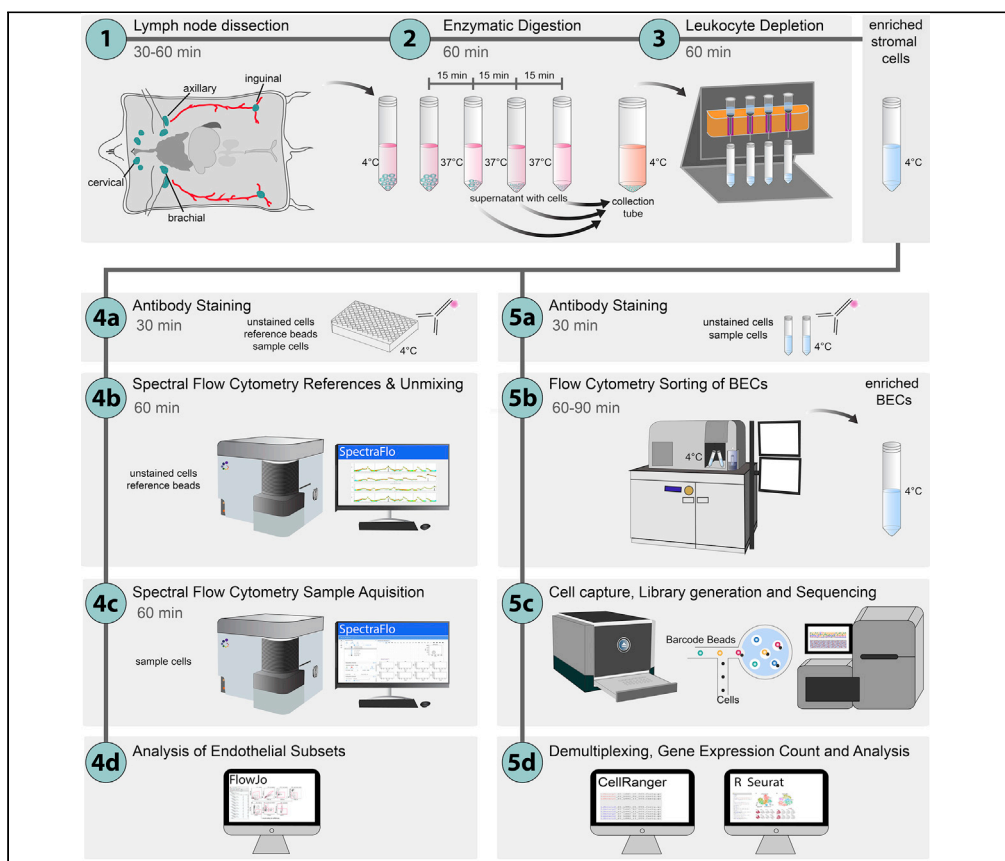


Protocol

Analyses of murine lymph node endothelial cell subsets using single-cell RNA sequencing and spectral flow cytometry



Blood endothelial cells (BECs) in lymph nodes are distinct stromal cells with a transcriptional profile allowing fast and specific adaptation to the functional requirements. Here, we describe a step-by-step protocol for the enzymatic digestion of lymph nodes, the enrichment of stromal cells, the sorting of BECs, and the processing of BEC-related data for modern analysis approaches as spectral flow cytometry and single-cell RNA sequencing (scRNA-seq).

Lutz Menzel, Maria Zschummel, Armin Rehm

arehm@mdc-berlin.de

Highlights

Protocols describing the isolation of murine lymph node endothelial cells

Isolation of lymphatic endothelial cells for spectral flow cytometry and scRNA-seq

Basic analysis of spectral flow cytometry data of endothelial cells and scRNA-seq

Menzel et al., STAR Protocols
3, 101267
June 17, 2022 © 2022 The Author(s).
<https://doi.org/10.1016/j.xpro.2022.101267>



Protocol

Analyses of murine lymph node endothelial cell subsets using single-cell RNA sequencing and spectral flow cytometry

Lutz Menzel,^{1,3} Maria Zschummel,² and Armin Rehm^{1,4,*}¹Translational Tumorimmunology, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany²Microenvironmental Regulation in Autoimmunity and Cancer, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany³Technical contact⁴Lead contact*Correspondence: arehm@mdc-berlin.de
<https://doi.org/10.1016/j.xpro.2022.101267>

SUMMARY

Blood endothelial cells (BECs) in lymph nodes are distinct stromal cells with a transcriptional profile allowing fast and specific adaptation to the functional requirements. Here, we describe a step-by-step protocol for the enzymatic digestion of lymph nodes, the enrichment of stromal cells, the sorting of BECs, and the processing of BEC-related data for modern analysis approaches as spectral flow cytometry and single-cell RNA sequencing (scRNA-seq).

For complete details on the use and execution of this protocol, please refer to Menzel et al. (2021).

BEFORE YOU BEGIN

Institutional permission for animal experimentation.

All animal experiments were conducted in compliance with the institutional guidelines of the Max-Delbrück-Center for Molecular Medicine and approved by the Landesamt für Gesundheit und Soziales, Berlin, Germany.

Coat/block collection tubes

⌚ Timing: 16–18 h

1. Coating/Blocking of cell sorting collection tubes.
 - a. Incubate 15 mL tubes for every sorting sample with 5 mL coating solution (5% BSA in PBS) 14–16 h at 4°C.

Prepare enzyme stock solutions

⌚ Timing: 15 min

2. For the preparation of 10 mL stock solution.
 - a. Dissolve 240 mg of Dispase II in 10 mL RPMI-1640 (specific activity ≥ 0.8 units/mg lyophilized protein) by vigorous vortexing.
 - b. Dissolve 60 mg of Collagenase P in 10 mL RPMI-1640 (specific activity 1.8 units/mg lyophilized protein).



- c. Aliquot solutions and store at -20°C for up to 6 months.

Prepare enzymatic digestion solutions

⌚ Timing: 5 min

3. For the preparation of 30 mL enzymatic digestion solution.
 - a. Dissolve enzyme stock solutions in RPMI-1640.
 - b. Store at 4°C for one day.

Prepare erythrocyte lysis buffer

⌚ Timing: 5 min

4. For the preparation of 500 mL erythrocyte lysis buffer.
 - a. Add NH_4Cl , KHCO_3 and EDTA to H_2O .
 - b. Store at 18°C – 25°C for up to 6 months.

Prepare MACS buffer

⌚ Timing: 5 min

5. For the preparation of 500 mL MACS buffer.
 - a. Add bovine serum albumin (BSA) and EDTA to PBS solution.
 - b. Store at 4°C for up to 3 months.

Prepare FACS buffer

⌚ Timing: 5 min

6. For the preparation of 500 mL FACS buffer.
 - a. Add fetal calf serum (FCS) and EDTA to PBS solution.
 - b. Store at 4°C for up to 3 months.

