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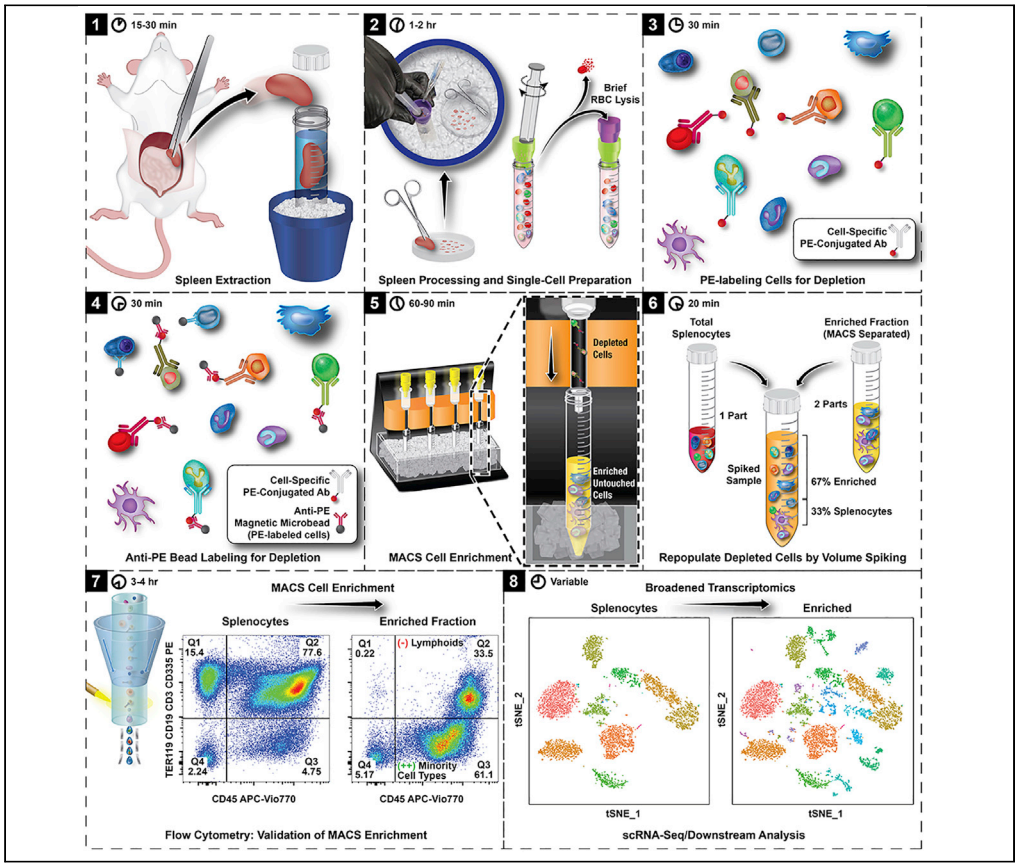
Mouse splenocyte enrichment strategies via negative selection for broadened single-cell transcriptomics



Mammalian splenic tissue is rich in functional immune cells, primarily lymphocytes which can mask low-abundance populations in downstream analyses. This protocol enriches minority immune cell populations from mouse spleen via immunomagnetic negative depletion to generate an untouched enriched cell fraction. Enriched cells are then spiked with untouched splenocytes in a controlled repopulation, validated by flow cytometry and results in a single-cell transcriptomic clustering analysis with a broadened cellular landscape.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to enrich minority cell types from total mouse spleen

Splenic cells are magnetically depleted of abundant lymphocytes

Abundant cells are partially spiked back to repopulate the cellular landscape

Single-cell transcriptomics permits expression analysis and confirmation of heterogeneity

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Mouse splenocyte enrichment strategies via negative selection for broadened single-cell transcriptomics

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SUMMARY

Mammalian splenic tissue is rich in functional immune cells, primarily lymphocytes which can mask low-abundance populations in downstream analyses. This protocol enriches minority immune cell populations from mouse spleen via immunomagnetic negative depletion to generate an untouched enriched cell fraction. Enriched cells are then spiked with untouched splenocytes in a controlled repopulation, validated by flow cytometry and results in a single-cell transcriptomic clustering analysis with a broadened cellular landscape.

BEFORE YOU BEGIN

While FACS approaches are valuable for separating cell populations, there are indications that this method introduces transcriptional changes in cell populations (Xiong et al., 2002; Romero-Santacreu et al., 2009; van den Brink et al., 2017; Nguyen et al., 2018). Density centrifugations (i.e., Ficoll and Percoll gradients) are also employed to obtain rarer cell types (i.e., neutrophils and eosinophils). However, these may be affected by imbalances of lower to higher density granulocytes, altered phenotypes affecting downstream function assays or marker expression by flow cytometry, transcriptional perturbations, and may ultimately result in less pure populations, especially when compared to improved magnetic cell isolation techniques, products, and protocols (Brattig et al., 1987; Sedgwick et al., 1996; Son et al., 2017).

Although others have used similar negative immunomagnetic cell separations (Hasenberg et al., 2011; Kamala, 2008), the present approach highlights the versatility of targeting cell populations by using a *single* fluorophore conjugated to antibodies that target undesired cells. This permits the use of fluorophore-targeted antibodies conjugated to magnetic beads that allow for negative depletion of undesired or highly abundant cell types. Importantly, negative selection by this method avoids any antibody-initiated binding to and activation of cell-specific receptors in the target population (i.e., "untouched"). Further, the use of a single, common fluorophore permits facile adjustment of the protocol to deplete different populations of cells. The outcome is the ability to study cell populations that were previously in such low frequencies as to make an analysis of these minor subsets unreliable.

A 10× Genomics single-cell RNA sequencing (scRNA-seq) dataset (named "Solvent") was generated by utilizing the method described herein from three 10 week old female Swiss-Webster (CFW) mice, treated via oral gavage with sterile 7% Tween-80, 3% ethanol, 90% deionized water (diH_2O), a common solvent. The resultant publicly available dataset can serve as a transcriptome control for comparison of immune-affecting treatments using strain-, age-, and sex-matched mice.

Institutional permissions

This protocol was developed using female CFW mice between 8–12 weeks of age from Charles River Laboratories, maintained on a 12-h light/12-h dark cycle, and allowed a minimum of three weeks to acclimate. All procedures were approved by the University of Nebraska Omaha Institutional Animal Care and Use Committee (IACUC, protocol #: 19-032).

Prepare solutions and buffers (day prior to experiment, day -1)

Timing: 2–4 h

Solutions and buffers should be prepared the day prior to the experiment (Day -1) due to the extensive procedures on the day of the experiment (Day 0).

Note: All required recipes to prepare solutions and buffers are provided in the materials and equipment section.

1. Prepare 50 mL aliquots of PBS, SES, ER Buffer, PEB Buffer, and 10×-SB per the recipes given in the materials and equipment section.

Flow cytometry antibody cocktails (day -1 or 0)

Timing: 2–4 h

Prepare the flow cytometry antibody staining cocktails before the experiment. These can be prepared earlier on the experiment day (Day 0) or the day prior to the experiment (Day -1).

⚠ CRITICAL: To preserve fluorophore brightness, prepare the flow antibody cocktail in a low light environment and keep all antibodies and cocktails on ice.

2. Prepare the Enriched flow antibody cocktail as shown below (Table 1).
   a. In this protocol, users will want to prepare for a total of 4 samples that receive the full panel cocktail in Table 1. These 4 samples are:
      i. Untouched splenocytes.
      ii. Enriched cells.
      iii. Spiked cells.
      iv. Viability fixable dye FMO sample (includes all the antibody-fluorophores but lacks the viability fixable dye which is added during the staining protocol).
   b. Also, prepare the FMOs by leaving out one unique fluorophore in the cocktail (i.e., FMO, "fluorescence minus one" fluorophore).
      i. This will result in 8 total Antibody-Fluorophore FMOs.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Ter-119 Antibody, anti-mouse, PE, REAfinity ™ Clone REA847	Miltenyi Biotec	Cat# 130-112-909; RRID: AB_2654115
CD3 Antibody, anti-mouse, PE, REAfinity™ Clone REA641	Miltenyi Biotec	Cat# 130-121-133; RRID: AB_2801803
CD19 Antibody, anti-mouse, PE, REAfinity™ Clone REA749	Miltenyi Biotec	Cat# 130-112-035; RRID: AB_2655822

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD335 (NKp46) Antibody, anti-mouse, PE, REAfinity™ Clone REA815	Miltenyi Biotec	Cat# 130-112-358; RRID: AB_2657602
CD45 Antibody, anti-mouse, APC-Vio® 770, REAfinity™ Clone REA737	Miltenyi Biotec	Cat# 130-110-800; RRID: AB_2658230
CD11c Antibody, anti-mouse, PerCP-Vio® 700, REAfinity™ Clone REA754	Miltenyi Biotec	Cat# 130-110-842; RRID: AB_2654717
CD11b Antibody, anti-mouse, VioBlue®, REAfinity™ Clone REA592	Miltenyi Biotec	Cat# 130-113-810; RRID: AB_2726327
Ly-6G Antibody, anti-mouse, FITC, REAfinity™ Clone REA526	Miltenyi Biotec	Cat# 130-120-820; RRID: AB_2784431
Siglec-F Antibody, anti-mouse, PE-Vio® 770, REAfinity™ Clone REA798	Miltenyi Biotec	Cat# 130-112-334; RRID: AB_2653445
CD193 (CCR3) Antibody, anti-mouse, APC, REAfinity™ Clone REA122	Miltenyi Biotec	Cat# 130-102-281; RRID: AB_2655879
FcεRIα Antibody, anti-mouse, PE-Vio® 615, REAfinity™ Clone REA1079	Miltenyi Biotec	Cat# 130-118-899; RRID: AB_2801723
CD117 Antibody, anti-mouse, APC, REAfinity™ Clone REA791	Miltenyi Biotec	Cat# 130-111-694; RRID: AB_2654592
Ly-6G Antibody, anti-mouse, PE, REAfinity™ Clone REA526	Miltenyi Biotec	Cat# 130-123-780; RRID: AB_2811549
CD11c Antibody, anti-mouse, PE, REAfinity™ Clone REA754	Miltenyi Biotec	Cat# 130-110-701; RRID: AB_2654708
CD115 Antibody, anti-mouse, PE, REAfinity™ Clone REA827	Miltenyi Biotec	Cat# 130-112-639; RRID: AB_2654553
Chemicals, peptides, and recombinant proteins		
Red Blood Cell Lysing Buffer Hybri-Max™	Sigma-Aldrich	Cat# R7757
autoMACS® Rinsing Solution	Miltenyi Biotec	Cat# 130-091-222
MACS® BSA Stock Solution	Miltenyi Biotec	Cat# 130-091-376
Hank's Balanced Salt Solution (HBSS), 1× without Calcium, Magnesium and Phenol Red	Corning	Cat# 21-022-CV
Cytiva HyClone™ HEPES 1 M Solution	Cytiva	Cat# SH30237.01
Trypan blue solution 0.4% (w/v) in PBS	Corning	Cat# 25-900-CI
Invitrogen™ UltraPure™ 0.5 M EDTA, pH 8.0, Sterile-filtered solution	Invitrogen	Cat# 15575020
Paraformaldehyde, 4% in PBS, Ready-to-Use Fixative	Biotium	Cat# 22023
PBS, Phosphate Buffered Saline, 10× Solution, Fisher BioReagents™	Fisher Scientific	Cat# BP3994
Fetal Bovine Serum, heat inactivated, certified, One Shot™, United States	Gibco	Cat# A3840001
Ethyl alcohol, Pure, 200 proof, for molecular biology	Sigma-Aldrich	Cat# E7023; CAS 64-17-5
"Spleen Extraction Solution" (SES)	This paper	Materials Section
"Enhanced Recovery Buffer" (ER Buffer)	This paper	Materials Section
Critical commercial assays		
MACS® Comp Bead Kit, anti-REA	Miltenyi Biotec	Cat# 130-104-693
Viobility™ 405/520 Fixable Dye	Miltenyi Biotec	Cat# 130-109-814
FcR Blocking Reagent, mouse	Miltenyi Biotec	Cat# 130-092-575
CD19 MicroBeads, mouse	Miltenyi Biotec	Cat# 130-121-301
Anti-PE MicroBeads UltraPure	Miltenyi Biotec	Cat# 130-105-639
Acridine Orange / Propidium Iodide (AO/PI) Cell Viability Kit	Logos Biosystems	Cat# F23001
HS NGS Fragment Kit (1–6,000 bp)	Agilent Technologies	Cat# DNF-474
Chromium Single Cell 3′ GEM, Library & Gel Bead Kit v3	10× Genomics	Cat# PN-1000092

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
NovaSeq 6000 S1 Reagent Kit (100 cycles)	Illumina	Cat# 20012865
Deposited data		
Mouse reference, mm10 (GENCODE vM23/Ensembl 98), dataset required for Cell Ranger	Obtained from 10× Genomics Software webpage	https://support.10xgenomics.com/single-cell-gene-expression/software/release-notes/build%22%20/l%20%22mm10_2020A
scRNA-seq reads referred to as "Solvent"	NCBI Repository	NCBI Sequence Read Archive: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA809678
Experimental models: Organisms/strains		
Mouse: CFW: Crl:CFW(SW)	Charles Rivers Labs	Strain code: 024; RRID: IMSR_CRL:024; Female 8–12 weeks old
Software and algorithms		
FlowJo Version 10.8.1	BD Biosciences	https://www.flowjo.com/solutions/flowjo/downloads
BD FACSDiva 8.0.2	BD Biosciences	https://www.bdbiosciences.com/en-us/products/software/instrument-software/bd-facsdiva-software
Ubuntu 20.04.2 LTS	Ubuntu	www.Ubuntu.com
R Software Version 4.0.3	(R Core Team, 2020)	https://www.r-project.org/
RStudio Version 1.4.1103	(RStudio Team, 2020)	https://rstudio.com/
Seurat Version 4.1.0 (R Package)	Paul Hoffman, Satija Lab and Collaborators	https://satijalab.org/seurat/
Cell Ranger Pipeline Version 4.0.0	10× Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/4.0
Loupe Browser Version 6.0.0	10× Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest#loupe
scCATCH v3.0 - Automatic Annotation on Cell Types of Clusters from Single-Cell RNA Sequencing Data	Shao et al., 2020, scCATCH: Automatic Annotation on Cell Types of Clusters from Single-Cell RNA Sequencing Data, iScience, Volume 23, Issue 3, 27 March 2020. https://doi.org/10.1016/j.isci.2020.100882.	https://github.com/ZJUFanLab/scCATCH
Fragment Analyzer Version #: 1.2.0.11	Advanced Analytical Technologies, Inc. (Has since been acquired by Agilent)	https://www.agilent.com/en/product/automated-electrophoresis/fragment-analyzer-systems/fragment-analyzer-systems-software
PROSize 3.0 (v. 3.0.1.6) Fragment Data Analysis Software	Advanced Analytical Technologies, Inc. (Has since been acquired by Agilent)	https://explore.agilent.com/Software-Download-Fragment-Analyzer-Prosize
Python versions 2.7 & 3.0	Python Software Foundation	https://www.python.org
Other		
MACS® SmartStrainers (30 µm)	Miltenyi Biotec	Cat# 130-098-458
MACS® SmartStrainers (70 µm)	Miltenyi Biotec	Cat# 130-098-462
Pre-Separation Filters (30 µm)	Miltenyi Biotec	Cat# 130-041-407
QuadroMACS Separator	Miltenyi Biotec	Cat# 130-090-976
MACS® MultiStand	Miltenyi Biotec	Cat# 130-042-303
MACS 15 mL Tube Rack	Miltenyi Biotec	Cat# 130-091-052
LD Columns	Miltenyi Biotec	Cat# 130-042-901
DNA LoBind® Tubes, 15 mL, conical tubes, PCR clean, colorless	Eppendorf	Cat# 0030122208
DNA LoBind® Tubes, 50 mL, conical tubes, PCR clean, colorless	Eppendorf	Cat# 0030122208
DNA LoBind® Tubes, 1.5 mL, PCR clean, colorless	Eppendorf	Cat# 022431021

