. Filled green, blue, and purple histograms represent CD45, CD11b, and Ly6G positive populations (n = 6).

2. Several widely used methods for isolating immune cells from intestinal tissues require the use of enzymes such as collagenases, dispases, and DNase I (Geem *et al.*, 2012; Goodyear *et al.*, 2014). These methods include digestion of intestinal tissues with these enzymes for 30-45 min at 37°C with vigorous shaking. The prolonged time required for cell isolation and the mechanical stress of shaking affect cell viability negatively (Figure 4) and, most importantly, can alter the activation state of innate immune cells (especially neutrophils and macrophages). The incubation time is the most prominent constraint. By reducing enzyme concentrations and exposure time together with an efficient extraction technique, we developed a fast and reliable method for the extraction of viable immune cell populations from the mouse intestine. In preliminary experiments, different incubation time and enzyme concentration was used, and we found the proposed duration and concentration optimal in our conditions. When establishing this protocol for the first time in the laboratory, crucial steps such as epithelium removal, tissue digestion, and dissociation using Medimachine II should be optimized for the specific tissue. For

example, when using a control (non-inflamed) colon, the duration of epithelium removal (step 2) should be increased and optimized to achieve complete removal of the epithelium. Likewise, if colonic tissue is expected to be severely damaged and massive loss of epithelium has been observed, it is recommended that the duration of the epithelial removal step (step 2) is decreased to prevent loss of lamina propria cells.

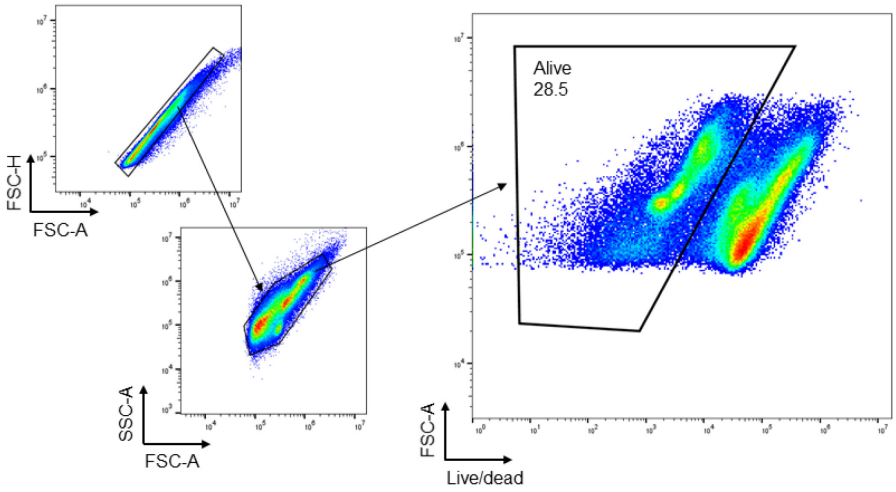

Figure 4. Representative images of flow cytometry analysis of cells extracted from TNBS-induced colitis mouse colon using the conventional method. Colonic tissues were harvested after 48 hours (day 2) of 2.5 % TNBS enema. After selecting singlets, total cells were gated, followed by gating live/dead cells. Fixable Viability Dye eFluor™ 780 was used to distinguish alive (negative staining) and dead (positive staining) cells (n = 3).

Recipes

1. Epithelial removal solution (500 ml)
   Store at 4°C for 2-3 weeks. Prewarm in the water bath at 37°C before use

Components	Volume required	Final concentration
1 M HEPES solution	5 ml	10 mM
500 mM EDTA	5 ml	5 mM
Penicillin-Streptomycin	1 ml	50 U/ml
HBSS	489 ml	--

2. Washing solution (500 ml)
   Store at 4°C for 2-3 weeks

1 M HEPES	5 ml	10 mM
Penicillin-Streptomycin	1 ml	50 U/ml
HBSS	494 ml	--

3. 2× Digestion solution (250 ml)
   Store at 4°C for 2-3 weeks

100× Non-essential amino acids	5 ml	1×
100 mM Sodium pyruvate	5 ml	2 mM
1 M HEPES	5 ml	20 mM
FBS (heat inactivated)	12.5 ml	10%
Penicillin-Streptomycin	1 ml	100 U/ml
RPMI-1640	221.5 ml	--

4. Complete RPMI with FBS/cRPMI (600 ml)

   Store at 4°C for 2-3 weeks

100× Non-essential amino acids	6 ml	1×
100 mM Sodium pyruvate	6 ml	1 mM
1 M HEPES	6 ml	10 mM
FBS (heat inactivated)	60 ml	10%
Penicillin-Streptomycin	1.2 ml	50 U/ml
RPMI-1640	520.8 ml	--

5. Digestion solution (10 ml)

   To be made fresh. Keep on ice until use. Prewarm in the water bath at 37°C before use

Dispase	2.5 ml	12.5 U/ml
Collagenase D	50 µl	250 µg/ml
Liberase TM	150 µl	37.5 µg/ml
DNase I	200 µl	200 µg/ ml
2× Digestion solution (Reagent 3)	5 ml	1×
RPMI-1640	2.1 ml	-

6. Flow Cytometry Staining Buffer/FACS buffer (100 ml)

FBS (heat inactivated)	2	2%
EDTA (500 mM)	0.4	2 mM
NaN_3 (100 mM)	2	2 mM
DPBS	95.6	-

Acknowledgments

Design of experiments and generation of data, AKS; Analysis of data, AKS and AB; Manuscript preparation, AKS and UGK; Specialized equipment, AB, RS; Financial support, RS and UGK.

This modified and optimized protocol is partially based on earlier works described by Goodyear *et al.* (2014) and Harusato *et al.* (2016).

Competing interests

AS received partial funding support from Syntec International for the project. RS is director of Syntec International.

Ethics

All animal experiments were performed in accordance with EU Directive 2010/63/EU, approved by the UCD Ethics Committee and authorized by the Irish Regulatory Authorities.

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