Prepare collection buffer

⌚ Timing: 5 min

7. For the preparation of 100 mL HBSS collection buffer.
 - a. Add BSA to HBSS buffer solution.
 - b. Store at 4°C for one day.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD31, clone: 390, Brilliant Violet 785 (1:200)	BioLegend	Cat# 102435 RRID: AB_2810334
CD31, clone: 390, FITC (1:200)	BioLegend	Cat# 102405 RRID: AB_312900

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD45, clone: 30-F11, Alexa Fluor 700 (1:200)	BioLegend	Cat# 103127 RRID: AB_493714
CD45, clone: 30-F11, Pacific Blue (1:200)	BioLegend	Cat# 103125 RRID: AB_493536
TER119, clone: Ter-119, Alexa Fluor 700 (1:200)	BioLegend	Cat# 116220 RRID: AB_528963
TER119, clone: Ter-119, Pacific Blue (1:200)	BioLegend	Cat# 116231 RRID: AB_2149212
PDPN, clone: 8.1.1, PE-Cy7 (1:200)	BioLegend	Cat# 127411 RRID: AB_10613294
CD36, clone: HM36, PerCP-Cy5.5 (1:100)	BioLegend	Cat# 102619 RRID: AB_2750187
CXCR4, clone: L276F12, PE (1:200)	BioLegend	Cat# 146505 RRID: AB_2562782
JAG-1, clone: HMJ1-29, APC (1:50)	BioLegend	Cat# 130913 RRID: AB_2561304
PNAd, clone: MECA-79, AF647 (1:200)	BioLegend	Cat# 120801 RRID: AB_493554
CD16/CD32, clone: 93, purified (1:100)	BioLegend	Cat# 101301 RRID: AB_312800
Chemicals, peptides, and recombinant proteins		
7-AAD	BioLegend	Cat# 420403
CD45 Microbeads	Miltenyi Biotec	Cat# 130-052-301
DNase-I	Thermo Fisher Scientific	Cat# EN0521
Dispase-II	Roche	Cat# 04942078001
Collagenase-P	Roche	Cat# 11213865001
Fetal calf serum	Gibco	Cat# A4766801
UltraComp eBeads™ Compensation Beads	Invitrogen	Cat# 01-3333-42
PBS (w/o Ca, w/o Mg)	Biowest	Cat# P0750-N10L
HBSS	Gibco	Cat# 14065-049
EDTA	Carl Roth	Cat# 8043.4
BSA, Fraction IV	Carl Roth	Cat# 8076.4
SpectroFlo QC Beads	Cytek	Cat# SKU B7-10001
UltraComp eBeads Compensation beads	Thermo Fisher Scientific	Cat# 01-2222-42
Deposited data		
scRNA-seq data of mouse lymph node blood endothelial cells	Menzel et al. (2021)	ArrayExpress: E-MTAB-10389
Software and algorithms		
FlowJo v10.7	BD	www.flowjo.com
R Studio v1.2.5042	RStudio	www.rstudio.com
Seurat package v2.3.4	(Satija et al., 2015; Butler et al., 2018)	https://satijalab.org/seurat/
CellRanger v3.0.2	10x Genomics	www.10xgenomics.com
Other		
Cytek Aurora spectral flow cytometer	Cytek	https://cytekbio.com/pages/aurora
AriaFusion	BD	https://www.bdbiosciences.com
15 mL tubes	Falcon	Cat# 352096
50 mL tubes	Falcon	Cat# 352070
Cell strainer 100 µm	Falcon	Cat# 352350
96 well plate, polystyrene, U-bottom	Sarstedt	Cat# 83.3925
FACS tubes	Sarstedt	Cat# 55.1579
Experimental models: Organisms/strains		
C57.BL/6N mice; male and female animals; 8–12 week old; experimental cohorts matched for gender and age	Charles River	Strain code: 027

MATERIALS AND EQUIPMENT

Enzymatic digestion solution		
Reagent	Final concentration	Amount
Dispase II	0.8 mg/mL	1 mL
Collagenase P	0.2 mg/mL	1 mL
DNase I	0.1 mg/mL	1 μ L
RPMI-1640	–	28 mL
Total		30 mL

Erythrocyte lysis buffer		
Reagent	Final concentration	Amount
NH ₄ Cl	9 mg/mL	4.5 g
KHCO ₃	1 mg/mL	500 mg
EDTA, stock: 0.5 M	10 mM	10 mL
H ₂ O	–	490 mL
Total		500 mL

MACS buffer		
Reagent	Final concentration	Amount
BSA	0.5%	2.5 g
EDTA, stock: 0,5 M	2 mM	2 mL
PBS	–	498 mL
Total		500 mL

FACS buffer		
Reagent	Final concentration	Amount
FCS	2%	10 mL
EDTA, stock: 0,5 M	2 mM	2 mL
PBS	–	488 mL
Total		500 mL

Collection buffer		
Reagent	Final concentration	Amount
BSA	0.04%	200 mg
HBSS buffer	–	500 mL
Total		500 mL

STEP-BY-STEP METHOD DETAILS

Isolation of stromal cells from murine lymph nodes

- ⌚ Timing: 30–60 min for step 1
- ⌚ Timing: 60 min for step 2
- ⌚ Timing: 60 min for step 3

Stromal cells and especially the endothelial subsets are rare populations with regard to all lymph node cells (approximately 0.1%). Obtain and pool as many lymph nodes as possible from several

mice of the same condition. However, consider if there might be reasons for obtaining only certain lymph node stations (e.g., tumor draining lymph nodes) as to avoid dilution of your expected effects by including lymph nodes not exposed to your tested “condition”, e.g., lymph nodes distant to tumor, or lymph nodes that only drain certain tissues not affected by your treatment. In order to generate high quality flow cytometry data, for optimal sorting yield and purity, leukocytes and erythrocytes are depleted before flow cytometry analysis or cell sorting. Lymph node stromal cell isolation has been established and validated previously (Fletcher et al., 2011).

1. Dissection and collection of lymph nodes.
 - a. Mice should be bred and maintained in a pathogen-free controlled environment (12 h light cycles, 22°C, 55% humidity) with food and water ad libitum.
 - b. Sacrifice mice using an authorized method.
 - c. Dissect axillary, brachial, cervical and inguinal lymph nodes.
 - d. Collect all lymph nodes in 5 mL ice cold RPMI-1640.
2. Enzymatic Digestion of the lymph nodes.
 - a. After lymph nodes are settled at the bottom of the tube, remove RPMI-1640 with a pipette and add 5 mL of enzyme-solution.
 - b. Place tubes in 37°C water bath to start enzymatic digestion and incubate for 15 min.
 - c. Aspirate and pipette out the solution 10 times using a 5 mL serological pipette to apply gentle mechanical force to the lymph node. This results in disruption of the capsule and will release most of the lymphocytes.
 - d. Allow large fragments to settle down, carefully harvest the supernatant and collect in 20 mL of ice-cold MACS buffer.
 - e. Add 5 mL fresh enzymatic digestion solution to the partially digested lymph node fragments and continue digestion in the 37°C water bath for 15 min.
 - f. Aspirate and pipette out the solution 10 times using a 5 mL serological pipette and obtain supernatant, pool in the MACS buffer collection tube and add new enzymatic digestion solution to the remaining node fragments every 15 min.
 - g. Once the lymph nodes are fully digested, centrifuge the pooled supernatants from all digestion steps at 300 g for 5 min at 4°C.
 - h. Resuspend cells in MACS buffer.
3. Enrichment of stromal cells by depletion of CD45⁺ leukocytes.
 - a. Count cells and label leukocytes with anti-CD45 magnetic beads as stated below and according to the manufacturer’s guidelines (<https://www.miltenyibiotec.com/DE-en/products/cd45-microbeads-mouse.html#gref>).
 - b. Use 10 µL of bead solution in 90 µL MACS buffer per 10⁷ cells.
 - c. Mix and incubate for 15 min at 4°C.
 - d. Add 20 mL of MACS buffer per tube and centrifuge at 300 g for 5 min at 4°C.
 - e. Discard supernatant and resuspend cells in 500 µL MACS buffer.
 - f. Apply cell suspension onto MACS buffer-equilibrated MACS LS columns.
 - g. Collect stromal cells in the flow through, leukocytes remain trapped on the column.

