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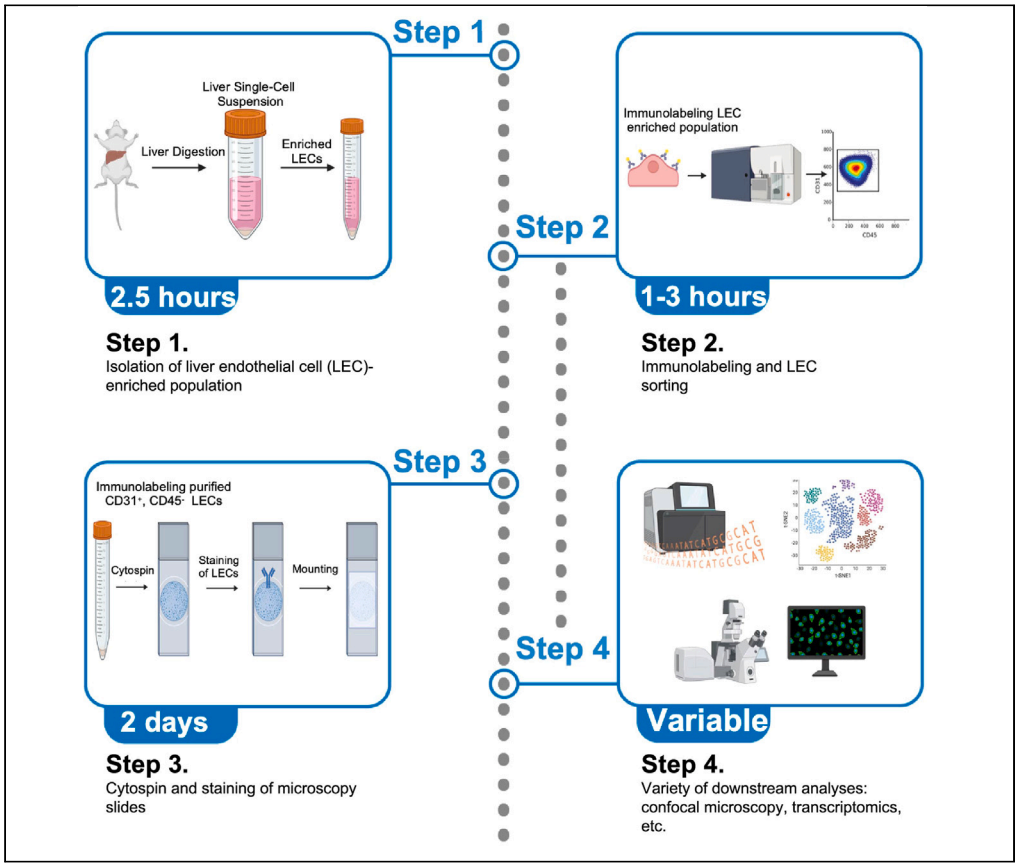
Protocol for enrichment, purification, and cytocentrifugation of mouse liver endothelial cells



Liver endothelial cells (LECs) are critical in maintaining liver homeostasis. To understand the mechanistic processes occurring in these cells, high-quality isolation protocols must be in place. Here, we present a protocol for LEC enrichment, subsequent LEC purification using fluorescence-assisted cell sorting, and cytocentrifugation of sorted LECs for imaging. We describe steps for isolation of LEC-enriched population from mouse livers, immunolabeling and sorting, and cytospin and immunostaining. We then mention procedures for downstream analysis.

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

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Highlights

Isolation of liver-endothelial-cells (LECs)-enriched population from mouse livers

Purification of LECs using FACS for downstream analysis

Cytocentrifugation of FACS-sorted LECs for imaging

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Protocol for enrichment, purification, and cytocentrifugation of mouse liver endothelial cells

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SUMMARY

Liver endothelial cells (LECs) are critical in maintaining liver homeostasis. To understand the mechanistic processes occurring in these cells, high-quality isolation protocols must be in place. Here, we present a protocol for LEC enrichment, subsequent LEC purification using fluorescence-assisted cell sorting, and cytocentrifugation of sorted LECs for imaging. We describe steps for isolation of LEC-enriched population from mouse livers, immunolabeling and sorting, and cytospin and immunostaining. We then mention procedures for downstream analysis.

