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Author/s:

Alexandre, YO;Mueller, SN

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An optimized protocol for the isolation of rare stromal cell populations from the mouse spleen

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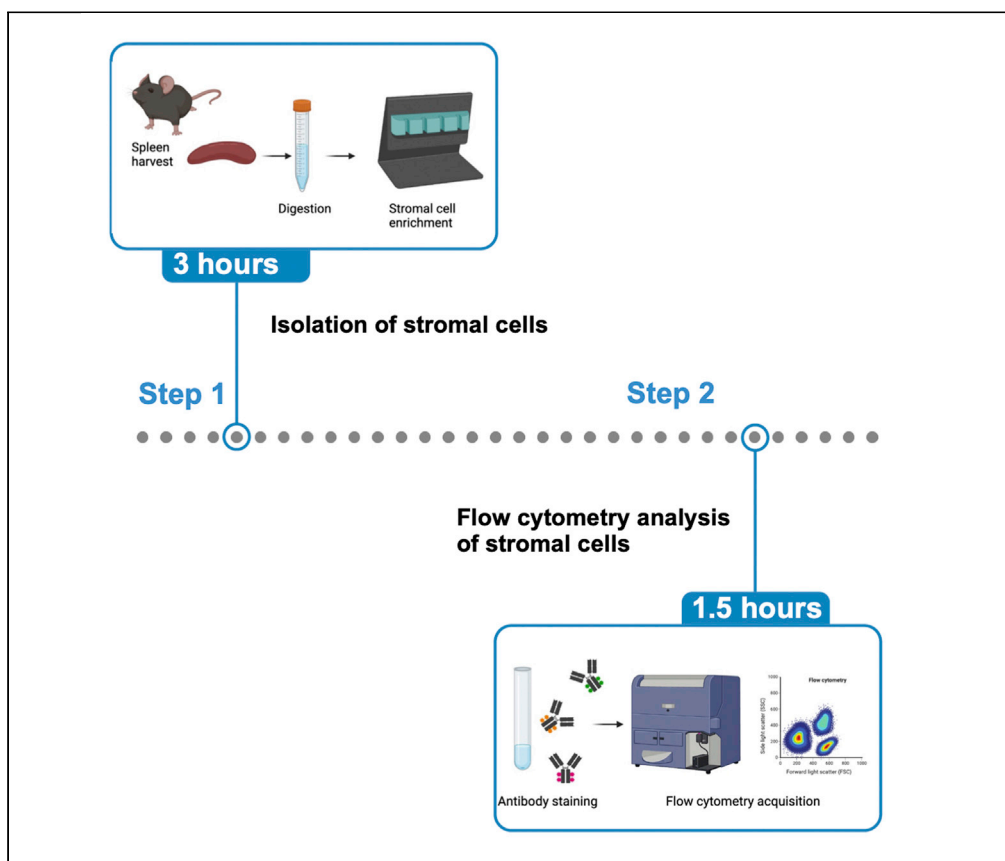
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Protocol

An optimized protocol for the isolation of rare stromal cell populations from the mouse spleen



Yannick O.
Alexandre, Scott N.
Mueller

yannick.alexandre@
unimelb.edu.au (Y.O.A.)
smue@unimelb.edu.au
(S.N.M.)

Highlights

Protocol for the
isolation and analysis
of mouse splenic
stromal cells

Digestion method to
release stromal cells

Enrichment and
antibody staining of
stromal cells

Details of FACS
analysis of splenic
stromal cell subsets

Lymphoid tissue stromal cells are important regulators of spleen homeostasis and immune responses. Here, we present an optimized protocol that describes the digestion and enrichment steps for the isolation and analysis of rare populations of stromal cells, including fibroblastic reticular cells, perivascular cells, and glial cells found in the spleen. This protocol is suitable for subsequent analysis of spleen stromal cells by flow cytometry or single-cell RNA sequencing and to analyze different disease models.

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

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Protocol

An optimized protocol for the isolation of rare stromal cell populations from the mouse spleen

Yannick O. Alexandre^{1,2,*} and Scott N. Mueller^{1,3,*}¹Department of Microbiology and Immunology, University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia²Technical contact³Lead contact*Correspondence: yannick.alexandre@unimelb.edu.au (Y.O.A.), smue@unimelb.edu.au (S.N.M.)
<https://doi.org/10.1016/j.xpro.2022.101923>

SUMMARY

Lymphoid tissue stromal cells are important regulators of spleen homeostasis and immune responses. Here, we present an optimized protocol that describes the digestion and enrichment steps for the isolation and analysis of rare populations of stromal cells, including fibroblastic reticular cells, perivascular cells, and glial cells found in the spleen. This protocol is suitable for subsequent analysis of spleen stromal cells by flow cytometry or single-cell RNA sequencing and to analyze different disease models.