Optional: Although the GentleMACS™ dissociator (Miltenyi) does not provide a dissociation program for lymph nodes, pilot experiments with lymph node dissociation by using the *spleen dissociation kit* (Miltenyi) and the *m_spleen_02* program yielded similar numbers of viable BECs as our manual protocol and might be used for the dissociation, if such an automatic device is available.

Note: Lymph nodes are usually fully digested (no large fragments visible) after 45 min (2–3 cycles of digestion buffer renewal). Make sure that all samples/conditions are treated equally.

△ **CRITICAL:** Do not exceed the binding capacity of the LS columns. Over 99% of the cells are CD45⁺ and remain bound to the resin. Consider the number of columns that can be handled simultaneously. In our hands, handling of up to 4 columns in parallel works without problems.

△ **CRITICAL:** In order to perform flow cytometry analysis of BEC subsets, a pool of 10 lymph nodes from a single mouse usually yields a sufficient amount of cells for a proper analysis (BEC count >10,000). However, it might be necessary to pool lymph nodes of several animals depending on the sex, age and condition of mice. Young mice and females tend to have smaller lymph nodes and yield a lower amount of isolated BECs. Disease models of infection or lymphoma can influence the amount of BECs (Mondor et al., 2016; Gloger et al., 2020) and cause changes of BEC subsets (Menzel et al., 2021).

△ **CRITICAL:** In order to perform single cell sequencing, a pool of 10 lymph nodes per mouse and from 10 mice per group/condition are required to obtain a sufficient number of cells. Such quantities are necessary to compensate for possible cell loss during the various centrifugation steps and cell sorting procedure, and for determination of the cell count and viability.

Antibody staining of lymph node endothelial cells

⌚ **Timing:** 30 min for step 4

⌚ **Timing:** 60 min for step 5

The stromal cell-enriched suspension may still contain remaining erythrocytes and leukocytes, which are dump-gated based on TER119 and CD45 expression. Further surface markers distinguish fibroblastic reticular cells (FRCs), lymphatic endothelial cells (LECs) and different subsets of blood endothelial cells (BECs), including tip cells, stalk cells, quiescent/phalanx cells and cells from high endothelial venules (HEVs) (Figure 1).

4. Prepare reference samples for spectral unmixing.
 - a. Retain unstained stromal cells as autofluorescence reference controls.
 - b. Staining of UltraComp eBeads with the exact antibodies applied in the assay are used as reference controls. If you employ models with fluorescent reporters or dyes that are not antibody dependent (e.g., 7-AAD, other viability dyes or cell tracers) you need to include cells that were stained with these fluorophores separately.
 - c. Incubate UltraComp eBeads with antibodies for 10 min at 4°C in the dark.
5. Antibody staining of lymph node cell populations.
 - a. Transfer all cells in 200 µL FACS buffer to a 96 well plate (U-bottom).
 - b. Centrifugation at 300 × g for 5 min at 4°C.

Optional: Lysis of erythrocytes with lysis buffer (can cause loss of sensitive endothelial cells).

- c. Perform Fc-receptor block for prevention of unspecific binding of staining antibodies by incubation with 50 µL of an CD16/CD32 antibody solution (1:100 in FACS buffer) for 10 min at 4°C in the dark.
- d. Prepare an antibody staining master mix in FACS buffer as follows and add 50 µL of the mix to each sample:
 - i. TER119-AlexaFluor 700 (Erythrocytes) 1:200 in FACS buffer.
 - ii. CD45-AlexaFluor 700 (Leukocytes) 1:200 in FACS buffer.
 - iii. CD31-BrilliantViolet 785 (Endothelial cells) 1:200 in FACS buffer.
 - iv. PDPN-PE/Cy7 (FRCs, LECs) 1:200 in FACS buffer.