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DNA LoBind® Tubes, 2.0 mL, PCR clean, colorless	Eppendorf	Cat# 022431048
Nunc 50 mL Conical Sterile Polypropylene Centrifuge Tubes, Pyrogen-free, RNase/DNase-Free	Thermo Scientific	Cat# 339653
10 mL BD Luer-Lok™ Syringe sterile, single use	Becton, Dickinson and Company (BD)	Cat# 302995
20 mL BD Luer-Lok™ Tip	Becton, Dickinson and Company (BD)	Cat# 302830
ART™ 200G Wide Bore Genomic Filtered Pipette Tips, Sterile, Certified Free of RNases, DNases, DNA, and Pyrogens	Thermo Scientific	Cat# 2069G
Fisherbrand™ 1,000 µL Large Orifice/ Genomic Aerosol-Barrier Tips, Filtered, Sterile	Fisher Scientific	Cat# 02-707-180
50 mL Steriflip-GP, 0.22 µm, Polyethersulfone (PES), Gamma irradiated	EMD Millipore	Cat# SCGP00525
Bel-Art™ SP Scienceware™ Flowmi™ Cell Strainers for 1,000 µL Pipette Tips, Mesh Size: 40 µm, Sterile	Bel-Art™	Cat# H13680-0040
DISTRIMAN Repetitive Pipette	Gilson	Cat# F164001
DISTRITIP Maxi ST, 12.5 mL, pre-sterile	Gilson	Cat# F164150
DISTRITIP Mini ST, 1,250 µL, pre-sterile	Gilson	Cat# F164140
DISTRITIP Micro ST, 125 µL, pre-sterile	Gilson	Cat# F164130
INCYTO C-Chip™, Disposable Hemacytometers, Neubauer Improved Grid, 100 µm Chamber	SKC Inc.	Cat# DHC-N015
Thermo Scientific™ BioLite 6-well Plates	Thermo Scientific	Cat# 130184
Thermo Scientific™ Sorvall™ ST 16R Refrigerated Centrifuge	Thermo Scientific	Cat# 75004381
Anodized Aluminum Chamber Block for 1.5 mL tubes, 80 tube capacity	Diversified Biotech	Cat# CHAM-8000
Lab Oscillator Orbital Rotator Shaker with Adjustable Speed at 0–210 RPM	CO-Z	https://www.amazon.com/CO-Z-Orbital-Adjustable-Oscillator-Platform/dp/B07FCY2S1P
Microcentrifuges - refrigerated or placed in 4°C fridge	N/A	N/A
Laboratory Inverted Microscope with brightfield and phase contrast	N/A	N/A
Sterile Surgical Scissors	N/A	N/A
Sterile Forceps	N/A	N/A
Ice Buckets/Pans	N/A	N/A
Lab Timers	N/A	N/A
BD LSR II YG (Green Profile) Flow Cytometer	BD Biosciences	BD LSR II YG (Green Profile)
LUNA-FL™ Dual Fluorescence Cell Counter	Logos Biosystems	Cat# L20001
PhotonSlides™	Logos Biosystems	Cat# L12005
10× Genomics Chromium Controller & Next GEM Accessory Kit	10× Genomics	Cat# 1000202
illumina NovaSeq 6000 Sequencing System	Illumina	NovaSeq 6000
Fragment Analyzer Automated CE System – 12 capillaries	Advanced Analytical Technologies, Inc.; (Has since been acquired by Agilent)	Cat# Fsv2-CE2F

MATERIALS AND EQUIPMENT

Spleen Extraction Solution ("SES") - 50 mL aliquots

Reagent	Final concentration	Stock concentration	Volume stock reagent for 50 mL
Hank's Balanced Salt Solution (HBSS), 1× without Calcium, Magnesium, and Phenol Red	N/A	N/A	48.5 mL
Heat inactivated Fetal Bovine Serum (HI-FBS)	2% HI-FBS	100% HI-FBS	1.0 mL
Cytiva HyClone™ HEPES 1 M Solution	10 mM HEPES	1 M HEPES	0.500 mL
Total Final Volume			**50 mL**

Five aliquots are prepared for each experiment. All reagents are sterile, and the resultant solution is again sterile-filtered in 50 mL aliquots using a Millipore 0.22 μm PES Steriflip vacuum filtration device (refer to key resources table). The prepared solution can be stored at 2°C–8°C, protected from light, for up to 1 week. On the day of the experiment, store at 2°C–8°C or on ice when not in use and always on ice when actively using the SES.

Enhanced Recovery Buffer ("ER Buffer") – 50 mL aliquots

Reagent	Final concentration(s)	Stock concentration(s)	Volume stock reagent for 50 mL
Hank's Balanced Salt Solution (HBSS), 1× without Calcium, Magnesium, and Phenol Red	N/A	N/A	39.8 mL
MACS BSA Stock Solution (10% BSA Stock)	2% BSA	10% BSA	10.0 mL
Invitrogen UltraPure EDTA, pH 8.0, Sterile-filtered solution (0.5 M EDTA)	2 mM EDTA	0.5 M EDTA	0.200 mL
Total Final Volume			**50 mL**

Six aliquots are prepared for each experiment. All reagents are sterile, and the resultant solution is again sterile-filtered in 50 mL aliquots using a Millipore 0.22 μm PES Steriflip vacuum filtration device (refer to key resources table). The prepared solution can be stored at 2°C–8°C, protected from light, for up to 1 week. On the day of the experiment, store at 2°C–8°C or on ice when not in use and always on ice when actively using the ER buffer.

Alternatives: Our final Enhanced Recovery (ER) Buffer recipe differs from Miltenyi Biotec's recommended cell isolation and flow staining buffer ("MACS Buffer"), the latter being composed of PBS, a lower concentration of BSA (0.5% BSA), yet an identical EDTA concentration (2 mM EDTA) compared to our ER buffer. The preparation of Miltenyi's MACS Buffer is provided in technical datasheets as follows: "Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA is made by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2°C–8°C)." Using our optimized ER buffer, we observed that HBSS (without phenol red, calcium, and magnesium), and an increased BSA concentration (2% BSA), provided a slight increase in cell viability compared to Miltenyi's MACS Buffer. In addition, a noticeable increase in cell recovery and pellet visibility was observed with increased BSA, up to 2%. This finding is consistent with previous studies and is potentially helpful for those working with limited cell quantities, granulocytes, similar to the protocol presented here, which requires numerous centrifugations and washing steps (Bedner et al., 1997; Phi-Wilson and Recktenwald, 1993, US Patent ID: US523828A). We have not tested the ER Buffer with any other species or any other murine organs, tissues, or cell types. Thus, it is appropriate to begin with the manufacturer's recommended buffer as any variations of this protocol would require the end user's validation of ER buffer compatibility.

Alternatives: Miltenyi Biotec currently states that "EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human

serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use."

PBS, EDTA, BSA Buffer ("PEB Buffer") - 50 mL aliquots

Reagent	Final concentration(s)	Stock concentration(s)	Volume stock reagent for 50 mL
autoMACS® Rinsing Solution (1× PBS, 2 mM EDTA)	1.6 mM EDTA	2 mM EDTA	40.0 mL
MACS BSA Stock Solution (10% BSA Stock)	2% BSA	10% BSA	10.0 mL
Total Final Volume			**50 mL**

Five aliquots are prepared for each experiment. All reagents are sterile, and the resultant solution is again sterile-filtered in 50 mL aliquots using a Millipore 0.22 μm PES Steriflip vacuum filtration device (refer to key resources table). The prepared solution can be stored at 2°C–8°C, protected from light, for up to 1 week. On the day of the experiment, store at 2°C–8°C or on ice when not in use and always on ice when actively using the PEB buffer.

Note: The increased BSA (2% BSA) in our PEB Buffer, compared to Miltenyi's MACS Buffer (0.5% BSA), was observed to maintain a higher level of viability and a significant increase in cell recovery throughout each step of the protocol.

10× Sequencing Buffer ("10×-SB") - 50 mL aliquots

Reagent	Final concentration(s)	Stock concentration(s)	Volume stock reagent to 50 mL
Hank's Balanced Salt Solution (HBSS), 1× without Calcium, Magnesium, and Phenol Red	N/A	N/A	45.0 mL
MACS BSA Stock Solution (10% BSA Stock)	1% BSA	10% BSA	5.0 mL
Total Final Volume			**50 mL**

Two aliquots are prepared for each experiment. All reagents are sterile, and the resultant solution is again sterile-filtered in 50 mL aliquots using a Millipore 0.22 μm PES Steriflip vacuum filtration device (refer to key resources table). The prepared solution can be stored at 2°C–8°C, protected from light, for up to 1 week. On the day of the experiment, store at 2°C–8°C or on ice when not in use and always on ice when actively using the 10×-SB.

Alternatives: Many alternative buffer formulations are compatible with 10× Genomics Chromium Single Cell 3' GEM, Library & Gel Bead Kit v3. However, it is critical to review the 10× Genomics buffer compatibilities found on their webpage. Some of these alterations include using PBS without Ca^{2+} and Mg^{2+} in place of HBSS without Ca^{2+} and Mg^{2+} and replacing BSA with FBS.

STEP-BY-STEP METHOD DETAILS

Preparation of splenocyte single-cell suspension

Timing: 1–2 h

Single-cell suspensions of splenocytes are isolated for evaluation and downstream RNA expression analysis using the targeted enrichment strategy. After this step, splenocytes will be isolated from mouse spleen, counted and viability-verified, and prepared to undergo selective antibody labeling and magnetic separation.

1. Euthanize three female CFW mice, 8–12 weeks old, via CO_2 asphyxiation followed by secondary cervical dislocation.

⚠ CRITICAL: This protocol was optimized for using 3 mouse spleens pooled in the single-cell suspension step. If fewer than 3 spleens are used, we recommend optimizing and

Table 1. Flow cytometry enriched antibody cocktail (22 μL) panel for analyzing rarer granulocyte and myeloid cells in mouse spleen for downstream scRNA-seq

Antibody	Marker	Fluorophore	Company	Cat#	Clone	Volume (μL)	Final dilution[a]
Ter-119, anti-mouse, PE, REAfinity™	Ter-119	PE	Miltenyi Biotec	130-112-909	REA847	2.0	1/55
CD3, anti-mouse, PE, REAfinity™	CD3	PE	Miltenyi Biotec	130-121-133	REA641	2.0	1/55
CD19, anti-mouse, PE, REAfinity™	CD19	PE	Miltenyi Biotec	130-112-035	REA749	2.0	1/55
CD335 (NKp46), anti-mouse, PE, REAfinity™	CD335 (Nkp46)	PE	Miltenyi Biotec	130-112-358	REA815	2.0	1/55
CD45, anti-mouse, APC-Vio® 770, RE-Afinity™	CD45	APC-Vio® 770	Miltenyi Biotec	130-110-800	REA737	2.0	1/55
CD11c, anti-mouse, PerCP-Vio® 700, RE-Afinity™	CD11c	PerCP-Vio® 700	Miltenyi Biotec	130-110-842	REA754	2.0	1/55
CD11b, anti-mouse, VioBlue®, REAfinity™	CD11b	VioBlue®	Miltenyi Biotec	130-113-810	REA592	2.0	1/55
Ly-6G, anti-mouse, FITC, REAfinity™	Ly-6G	FITC	Miltenyi Biotec	130-120-820	REA526	2.0	1/55
Siglec-F, anti-mouse, PE-Vio® 770, REAfinity™	Siglec-F	PE-Vio® 770	Miltenyi Biotec	130-112-334	REA798	2.0	1/55
FcεRIα, anti-mouse, PE-Vio® 615, REAfinity™	FcεRIα	PE-Vio® 615	Miltenyi Biotec	130-118-899	REA1079	2.0	1/55
CD117, anti-mouse, APC, REAfinity™	CD117	APC	Miltenyi Biotec	130-111-694	REA791	2.0	1/55
Total Cocktail Volume						**22.0 μL**	

Abbreviations: PE, Phycoerythrin; APC, Allophycocyanin; FITC, Fluorescein isothiocyanate.

[a]Final dilution is based on a final total volume of 110 μL, where the amount of Viobility™ 405/520 Fixable Dye is equal to the difference of 110 μL and the volume (μL) of antibody cocktail.

validating the procedures. If using more than 3 spleens, we recommend doing them in groups of 3 pooled spleens, as this allows the user to adhere to all the specifics (i.e., volumes and dilution factors) presented in this protocol.

⚠ CRITICAL: Unless the technician is skilled and quick at dissecting spleens, we recommend sacrificing one mouse at a time. This should help prevent any transcriptional drift due to post-mortem processes.

Note: The use of three spleens allows the user to capture the heterogeneity of biological replicates without expanding sequencing. Fortunately, 10× Genomics has updated chemistry kit versions that allow multiplexing biological replicates within the same sample prep.

2. Make an incision in the abdominal cavity and remove spleen (located to the left side of the abdomen, inferior to the stomach).
3. Immediately extract the three spleens, placing them all in a single 50 mL tube of SES on ice, as previously prepared (before you begin, Prepare Solutions and Buffers).
4. To obtain a single-cell suspension, cut spleens into small pieces with sterile scissors in a 6-well plate on ice in a sterile environment.

Alternatives: Petri dishes or other alternatives can be used to dice the spleen. However, a 6-well plate provides an optimal area and volume to efficiently cut the spleen while ensuring that the entirety of each spleen is incorporated into the dissociation.

5. Place a 70 μm MACS® SmartStrainer on top of a 50 mL LoBind tube on ice and pre-wet the cell strainer with 5 mL of cold SES. Discard the flow-through.
6. Place minced pieces of spleens in the 70 μm cell strainer and gently dissociate by moving the rubber plunger of a 10- or 20-mL sterile syringe in a circular motion with exceptionally light pressure. It is vital to add ice-cold SES continuously as the dissociation is performed. The total SES added during this step should range between 40 and 45 mL. This equates to roughly 2.5–3 mL of SES added per minute during the dissociation.

⚠ CRITICAL: Constant addition of cold SES to the minced spleen, strainer, and plunger ensures that dissociated cells flow through the strainer and do not require extreme pressure to force them through. This also prevents cells from getting stuck or deprived of liquid. There should be little pressure applied to the spleen. This process should take roughly 10–15 min to gently dissociate all 3 spleens through the cell strainer.

7. Using approximately 5 mL of SES, wash all excess cells off the plunger into the strainer and ensure all cells have been thoroughly washed from the strainer, passing through to the 50 mL LoBind flow-through tube on ice, that now contains the 3 dissociated spleens creating a single-cell suspension that is 45–50 mL in total.
8. Centrifuge the resulting single-cell suspension at 4°C for 8 min at 300 × *g*. Aspirate the supernatant, being careful to avoid any disturbance of the cell pellet, and place the tube containing the cell pellet on ice.
9. Lyse red blood cells (RBCs) with 3 mL of Sigma Hybri-Max Red Blood Cell Lysing Buffer (1 mL/spleen).
   a. Start a timer precisely when the gentle pipetting begins to lift and resuspend the pellet in the 3 mL of RBC lysis buffer. Pipetting is done with the tube in ice but angled so the user can visualize their pipetting.
   b. Keep pipetting to a minimum, with intermittent swirling. Only take the suspension out of ice when swirling and ensure placement on ice in between swirls.