For complete details on the use and execution of this protocol, please refer to Alexandre et al. (2022).¹

BEFORE YOU BEGIN

The spleen is a secondary lymphoid organ where immune responses against blood-borne infections are initiated. It is divided in two main compartments: the red pulp and the white pulp. The red pulp is responsible for the removal of old red blood cells, iron recycling and is rich in red blood cells, macrophages and other innate immune cells that participate in the control of infection. The white pulp is organized into lymphoid compartments, including the T cell zone and B cell follicles where antigen presentation and activation of lymphocytes occur. Both the white pulp and the red pulp are supported by intricate networks of stromal cells. These stromal cells not only support the architecture of the spleen, but also contribute to immune cell homeostasis and the coordination of immune responses.² Within the white pulp, specialized subsets of stromal cells called Fibroblastic Reticular Cells (FRCs) create a scaffold for immune cell migration. Our recent work highlighted strong heterogeneity within FRCs that also respond dynamically to viral infection and influence T cell responses. We also provided a gating strategy to phenotypically analyze other stromal cells found in the spleen such as adventitial cells, glial cells, and mesothelial cells.¹

The step-by-step protocol below describes how to isolate and analyze by flow cytometry the stromal cell landscape found in the spleen of mice at steady state and during systemic viral infection. This protocol is related to, but distinct from that used to isolate stromal cells from lymph nodes.³

It is important to prepare all reagents before starting tissue dissection.

Mice

We have applied this protocol to 8–16 week old male and female C57BL/6J mice.



Cell enrichment and reagents

This protocol requires an enrichment step to deplete hematopoietic cells. This is performed using a QuadroMACS™ Separator attached to a MACS® Multistand to hold the LS column (Miltenyi Biotec). We have not tried alternate enrichment approaches.

Institutional permissions

Animal experiments were approved by the Animal Ethics Committee of the University of Melbourne.

Note: All mouse experiments must be approved by an Animal Care Committee in your research institution.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|---|--------------------------------------|
| Antibodies | | |
| CD45-APC-R700 (Clone 30-F11, dilution 1/100) | BD | Cat # 565478, RRID: AB_2739257 |
| Ter119-A700 (Clone TER119, dilution 1/100) | Thermo Fisher | Cat# 45-5921-82, RRID: AB_925765 |
| CD31-BV421 (Clone 390, dilution 1/500) | BioLegend | Cat# 102423, RRID: AB_2562186 |
| Podoplanin-PE-Cy7 (Clone 8.1.1, dilution 1/500) | BioLegend | Cat# 127411, RRID: AB_10613294 |
| CD140a-BV605 (Clone APA5, dilution 1/200) | BioLegend | Cat# 135916, RRID: AB_2721548 |
| CD157-BV650 (Clone BP-3, dilution 1/500) | BD | Cat# 740611, RRID: AB_2740311 |
| CD21/35-PerCP-Cy5.5 (Clone 7G6, dilution 1/100) | BioLegend | Cat# 123416, RRID: AB_1595490 |
| CD106-AlexaFluor488 (Clone 429, dilution 1/200) | BioLegend | Cat# 105710, RRID: AB_493427 |
| CD146-APC (Clone ME-9F1, dilution 1/500) | BioLegend | Cat# 134712, RRID: AB_2563088 |
| Ly6C-BV785 (Clone HK1.4, dilution 1/500) | BioLegend | Cat# 128041, RRID: AB_2565852 |
| MAcCAM-1-Biotin (Clone MECA-367, dilution 1/100) | BioLegend | Cat# 120706, RRID: AB_493397 |
| Streptavidin-PE, dilution 1/1000) | BD | Cat# 554061, RRID: AB_10053328 |
| Chemicals, peptides, and recombinant proteins | | |
| Bovine serum albumin | Sigma-Aldrich | Cat# A7906 |
| EDTA | Sigma-Aldrich | Cat# E5134 |
| Collagenase D | Sigma-Aldrich | Cat# COLLD-RO |
| DISPASE II (NEUTRAL PROTEASE GRADE II) | Sigma-Aldrich | Cat# 4942078001 |
| Deoxyribonuclease I from bovine pancreas (DNAse I) | Sigma-Aldrich | Cat# DN25 |
| RPMI-1960 | GIBCO | Cat# 10010023 |
| PBS 1x | GIBCO | Cat# 11875176 |
| DAPI | Thermo Fisher | Cat# D1306 |
| Fetal bovine serum | Sigma-Aldrich | N/A |
| RBC lysis buffer | Thermo Fisher | Cat# 00-4333-57 |
| Critical commercial assays | | |
| Sphero calibration particles | BD Bioscience | Cat# 556296 |
| eBioscience Foxp3/Transcription Factor Staining Buffer Set | Thermo Fisher | Cat# 00-5523-00 |
| CD45 microbeads, mouse | Miltenyi Biotec | Cat# 130-052-301 |
| Ter-119 microbeads, mouse | Miltenyi Biotec | Cat# 130-049-901 |
| DAPI | Sigma-Aldrich | Cat# D9542 |
| Propidium iodide | Sigma-Aldrich | Cat# P4170 |
| Experimental models: Organisms/strains | | |
| Mouse: C57BL/6J (8–16 weeks old, female and male) | The Jackson Laboratory | Stock# 000644; RRID: IMSR_JAX:000664 |
| Software and algorithms | | |
| FlowJo version 10 (Treestar inc) | https://www.flowjo.com | RRID: SCR_008520 |
| Other | | |
| LS Columns | Miltenyi Biotec | Cat# 130-042-401 |
| MACS Multistand | Miltenyi Biotec | Cat# 130-042-303 |
| QuadroMACS™ Separator | Miltenyi Biotec | Cat# 130-090-976 |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|------------------------------------|-----------------------|---------------------|
| Petri dish | Falcon | Cat# 353002 |
| 1 mL Syringes Slip Tip | Terumo | Cat# SMSLIP1T |
| Terumo Agani Hypodermic Needle 26G | Terumo | Cat# AN*2613R1 |
| 75 μ M nylon mesh | Clear Edge Filtration | Cat# Mon PA 75-1750 |