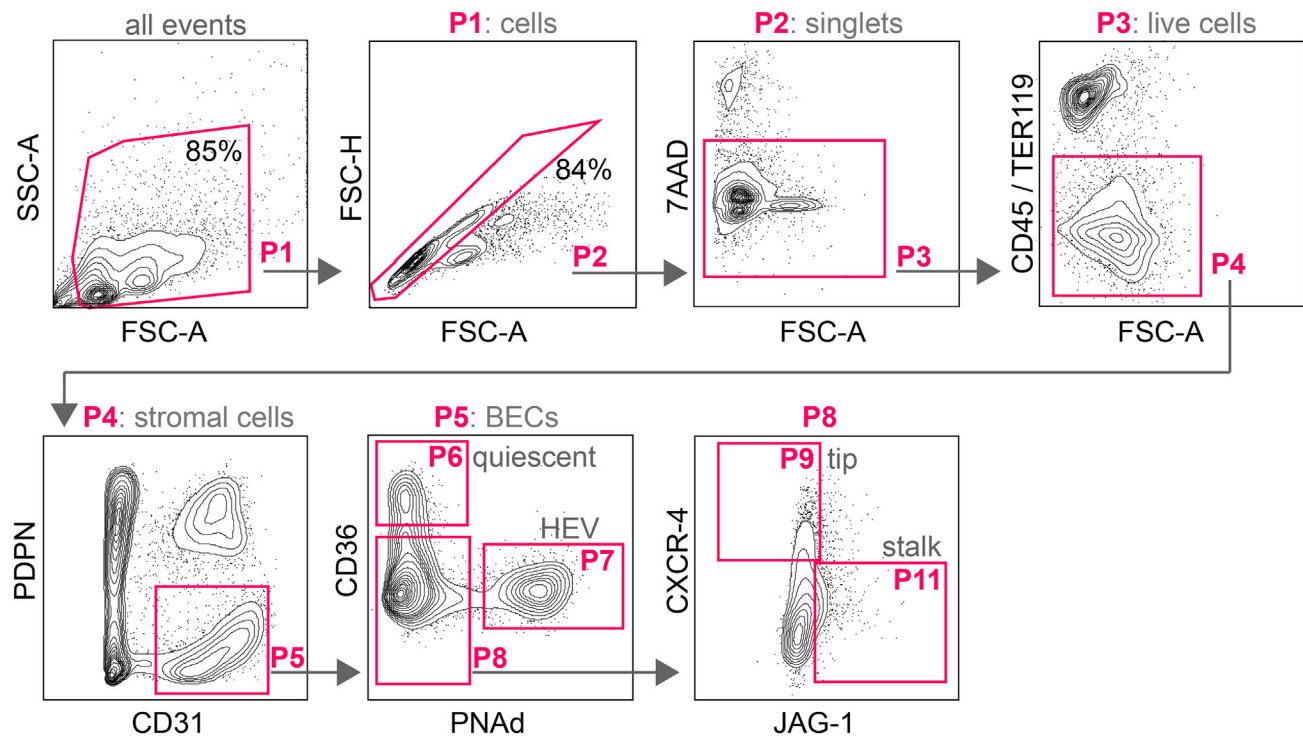


Figure 1. Gating strategy for flow-cytometry-based analysis of BECs

Flow cytometry gating strategy of BECs and their subsets. Gates depict events in a hierarchical order, FSC-A/SSC-A (P1: cell size and granularity), FSC-A/FSC-H (P2: singlets), FSC-A/7-AAD (P3: live cells), FSC-A/CD45-Ter119 (P4: stromal cells) and CD31/PDPN (P5: BECs), PNAAd/CD36 (P6: PNAAd/CD36⁺ quiescent cells, P7: PNAAd⁺/CD36⁺ HEV cells, P8: PNAAd⁺/CD36⁻), JAG-1/CXCR4 (P9: JAG-1⁺/CXCR4⁺ tip cells, P10: JAG-1⁺/CXCR4⁻ stalk cells).

- v. PNAAd-AlexaFluor 647 (HEVs) 1:200 in FACS buffer.
- vi. CD36-PerCP/Cy5.5 (quiescent BECs) 1:100 in FACS buffer.
- vii. CXCR4-AlexaFluor 488 (tip BECs) 1:200 in FACS buffer.
- viii. JAG1- APC (stalk BECs) 1:50 in FACS buffer.
- e. Mix cells and antibodies using a plate shaker at 1,500 rpm for 2 min.
- f. Incubate antibodies with cells for 20 min at 4°C, in the dark.
- g. Wash cells twice with FACS buffer by centrifugation at 300 × g for 5 min at 4°C.
- h. Resuspend cells in 300 μL FACS buffer and transfer cells into FACS tubes.
- i. For dead-cell exclusion, add 7-AAD (1:100) solution immediately before analysis.

Pause point: If cells are meant to be analyzed by flow cytometry only, without further downstream analysis, you can consider fixation of your cells post surface antibody staining. For this purpose, perform surface staining as usual and incubate cells in 2% PFA in PBS for 10 min on ice. Wash twice with PBS and store at 4°C until analysis. Data should be recorded as soon as possible thereafter, but at the latest within 2–3 days post fixation. Since 7-AAD is a non-fixable dye, consider using fixable viability dyes (e.g., Zombie dyes from BioLegend) to exclude dead cells.

CRITICAL: Fixation of the cells might affect some of the used dyes. Extended PFA fixation might influence the integrity and signal intensity of fluorescent proteins like PE and APC and the Cy7 moiety in tandem dyes.

CRITICAL: Fixation might affect the spectrum of the autofluorescence of these cells. The unstained reference control should be treated exactly in the same way as the fixated and stained cells.

Note: Consider that the CD45 expression might be low or absent in a fraction of plasma cells or in cells of hematopoietic cancer. Other or additional markers like CD90 or leukocyte subset-specific markers might be necessary.

Note: Fluorescent tandem dyes like PE-Cy7 and PerCP-Cy5.5 are not stable over long periods of time, which can cause changes of the spectral fingerprint of these dyes. The antibody mastermix should therefore be generated immediately before use to avoid issues with the spectral unmixing or signal loss. Tandem dyes also require reference controls stained and recorded at the day of experiment and should not be used from the reference library.

Flow cytometry detection and analysis of lymph node endothelial cells

⌚ Timing: 60 min for step 6

⌚ Timing: 60 min for step 7

Since flow cytometry methods and devices are usually optimized for the analysis of leukocytes, endothelial cell analysis requires extended preparation procedures, cell-specific detection adjustments and thorough sample-by-sample, gate-by-gate analysis.

6. Record reference samples for spectral unmixing.
 - a. Unstained stromal cells are used as unstained controls.
 - b. Make sure all reference control spectra are accurate and of high quality, unmix the experiment and use live unmixing during the recordings. You can always re-unmix recorded samples later during the analysis in case you are not satisfied with your reference controls.

△ CRITICAL: Spectral unmixing crucially relies on the good quality and choice of your reference samples. Your unstained control represents the “baseline” for autofluorescence subtraction. Since different cell types can exhibit distinct spectral features, it is vital to use the cell type you will be analyzing later on (e.g., stromal cells) as your unstained control for optimal unmixing performance.

Perform Quality Check runs with SpectroFlo QC beads on your device on a regular basis, as this ensures reliability and reproducibility of your experiments.

Check your staining panel beforehand using the similarity matrix option in the SpectroFlo software. In this protocol they are providing you with a full flow cytometry panel that works well with spectral unmixing using the 5 laser Aurora device (Cytek). However, should you decide to use alternative fluorophores or include additional dyes, check the compatibility of your fluorophores using either the similarity index in SpectroFlo or use full spectrum viewer (<https://spectrum.cytekbio.com>). Do not refer to conventional spectrum viewers since they are usually optimized for conventional filter-based flow cytometry displaying only the most pronounced emission peak with single laser excitation. The analysis method introduced in this protocol requires a full spectral signature of fluorophores during simultaneous multi-laser excitation.

Note: As spectral flow cytometry gets increasingly recognized and appreciated, most antibody manufacturers offer many antibodies with an increasing number of coupled fluorophores. A suggestion of other fluorophores suited to be used for the acquisition with the 5 laser Aurora device (Cytek) is listed in the following table. Find further information in the fluorochrome selection guides of Cytek. Consider that polymer dyes such as Brilliant Violet can interact with each other and cause artifacts that can be avoided by using commercially available staining buffers such as Brilliant Stain buffer by BD.

Ultra violet ex.	Violet ex.	Blue ex.	Yellow/Green ex.	Red ex.
BUV395	BV421	BB515	7-AAD	APC
BUV486	BV480	AF488	PE	AF647
BUV563	BV510	AF532	PE-Dazzle594	AF700
BUV661	BV570	PerCP-Cy5.5	PE-Cy5	APC-Cy7 (Fire750)
BUV737	BV605	PerCP-eFluor710	PE-Cy7	
BUV805	BV650			
	BV711			
	BV750			
	BV785			

Note: The Cytex Aurora flow cytometer is susceptible to a clogged or partly blocked sample line if the device is not rinsed properly on a regular basis. Pay attention to stable flow rates during acquisition and consider activation clog and bubble detection.

Note: Once the antibody staining and the reference controls are established, it is also appropriate to use the reference sample library. However, please note that not all fluorescent conjugates are stable over time. Especially tandem dyes (e.g., Pe-Cy7, APC-Cy7) differ between aliquots, tend to change their emission spectrum over time, and should be included to the reference controls on a regular basis.