⚠ CRITICAL: To ensure reproducibility in experimental replicates, we use a 1,000 μL wide-bore pipette tip and quickly pipette 3 volumes of 1,000 μL of RBC lysis buffer down the side of the tube containing the cell pellet on ice. The RBC lysis must proceed for precisely 45 s. This minimizes stress to the other cell populations, results in high viability, minimizes cell aggregates, and enhances reproducibility amongst experimental replicates (see expected outcomes).

⚠ CRITICAL: Although we minimize the time cells are exposed to RBC lysis buffer, cells can still experience stress. To reduce any further stress to the cells, it is critical that the user conducts gentle and controlled pipetting to minimize the lysis of cells yet produce a homogenous solution. White fibrotic aggregates may form due to the lysed RBCs, but these are removed in steps #11–13 by passing the solution through cell strainers, so there is no need to attempt to dissociate these cell debris aggregates.

10. After 45 s, pour 45–50 mL of cold SES (15 mL/spleen) down the side of the tube while gently swirling to neutralize the RBC lysis buffer, stopping the RBC lysis reaction. Immediately place on ice and gently pipette to dissociate any aggregates.

Note: A more concentrated salt solution, such as 10× HBSS without phenol red, Ca^{2+}, and Mg^{2+}, can be used to minimize the total volume of the neutralized suspension (Son et al., 2017). This may reduce cell loss in the subsequent centrifugation (step #14).

11. Stack strainers, 70 μm on top of 30 μm, and place on top of new 50 mL LoBind tube on ice.
12. Pre-wet the strainer stack with roughly 5 mL of SES and discard the flow-through. Use the same tube in the next step.

Note: Using the same tube serves to coat the tube with proteins to retain cells.

13. Gently pour cells slowly into the strainer stack, and single cells flow through into the 50 mL LoBind tube. The strainer stack is then discarded, and the tube cap is re-placed on the 50 mL LoBind tube.
14. Centrifuge at 4°C for 8 min at 300 × *g* with the brake/deceleration reduced by 30% with respect to the highest setting. The acceleration does not need to be altered from the highest setting.
    a. During this centrifuge, prepare tubes for steps #16, #17, and #18.
        i. For step #16, add the 27 mL of cold SES into a 50 mL LoBind tube on ice and label as 1:10 splenocytes.
        ii. For step #17, add the 18 mL of cold SES into a 50 mL LoBind tube on ice and label as 1:100 splenocytes.
        iii. For step #18, to two 2.0 mL LoBind tubes, add 200 μL of 0.4% Trypan Blue solution per tube.

Note: Preparing these tubes during the centrifuge step avoids unnecessary time lapses that could affect downstream analyses (i.e., by preserving the cells' transcriptomes for scRNA-seq).

15. Aspirate supernatant and resuspend in 4.0 mL of cold SES (1.33 mL/spleen).

Note: For accuracy, use a pipette to deliver 4.0 mL as opposed to a serological pipet.

16. Make a 1:10 splenocyte dilution of splenocytes by diluting 3.0 mL of undiluted cell suspension into 27 mL of cold SES (from step #14-a-i) for a total of 30 mL of 1:10 diluted splenocytes. Place on ice.
17. Create a 1:100 dilution of cells by adding 2.0 mL of the 1:10 splenocytes dilution into 18 mL of SES (from step #14-a-ii) to create a total of 20 mL of 1:100 diluted splenocytes. Place back on ice.
18. Add 200 μL of the 1:100 splenocytes dilution into each of the 2 mL LoBind tubes pre-aliquoted with 200 μL of trypan blue (from step #14-a-iii), generating a 1:1 mixture of trypan to cell suspension ratio.

Note: The dilution factor of 2 must be considered when calculating cell concentrations (if prepared as described in this protocol).

19. Load 10 μL of the 1:1 mixture of trypan:cells to each chamber of a hemocytometer and count viable and total cells. Calculate and record the concentrations and viability of the undiluted, 1:10, and 1:100 splenocyte dilution samples. Troubleshooting 1 and 2.

⚠ CRITICAL: Calculating the total number of cells per sample for the undiluted cell concentration and viability is essential for experimental reproducibility and applicability (Expected outcomes).

Alternatives: An automated cell counter can be used in place of a hemocytometer. This would be preferred as it is more efficient, allowing users to quickly proceed with the next step.

20. Save the 1:10 and 1:100 splenocyte dilutions created above for downstream flow cytometry and spiking splenocytes back into the enriched cell population. Troubleshooting 5.

a. Place the 1:100 splenocyte dilutions in a 4°C fridge, tilted on their side on an orbital rotator, rotating at 30 RPM.
b. Keep the 1:10 splenocyte dilution on ice and use it for the next step (step #21).

⚠ CRITICAL: If the splenocytes are neither diluted nor shaken gently, the viability drastically decreases over time. The dilutions and gentle shaking minimize cell clumping and settling, which can result in aggregation and cell death. Troubleshooting 5.

21. Using the 1:10 diluted splenocytes, add 400 × 10^6 cells to a 15 mL LoBind tube, centrifuge at 4°C for 5 min at 300 × *g*, then aspirate (leaving roughly 30 μL supernatant) and bring up to a final volume of 2.4 mL with ER buffer. Gently mix and promptly place the tube back in ice.
22. Proceed immediately with the MACS Immunomagnetic Enrichment of Untouched Target Cells method.

⚠ CRITICAL: This cell suspension must proceed to cell isolation immediately as cell aggregates can form when left stationary too long on ice. This may result in decreased enriched cell recovery, purity, and viability.

MACS immunomagnetic enrichment of untouched target cells

Timing: 1.5–2.5 h

Unwanted cell populations are removed by targeting antigens specific to the unwanted cells with fluorophore-conjugated antibodies, followed by immunomagnetic beads which bind to the fluorophore. These undesired cells will be retained in the immunomagnetic column while the desired cell population(s) will be enriched in flow-through. There are considered "untouched" as the antibodies used for targeting exclusively bound the unwanted population, leaving the flow-through cell populations "untouched" by antibodies. At the conclusion of this step, enriched "untouched" cell populations will be purified and ready for evaluation (i.e., via flow cytometry) and RNA capture (i.e., scRNA-seq).

23. Add the previously prepared 570 μL of PE-Conjugated Antibody Depletion Cocktail (Table 2) directly to the cell suspension prepared in step #21 and mix gently by pipetting.
24. Incubate for 10 min in a light-protected 4°C fridge, with the tube slightly slanted on an orbital rotator, rotating at 30 RPM.

⚠ CRITICAL: Miltenyi Biotec states that incubating on ice requires a longer incubation time; it is highly recommended to incubate for 10 min in a refrigerator that maintains 2°C–8°C (Miltenyi cell surface staining protocol).

⚠ CRITICAL: Although this incubation includes PE-conjugated antibodies, it is not essential to do this incubation step protected from light (i.e., in a dark 4°C fridge), as the PE-conjugated antibodies serve as the ligand of the immunomagnetic anti-PE microbeads which deplete the undesired cell populations. However, analyzing the unstained enriched sample using flow cytometry can provide detection of PE-labeled cells that were not retained, a likely indication that more anti-PE microbeads are needed in the microbead cocktail to deplete these populations efficiently. The PE-conjugated fluorophores used in this protocol have been extensively tested. Miltenyi's PE-conjugated antibodies' exposure time to light from this depletion procedure is minimal when considering Miltenyi's extended validation of these recombinant antibody-PE-fluorophore conjugates. Refer to troubleshooting 1 and 2.

25. After the 10-min incubation, add 12 mL of ER buffer directly to the antibody-cell mixture.

Table 2. PE-conjugated antibody depletion cocktail (570 μL)

Block or PE-Conjugated antibody	Target (major cell type)	Fluorophore	Company	Cat#	Clone	Volume (μL)	Final dilution[a]
FcR Blocking Reagent, mouse	N/A	N/A	Miltenyi Biotec	130-092-575	N/A	200	1/15
Ter-119, anti-mouse, PE, REAfinity ™	Ter-119 (RBC)	PE	Miltenyi Biotec	130-112-909	REA847	160	1/18.75
CD3, anti-mouse, PE, REAfinity™	CD3 (T cells)	PE	Miltenyi Biotec	130-121-133	REA641	160	1/18.75
Ly-6G, anti-mouse, PE, REAfinity™	Ly-6G (Neutrophil)	PE	Miltenyi Biotec	130-123-780	REA526	15	1/200
CD335 (NKp46), anti-mouse, PE, REAfinity™	CD335/ Nkp46 (NK cells)	PE	Miltenyi Biotec	130-112-358	REA815	15	1/200
CD115, anti-mouse, PE, REAfinity™	CD115 (Monocyte)	PE	Miltenyi Biotec	130-112-639	REA827	10	1/300
CD11c, anti-mouse, PE, REAfinity™	CD11c (Dendritic cells)	PE	Miltenyi Biotec	130-110-701	REA754	10	1/300
Total Cocktail Volume						**570 μL**	

Abbreviations: NK, Natural Killer; PE, Phycoerythrin; RBC, red blood cell.

Antibody cocktail targets cell-specific antigens for cell populations that are over-represented (i.e., T & B cells). All cell-specific antigen-targeting antibodies are conjugated to a PE fluorophore which serves as the antigen for the anti-PE magnetic microbeads (step #28). This allows for negative depletion for enriched and untouched rarer granulocytic and myeloid cells for downstream scRNA-seq analysis.

[a]Final dilution was calculated from a final total volume of 3.0 mL, as roughly 30 μL of supernatant is left after the spin down to avoid disturbance of the pellet. The 3.0 mL final total volume breakdown is 0.030 mL (leftover from aspiration) + 2.4 mL ER buff (resuspension step #21) + Enrichment Antibody Cocktail #1 (0.570 mL or 570 μL). The desired final dilutions are initially based on the estimated total nucleated cell number and then adjusted for the volume.

a. This step serves to significantly pre-dilute and washes off unbound and loosely bound antibodies.

Note: Due to the time-sensitive nature of scRNA-seq, the direct addition of ER buffer serves as a sufficient wash to proceed without an additional wash and centrifugation step, thus saving time and cell loss.

26. Centrifuge the sample at 300 × *g*, 8 min, 4°C. Aspirate as much supernatant as possible without disturbing the cell pellet.
27. Resuspend cells in 3.2 mL ER buffer (80 μL ER buffer/10 × 10^6 cells).
28. Add 500 μL of anti-PE UltraPure microbeads (12.5 μL anti-PE beads/10 × 10^6 cells) and 400 μL anti-CD19 microbeads (10 μL anti-CD19 microbeads/10 × 10^6 cells) to the cell suspension (Table 3). Mix gently with minimal pipetting, followed by swirling.
29. Incubate 15 min at 4°C in the fridge, with the tube slightly slanted on an orbital rotator, rotating at 30 RPM.
    a. During this incubation, proceed to steps #33 (assembly of QuadroMACS unit), #34, and #35, as it takes a while to pre-wash the LD columns via gravity flow.

Table 3. Microbead cocktail for immunomagnetic negative depletion of dominant spleen cell types

MicroBeads	Target(s)	Company	Cat#	Volume (μL)	Final dilution[a]
Anti-PE MicroBeads UltraPure	PE (PE-labeled cells)	Miltenyi Biotec	130-105-639	500	500/4100
CD19 MicroBeads, mouse	CD19 (B cells)	Miltenyi Biotec	130-121-301	400	400/4100
Total Cocktail Volume				**900 μL**	

Abbreviations: PE, Phycoerythrin.

This cocktail is added to the cells from step #27 to deplete the more dominating cell types that constitute the spleen.

[a]Final dilutions are calculated as used in the protocol (steps #27 and #28). These dilutions were optimized based on the specific input of 400 × 10^6 cells in a volume of 3.2 mL ER Buffer, which upon addition of the bead cocktail yields a total volume of 4.1 mL.

30. After the 15-min incubation, add 12 mL of ER buffer directly to the antibody-cell mixture. This serves to significantly pre-dilute and washes off any unbound or loosely bound microbeads.

Note: Due to the time-sensitive nature of scRNA-seq, the direct addition of ER buffer serves as a sufficient wash to proceed without an additional wash and centrifugation step. Thus, saving time and reducing cell loss.

31. Centrifuge at 300 × *g*, 8 min, at 4°C. Aspirate as much supernatant as possible without disturbing the cell pellet.

Note: To minimize transcriptional drift, we do not wash the cells twice following step #30. We have not noticed any significant differences in washing twice.

32. Resuspend cells in 4.0 mL of ER buffer (500 µL ER buffer/100 × 10^6 cells).
33. Proceed to magnetic separation using the previously assembled 8 LD columns secured in two QuadroMACS magnetic stand units (4 LD columns per QuadroMACS unit). Ensure that all 15 mL collection tubes are in ice and secured, as these will be retrieving the flow-through that contains the enriched target cell population. Figure 1 shows an illustration of this setup.
34. Pre-wet the 30 µm pre-separation filters (on top LD columns) with 2 mL of ER buffer per column (8 columns) and allow this solution to flow-through (exit) via gravity flow the filter before any cell suspension is added. Discard this pre-wetting solution from the collection tubes (8 columns).
    a. Use these same tubes for the next step (step #35).

⚠ CRITICAL: ER buffer has a 2% BSA concentration that serves to precoat collection tubes with BSA, which has been proven to increase the recovery and viability of cells (Phi-Wilson and Recktenwald, 1993, US Patent ID: US523828A).

35. Re-place the same 15 mL LoBind tubes used in step #34, positioned below each LD column.
36. Slowly pipette 500 µL of cell suspension into the Miltenyi 30 µm pre-separation filters atop the LD column and allow the cell suspension to flow through via gravity. The flow-through contains the enriched cell population.

⚠ CRITICAL: Each LD column comes pre-packaged with a plunger. Do not plunge the cell suspension to speed up the immunomagnetic separation, resulting in the undesired

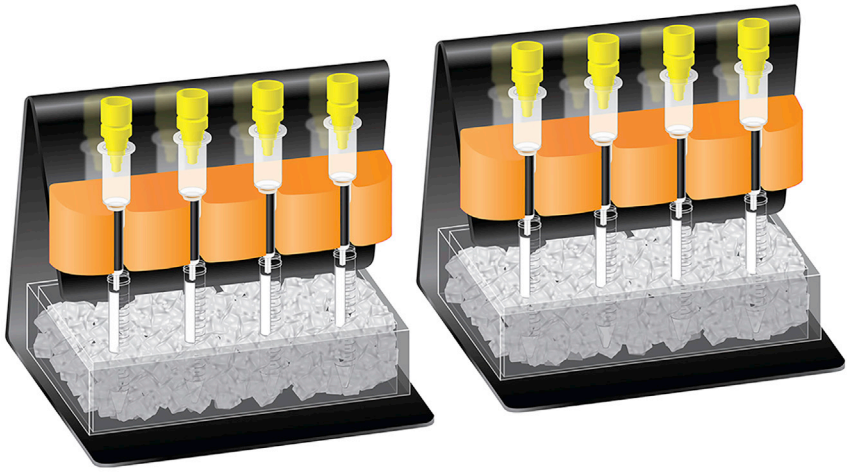


Figure 1. Illustration of the MACS stands experimental setup for immunomagnetic cell enrichment procedure

microbead labeled cells being forced through the column and contaminating the targeted enriched population. Additionally, it will subject all the cells to substantial shear forces and result in high debris, low viability, and poor candidates for scRNA-seq. See troubleshooting 1 and 2.