MATERIALS AND EQUIPMENT

Harvest media

| Reagent | Final concentration | Amount |
|--------------|---------------------|--------------|
| RPMI-1640 | 1 \times | 49 mL |
| FBS | 2% | 1 mL |
| Total | N/A | 50 mL |

Keep sterile and store at 4°C for up to one month. This is for 20–25 samples – adjust volumes accordingly.

Digestion media

| Reagent | Final concentration | Amount |
|---------------|---------------------|--------------|
| RPMI-1640 | 1 \times | 19.6 mL |
| FBS | 2% | 400 μ L |
| Collagenase D | 2 mg/mL | 40 mg |
| Dispase II | 0.8 mg/mL | 16 mg |
| DNase I | 0.1 mg/mL | 2 mg |
| Total | N/A | 20 mL |

Prepare fresh and keep at 20°C–22°C. Discard any leftover at the end of the experiment. This is for 8–10 spleens - Adjust volumes and weight accordingly. Alternatively, concentrated stock solutions for Collagenase D, Dispase II and DNase I can be prepared in RPMI and stored at –20°C for a maximum of 6 months.

FACS buffer

| Reagent | Final concentration | Amount |
|----------------------|---------------------|---------------|
| PBS | 1 \times | 500 mL |
| Bovine serum albumin | 2% | 10 g |
| EDTA (0.5 M) | 5 mM | 5 mL |
| Total | N/A | 500 mL |

Store the solution at 4°C for up to 2 months. Bovine serum albumin can be substituted with Fetal Bovine Serum.

Counting beads

| Reagent | Final concentration | Amount |
|------------------------------|---------------------|-------------|
| Sphero calibration particles | 1/10 | 500 μ L |
| FACS buffer | N/A | 4.5 mL |
| Total | N/A | 5 mL |

We use counting beads to calculate cell counts in our samples. To prepare the beads, vortex for at least 30 s the vial. Once the beads are made, vortex, count and calculate the concentration of beads. This is for 250 samples and is stored for up to 6 months at 4°C.

STEP-BY-STEP METHOD DETAILS

Digestion of mouse spleens

⌚ Timing: 3 h

In this section, spleens are harvested and digested with an enzyme cocktail mix to release stromal cells. Upon completion of the digestion, the stromal cell fraction is separated from the hematopoietic cells.

1. Harvest spleens.
 - a. Euthanize mice using either CO₂ or other approved methods.
 - b. Lay euthanized mice on their right flank and spray body area with 70% ethanol.
 - c. Using scissors, cut through the skin and peritoneum to expose the spleen.
 - d. Harvest the spleen with forceps and place individual spleens in 1–2 mL of harvest media and keep on ice.

Note: Remove external tissue connected to the spleen.

2. Place one spleen in the middle of a Petri dish.
3. Load two 1 mL syringes equipped with 26G needles with digestion media.
4. Inject 2 mL of digestion media evenly across the spleen.

Note: We inject around 200 μ L in one area of the spleen then inject in another area until the entire spleen is injected with 2 mL of digestion media. Spleens should swell and become lighter in color due to the injection of the enzyme mix ([Figures 1A–1C](#) and [Methods video S1](#)).

5. Transfer the spleen and digestion media into a 15 mL tube.

Note: If more than one spleen is processed, use a new petri dish and repeat steps 2–5 for the next spleen. Continue until all spleens are injected and transferred with the digestion media into tubes.

△ CRITICAL: We routinely digest up to 16 individual mouse spleens within one experiment. However, when processing more than 5–6 spleens, we recommend splitting samples in two or more batches for the processing to ensure a standard digestion time for each sample. For example, to process 10 spleens, we suggest injecting the enzyme mix in the first 5 spleens, transfer them in the water bath and start a timer, then continue with the next 5 spleens.