7. Recording of spectral flow cytometry data with a Cytex Aurora device.

a. Gating (Figure 1):

- i. FSC-A/SSC-A: P1 typical cell size and granularity →
- ii. FSC-A/FSC-H: P2 single cells →
- iii. FSC-A/7-AAD: P3 live cells →
- iv. FSC-A/Ter119, CD45: P4 stromal cells →
- v. CD31/PDPN: P5 BECs →
- vi. PNA/CD36: P6 HEV cells/quiescent cells →
- vii. JAG-1/CXCR4 P7 stalk cells/tip cells.

b. Recording of at least 10,000 BECs per sample is recommended. If the amount of cells allows to increase the amount of recorded cells, we recommend to record as much cells as possible, since a higher number of cells improves the further gating and analysis of the hierarchical BEC subsets over several levels (see note below).

Note: When the expression level of surface molecules in terms of the mean fluorescence intensity (MFI) per cell is a desired readout, consider adjusting the recording of BECs to a specific amount of BECs in all samples. Larger variances of the cell count between samples/groups can influence the MFI and might distort comparisons of expression levels.

Note: Consider adding isotype controls for targets known to not have a clear positive population but rather an intensity shift (e.g., JAG-1, CXCR4).

Optional: In order to avoid computer performance issues when applying FlowJo analysis on an extended data size per sample, consider using the stromal gate as storage gate to exclude events from cell debris and the TER119/CD45 dump-gate. Depending on the depletion of leukocytes, storing stromal cells only can result in a remarkable reduction of file sizes, facilitates fluent data analysis, and avoids computer crash.

Flow cytometry sorting of BECs for scRNA-seq processing

⌚ Timing: 30 min for step 8

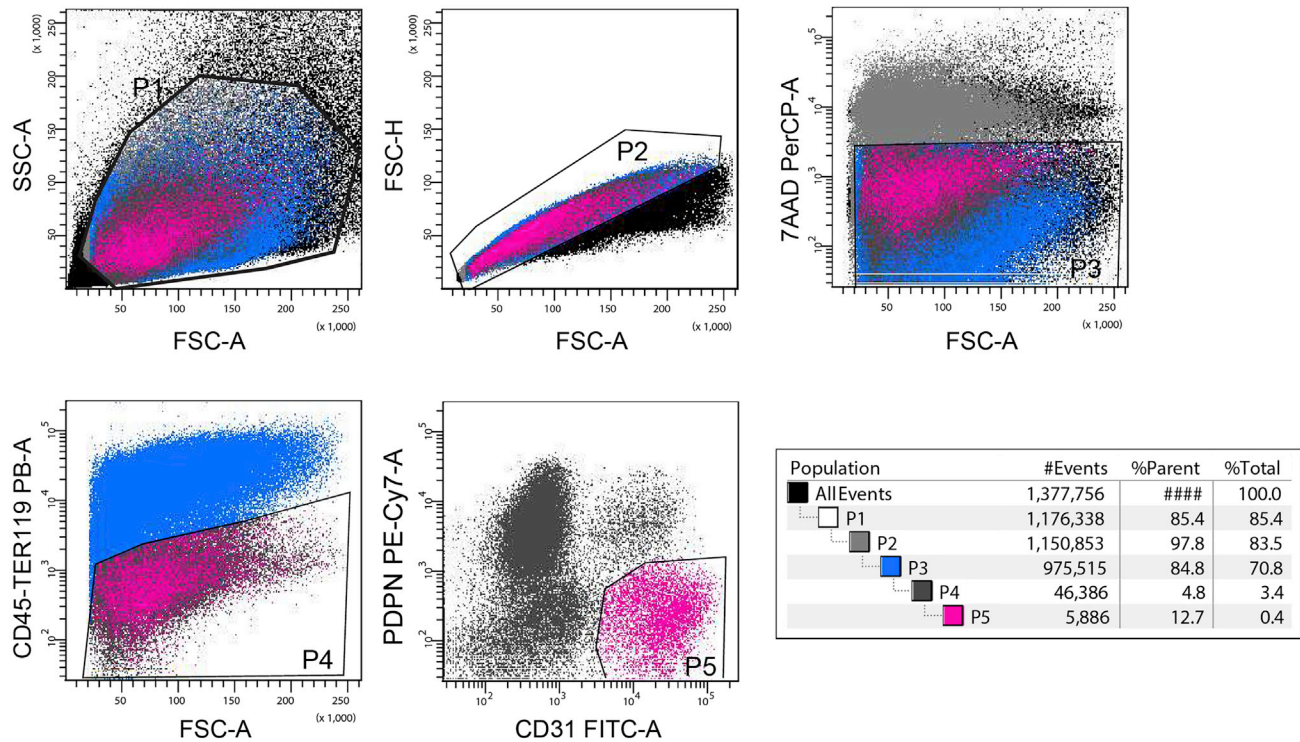


Figure 2. Gating strategy for flow-cytometry-based sorting of BECs

(P1-P5) Flow cytometry gating strategy of BECs (P5). Gates depict events in a hierarchical order, (P1) FSC-A/SSC-A (cell size and granularity), (P2) FSC-A/FSC-H (single cells), (P3) FSC-A/7-AAD (7-AAD⁺ live cells), (P4) FSC-A/CD45-Ter119 (CD45⁺ stromal cells), (P5) CD31/PDPN (BECs). Bottom right, population hierarchy.

⌚ Timing: 30 min for step 9

⌚ Timing: ~30 min per sample for step 10

⌚ Timing: 8 h for step 11

⌚ Timing: variable for step 12

Consider that the quantity of BECs isolated from murine lymph nodes is very low (10%–20% of all CD45⁺ stromal cells), and cell sorting and processing for scRNA-seq is often prone to a loss of cells. In our experience, if a total number of approximately 1×10^4 cells to be analyzed is desired, pooled LNs of at least five mice per group are necessary. Use fluorescence coupled antibodies according to the configuration of the cell-sorting device available (Figure 2).

8. Perform antibody staining of lymph node cell populations.
 - a. Incubation with blocking antibody for 10 min at 4°C.
 - i. CD16/CD32 1:100 in FACS buffer.
 - b. Incubation with staining antibodies for 20 min at 4°C in the dark.
 - i. TER119-Pacific Blue (Erythrocytes) 1:200 in FACS buffer.
 - ii. CD45-Pacific Blue (Leukocytes) 1:200 in FACS buffer.
 - iii. CD31-FITC (Endothelial cells) 1:200 in FACS buffer.
 - iv. PDPN-PE/Cy7 (FRCs, LECs) 1:200 in FACS buffer

9. Prepare the flow cytometry sorter.

- a. Pre-cool collection tube array to 4°C.
 - b. Use 100 µm sorting nozzle to minimize shear stress for sorted cells.
 - c. Gating (Figure 2):
 - i. FSC-A/SSC-A: P1 typical cell size and granularity →
 - ii. FSC-A/FSC-H: P2 single cells →
 - iii. FSC-A/7-AAD: P3 live cells →
 - iv. FSC-A/Ter119, CD45: P4 stromal cells →
 - v. CD31/PDPN: P5 BECs (collected cell population).
10. Cell sorting and adjustment.
- a. Sort cells into ice-cold HBSS (0.04% BSA) in pre-coated collection tubes. Adjust flow speed to a maximum of 12,000 events/sec.
 - b. Centrifuge cells at 300 g for 5 min at 4°C.
 - c. Determine cell count and viability, adjust the cell concentration to 10⁴ cells per 10 µL.
11. Sample and library preparation.
- a. Use the recommended protocol of the 10× genomics single cell gene expression workflow and the chromium controller (Protocol: <https://support.10xgenomics.com/permalink/OAXXHQmeWa60lWK66uqSo>).
12. Sequencing and processing of reads.
- a. Sequence samples until the desired sequencing depth or sequencing saturation is reached.