⚠ CRITICAL: Do not proceed to step #37 until all volume has exited the column.

37. Wash pre-separation filter and column twice, with 1 mL of ER buffer per wash.

⚠ CRITICAL: Only add additional buffer (the second wash in step #37) when the column reservoir is completely emptied of buffer.

Note: This step serves to retrieve any remaining enriched target cells that may have been retained in the column.

38. These resulting flow-throughs in the 15 mL LoBind tubes contain the untouched, "enriched" cell populations. See troubleshooting 3.
39. Combine the 8 enriched 15 mL collection tubes into 2 of the 15 mL LoBind tubes by pipetting each of 3 tubes into the 4th collection tubes.
40. Centrifuge both tubes 300 × *g*, 8 min, at 4°C. Aspirate the ER buffer while leaving approximately 50 μL in both tubes to minimize cell loss. Place both tubes on ice.
41. Take one of the two tubes and resuspend the cell pellet with 400 μL of 10×-Sequencing Buffer (10×-SB) using a wide-bore tip and careful pipetting to preserve cell viability. Once resuspended, transfer this cell suspension (volume approximately 450 μL) into the second tube and use the volume to resuspend the second tube's cell pellet.

⚠ CRITICAL: At this step (step #41) in the workflow, the cell suspension buffer is transitioned from ER buffer to 10×-SB to remove EDTA and reduce BSA for compatibility with the Chromium instrument and library prep chemistry (see Chromium Single Cell 3' Reagent Kits v3).

Note: The resuspension process described here is designed to maximize cell recovery and the concentration (cells/mL) of enriched cells, which can be limiting. Additionally, the cell concentration must be carefully chosen for capturing the desired number of cells with single-cell sequencing.

42. Immediately begin counting cells and assessing viability for the enriched fraction using a 1:1 dilution of cells to trypan blue solution. Load the 1:1 suspension in a hemocytometer and count as previously mentioned (step #19). See troubleshooting 1, 2, and 3.

Spiking: Repopulation of depleted cells for broadened transcriptomics

Timing: 20 min

The spiking procedure is used to repopulate the more abundant spleen cells that were deliberately depleted during the magnetic cell enrichment procedure. Spiking with the untouched splenocytes allows the reintroduction of abundant spleen cell types that were minimally perturbed, a crucial factor for the sensitivity of scRNA-seq.

43. To partially repopulate the highly abundant spleen cells depleted during the enrichment (e.g., T and B Lymphocytes), use the 1:10 and/or 1:100 untouched splenocytes previously prepared (steps #14–19). Create the "Spiked" sample so that its final total cell composition consists of 33% cells from the untouched splenocytes and 67% of cells from the enriched cell fraction.

Volumes will vary based on cell counts and concentrations of the 1:10 and 1:100 splenoctyes (see steps #19 and #20).

a. For the intended Spiked sample to undergo scRNA-seq, aliquot a desired number of cells for the user(s) target sequencing depth (see Chromium Single Cell 3′ Reagent Kits v3), and centrifuge at 300 × *g*, 8 min, at 4°C.
b. Aspirate and add a volume of 10×-SB to obtain a Spiked sample with 800-1,200 Spiked cells/μL (recommended concentrations from the 10× Genomics Chromium Single Cell 3′ Reagent Kits v3).

⚠ CRITICAL: An aliquot of the Spiked sample intended for 10× scRNA-seq must be spun down and replaced with 10×-SB to remove EDTA and reduce BSA for compatibility with the Chromium instrument and library prep chemistry (see Chromium Single Cell 3′ Reagent Kits v3).

Note: At this stage in the workflow, the user has generated untouched splenocytes, an enriched fraction, and a spiked fraction.

44. The Spiked sample (prepared in stepa #43a and b) should be processed immediately for scRNA-seq using the 10× Genomics Chromium Single Cell 3′ Reagent Kits v3 User Guide.
    a. It is beyond the scope of this protocol, but ensure that the concentration of cells for sequencing, sequencing depth, and other parameters are followed in accordance with the 10× Genomics Chromium Single Cell 3′ Reagent Kits v3 User Guide.
    b. The Spiked sample should also be analyzed in the Flow Cytometry Staining procedure to ensure the user(s) repopulated prevalent spleen cell types depleted in the enrichment.

⚠ CRITICAL: The sample should be processed immediately to preserve the transcriptome. It is best if one user begins the 10× Genomics sample preparation and the other user(s) proceed to the flow cytometry procedure.

Note: Fundamentally, this protocol is designed to produce unperturbed and highly viable cells. These could be utilized for various downstream applications, including but not limited to scRNA-seq (i.e., *ex vivo* functional assays, proteomic analyses, mechanistic drug studies, etc.).

Flow cytometry staining

Timing: 3–4 h

Flow cytometry can be used as a quality control tool to give insight into the enrichment strategy's success, failure, or anomalies. This is especially useful for scRNA-seq, as it allows one to assess if costly downstream NGS should be pursued based on the users' expected target cell populations.

45. Aliquot 1 × 10^6 cells into 1.5 mL LoBind tubes for all samples, including controls. Troubleshooting 4.

Note: 2.0 mL LoBind tubes can be used in place of 1.5 mL LoBind tubes. This usually comes down to the user preference when viewing/protecting the cell pellet during aspiration steps.

46. Centrifuge samples at 4°C and 300 × *g*, for 10 min.
    a. During this centrifuge, prepare 1× Viobility™ 405/520 Fixable Dye per the manufacturer's instructions.
        i. For every 1 μL of 100× stock Viobility™ 405/520 Fixable Dye, add 100 μL of PBS.

⚠ CRITICAL: Per Miltenyi's manufacturer instructions, Viobility Fixable Dye is not compatible with BSA during the Viobility Dye Incubation. Therefore, only PBS must be used to dilute the 100× stock to a working 1× Viobility Fixable Dye concentration.

47. Resuspend each sample in the designated volume of freshly prepared 1× Viobility™ 405/520 Fixable Dye, according to the sample type:
    a. Stained samples: 88 μL of 1× Viobility™ 405/520 Fixable Dye.
    b. FMOs: 90 μL of 1× Viobility™ 405/520 Fixable Dye.
    c. FM4 (FMO for the 4 PE-antibodies): 96 μL of 1× Viobility™ 405/520 Fixable Dye.
48. In the dark, mix well and incubate in 1× Viobility™ 405/520 Fixable Dye for 5 min at room temperature (18°C–23°C).
49. Add the previously prepared Flow Cytometry Enriched Antibody Cocktails (see Table 1 and before you begin step #2) directly to their respective samples as described below and incubate in the dark for 10 min at room temperature (18°C–23°C).
    a. Stained samples: Add 22 μL of Enriched Flow Antibody Cocktail.
    b. FMOs: add 20 μL of each FMO cocktail to one cell sample (one sample per FMO cocktail).
    c. FM4 (fluorescence minus four control for all four PE-conjugated antibodies): add 14 μL of FM4 panel.

⚠ CRITICAL: In this protocol, follow Miltenyi's manufacturer instructions, "Staining with Viobility™ Fixable Dye and staining surface markers simultaneously," section 2.4. By following these instructions, the final total volume of Viobility Fixable Dye plus the volume of the antibody cocktail in a sample should always equal 110 μL.

Note: We do not use isotype fluorophore-conjugated controls due to Miltenyi Biotec's extended validations, which show their REA recombinant antibodies have negligible non-specific binding to the commonly problematic FcgRI (CD65), FcgRIII (CD16), and FcgRII (CD32). Please refer to Miltenyi's scientific poster, "Recombinant antibodies for improved standardization in flow cytometry," for more information. Due to the high cost of using isotype controls given the extended validation of the recombinant REA antibodies, it is practical to proceed without, although we still encourage isotype fluorophore-conjugated antibodies in flow cytometry.

50. Add PEB to bring the volume up to 1,000 μL.

Note: This dilution serves as a pre-wash, diluting the loosely labeled or unbound antibodies to maximize cell recovery by side-stepping additional centrifugation and wash steps.

51. Centrifuge at 4°C and 300 × *g*, for 8 min. Aspirate supernatant.
52. Add 150 μL of cold PBS and resuspend cells gently.

⚠ CRITICAL: It is essential to resuspend the cells completely in this step. There should be no aggregates resulting in a homogenous cell suspension. Failure to do so can result in cells getting fixed as aggregates in the next step, step #53, which will negatively impact the flow cytometry results and interpretations.

53. Add 150 μL of 4% PFA in PBS to the samples to obtain a final 2% paraformaldehyde (PFA) concentration and gently mix. Allow samples to be fixed by incubating them in a 2°C–8°C fridge for 20 min, protected from light.

⚠ CRITICAL: Using an aqueous 4% PFA solution, rather than the 4% PFA in PBS solution (used here), could impact cell viability as it results in a decreased salt concentration that

keeps the 1× PBS isotonic to cells. An aqueous PFA solution could result in a hypotonic solution that can cause cell swelling and cytolysis.

Note: PFA fixation incubations can be performed at room temperature (18°C–23°C), in a fridge (2°C–8°C), or on ice. Room temperature (18°C–23°C) incubations require less time for fixation, and incubations on ice require slightly increased fixation times.

54. Pre-dilute the 2% PFA concentration in the samples by directly adding 600 μL of PEB to the sample.

⚠ CRITICAL: PEB buffer is used here as the 2% BSA in this buffer improves cell recovery. There will be a significant decrease in the recovery of cells if PBS is used in place of PEB buffer in this step.

Note: By directly diluting the 2% PFA containing sample, this step serves as a pre-washing step and reduces the number of wash-centrifuge cycles to one.

55. Centrifuge samples at 4°C, 700 × *g*, for 10 min.
56. Aspirate supernatant, leaving a small volume (50 μL) if the pellet is hard to distinguish and resuspend each sample in 150 μL of cold PEB buffer.
57. Immediately preserve the fixed flow samples by storing them at 2°C–8°C, protected from light.

⚠ CRITICAL: Failure to protect the samples from sunlight or visible light can reduce the fluorophore staining intensity. This can result in diminished staining indexes and can negatively impact the data analysis and interpretation of the acquired flow data. In the event, it is recommended to redo the flow procedure rather than analyze cells that sit overnight at room temperature (18°C–23°C) or were exposed to light for more than 4 h.

Note: We prefer to perform the flow cytometry on the fixed samples no longer than 24 h after fixation. However, others have stored fixed flow samples for more extended periods. We have not attempted to analyze after 24 h and do not recommend doing so unless the users independently test the conditions.

Pause point: The antibody-labeled fixed cell samples can be stored at 2°C–8°C, protected from light, and analyzed within 24 h post-fixation. The single-stained compensation bead controls should be prepared immediately before submitting/running the fixed flow samples (see step #58, below).

58. 30–90 min before submitting the flow samples, prepare single-stained controls using the Miltenyi Compensation Beads Anti-REA Kit following the manufacturer's instructions.
    a. Users should have a total of 8 single-stained bead controls, one per unique fluorophore, for this particular protocol.

⚠ CRITICAL: Each unique fluorophore must have a single-stained compensation bead control sample. Therefore, the number of single-stained compensation bead control samples will equal the unique number of fluorophores in the antibody cocktail panel.

59. Perform flow cytometry on the cell samples using a BD LSRII YG (Green Profile) flow cytometer. Troubleshooting 1 and 2.

Note: Flow cytometry data was acquired on a BD LSR II YG (BD Biosciences) flow cytometer. Compensation beads (Miltenyi MACS® Comp Bead Kit, anti-REA), single-stained cell controls and unstained cell controls were used to perform and construct a compensation matrix. The resulting data were analyzed using FlowJo (Version 10.8.1, BD Biosciences) with plots constructed in the FlowJo layout editor. Specific details on the analysis and processing of the acquired flow cytometry data are beyond the scope of this protocol. However, numerous recommended resources are available for good practices in the processing and analysis (Cossarizza et al., 2019; DiPiazza et al., 2019; Kalina, 2020; Liechti et al., 2021; Lucas et al., 2020; Maciorowski et al., 2017; Mair and Tyznik, 2019; Sun et al., 2021; Wang and Hoffman, 2017).

EXPECTED OUTCOMES

This protocol describes an adjustable method for splenocyte enrichment that may be useful for broadened and sensitive transcriptomic applications with comprehensive discussion on sample-prep considerations. In this approach, underrepresented cell types of the spleen were enriched via negative selection with immunomagnetic technology to control for any transcriptional alterations that may be induced through positive selection strategies. The addition of a spiking approach was also used to re-populate cells removed (and labeled) during enrichment with unperturbed "untouched" splenocytes to capture the full spleenome. During development we found the protocol to be reproducible and reliable (Table 4). Further, this protocol has the potential for wider usability with alternative downstream applications where untouched cells may be desirable such as functional assays. As this depletion system uses PE-labeled antibody cocktails the user can quickly adjust (and verify via flow cytometry) the antibody depletion cocktail to obtain their desired cell populations.

Table 5 provides an overview of how the spleen cells populations changed in response to the enrichment procedure. For example, this protocol enriched basophils from <1% to over 7%, measured via flow cytometry and identified in terminal gates by FcER1a^{+}/CD117^{-} expression (Figures 2, 3, and 4). Additional cell types are reported in Table 5.

Figures 2, 3, and 4 demonstrate flow cytometry analysis of the untouched mouse spleen, enriched fraction, and spiked fraction, respectively, for which Table 5 shows the cell population comparisons (Table 5). A variety of granulocytes were identified and other cells such as innate lymphoid cells (ILCs) of subtypes ILC2 and lymphoid tissue inducer (LTi) cells (Bar-Ephraïm and Mebius, 2016; Brestoff et al., 2015; Buettner and Lochner, 2016; Shiu et al., 2015; Zeng et al., 2019). It is recommended to use flow cytometry as a validation component when any enrichment strategies

Table 4. Expected undiluted single-cell splenocyte suspension concentrations upon completion of the single-cell preparation method

Experimental replicate	Undiluted splenocyte concentration[a] (cells/mL)
Trial #1	266,500,000
Trial #2	262,000,000
Trial #3	266,000,000
Statistical summary	
Mean (Average) =	264,833,333 cells/mL
Standard Deviation =	2,466,441 cells/mL
% Relative Standard Deviation (%RSD) =	0.93%
Relative Standard Deviation (RSD) =	0.0093

The presented data is from three experimental replicates in which we prepared the single-cell splenocyte suspensions using this protocol and executed the cell counts in step #19. The 1:100 dilution was counted, and the undiluted single-cell splenocyte suspension was back-calculated as described in step #19.

[a]Cell numbers and calculations from cell counts at step #19.

Table 5. Percent population comparison of samples by flow cytometry analysis

Flow cytometry defined cell populations	Total splenocytes (%)	Enriched (%)	Spiked[a] (%)
Dump Gate $Ter119^{-}CD3^{+}CD19^{+}CD335^{+}$	70.9	28.1	40.5
$Ter119^{+}CD45^{-}$	14.1	0.18	1.02
$CD45^{+}CD11b^{+}$	2.51	15.5	11.6
Dendritic	0.90	0.36	0.58
Neutrophil	1.72	3.14	2.60
Eosinophil	0.33	9.03	6.38
Basophil	0.087	7.86	6.21
Mast	0.00167	0.32	0.16
Putative ILC2[b]	0.49	24.3	16.9

Abbreviations: ILC, Innate lymphoid cell; LTi, Lymphoid Tissue inducer.