6. Transfer tubes into a pre-warmed water bath set to 37°C and incubate for 30 min.
7. After incubation, remove tubes from the water bath and place them back at 20°C–22°C.
8. Using a 1 mL pipette, thoroughly gently mix spleens 90 times.

△ CRITICAL: The mixing step is indispensable to achieve optimal cell recovery.

Note: Spleens should be digested completely with no fragments left. If not, leave undisturbed until remaining visible pieces of tissue settle to the bottom of the tube. Transfer supernatant into a 15 mL tube containing 10 mL of FACS buffer without taking the undigested pieces of spleen and keep this on ice. Repeat digestion by adding 1 mL of digestion media to the undigested pieces of spleen and incubate for 15 min at 37°C. Repeat step 8 following the second incubation and pool together the two digested fractions in the 15 mL tube that contains the

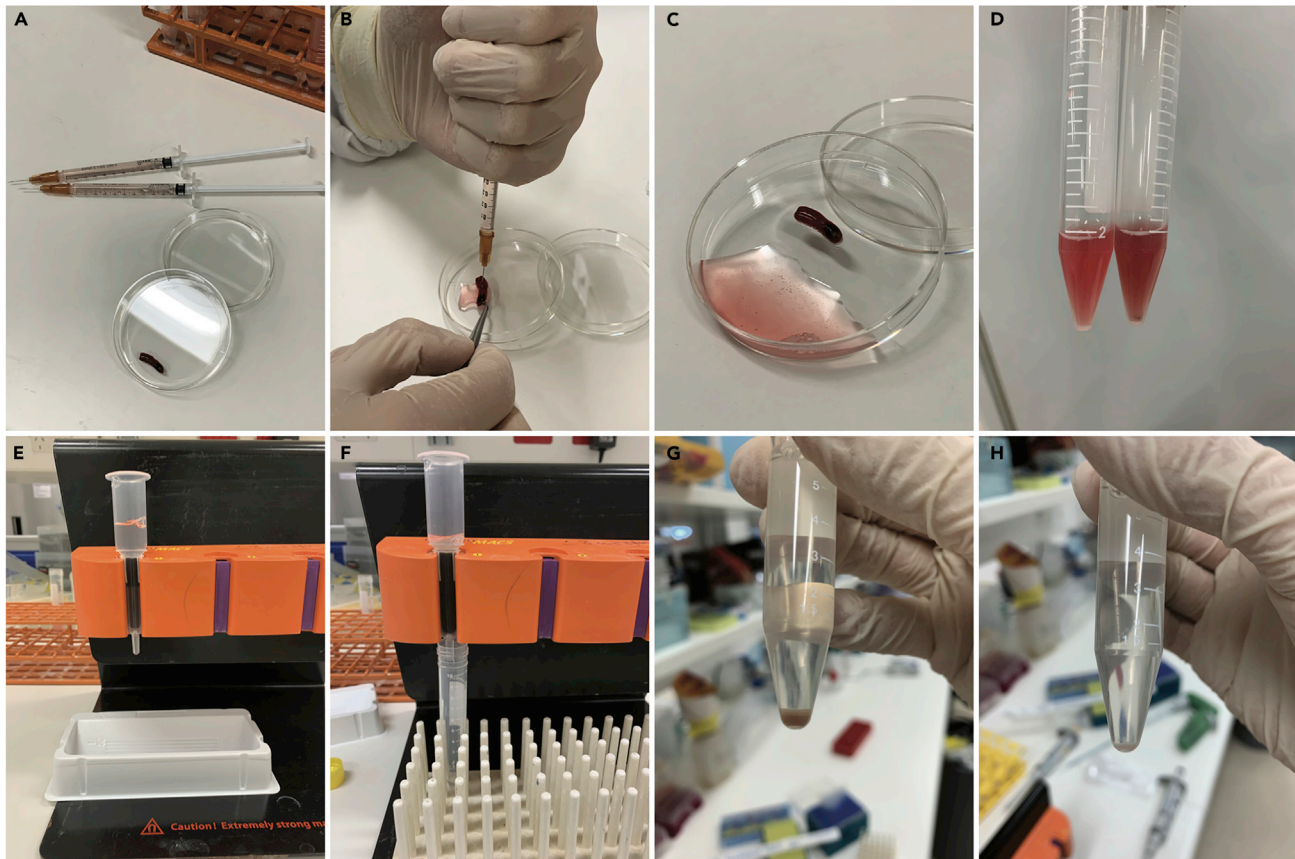


Figure 1. Spleen stromal cell isolation protocol

- (A) Set up before injecting spleens with enzyme mix.
 (B) Injection of the enzyme mix in the spleen.
 (C) Spleen after injection of the enzyme mix.
 (D) Example of complete digestion (left tube) and incomplete digestion (right tube) following one round of digestion.
 (E) Washing of the LS column.
 (F) Separation of the stromal cell fraction from the hematopoietic fraction.
 (G and H) Cell pellet before (left) and after (right) enrichment.

first fraction in FACS buffer. Skip step 9 (Figure 1D). See also [problem 1](#) in [troubleshooting](#) section.