Optional: For cell sorting, apply cells on a 40 µm cell strainer to avoid clogging of the sorting nozzle. When sorting endothelial cells, we recommend configuring your sorter to a larger inner nozzle diameter (e.g., 100 µm). The standard 70 µm nozzle can cause shear stress on endothelial cells and decreases cell viability.

△ CRITICAL: Once disintegrated from the vessel structure, endothelial cells are very sensitive and prone for apoptosis and cell death. Additionally, mRNAs are relatively unstable and rapidly degraded molecules. Following the cell sorting procedure, avoid delays of the procedure and process cells as quickly as possible to preserve cell viability and mRNA integrity. Prolonged cell storage or cryopreservation is not recommended.

△ CRITICAL: Consider that 10× reagent kits and protocols are not cross-compatible between versions. Take care that you use the suitable protocol for the type and version of your 10× chemistry.

Note: Given antibody dilutions are suggestions and might be changed or titrated to avoid adverse signal spread and compensation requirements.

Note: Non-viable cells (7-AAD⁺) are removed during the cell sorting process. However, endothelial cells are sensitive and the viability can decline between cell sorting and the preparation of the single cell libraries. We recommend to determine the cell viability immediately before single cell capture and proceed only with samples with a cell viability above 80%.

Note: In contrast to many other applications of flow cytometry sorted cells, we highly recommend to not set the gates of BECs determined for scRNA-seq in a very strict fashion. An enrichment of BECs with more generous/relaxed gates ensures that all BEC subsets and BECs with different differentiation grades are covered by the data set. All events contributed by non-BECs can be excluded during the computational pre-processing and analysis of the scRNA-seq data.

Note: It is usually recommended to sort into FCS-containing medium or directly into FCS in order to preserve cell viability. However, the performance of the downstream 10× genomics single cell protocols might be hampered by ingredients of the serum. In our experience, using HBSS with 0.04% BSA resulted in good cell viability, low cell aggregates and a sufficient cell recovery rate.

Analysis of scRNA-seq data from murine lymph node BECs

⌚ Timing: undefined

The Seurat package for R provides all basic tools and functions for quality control, analysis and illustration of scRNA-seq data (Satija et al., 2015; Butler et al., 2018; Stuart et al., 2019; Hao et al., 2021).

Note: Especially for researchers with a profession offsite from bioinformatics and with a low level of experience in using statistics software (e.g., R, Matlab, Python), the Satija lab (New York genome center, USA) provides online step-by-step tutorials for scRNA-seq data processing and analysis (<https://satijalab.org/seurat>). The following code lines and parameters provided convincing results with our set up and our data set and might have to be adjusted individually for other data sets.

13. Use the CellRanger analysis pipeline to align reads, generate feature-barcode matrices and aggregate data from different samples.
14. Further processing and analysis are performed in the R studio software with the following libraries installed and initialized:

```
> library (Seurat)
> library (SeuratData)
> library (ggplot2)
> library (dplyr)
```

15. Load the data package from the CellRanger output.

```
> data_set <- Read10X(data.dir =
"HD.../outs/filtered_gene_bc_matrices/mm10")
```

16. Create a Seurat object.

```
> Seurat_BECs <- CreateSeuratObject (counts = data_set, project =
"Endothelial cells", min.cells = 3, min.features = 100)
```

17. Perform quality control pre-processing.
 - a. Remove all cells with unique feature counts above 5,000 or less than 100.
 - b. Remove cells that have above 5% mitochondrial counts.

```
> Seurat_BECs[["percent.mt"]]
Seurat_BECs <- PercentageFeatureSet (Seurat_BECs, pattern="^MT- ")
> Seurat_BECs <- subset (Seurat_BECs, subset = nFeature_RNA > 100 &
nFeature_RNA < 5000 & percent.mt < 5)
```

18. Normalize Data using the *NormalizeData* function of Seurat.

```
> Seurat_BECs <- NormalizeData(Seurat_BECs, normalization.method =  
"LogNormalize", scale.factor = 10000)
```

Optional: If required, remove non-BECs from the data set to avoid influence on the analyses. Non-BECs can be filtered along the following markers:

```
Cdh5+ (Endothelial marker)  
Pecam1+ (coding for CD31, Endothelial marker)  
Lyve1- (lymphatic endothelial cell marker)  
Prox1- (lymphatic endothelial cell marker)  
Acta2- (mesenchymal marker)  
Pdpn- (mesenchymal marker)  
Pdgfrb- (mesenchymal marker)  
Ptprc- (leukocyte marker)  
Cd52- (lymphocyte marker)  
  
> real_BEC <- subset(Seurat_BECs, subset = Cdh5>1 & Pecam1>1 & Cd52 ==  
0 & Ptprc == 0 & Acta2 == 0 & Prox1 == 0 & Pdgfrb == 0)
```

19. Identify highly variable genes using the *seurat FindVariableGenes* function.

```
> real_BEC <- FindVariableFeatures(real_BEC, selection.method = "vst",  
nfeatures = 5000)
```

20. Scale data for an equal weighting of genes during dimensional reduction processing using the *ScaleData* function.

```
> all_genes <- rownames(real_BEC)  
> real_BEC <- ScaleData(real_BEC, features = all_genes)
```

21. Perform linear dimensionality reduction using the *RunPCA* function.

```
> real_BEC <- RunPCA(real_BEC, features = VariableFeatures(object =  
real_BEC))
```

22. Determine the dimensionality of the data set using the *ElbowPlot* function.

```
> ElbowPlot(real_BEC)
```


23. Cluster cells using the *FindNeighbors* and *FindCluster* function.

```
> real_BEC <- FindNeighbors(real_BEC, dims = 1:5)
> real_BEC <- FindClusters(real_BEC, resolution = 0.4)
```

24. Run nonlinear dimensionality reduction using uniform manifold approximation and projection (UMAP) or the t-distributed stochastic neighbor embedding (t-SNE). (Figure 3).

```
> real_BEC <- RunTSNE(object = real_BEC, dims = 1:5)
> real_BEC <- RunUMAP(object = real_BEC, dims = 1:5)
```

Note: The cell ranger pipeline and the analysis of large data sets require extensive computational resources and the usage of a high performance computational cluster or cloud-based computing.