Cell populations shown here are reported as a percentage of single cells and calculated in FlowJo software version 10.8.1. The spiked sample described here is prepared from the untouched splenocytes and enriched fraction. It enables the users to perform broadened transcriptomics (i.e., capture transcriptomes of underrepresented cell types), an example use case described in this protocol.

[a]To create the spiked sample, cells should be added accordingly so that the spiked cells sample is composed of 67% of the enriched cells and 33% of the untouched splenocytes, allowing the inclusion of the dominant spleen cells that are depleted in the enrichment procedure (i.e., T and B cells).

[b]ILC2 population likely includes an LTi cell population.

are attempted to determine if the expected cell populations were successfully obtained. Validation via flow can guide the users prior to committing to expensive library preparation(s) and sequencing.

Using the methods described herein, we provide an example scRNA-seq dataset and traditional Seurat analysis (Figure 5). The specifics of single-cell sequencing are outside the scope of this work; however, protocol resources are currently available. Briefly, the 10× Genomics recommended guidelines for cell preparation were utilized for this analysis. An overview of our sequencing results is available in Table 6, including comprehensive information on read yield, quality, mapping, and additional metrics. Standard pre-processing inspections (Figures 6 and 7) were performed with the Seurat R package and are also included to demonstrate the quality one can expect with these methods (following the Seurat pbmc3k Vignette available on Github; compiled January 11, 2022). Additional reference information on cDNA library quality checks is also included (Figure 8). It is recommended for users to consider similar quality checks when performing scRNA-sequencing for best results.

Details of a typical non-integrated Seurat analysis have been described with additional detail recently by Ji & Sadreyev (Ji and Sadreyev, 2019). For this example analysis, parameters suggested by Seurat are used with dimensions ("dims") and resolution (which are dataset dependent) set at 1:43 and 1 respectively. See example:

```
>seurat.object <- FindNeighbors(seurat.object, dims = 1:43)

>seurat.object <- FindClusters(seurat.object, resolution = 1)

>seurat.object <- RunUMAP(seurat.object, dims = 1:43)

>seurat.object <- RunTSNE(seurat.object, dims = 1:43)
```

Dimensions were informed from a jackstraw analysis (Figure 7), discussed in the Seurat vignettes, and the relevant reference (Ji and Sadreyev, 2019). Current splenic scRNA datasets are typically able to reconstruct major lymphoid (T, B, NK) and myeloid (Macrophage/Dendritic) cell populations that make up much of the spleen (Tabula Muris Consortium et al., 2018). However, an increased assortment of cell identities can be characterized through strategic enrichment, enabling more comprehensive

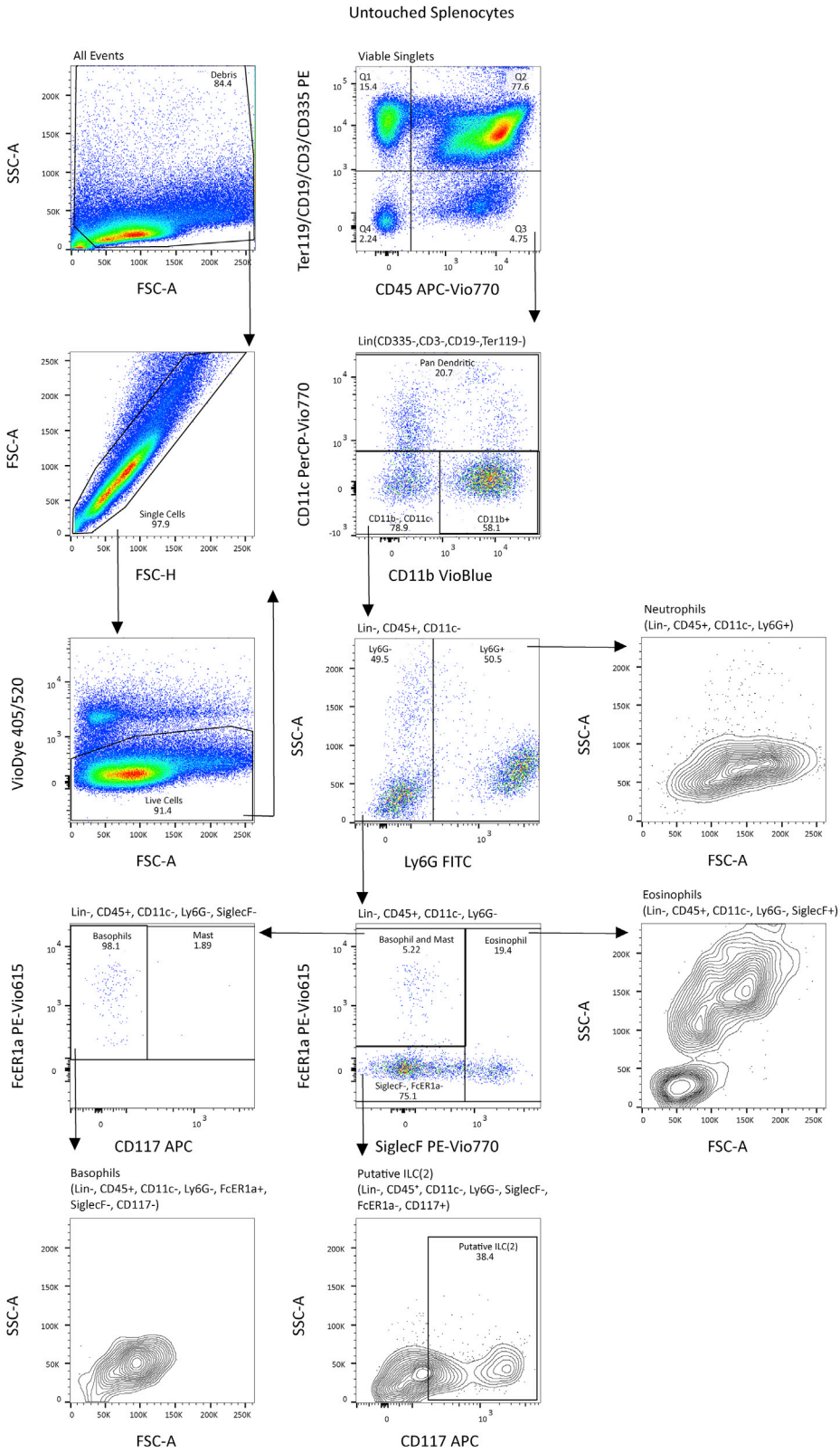


Figure 2. Flow analysis and gating strategy of untouched splenocytes

At gate Lin-, CD45+, CD11c- and thereafter, the populations are not gated on CD11b, however, CD11b counts are included for comparison purposes.

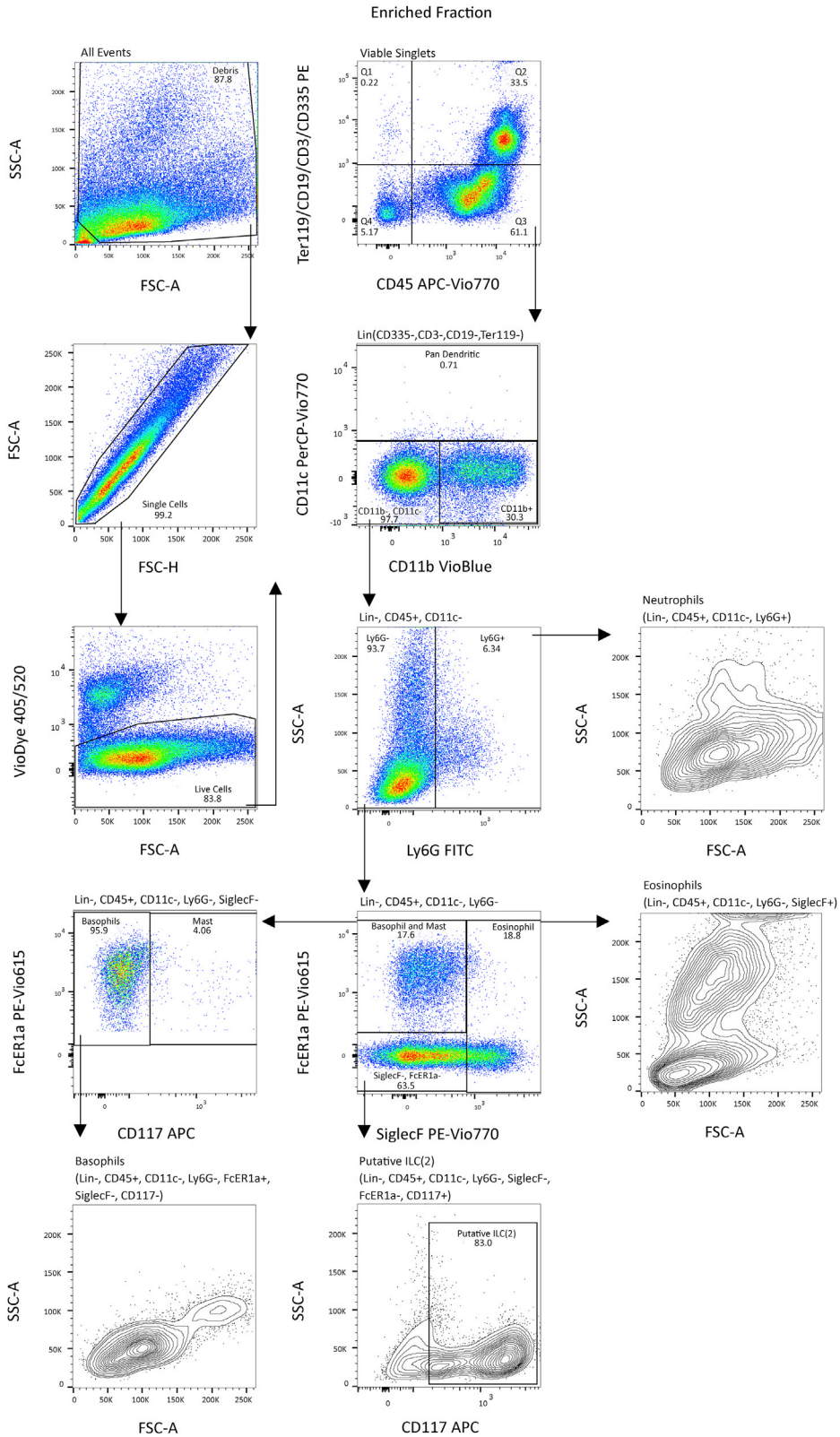


Figure 3. Flow analysis of the enriched population resulting from the immunomagnetic MACS separation procedure

At gate Lin-, CD45+, CD11c- and thereafter, the populations are not gated on CD11b, however, CD11b counts are included for comparison purposes.

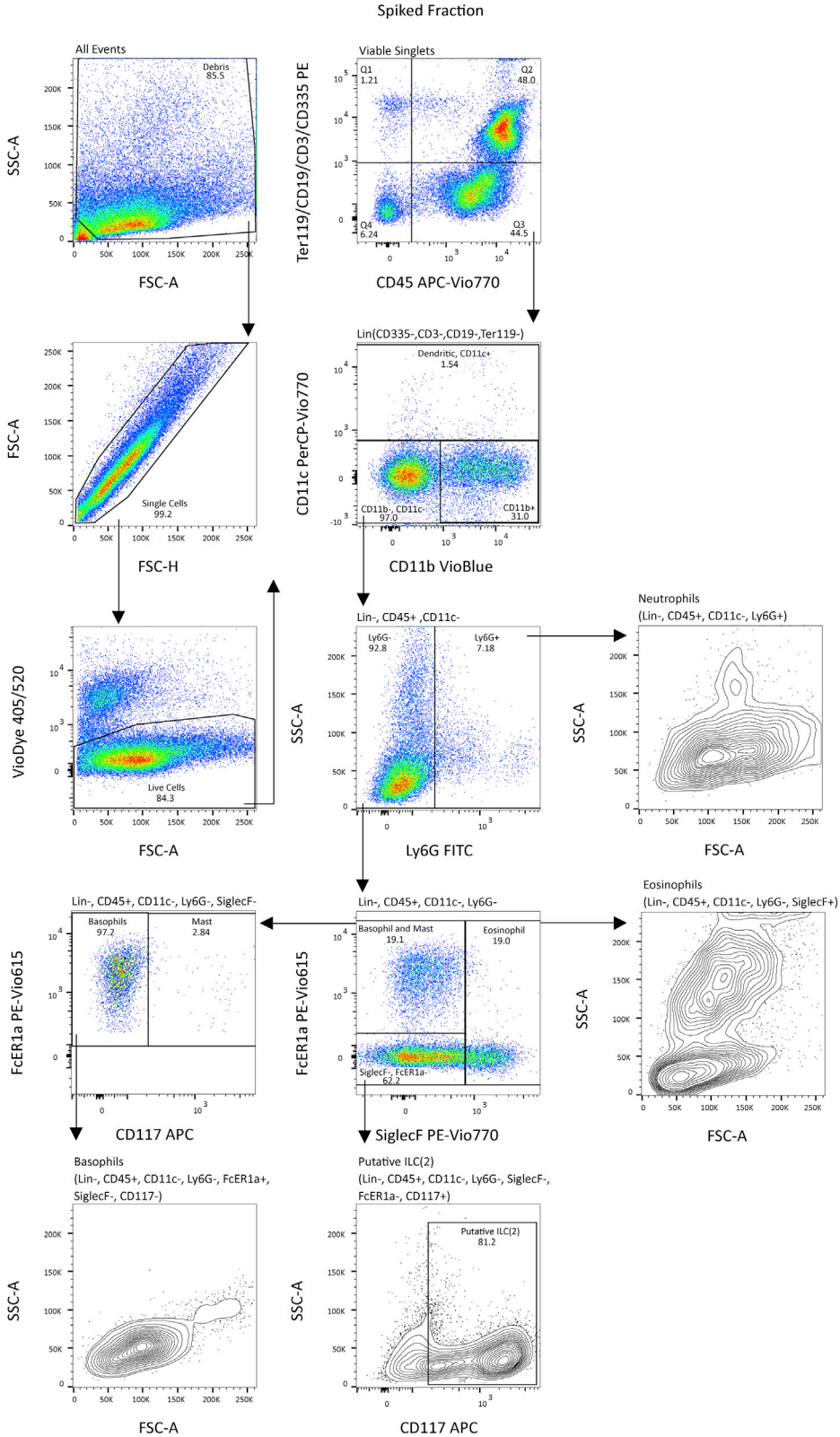

Spiked Fraction
All Events
Debris
85.5
SSC-A
FSC-A
Viable Singlets
Q1
1.21
Q2
48.0
Q4
6.24
Q3
44.5
Ter119/CD19/CD3/CD335 PE
CD45 APC-Vio770
FSC-A
Single Cells
99.2
FSC-H
Lin(CD335-,CD3-,CD19-,Ter119-)
Dendritic, CD11c+
1.54
CD11b-, CD11c-
97.0
CD11b+
31.0
CD11c PerCP-Vio770
CD11b VioBlue
VioDye 405/520
Live Cells
84.3
FSC-A
Lin-, CD45+ ,CD11c-
Ly6G-
92.8
Ly6G+
7.18
SSC-A
Ly6G FITC
Neutrophils
(Lin-, CD45+, CD11c-, Ly6G+)
SSC-A
FSC-A
Lin-, CD45+, CD11c-, Ly6G-, SiglecF-
Basophils
97.2
Mast
2.84
FcER1a PE-Vio615
CD117 APC
Lin-, CD45+, CD11c-, Ly6G-
Basophil and Mast
19.1
Eosinophil
19.0
SiglecF-, FcER1a-
62.2
FcER1a PE-Vio615
SiglecF PE-Vio770
Eosinophils
(Lin-, CD45+, CD11c-, Ly6G-, SiglecF+)
SSC-A
FSC-A
Basophils
(Lin-, CD45+, CD11c-, Ly6G-, FcER1a+, SiglecF-, CD117-)
SSC-A
FSC-A
Putative ILC(2)
(Lin-, CD45+, CD11c-, Ly6G-, SiglecF-, FcER1a-, CD117+)
Putative ILC(2)
81.2
SSC-A
CD117 APC

Figure 4. Flow analysis of the spiked cell populations resulting from the enriched sample being spiked with untouched splenocytes

To create the spiked sample, cells were added accordingly so that the sample was composed of 67% of the enriched cells and 33% is composed of untouched splenocytes, allowing inclusion of the dominant spleen cells that are depleted in the enrichment procedure (i.e., T and B cells). At gate Lin-, CD45+, CD11c- and thereafter, the populations are not gated on CD11b, however, CD11b counts are included for comparison purposes.

transcriptomics. With the example splenic single-cell dataset included here (Figure 5), 25 clusters, or distinct cell types and/or subtypes, were discovered with robust statistics. We utilized a recently published predictive annotation approach, scCATCH (Shao et al., 2020), that uses published studies and markers for cluster-calling, and these are detailed in Table 7. Notably, this approach used multiple tissues with scCATCH (spleen, peripheral blood, and bone marrow) to annotate this dataset. It may be revised in the future as these methods are rapidly progressing, particularly as under-sequenced cell types and corresponding transcription markers become better characterized. Overall, the intention of providing this example transcription dataset is to illustrate what the user can expect to achieve with the enrichment and processing procedures presented here.