9. Add 14 mL of FACS buffer to each digested spleen and centrifuge for 5 min, $500 \times g$, 4°C .
10. Remove supernatant and perform red blood cell lysis by resuspending spleen cells with 1 mL of RBC lysis buffer.
11. After 2 min incubation at 20°C – 22°C , wash cells by adding 10 mL of FACS buffer and centrifuge for 5 min, $500 \times g$, 4°C .
12. Remove supernatant, resuspend spleen cells in 5 mL of FACS buffer and filter cell suspension through $75 \mu\text{M}$ nylon mesh into a new 10 or 15 mL tube.
13. Centrifuge cells for 5 min, $500 \times g$, 4°C .
14. Aspirate supernatant using a vacuum trap system.

Note: It is important here to use a vacuum trap to remove all the supernatant from the tubes as the next step requires low volume of buffer.

15. Add $250 \mu\text{L}$ of FACS buffer into each tube then add $35 \mu\text{L}$ of anti-mouse CD45 microbeads.

Note: we found there is no need to strictly follow the [manufacturer's instructions](#) for the volume of beads that is required for CD45⁺ cell depletion. The amount and dilution of microbeads used here is enough to perform a robust depletion of CD45⁺ cells for stromal cell enrichment and further analysis. If working with spleens with higher total cellularity than steady state spleens (for example from infected mice), we recommend to manually count and determine the total cellularity in the samples that need to be enriched. Users can then scale up the volume of FACS buffer and beads. As an example, we typically resuspend spleen cells in 500 μ L of FACS buffer and add 60 μ L of CD45 microbeads during the peak of the immune response following LCMV infection.

Note: If the goal of the experiment is to perform cell sorting of stromal cells for subsequent analysis, we recommend including anti-mouse Ter119 microbeads in addition to CD45 microbeads to deplete residual red blood cells and their precursors that will be enriched in the stromal cell fraction. There is no need to include Ter119 microbeads if stromal cells are analyzed by flow cytometry.

16. Resuspend cell pellets and incubate for 15 min on ice.
17. Wash cells by adding 5 mL of FACS buffer and centrifuge cells for 5 min, 500 \times g, 4°C.
18. Aspirate supernatant using vacuum trap and resuspend cells in 500 μ L of FACS buffer. Keep cells on ice.
19. Attach the QuadroMACS Separator to the MACS MultiStand and place an LS Column in the separator for each spleen sample. Place a reservoir under the LS Column.

Note: We slightly modified the depletion protocol from [manufacturer's instructions](#), as indicated below in steps 20–25 ([Figure 1E](#)).

20. Prepare the LS Column by rinsing with 3 mL of FACS buffer on top of the column and let the buffer run through ([Figure 1E](#)).
21. Wait until the buffer has completely run through and remove the reservoir. Place a 10 or 15 mL collection tube under the LS Column.
22. Apply the spleen cell suspension onto the prepared LS Column ([Figure 1F](#)).

△ CRITICAL: Stromal cells do not express CD45 and are not retained within the column. It is critical to collect the negative fraction that flows through the column because it contains the enriched stromal cells. See also [problem 2](#) in [troubleshooting](#) section.

23. Once the cells have completely run through, add 500 μ L of FACS buffer to the tube that had the cell suspension in step 18. to collect any remaining cells and apply this supernatant onto the column.
24. Once the buffer has completely run through, wash the column once with 1 mL then 2 times with 3.5 mL of FACS buffer. The total volume of in the collection tube should be 9 mL.
25. Put collection tubes on ice. Those contain the enriched stromal cells ([Figures 1G](#) and [1H](#)).

Note: We expect to obtain between 1 to 5 million cells following enrichment.

Note: To collect the CD45⁺ cell fraction, remove the column from the QuadroMACS Separator and flush with FACS buffer into a separate tube.