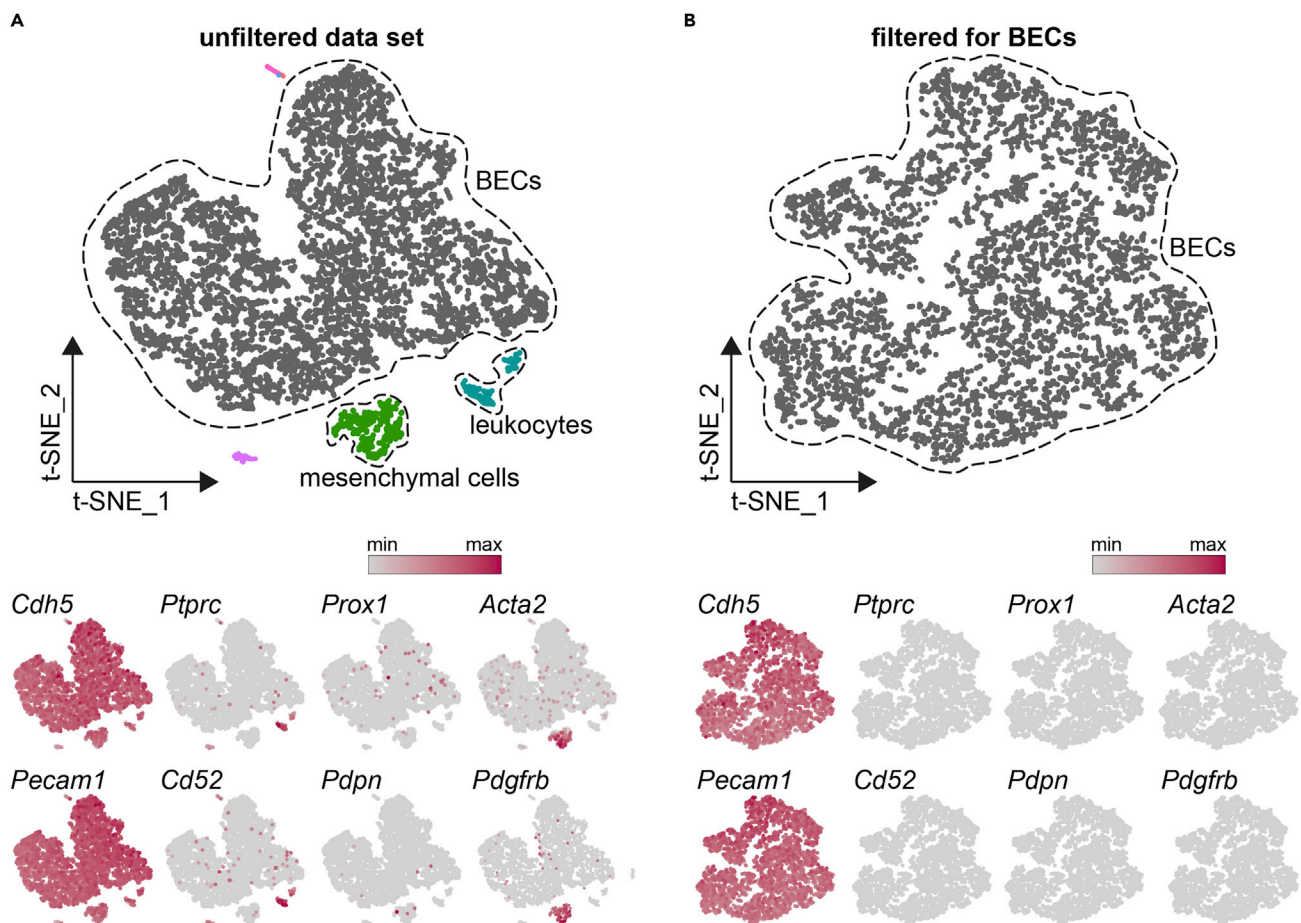


Figure 3. Characterization of contaminating cells and filtering for BECs

(A) t-SNE non-linear dimensionality reduction of the scRNA-seq analysis of BECs from mouse lymph nodes. Bottom, canonical markers of endothelial cells (*Cdh5*, *Pecam1*), leukocytes (*Ptprc*, *Cd52*), lymphatic endothelial cells (*Prox1*, *Pdpn*), and mesenchymal cells (*Acta2*, *Pdgfrb*).

(B) t-SNE non-linear dimensionality reduction of the scRNA-seq analysis of BECs from mouse lymph nodes after filtering BECs (*Cdh5*, *Pecam1*) and removal of non-BEC contaminating cells (*Ptprc*, *Cd52*, *Prox1*, *Pdpn*, *Acta2*, *Pdgfrb*). Color scales indicate the relative gene expression.

Note: It is recommended to perform a quality control analysis and exclude low quality cells from further analysis. Quality control metrics commonly include the number of unique genes detected in each cell (excludes empty droplets and multiplets) and the percentage of reads that map to the mitochondrial genome (low quality cells with low viability). The appropriate threshold should be determined individually according to the respective samples or runs. We included cells with 100–5,000 unique genes (nFeature_RNA) and a percentage of mitochondrial genes below 5%.

Note: Check the expression of typical non-BEC and BEC marker after removal of non-BECs from the scRNA-seq data set. The filtered subset should not include cells expressing non-BEC marker but just cells expressing the pan-endothelial markers *Cdh5* and *Pecam1*. The latter encodes for the surface marker CD31.

Optional: Find further analysis suggestions and display formats in the vignettes and archive section of the Seurat website (<https://satijalab.org/seurat/index.html>).

EXPECTED OUTCOMES

The following figures illustrate examples of data from our recent study ([Menzel et al., 2021](#)).

LIMITATIONS

We acknowledge some limitations of the described protocols. The protocols were established using healthy mice of both sex at an age of 8–12 weeks. Adjustments might be needed, since the lymph node is a highly plastic organ. Size, morphological structure and BEC content differ between different mouse ages and disease models.

We would like to point out that our protocols' advantages and novelty mostly rely on the combination of optimized and economic process timing, reproducibility and optimized quantity and quality of BECs after the isolation. Additionally, the estimated timing of the protocols was established for isolation of 10 lymph nodes per mouse, and 10 mice per condition in total. Depending on the age of the mice, the disease model, the condition of the lymph nodes, and the experience of the experimenter, detection and dissection of a sufficient number of lymph nodes can be challenging and may increase the time consumption drastically.

Of note, the protocols may differ slightly from the isolation processes described in ([Gloger et al., 2020](#); [Menzel et al., 2021](#)) due to further optimization and the individual requirements of the disease model employed.

TROUBLESHOOTING

Problem 1

Problems while unmixing your samples (bad signal-to-noise ratio, subjective "wrong" populations or strange shape of the populations) (step 6).