LIMITATIONS

Regarding flow cytometry, outcomes can differ between labs due to variations in equipment and reagents (e.g., antibodies). Users who decide to integrate flow cytometry verification in their approach may need to optimize the workflow for their reagents and equipment (Rødahl et al., 2021). Unstained, live/dead, and Fluorescent Minus One (i.e., FMO) controls are highly recommended for all flow cytometry experiments, but this can be costly and sometimes impractical. However, immune cells, particularly granulocytes, are known for their autofluorescence which can impact compensation and result in misinterpretations of the data and conclusions (Dorward et al., 2013). Notably,

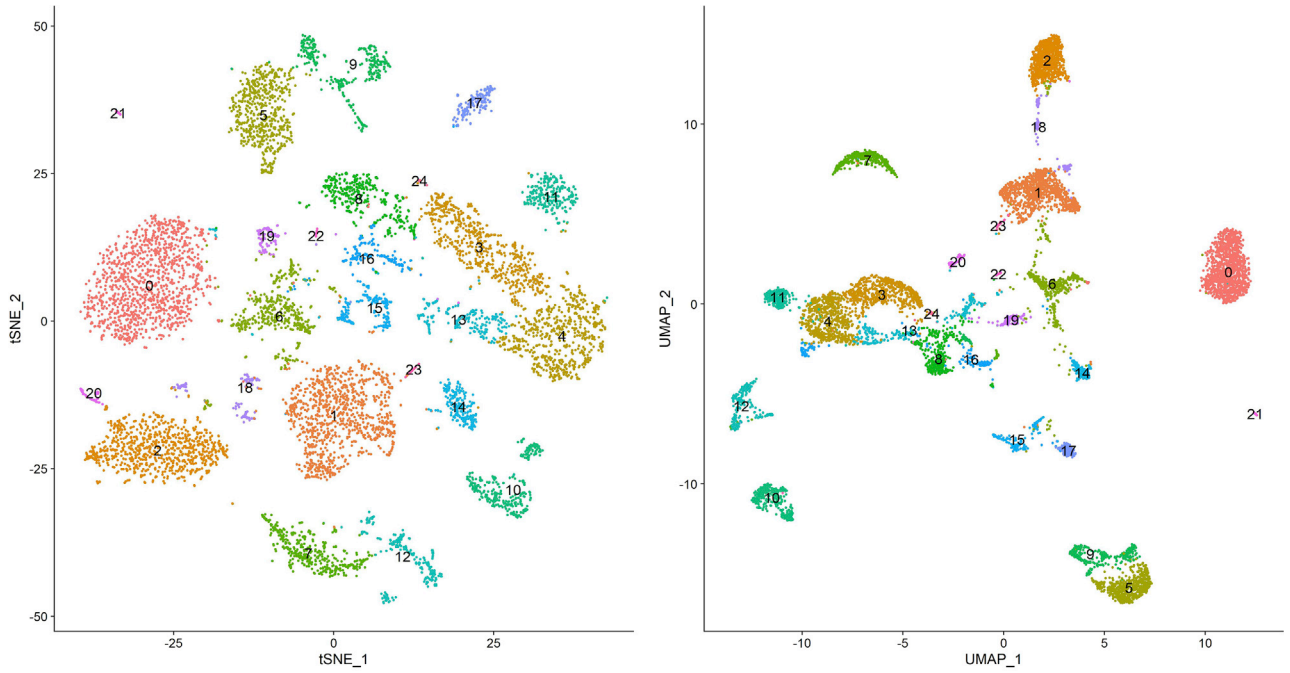


Figure 5. Dimensional reduction plots (DimPlots) outputs generate graphs of tSNE and UMAP dimensional reduction techniques

Dimensional Reduction plots in t-distributed stochastic neighbor embedding (tSNE; left) and Uniform Manifold Approximation and Projection (UMAP; right) were constructed using the DimPlot function in the Seurat R Package (version 4.1.0; Hao et al., 2021). Each plot consists of a 2-dimensional scatter plot where each dot represents a cell, and its location is determined based on the reduction technique used. In total, 25 clusters were generated using a resolution parameter of 1.0 and the dimensions ("dims") of 43 determined by the JackStraw analysis (Figure 6). It is recommended to use higher than default (default resolution = 0.5) resolutions with larger datasets (i.e., more cells and/or more expected cell populations) such as this dataset that is expected to contain more diverse cell types.

Table 6. Read library statistics	
CellRanger (10× genomics) pipeline quality statistics	
Estimated Number of Cells	8,506
Mean Reads per Cell	119,390
Median Genes per Cell	765
Number of Reads	1,015,534,648
Valid Barcodes	98.20%
Sequencing Saturation	94.10%
Q30 Bases in Barcode	97.00%
Q30 Bases in RNA Read	94.60%
Q30 Bases in UMI	96.80%
Reads Mapped to Genome	94.30%
Reads Mapped Confidently to Genome	87.40%
Reads Mapped Confidently to Intergenic Regions	2.70%
Reads Mapped Confidently to Intronic Regions	15.20%
Reads Mapped Confidently to Exonic Regions	69.50%
Reads Mapped Confidently to Transcriptome	66.80%
Reads Mapped Antisense to Gene	1.40%
Fraction Reads in Cells	89.70%
Total Genes Detected	19,924
Median UMI Counts per Cell	1,793
NovaSeq6000 run statistics	
Mean Quality Score	36.17
% ≥ Q3 Bases	95.16%
Yield (Mbases)	118,819

Pertinent sequencing read library information. The reference transcriptome used for analysis was "mm10-2020-A" (see key resources table). CellRanger (Version 4.0.0) developed by 10× Genomics was used to generate quality control analytics. Reporting these metrics is standard practice with sequencing data sets for transparency. This protocol produced typical quality statistics, and a high degree of sequencing saturation was achieved with the chosen read depth.

the flow cytometry work reported here has the potential to be improved using recent advances that employ more sophisticated compensation methods, such as AutoSpill (Roca et al., 2021).

Additionally, this protocol utilizes manual mechanical disruption with minimal force applied to the minced spleens by a soft rubber syringe plunger through a 70 μm cell strainer to facilitate the dissociation and generate the single-cell suspensions. Although this method is widely used, results may vary from lab to lab or between users (Lombardo et al., 2021). In cases where multiple experiments are repeated, it is best to have one user perform the spleen dissociation step that requires the syringe plunger and strainer for consistency. This may help minimize sample processing variation and result in more reliable comparisons between samples in downstream analyses, such as scRNA-seq. Alternatives to manual mechanical dissociation are commercially available automated instruments, such as the Miltenyi GentleMACS Dissociator. This instrument can be used along with Miltenyi's Mouse Spleen Dissociation Kit (Cat# 130-095-926), composed of a cocktail of enzymes optimized to gently dissociate mouse spleen using an optimized pre-programmed Miltenyi-designed protocol gently. Furthermore, others have used enzymatic dissociations in addition to manual mechanical dissociations. More recent methods have been explored using microfluidic systems to dissociate tissues for sensitive downstream analyses, such as scRNA-seq (Lombardo et al., 2021). Considering the sensitivity of scRNA-seq, it is critical to know how each of the different dissociation and cell enrichment strategies could affect your downstream data analyses and interpretations (Nguyen et al., 2018).

TROUBLESHOOTING

Problem 1

Unexpected fluorescent signal in the PE channel of the unstained and/or FMO samples (step #59). An example from our own experience can be seen and described in Figure 9. In addition, please see problem 2, as problem 1 and problem 2 are likely to be complications that coexist.

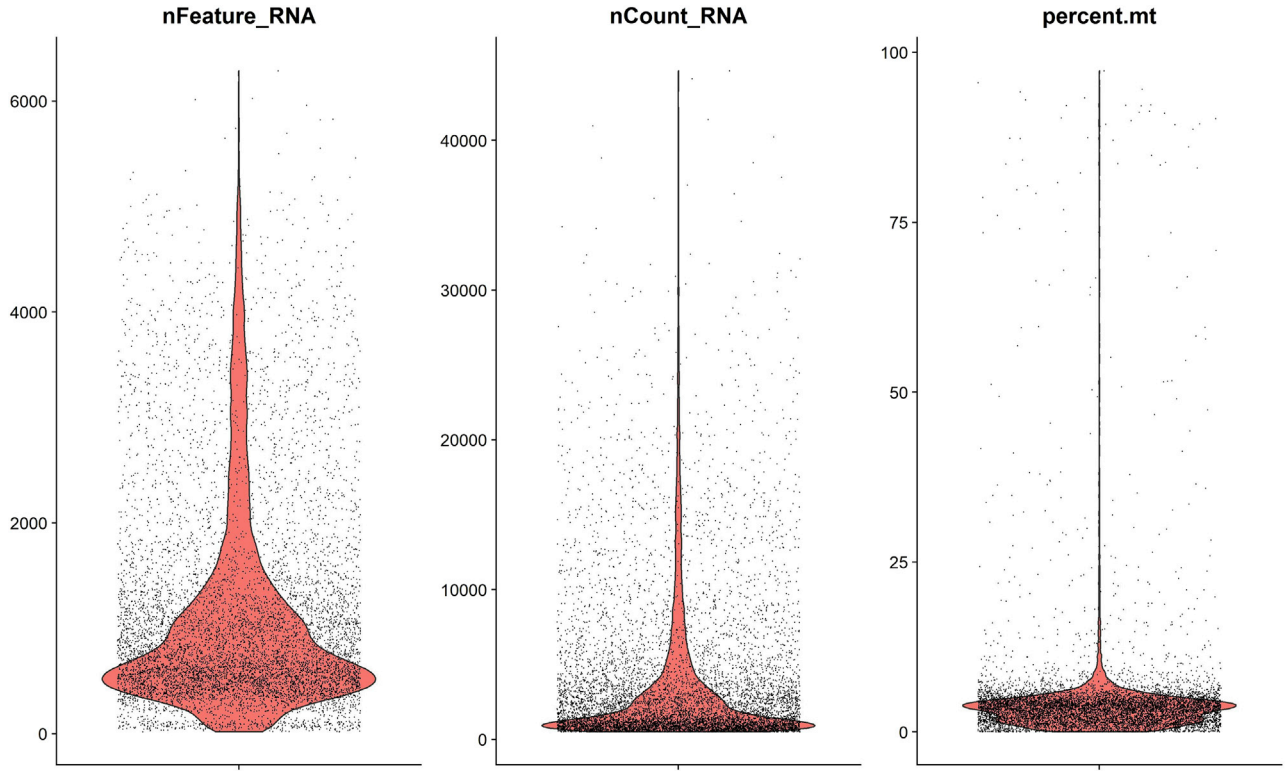


Figure 6. Quality control metrics of genes, reads, and mitochondrial transcripts per cell as determined by standard Seurat pre-processing

Violin plots illustrate the number of genes detected per cell (nFeature_RNA), number of reads per cell (nCount_RNA), and the percent of mitochondrial transcripts per cell (percent.mt). Deep sequencing produced an abundance of features and read counts with acceptable levels of RNA from mitochondrial genes. Notably, differences in sample preparation protocols can cause unexpected and profound differences in these parameters, demonstrating their value for consideration (Wohnhaas et al., 2019).

Potential solution

- This problem can be resolved by adding additional PE beads or decreasing the input number of cells subjected to the enrichment separations. It is vital to consider controls such as these during initial protocol development to ensure the separation is efficient performing as expected.
- Review problem 2 and its Potential solutions.

Problem 2

Detecting substantial unwanted or depleted cell populations in the enriched population from the LD column flow-through (steps #36–42 and #59). In addition, please see problem 1, as problem 2 and problem 1 are likely to be complications that coexist.

Potential solution

- Be sure to review the proper use of the plunger when using the MACS LD columns (steps #36 and #37). During binding or washing steps, plunging can push through cells bound via magnetic attraction (undesired PE-microbead-labeled cells).
- Ensure all antibodies and microbeads used for depleting cell populations are not expired, as this can reduce their binding effectiveness to their targets.
- This protocol uses LD columns, which are made for depleting unwanted (labeled) cells while obtaining unlabeled desired cells that are "untouched" (negative selection), rather than the LS columns, which are used when the desired cells are labeled and retained by the column (positive selection). The plunger is used to elute positively retained cells from the column matrix after magnetic separation. The plunger is not used in any part of this presented protocol.