Flow cytometry staining of spleen stromal cells

⌚ Timing: 1.5 h

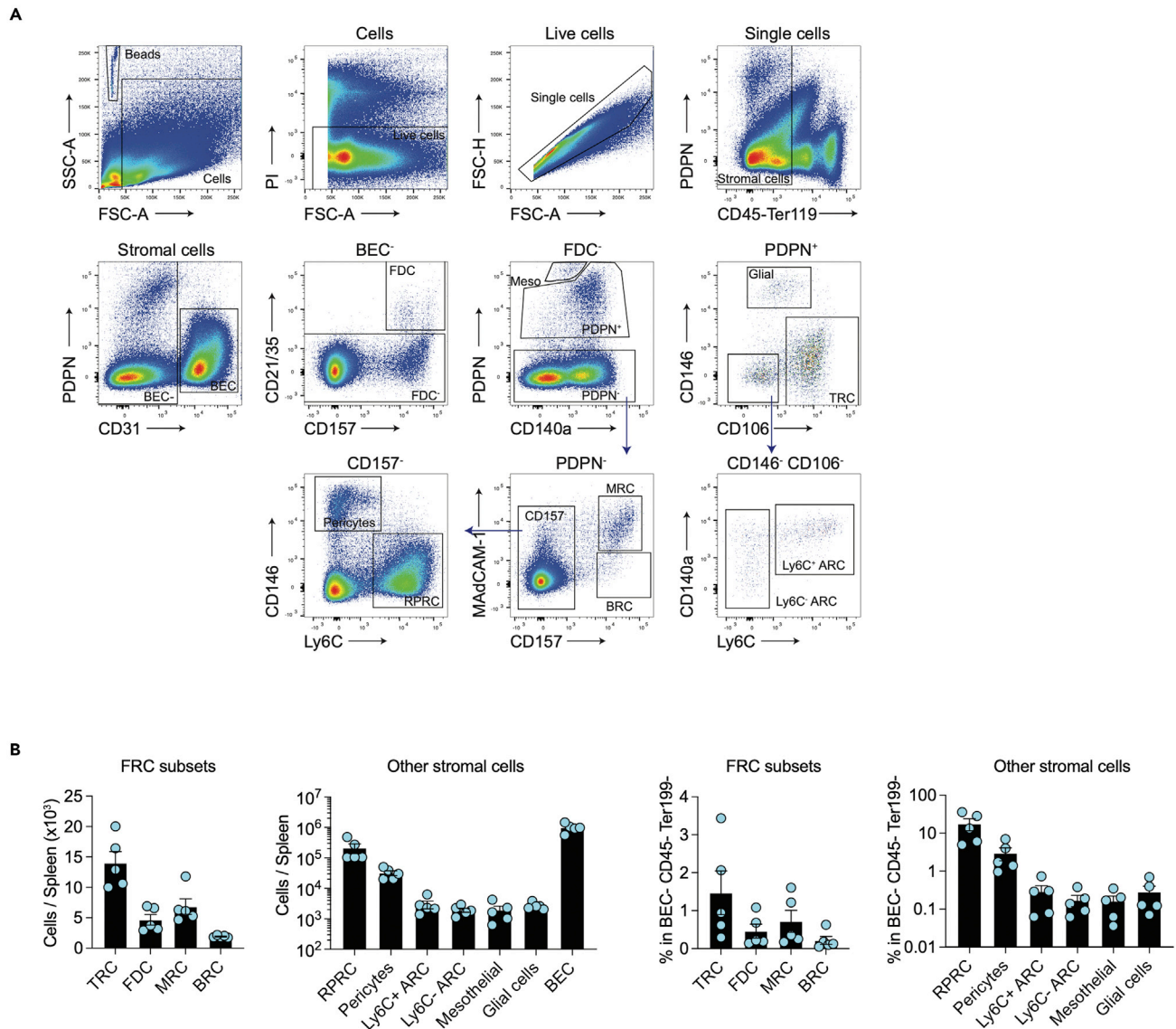


Figure 2. Stromal cell subset cellularity from the mouse spleen

(A) Gating strategy to analyze stromal cell subsets from mouse spleen. BEC: blood endothelial cells. FDC: Follicular Dendritic Cells. Meso: mesothelial cells. Glial: glial cells. TRC: T-zone Reticular Cells. ARC: Adventitial Reticular Cells. MRC: Marginal zone Reticular Cells. BRC: B cell follicles Reticular Cells. RPRC: Red Pulp Reticular Cells. Please refer to Alexandre et al., 2022 to learn more about the identity of the different stromal cell subsets present in this gating strategy.¹

(B) Cellularity as estimated using counting beads and percentage of stromal cell subsets within the mouse spleen. Graphs show representative data (means \pm SEM) with $n = 5$ mice.

Enriched stromal cells will be stained with antibodies to phenotype diverse subsets present in the spleen. We stain our samples in FACS tubes. Staining can also be performed in a round-bottom 96-well plate. If analyzing mouse white pulp stromal cells, we recommend using one spleen per staining. This is because some white pulp stromal cell subsets are numerically low in the spleen (Figure 2). For live versus dead cell discrimination, we recommend using a bright marker such as DAPI or Propidium Iodide (PI). These two markers are not compatible with intracellular staining (ICS). If ICS is required, we routinely use Zombie fixable viability dye that is added directly into the surface stain cocktail mix (refer to [manufacturer's instructions](#)). We provide a table below with details of the antibodies used to identify the main subsets of white and red pulp stromal cells from the mouse spleen.