Potential solution

The use of tandem dyes such as PE-Cy7 and PerCP-Cy5.5 in flow cytometry experiments requires careful consideration. Since fluorescence resonance energy transfer (FRET) between the acceptor and donor dye is never 100% efficient, you will encounter spillover into the donor dye channel (PE or PerCP). There are certain factors that influence FRET efficacy and, thus, the spectral fingerprint of the dyes, potentially hampering detection of the dye by the cytometer. Tandems are heavily sensitive to oxidation and therefore should be kept from light and other sources of oxidative stress at all times during the staining process. Fixatives and permeabilization buffers can also influence signal detection. Some methanol-based fixatives can degrade proteins such as PerCP, which form the backbone of some tandem dyes, whereas the acceptor moiety remains unaltered. Note that this

protein degradation also takes place after freezing, therefore antibodies coupled to these dyes should be stored frozen. Some non-fixated cells are prone to cell-intrinsic uncoupling of tandem dyes. To keep this effect at a minimum, stain and hold the cells at 4°C to minimize cell metabolism. To avoid issues with spectral unmixing or signal loss, incubate cells in buffers only as long as absolutely necessary and avoid denaturing agents or storage conditions when working with tandem dyes. Make sure to use reference controls that were treated and stained exactly like your sample (dilution, staining time, temperature, staining buffer) and record them at the day of the experiment.

Consider that polymer dyes such as Brilliant Violet can interact with each other and cause artifacts that can be avoided by using commercially available staining buffers such as Brilliant Stain buffer available from BD.

Problem 2

Different cell populations during the flow cytometry analysis or during cell sorting are not clearly separated from each other and appropriate gating is unclear (steps 7 and 10).

Potential solution

Optimization or titration of the antibody labeling can help to reduce the signal-to-noise ratio and the signal spread. The digestion process can result in increased cell debris and dead cells, both of which tend to have a high autofluorescence and should be excluded thoroughly according to their size, granularity and live data staining. Furthermore, stromal cells are often more heterogeneous than other cell types commonly used in flow cytometry (e.g., lymphocytes). Gating can therefore be important to optimize the staining and recording process. Using contour plots can help to identify and to differentiate cell populations and to set appropriate gates.

Problem 3

Low yield of isolated BECs due to incomplete digestion of the lymph nodes (step 2).

Potential solution

Optimize mechanical handling of the digestion process. Make sure that the forces during extensive pipetting are not too strong, but strong enough to support the digestion. Adjust the volume of the digestion solution to the number of pooled lymph nodes. We use 5 mL of digestion solution for up to 50 pooled lymph nodes.

Problem 4

Low yield of isolated BECs during CD45⁺ cell MACS depletion due to clogging of the MACS columns (step 3).

Potential solution

Make sure the whole cell pellet is resuspended properly and use a second cell strainer during transfer of the cell solution onto the MACS columns.

Problem 5

Low yield of isolated BECs during analysis or cell sorting due to poor antibody labeling (step 5).

Potential solution

Optimize antibody labeling by titrating your reagents to saturating concentrations and increase the antibody concentration if necessary. Make sure that you stain your cells in a suitable volume and vessel. Try to avoid cell densities > 1*10⁷/mL and consider shaking your vessel gently while staining in order to avoid cell aggregation and insufficient distribution of antibodies. Stain at 4°C rather than on ice to avoid cell loss during staining.

Problem 6

Low yield of isolated BECs or loss of BECs during sorting due to poor recovery within the collection tube (step 10).

Potential solution

The distribution of cells into the collection tube during the sorting process can be optimized with calibration beads (e.g., AccuDrop Beads by BD) before the sorting procedure. Cells which are not properly dropped in the center of the tubes may stick to the upper wall of the collection tube due to the electrostatic charge of the tubes' plastic and dry out over time. In order to prevent this, we here use BSA-coated collection tubes for cell sorting to avoid cell adherence to the plastic wall of the tube.

Problem 7

Low viability of isolated BECs. Low viability of BECs could be caused by an extended time demand for the lymph node isolation procedure, extended enzymatic digestion time or insufficient inhibition of digestion. Attention should be paid to proper storage of collected cells (step 10).

Potential solution

The most important step to increase BEC viability during the process is to optimize the sequential schedule of the process and keep the processing time from isolation to measurement at a minimum. Experience with lymph node detection and dissection helps to process all samples fast and to reduce unnecessary storage time of organs and cell solutions. It helps a lot to prepare and cool down all necessary equipment before starting the dissection. Always work with cooled buffers and reagents.

Problem 8

Discontinuous sorting process due to clogging nozzle (step 10).

Potential solution

Large cells such as endothelial cells tend to aggregate in suspension and are prone to clog the cytometer nozzle, which can also lead to lower yield. Always sieve your cells immediately prior to the sorting process (e.g., by using polystyrene FACS tubes with cell strainer snap cap) and keep your cells at a density of about 1×10^6 / mL. Gently mix the suspension by pipetting shortly before running the sample. Continuously monitor the stability of your sorting efficacy. Consider in which mode you want to run the sorting process. The "purity" mode will more precisely exclude contaminating cells outside of the desired sorting gates. The "yield" or "recovery" mode will ensure a higher yield of cells but is more prone to include contaminating non-target cells.

Problem 9

Extensive loss of cells during the quality control process of the scRNA-seq analysis (step 17).

Potential solution

The threshold values excluding low quality cells can differ between samples and runs and should be considered in an individual manner. Displaying the unique gene count, number of detected molecules and the percentage of mitochondrial genes in violin plots (per sample) helps to identify the appropriate threshold. Please find the detailed tutorial with explanations and examples at the Seurat website (https://satijalab.org/seurat/articles/pbmc3k_tutorial.html).

Problem 10

No mitochondrial genes were found during the quality control process of the scRNA-seq analysis (step 17).

Potential solution

The prefix of mitochondrial genes is different in your data set. The command `(>Seurat_BECS [['percent.mt']] <- PercentageFeatureSet(Seurat_BECS, pattern='^MT-'))` searches for all gene names starting with MT- and adds the results as a new column to the object metadata. However, depending on the vendor and the version and the used reference genome during the decomplexing pipeline (e.g., CellRanger with mouse MM10 reference genome) the prefix can vary. The prefix in the command has to be exactly identical to the prefix used for the gene names in your data set. Consider case sensitivity.

RESOURCE AVAILABILITY

Lead contact

Armin Rehm, arehm@mdc-berlin.de.

Materials availability

There are no newly generated materials associated with this protocol.

Data and code availability

scRNA-seq data have been deposited in the ArrayExpress database (ArrayExpress DatabaseID: E-MTAB-10389). There is no original code associated with this protocol.

ACKNOWLEDGMENTS

The authors thank K. Gerlach, K. Krüger, A. Golz, and M. Schrimpf for expert technical assistance in immunohistology and mouse genotyping. We thank Uta E. Höpken for critical discussions on the manuscript. We thank C. Fischer and V. Franke for help in scRNA-seq data acquisition, and H.-P. Rahn and K. Rautenberg for assistance in flow cytometry. This work was funded in part by a grant from Deutsche Krebshilfe (project #111918) awarded to Armin Rehm and Uta E. Höpken.

AUTHOR CONTRIBUTIONS

L.M. performed all experiments associated with this protocol. L.M., M.Z., and A.R. wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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