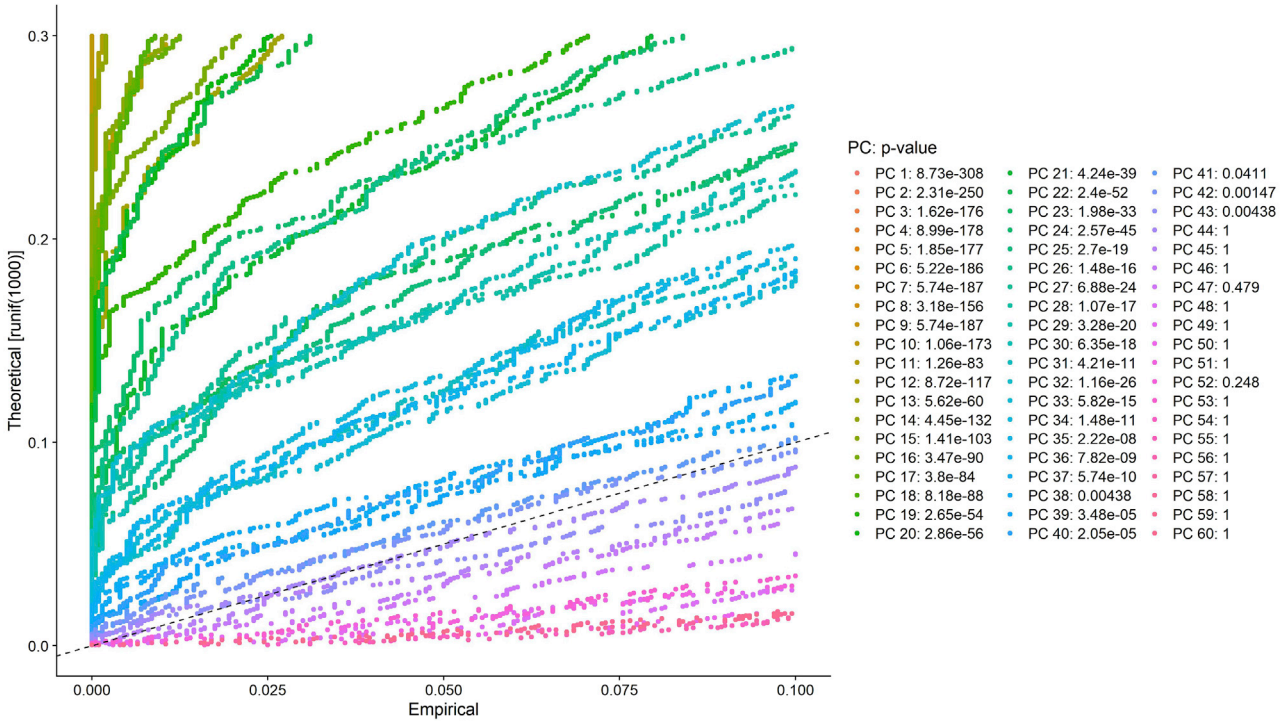


Figure 7. Scored JackStraw plot assists in determining the dimensionality of the scRNA-seq dataset for subsequent Seurat clustering analysis

During Seurat cluster analysis, principal components (PCs) were constructed and statistically analyzed with heuristic methods such as the Seurat functions JackStraw and/or ElbowPlots (Macosko et al., 2015). The JackStraw function analyzes the input dataset and returns a plot of PCs and their corresponding p-values which allows for the determination of the dimensionality, or the number of "real" dimensions, in the data set. The distinction between insignificant and significant PCs is used in determining the dimensions ("dims") which is subsequently used as the input for the "dims". We executed the JackStraw function using our dataset as the input which generated the JackStraw plot with the corresponding PCs and their respective p-values as shown in this figure. It is noteworthy that our dataset generated an exceptionally high number of dimensions, indicating that a substantial number of significantly distinct cell populations were able to be confidently identified. The highly significant PCs with a distinct cut-off between significant and insignificant PCs, shown as a black dotted line on the plot between PCs 43 and 44 implies that the JackStraw function identified 43 dimensions meeting statistical significance (p-value < 0.05; 95% confidence interval) and this value was used for the dimensions, or "dims", in the subsequent Seurat clustering analysis of the spiked scRNA-seq dataset.

- Ensure the LD columns are secured and locked into their proper position in the MACS stand. The columns should fit snugly into the magnetic column holders.
- Review problem 1 and its Potential solutions.

Problem 3

Low/no cell recovery in the expected enriched population that is eluted in the immunomagnetic depletion column (steps #38–42).

Potential solution

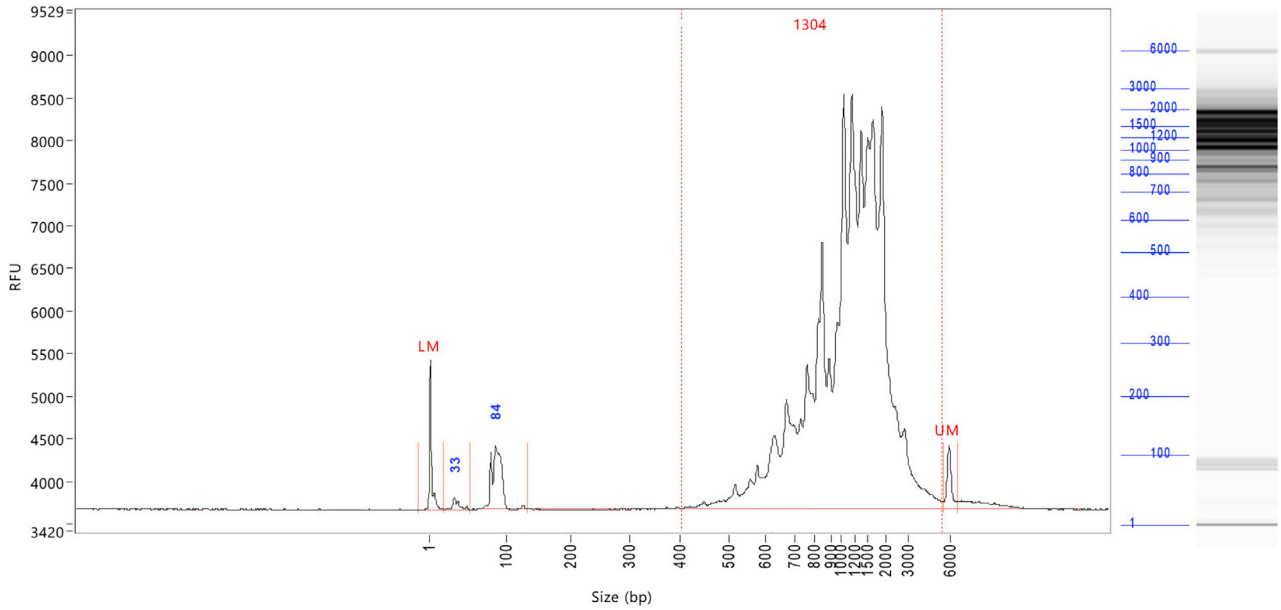
- Some bead combinations, or too many beads in a cocktail, may lead to unexpected cell losses or inappropriate cell population removal. We do not recommend more than two beads unless verified. Additionally, ensure that the antibodies and/or beads are not past their expiration dates.
- Make sure to use the FcR blocking reagent in this step. Although other blocking solutions can be made or purchased from other companies, we highly recommend Miltenyi's FcR Block mouse reagent. We have not tried any other blocking reagents and would advise against using other blocking reagents. If users choose to use a different blocking reagent, it should be validated in comparison to the Miltenyi mouse FcR blocking buffer prior to further downstream applications.

Table 7. Cluster gene markers and predicted identities as annotated with scCATCH package

Cluster	Cells	% Of total cells	Markers	scCATCH predicted identity	Score	Tissue
0	1163	13.67	Ccl5, Gzma, AW112010, Nkg7, Ncr1, Klra8, Klra1, Klre1, Klrd1, Klra4	Natural Killer Cell	0.63	PB
1	1000	11.76	Cd79a, Ebf1, Cd79b, Ighd, Ms4a1, Ly6d, Ifi30, Cd74, H2-Eb1, H2-Aa	Marginal Zone B Cell	0.61	SPLN
2	799	9.39	Ccl6, Ccl9, Gm12840, Ifitm1, Ms4a2, Lilr4b, Cyp11a1, Alox5ap, Cyp4f18, Ccl3	Macrophage/Neutrophil	0.58/0.58	PB/BM
3	610	7.17	Car1, Myb, Apoe1, Cdk6, Vamp5, Mfsd2b, Ass1, Muc13, Clec4d, Scin	ND		
4	599	7.04	Car11, Mt11, Mt21, Hspe11, Hsp90aa11, Ncl1, Car21, Npm11, Tuba1b1, Hdgf1	Macrophage	0.81	SPLN
5	570	6.70	Camp, Lcn2, Ngp, S100a9, Chil3, Ltf, Lyz2, S100a8, Pglyrp1, Hp	ND		
6	468	5.50	Trbc2, Cd3e, Cd3g, Cd3d, Trac, Bcl11b, Thy1, Ms4a6b, Trbc11, Gimap3	T Cell	0.75	SPLN
7	412	4.84	Hba-a1, Hbb-bt, Bpgm, Alas2, Hba-a2, Snca, Rsad2, Hbb-bs, Fam220a, Mkrn1	Erythroblast	0.5	SPLN
8	368	4.33	Myl10, Hlf, Meis1, Mecom, Adgrl4, Cd34, Adgrg1, Tmem176b, Mycn, Gcnt2	HSC/Stem Cell	0.58/0.58	BM
9	342	4.02	Wfdc21, Ltf, Ngp, Chil3, Lyz2, Lcn2, Ifitm6, Hp, Cybb, F630028O10Rik	Neutrophil/Macrophage	0.86/0.83	BM/SPLN
10	317	3.73	Jchain, Iglv1, Mzb1, Iglc1, Edem1, Eaf2, Trp53inp1, Txndc11, Derl3, Tnfrsf17	Plasma Cell	0.78	SPLN
11	259	3.04	Car1, Mt1, Blvrb, Rpl14, Npm1, Dut, Hsp90aa1, Rpl8, Rpl18, Rpl13	ND		
12	254	2.99	Slc4a1, Slc25a37, Gypa, Rsad2, Tmcc2, Alas2, Snca, Apol11b, Gch1, Trim10	Erythrocyte/B Cell	0.67/0.63	BM/SPLN
13	249	2.93	Nusap1, Cep55, Top2a, Hist1h2ak, Mki67, Kif11, Hist1h2ae, Cenpf, Stmn1, Tpx2	HSC	0.5	BM
14	211	2.48	Ncoa7, Il7r, S100a4, Tmem176a, Ccr6, Cxcr6, Ckb, Lingo4, Tmem176b, Asb2	HSC/Plasmacytoid DC	0.67/0.61	BM/SPLN
15	184	2.16	Fn1, F13a1, Clec4a3, Clec4a1, Sirpb1c, Pid1, Csf1r, S100a4, Ifi202b, Tifab	Monocyte	0.61	BM
16	181	2.13	mt-Cytb, Gm42418, mt-Nd1, mt-Atp6, mt-Nd2, mt-Co1, mt-Nd3, mt-Nd4, mt-Co3, mt-Co2	ND		
17	166	1.95	Siglech, Cd209d, Mpeg1, Cox6a2, Ccr9, Klk1, Pld4, Cadm1, Pltp, Cd209a	Dendritic Cell	0.77	BM
18	113	1.33	Cyp11a1, Fcer1a, Ccl6, Lilr4b, Ms4a2, Klra8, Csrp3, Cyp4f18, Il18r1, Ighd	ND		
19	90	1.06	Dntt, Bcl11b, Arpp21, Tcrg-C2, Myl10, Tcrg-C4, Thy1, Cd3g, Tcf7, Trbc1	T Cell	0.8	SPLN
20	58	0.68	Prss34, Ctsg, Fcer1a, Mcpt8, Cpa3, Mpo, Ms4a3, Alox5, Ms4a2, 4930519L02Rik	Basophil	0.86	BM
21	32	0.38	Il1b, Csf3r, Retnlg, Osgin1, Mmp9, Pla2g7, Gm5150, Il1f9, Sirpb1b, Cd300ld	Neutrophil/Granulocyte	0.67/0/63	BM/SPLN
22	23	0.27	Ppbp, Clec1b, Myl9, Thbs1, Clu, Tpm2, Alox12, Cdc42ep5, Cald1, Fhl1	HSC	0.88	BM
23	21	0.25	Mef2b, Rgs13, Aicda, Lipc, Mybl1, Gcsam, Nuggc, S1pr2, Neil1, Smagp	Dendritic Cell	0.82	BM
24	17	0.20	Scin, Mitf, Gzmb, Papss2, Ahr, Cpa3, Angpt1, Slc45a3, Gm43445, Csrp3	Basophil	0.75	BM

Abbreviations: BM: Bone Marrow; HSC: Hematopoietic Stem Cell; PB: Peripheral Blood; ND, Not Determined; SPLN: Spleen.

Cluster numbers and corresponding cell numbers and percentages are reported. The top 10 gene markers are reported for each cluster and determined with Seurat function FindAllMarkers. Top markers are positive in expression and are ordered by significance (most significant first, all markers statistically significant). Cells were annotated with scCATCH (version 3.0), allowing multiple annotation tissues to be selected (Shao et al., 2020). Tissues used for annotations included Spleen (SPLN), Bone Marrow (BM), and Peripheral Blood (PB). Annotations with the highest cell scores were reported, and, in some instances, multiple annotations are reported in the case of similar scores.



Peak	Size (bp)	Conc. (ng/uL)	From (bp)	To (bp)	Avg. Size (bp)	CV%	RFU	Corr. Peak Area
1	1 (LM)	0.0186	0	20	2	142.07	1757	11.340
2	33	0.0431	20	53	34	15.91	137	2.194
3	84	0.3889	53	133	86	9.16	748	19.792
4	6000 (UM)	0.0073	5600	6596	6018	3.32	737	4.447
	TIC:	0.4320	ng/uL					
	TIM:	9.4950	nmole/L					
	Total Conc.:	8.6116	ng/uL					
Smear Analysis	400 bp to 5500 bp	8.1659 ng/ul	94.8 %Total	10.3047 nmole/L	1304 Avg. Size (bp)	50.20 %CV		

Figure 8. cDNA library spectrum analysis

Prior to sequencing on the NovaSeq 6000 (this report) or similar platforms, the cDNA library was visualized on a Fragment Analyzer Automated CE System. When working with granulocytes and other RNA-poor cell types additional cycle-times during cDNA preparation can be beneficial (10× Genomics webpage [https://kb.10xgenomics.com/hc/en-us/articles/360004024032-Can-I-process-neutrophils-or-other-granulocytes-using-10x-Single-Cell-applications]). In our cDNA preparation, we included two additional cycles as we expected RNA-poor cell types as a result of cells like eosinophils being enriched and the presence of neutrophils. Tall and distinct peaks are desirable and indicate successful preparation.

Problem 4

User(s) obtained an expected yield for the enriched cell population but experienced low or diminishing cell recovery during the enriched cell fraction processing for downstream applications (i.e., in the many washing/centrifugation steps for flow prep samples and/or the scRNA-seq sample; step #45 onward).

Potential solution

BSA improves cell recovery and pellet visibility (see Enhanced Recovery Buffer - Alternatives). Be sure to consider the addition of this reagent at an increased concentration (2% BSA, as used in our ER Buffer) and verify compatibility with single-cell sequencing preparation workflows. Of note, this may increase debris detected in flow cytometry and should be a careful consideration of the user. The use of an ultra-pure BSA reagent (as used in this protocol, Miltenyi BSA Stock Solution) may attenuate this and is highly recommended.

Problem 5

Cells have low viability (less than 85%) in either the splenocyte single-cell suspension prep (step #19) or in the enriched fraction (step #42).