Table 1. Stromal cell antibody mix 1 (See key resources table for information about clone and supplier)

| Marker | Fluorochrome | Dilution |
|------------|------------------|----------|
| CD45 | APC-R700 | 1/100 |
| Ter119 | A700 | 1/100 |
| CD31 | BV421 | 1/500 |
| Podoplanin | PE-Cy7 | 1/500 |
| CD140a | BV605 | 1/200 |
| CD157 | BV650 | 1/500 |
| CD21/35 | PerCP-Cy5.5 | 1/100 |
| CD106 | Alexa Fluor® 488 | 1/200 |
| CD146 | APC | 1/500 |
| Ly6C | BV785 | 1/500 |
| MAdCAM-1 | Biotin | 1/100 |

Users should check their own FACS configuration and adapt the antibodies accordingly. To enumerate cellularity, we add counting beads (Sphero calibration particles) just before acquisition in each sample. Alternatively, users can manually count their samples.

26. Take a 500 μ L aliquot of unstained stromal cells from each sample, pool together and set aside.

Note: Stromal cells are bigger and more autofluorescent than lymphocytes. We recommend using unstained stromal cells as unstained negative control on the FACS machine. We use compensation beads and not enriched stromal cells for single stain controls to adjust compensations on the flow cytometer.

27. Centrifuge remaining cells for 5 min, 500 \times *g*, 4°C.

28. Remove supernatant, resuspend stromal cells in 1 mL of FACS buffer and transfer into FACS tubes.

29. Centrifuge cells for 5 min, 500 \times *g*, 4°C.

30. Aspirate supernatant and resuspend each sample with 100 μ L of stromal cell cocktail mix 1, made in FACS buffer (Table 1).

Note: We tested directly conjugated anti-MAdCAM-1 antibodies and found that the positive signal was too dim for ready analysis. We now use Biotin-conjugated MAdCAM-1. If the experiment does not require a stain for marginal zone reticular cells, the use of this antibody is not necessary and the second incubation with streptavidin is not required.

31. Incubate for 30 min at 4°C in the dark.

32. Following incubation, wash cells by adding 1 mL of FACS buffer and centrifuge cells for 5 min, 500 \times *g*, 4°C.

33. Aspirate supernatant and resuspend cells with 100 μ L stromal cell cocktail mix 2, made in FACS buffer (Table 2).

34. Incubate for 30 min at 4°C in the dark.

35. Following incubation, wash cells by adding 1 mL of FACS buffer and centrifuge cells for 5 min, 500 \times *g*, 4°C.

Optional: If intracellular staining is required, prepare Fixation/Permeabilization buffer by diluting 1 part of the Fixation/Permeabilization Concentrate with 3 parts of Fixation/Permeabilization Diluent from the eBioscience Foxp3/Transcription Factor staining buffer set. Centrifuge cells for 5 min, 500 \times *g*, 4°C, aspirate supernatant and resuspend cells with 100 μ L of Fixation/Permeabilization working solution. Incubate for 45 min at 20°C–22°C, in the dark. Prepare a 1 \times working solution of Permeabilization Buffer by mixing 1 part of 10 \times

Table 2. Stromal cell antibody mix 2

| Marker | Fluorochrome | Dilution |
|--------------|--------------|----------|
| Streptavidin | PE | 1/1,000 |

Permeabilization Buffer from the eBioscience Foxp3/Transcription Factor staining buffer set with 9 parts of distilled water. Following the fixation incubation, wash cells by adding 1 mL of Permeabilization Buffer and centrifuge cells for 5 min, 500 × g, 4°C. Aspirate supernatant and resuspend cells in 100 μL of intracellular antibody mix, diluted in Permeabilization buffer. Incubate cells for 45 min at 4°C and in the dark. Wash cell by adding 1 mL of Permeabilization Buffer and centrifuge cells for 5 min, 500 × g, 4°C. Aspirate supernatant and resuspend cells in 200 μL of FACS buffer. Keep samples at 4°C and in the dark until acquisition of the flow cytometer. Skip steps 36 and 37, below.

36. Aspirate supernatant and resuspend cells with 150 μL of FACS buffer.
37. Add 20 μL of 10 μg/mL PI or 20 μL of 5 μM DAPI to each sample and keep samples at 4°C and in the dark until acquisition of the flow cytometer.
38. Just prior to acquisition, vortex and add 20 μL of counting beads per sample.
39. Acquire samples on a FACS machine.

Note: We have used both FACSFortessa (BD) and Cytex Aurora machines for our studies, and FlowJo software for analysis.