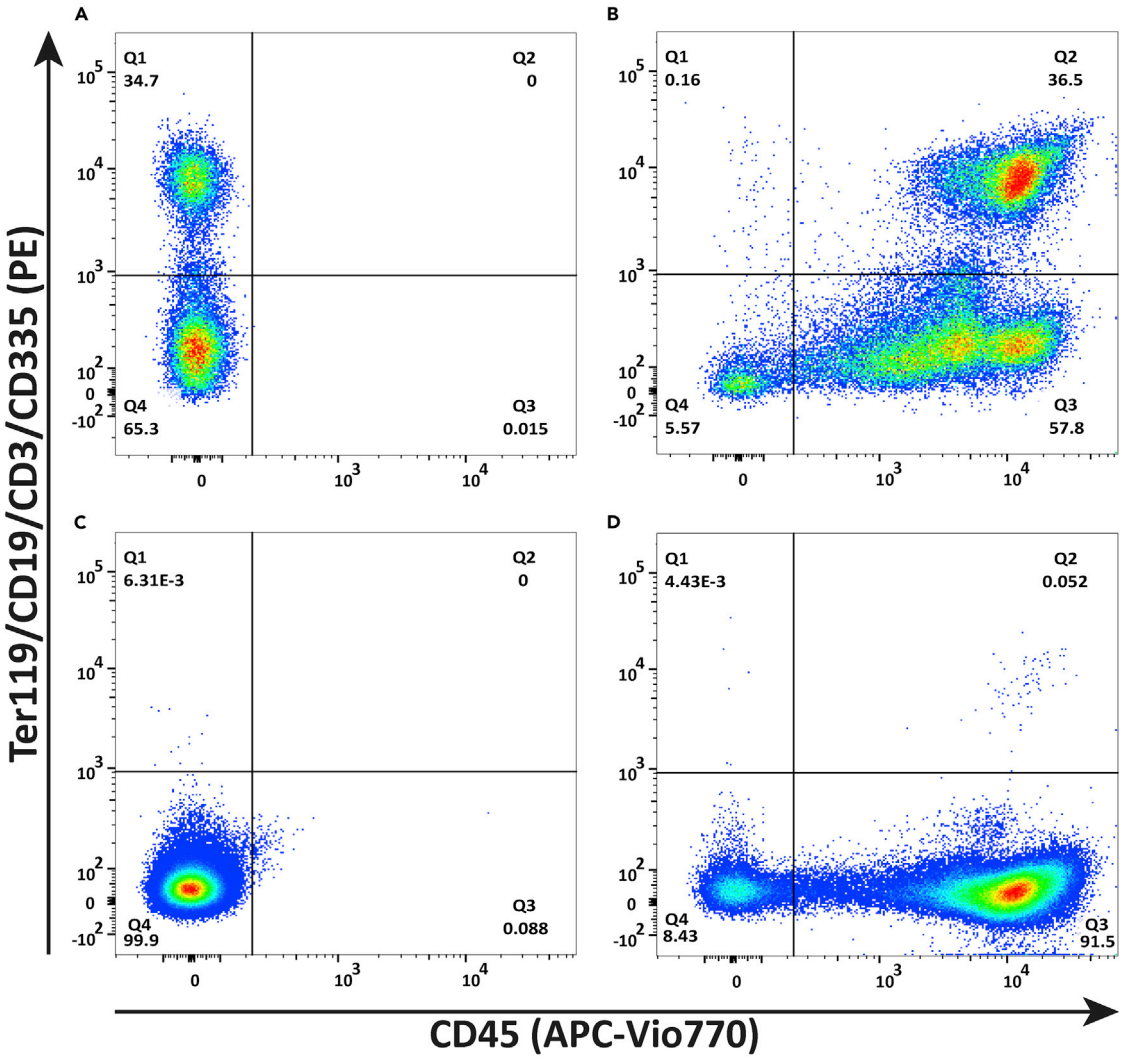


Figure 9. PE-labeled cell contamination in unstained and FMO gates

Example plots of unstained and FMO controls with PE-labeled cell flow-through from depletion cocktails with comparisons.
(A) indicates an unstained control cell sample with a PE-positive population that is carried over from the MACS separation.
(B) indicated the same PE-labeled cells in the FMO control for PE.
(C and D) Example plots (C) and (D) illustrate proper unstained and FMO controls, respectively.

Potential solution

- If low viability is observed in the splenocyte single-cell suspension (step #19), ensure that all timing is strictly followed, cells are kept on ice, and all solutions used are maintained on ice.
- The spleen homogenization process should be executed with negligible downward pressure with the rubber plunger. It is also critical that the spleen solution is constantly added during this process.
- Ensure that the user(s) extracting the spleens can quickly remove them after secondary cervical dislocation and immediately place them in ice-chilled SES. It is best to extract the three spleens separately if the user is less experienced in mouse surgical techniques.
- Low viability in either or both steps (#19 and/or #42) can be observed if the users do not use wide-bore pipette tips when cells are being resuspended during the entire protocol. We observed improved cell viability after switching to wide bore tips (+10%–15%). This was more pronounced in the enriched fraction as many cell types (i.e., neutrophils) are delicate and must be handled gently.

- Confirm that the PBS and/or HBSS used are calcium- and magnesium-free. Use of EDTA in the buffer starting at step #21 until the change to 10×-SB (step #41). These are all recommendations that Miltenyi suggests for immunomagnetic cell separations using their MACS technology.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Paul H. Davis (pdavis@unomaha.edu).

Materials availability

This study did not generate new unique reagents. Please contact Dr. Paul H. Davis (pdavis@unomaha.edu) to inquire about access to other materials in this manuscript.

Data and code availability

The accession number for the 10× scRNA-seq dataset files (fastq) reported in this papaer is NCBI Sequence Read Archive Bioproject PRJNA809678: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA809678. This study did not generate any new code.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.J.N. and T.T.S.; Protocol Development and Execution, A.J.N. and T.T.S.; Data Analysis, A.J.N., T.T.S., and R.C.; Writing – Original Draft, A.J.N. and T.T.S.; Reviewing and Editing, R.C., A.J.N., T.T.S., P.H.D.; Supervision, P.H.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Bar-Ephraïm, Y.E., and Mebius, R.E. (2016). Innate lymphoid cells in secondary lymphoid organs. Immunol. Rev. *271*, 185–199. https://doi.org/10.1111/imr.12407.

Bedner, E., Burfeind, P., Gorczyca, W., Melamed, M.R., and Darzynkiewicz, Z. (1997). Laser scanning cytometry distinguishes lymphocytes, monocytes, and granulocytes by differences in their chromatin structure. Cytometry *29*, 191–196.

Brattig, N.W., Garza, C.E.M., and Tischendorf, F.W. (1987). Comparative study of eosinophil purification on Nycodenz, Metrizamide and Percoll density gradients. Eur. J. Haematol. *39*, 148–153. https://doi.org/10.1111/j.1600-0609.1987.tb00745.x.

Brestoff, J.R., Kim, B.S., Saenz, S.A., Stine, R.R., Monticelli, L.A., Sonnenberg, G.F., Thome, J.J., Farber, D.L., Lutfy, K., Seale, P., and Artis, D. (2015). Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. Nature *519*, 242–246. https://doi.org/10.1038/nature14115.

Buettner, M., and Lochner, M. (2016). Development and function of secondary and tertiary lymphoid organs in the small intestine and the colon. Front. Immunol. *7*, 342. https://doi.org/10.3389/fimmu.2016.00342.

Cossarizza, A., Chang, H.D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W.W., Aghaeepour, N., Akdis, M., Allez, M., et al. (2019). Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur. J. Immunol. *49*, 1457–1973. https://doi.org/10.1002/eji.201970107.

DiPiazza, A.T., Hill, J.P., Graham, B.S., and Ruckwardt, T.J. (2019). OMIP-061: 20-color flow cytometry panel for high-dimensional characterization of murine antigen-presenting cells. Cytometry A *95*, 1226–1230. https://doi.org/10.1002/cyto.a.23880.

Dorward, D.A., Lucas, C.D., Alessandri, A.L., Marwick, J.A., Rossi, F., Dransfield, I., Haslett, C., Dhaliwal, K., and Rossi, A.G. (2013). Technical advance: autofluorescence-based sorting: rapid and nonperturbing isolation of ultrapure neutrophils to determine cytokine production. J. Leukoc. Biol. *94*, 193–202. https://doi.org/10.1189/jlb.0113040.

Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W.M., Zheng, S., Butler, A., Lee, M.J., Wilk, A.J., Darby, C., Zager, M., et al. (2021). Integrated analysis of multimodal single-cell data. Cell *184*, 3573–3587.e29. https://doi.org/10.1016/j.cell.2021.04.048.

Hasenberg, M., Köhler, A., Bonifatius, S., Borucki, K., Riek-Burchardt, M., Achilles, J., Männ, L.,

Baumgart, K., Schraven, B., and Gunzer, M. (2011). Rapid immunomagnetic negative enrichment of neutrophil granulocytes from murine bone marrow for functional studies in vitro and in vivo. PLoS ONE *6*, e17314. https://doi.org/10.1371/journal.pone.0017314.

Ji, F., and Sadreyev, R.I. (2019). Single-cell RNA-seq: introduction to bioinformatics analysis. Curr. Protoc. Mol. Biol. *127*, e92. https://doi.org/10.1002/cpmb.92.

Kalina, T. (2020). Reproducibility of flow cytometry through standardization: opportunities and challenges. Cytometry A *97*, 137–147. https://doi.org/10.1002/cyto.a.23901.

Kamala, T. (2008). An optimized immunomagnetic bead-based negative selection protocol for CD4 T-cell isolation from mouse lymph nodes and spleen. Scand. J. Immunol. *67*, 285–294. https://doi.org/10.1111/j.1365-3083.2007.02066.x.

Liechti, T., Weber, L.M., Ashhurst, T.M., Stanley, N., Prlic, M., Van Gassen, S., and Mair, F. (2021). An updated guide for the perplexed: cytometry in the high-dimensional era. Nat Immunol *22*, 1190–1197. https://doi.org/10.1038/s41590-021-01006-z.

Lombardo, J.A., Aliaghaei, M., Nguyen, Q.H., Kessenbrock, K., and Haun, J.B. (2021). Microfluidic platform accelerates tissue processing into single cells for molecular analysis and primary culture models. Nat. Commun. *12*, 2858. https://doi.org/10.1038/s41467-021-23238-1.

Lucas, F., Gil-Pulido, J., Lamacchia, J., Preffer, F., Wallace, P.K., and Lopez, P. (2020). MiSet RFC standards: defining a universal minimum set of standards required for reproducibility and rigor in research flow cytometry experiments. Cytometry A *97*, 148–155. https://doi.org/10.1002/cyto.a.23940.

Maciorowski, Z., Chattopadhyay, P.K., and Jain, P. (2017). Basic multicolor flow cytometry. Curr. Protoc. Immunol. *117*, 5.4.1–5.4.38. https://doi.org/10.1002/cpim.26.

Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell *161*, 1202–1214. https://doi.org/10.1016/j.cell.2015.05.002.

Mair, F., and Tyznik, A.J. (2019). High-dimensional immunophenotyping with fluorescence-based cytometry: a practical guidebook. Methods Mol. Biol. *2032*, 1–29. https://doi.org/10.1007/978-1-4939-9650-6_1.

Nguyen, Q.H., Pervolarakis, N., Nee, K., and Kessenbrock, K. (2018). Experimental considerations for single-cell RNA sequencing approaches. Front. Cel. Dev. Biol. *6*, 108. https://doi.org/10.3389/fcell.2018.00108.

Phi-Wilson, J.T., and Recktenwald, D.J. (1993). Coating agents for cell recovery. United States Patent Number 5232828. https://patentcenter.uspto.gov/#!/applications/07848513.

R Core Team (2020). R: A language and environment for statistical computing (Vienna, Austria: R Foundation for Statistical Computing). https://www.R-project.org/.

Roca, C.P., Burton, O.T., Gergelits, V., Prezzemolo, T., Whyte, C.E., Halpert, R., Kreft, Ł., Collier, J., Botzki, A., Spidlen, J., et al. (2021). AutoSpill is a principled framework that simplifies the analysis of multichromatic flow cytometry data. Nat. Commun. *12*, 2890. https://doi.org/10.1038/s41467-021-23126-8.

Rødahl, I., Gotley, J., Andersen, S.B., Yu, M., Mehdi, A.M., Christ, A.N., Hamilton-Williams, E.E., Frazer, I.H., Lukowski, S.W., and Chandra, J. (2021). Acquisition of murine splenic myeloid cells for protein and gene expression profiling by advanced flow cytometry and CITE-seq. STAR Protoc. *2*, 100842. https://doi.org/10.1016/j.xpro.2021.100842.

Romero-Santacreu, L., Moreno, J., Pérez-Ortín, J.E., and Alepuz, P. (2009). Specific and global regulation of mRNA stability during osmotic stress in Saccharomyces cerevisiae. RNA *15*, 1110–1120. https://doi.org/10.1261/rna.1435709.

RStudio Team (2020). RStudio: Integrated Development for R. RStudio (Boston, MA: PBC). http://www.rstudio.com/.

Sedgwick, J.B., Shikama, Y., Nagata, M., Brener, K., and Busse, W.W. (1996). Effect of isolation protocol on eosinophil function: Percoll gradients versus immunomagnetic beads. J. Immunol. Methods *198*, 15–24. https://doi.org/10.1016/0022-1759(96)00139-1.

Shao, X., Liao, J., Lu, X., Xue, R., Ai, N., and Fan, X. (2020). scCATCH: automatic annotation on cell types of clusters from single-cell RNA sequencing data. iScience *23*, 100882. https://doi.org/10.1016/j.isci.2020.100882.

Shiu, J., Piazuelo, M.B., Ding, H., Czinn, S.J., Drakes, M.L., Banerjee, A., Basappa, N., Kobayashi, K.S., Fricke, W.F., and Blanchard, T.G. (2015). Gastric LTi cells promote lymphoid follicle formation but are limited by IRAK-M and do not alter microbial growth. Mucosal Immunol. *8*, 1047–1059. https://doi.org/10.1038/mi.2014.132.

Son, K., Mukherjee, M., McIntyre, B.A., Eguez, J.C., Radford, K., LaVigne, N., Ethier, C., Davoine, F., Janssen, L., Lacy, P., and Nair, P. (2017). Improved recovery of functionally active eosinophils and neutrophils using novel immunomagnetic technology. J. Immunol. Methods *449*, 44–55. https://doi.org/10.1016/j.jim.2017.06.005.

Sun, J., Kroeger, J.L., and Markowitz, J. (2021). Introduction to multiparametric flow cytometry and analysis of high-dimensional data. Methods Mol. Biol. *2194*, 239–253. https://doi.org/10.1007/978-1-0716-0849-4_13.

Tabula Muris Consortium; Overall Coordination; Logistical Coordination; Organ Collection and Processing; Library Preparation and Sequencing; Computational Data Analysis; Cell type Annotation; Writing Group; Supplemental Text Writing Group; Principal Investigators (2018). Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. Nature *562*, 367–372. https://doi.org/10.1038/s41586-018-0590-4.

van den Brink, S.C., Sage, F., Vértesy, Á., Spanjaard, B., Peterson-Maduro, J., Baron, C.S., Robin, C., and van Oudenaarden, A. (2017). Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. Nat. Methods *14*, 935–936. https://doi.org/10.1038/nmeth.4437.

Wang, L., and Hoffman, R.A. (2017). Standardization, calibration, and control in flow cytometry. Curr. Protoc. Cytom. *79*, 1.3.1–1.3.27. https://doi.org/10.1002/cpcy.14.

Wohnhaas, C.T., Leparc, G.G., Fernandez-Albert, F., Kind, D., Gantner, F., Viollet, C., Hildebrandt, T., and Baum, P. (2019). DMSO cryopreservation is the method of choice to preserve cells for droplet-based single-cell RNA sequencing. Sci. Rep. *9*, 10699. https://doi.org/10.1038/s41598-019-46932-z.

Xiong, L., Lee, H., Ishitani, M., and Zhu, J.K. (2002). Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis. J. Biol. Chem. *277*, 8588–8596. https://doi.org/10.1074/jbc.M109275200.

Zeng, B., Shi, S., Ashworth, G., Dong, C., Liu, J., and Xing, F. (2019). ILC3 function as a double-edged sword in inflammatory bowel diseases. Cell Death Dis. *10*, 315. https://doi.org/10.1038/s41419-019-1540-2.