Optional: For single cell RNA sequencing, we sorted populations of interest with a BD FACS Aria III. Spleen stromal cells are fragile and we recommend sorting cells with a 100 μM nozzle and 20 psi, without exceeding a speed rate of 5,000 events per second. We sorted cells directly in RPMI supplemented with 10% FBS. Following cell sorting, cells were centrifuged and resuspended in PBS with 0.01% BSA and single cell RNA sequencing was performed according to [manufacturer's instructions](#) (10X Genomics).

EXPECTED OUTCOMES

We expect to obtain a sufficient number of spleen stromal cells for immunophenotyping by flow cytometry. The gating strategy in [Figure 2A](#) shows how to identify the different populations of stromal cells in the mouse spleen. If there are problems with the staining, please refer to [problem 3](#) in [troubleshooting](#) section.

[Figure 2B](#) shows the enumeration of the identified stromal cell populations in the spleen and expected numbers. If you have lower yield please refer to [problem 4](#) in [troubleshooting](#) section.

QUANTIFICATION AND STATISTICAL ANALYSIS

We provide here an example of quantification using counting beads ([Table 3](#)). After analysis of the data in Flowjo, export count statistics from beads and any population of interest. To calculate the amount of cells per sample, simply multiply the count statistic from the population of interest by the total amount of beads that was added to the sample and divide by the count statistic of beads that was acquired on the FACS. In the example below, 23,400 beads were added in each sample. Then calculate the number of cells based on the volume of cells after the enrichment step (the volume after enrichment of stromal cells was 9 mL and 500 μL was taken out for the negative control).

Table 3. Example of quantification using counting beads

| | Count events extracted from Flowjo analysis | | |
|----------------------------------|--|---------|--|
| | Beads | BEC | TRC |
| Sample 1 | 7398 | 332,294 | 6,335 |
| Sample 2 | 11452 | 273,331 | 4,901 |
| Formula to enumerate cellularity | | | |
| Sample 1 | $= 332,294 \times 23,400 / 7398 / 8.5 \times 9$ | | $= 6,335 \times 23,400 / 7398 / 8.5 \times 9$ |
| Sample 2 | $= 273,331 \times 23,400 / 11452 / 8.5 \times 9$ | | $= 4,901 \times 23,400 / 11452 / 8.5 \times 9$ |
| Cellularity for both samples | | | |
| Sample 1 | 1112878 | | 21216 |
| Sample 2 | 591353 | | 10603 |

LIMITATIONS

The efficiency of digestion and staining could be impacted depending on the strain of mice used, the age of the mice or treatment conditions. In addition, Dispase treatment can cleave certain cell surface markers. The markers presented in the staining table are not affected by our enzymatic digestion method. Refer to [problem 5](#) in [troubleshooting](#) section if there are issues with staining of markers.

TROUBLESHOOTING

Problem 1

There are pieces of undigested spleen left (step 9).

Potential solution

Make sure the enzymes are not expired, that these enzymes were freshly prepared and digestion is performed at 37°C. We found that preparing the enzymes fresh each time gives better results than using frozen aliquots. We do not recommend going further than two rounds of digestion because this can lead to lower cell viability.

Problem 2

The cell pellet following CD45+ cell depletion is very large (step 23).

Potential solution

This likely reflects suboptimal CD45 microbead staining. Increase the volume of beads used per sample.

Problem 3

The intensity of the staining is weak for some markers in the FACS analysis.

Potential solution

Each antibody should be titrated before use to ensure optimal staining. We also found that for the same antibody, some fluorochromes are not optimal. As an example, podoplanin staining is best with bright fluorochromes (PE, PE-Cy7, BV421). We found weak staining when used in Alexa Fluor 488 or 647.

Problem 4

The cell viability is low and/or the stromal cell recovery is low during flow cytometry analysis.

Potential solution

There could be several reasons for this issue. A good stromal cell preparation should have a cell viability range between 80%–90%.

- Following the incubation with enzymes, mix 90 times gently to avoid unnecessary shear force on cells.
- Limit the time cells are incubated with enzymes (no longer than one hour).
- Dispase enzyme activity could be poor if the reagent is old or has been improperly stored. We found that the condition of this enzyme is critical for a good stromal cell digestion. Dispase is sensitive to humidity and not suitable for long-term storage once the vial has been opened, even if reconstituted and stored at -20°C . We recommend using a fresh vial of the enzyme and store it in dry conditions. We discard any leftover Dispase after 6 months of opening a vial.

Problem 5

Cell markers are cleaved off after enzymatic digestion.

Potential solution

Dispase treatment can cleave certain cell surface markers. The markers presented here for phenotyping mouse spleen stromal cells are not affected by our enzymatic digestion method. We recommend testing your own markers first for sensitivity to the Dispase and look for alternative markers if required.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Scott Mueller (smue@unimelb.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101923>.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.O.A. and S.N.M.; Methodology, Y.O.A.; Writing – original draft, Y.O.A.; Writing – review and editing, Y.O.A. and S.N.M.; Funding acquisition, S.N.M.; Supervision, S.N.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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