For complete details on the use and execution of this protocol, please refer to Drzewiecki et al. (2021).[1]

BEFORE YOU BEGIN

The preparation of mouse liver tissue for this protocol requires collagenase digestion to generate a single-cell suspension composed of both liver parenchymal and non-parenchymal cells (NPCs). The method to digest mouse liver with or without liver perfusion is beyond the scope of this protocol. Excellent protocols detailing the procedure for perfusion and enzymatic digestion can be found in.[2–5] This protocol focuses on the isolation of mouse liver endothelial cells (LECs)-enriched NPCs using a Percoll gradient. Subsequently, we outline how LEC-enriched population can be further purified through fluorescence-activated cell sorting (FACS), by isolating cells that are alive (negative for DAPI), negative for the hematopoietic marker CD45 and positive for the endothelial cell marker CD31. Lastly, we describe how these purified LECs ($DAPI^-CD45^-CD31^+$) can be used for confocal microscopy after cytocentrifugation or for a variety of other applications, such as transcriptomics.[1] Our protocol allows investigators to perform liver endothelial cell-enrichment and purification steps while retaining the flexibility to utilize other hepatic cell types.

Institutional permissions

All mice used for developing this protocol were maintained under specific pathogen-free conditions at Yale Animal Resources Center and used according to the Yale University Institutional Animal Care and Use Committee (IACUC).

Preparation 1. Microscope slides for cytocentrifugation protocol

Timing: ~6 h

Timing: 10 min to make solution of 30% heat inactivated FBS in 1× PBS solution

Timing: 4–6 h to dry

Prepare microscope slides key resources table for cytocentrifugation protocol the evening prior or the morning of LEC-enriched NPC isolation. Using slides previously coated with 30% fetal bovine serum (FBS) in phosphate buffered solution (PBS 1X, pH 7.4) will maximize adherence of cells onto the slide. We recommend 30% FBS in 1X PBS to be freshly made on the day the microscope slides are coated. If necessary, the solution can be stored at 4°C for one week. Longer storage is not recommended.

1. Prepare 30% heat inactivated FBS in 1× PBS (~1 mL per slide).
2. Label slides for experiment and coat each slide with ~1 mL of 30% FBS in 1× PBS solution.
3. Place slides in an incubator at 37°C to allow the 30% FBS in 1× PBS to dry.

Note: Slides are ready to use once the layer of 30% FBS in 1× PBS has dried on the slides, which facilitates adherence of the cells during the cytocentrifugation step.

Preparation 2. Percoll solutions and gradient

Timing: 20 min for ~4 Percoll gradients; 5 min per 1 Percoll gradient

Note: Timing depends on user experience with Percoll gradient preparation and the required number of gradients for the investigator's experiment. Percoll is used for density gradient experiments due to its low viscosity compared to alternatives like Ficoll, along with its low osmolality and non-toxicity towards cells. Studies using Percoll achieved 95% viability when separating mouse liver and spleen cells. The success of LEC-enriched isolation relies on their efficient separation from other cell types, such as hepatic stellate cells and red blood cells.

The Percoll gradient can be made up to 30 min ahead of centrifugation. Please see materials and equipment for recipes of Percoll solutions. It is not recommended to make the gradient any earlier. Once Percoll solutions are made, they should be stored at RT (20–22°C). The Percoll gradients are also kept at RT (20–22°C) until use.

4. Pipette 5 mL of 50% Percoll solution into a 15 mL conical tube.

Optional: In our experiments, we have used conical tubes from the Corning brand. We expect other conical tube brands to perform in a similar manner.

5. Carefully layer 6.6 mL of 25% Percoll solution, by setting the automatic pipettor to 0 or at the slowest speed. For additional guidance please see Methods video S1.

Note: Optimal layering of 25% Percoll solution over 50% layer can take up to 1–2 min. The first droplet may result in mixing, but subsequent slow pipetting of the 25% solution will result in the formation of a distinct layer. For visual representation of gradients please see Figure 1.

⚠ CRITICAL: If there is mixing between the 50% and 25% layers, we recommend remaking the gradient, as it will negatively impact the yield of LEC-enriched population.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Allophycocyanin/cyanine 7 anti-mouse CD45 (1:100)	BioLegend	Cat#103116
BV605 anti-mouse CD31 (1:100)	BioLegend	Cat#102427

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Purified Rat Anti-Mouse CD16/CD32 Clone 2.4G2 (RUO) (1:100)	BD Pharmingen™	Cat #553142
Chemicals, peptides, and recombinant proteins		
10× Tris buffered saline (TBS)	Bio-Rad	Cat#1706463
16% Paraformaldehyde (formaldehyde) aqueous solution, EM grade	Thomas Scientific	Cat# C993M26
Ammonium chloride (NH_4Cl), crystal	VWR Chemicals	Cat#12125-02-9
Fetal bovine serum (FBS)	Gibco	Cat#10437-028
DAPI (1:100)	BioLegend	Cat#422801
PBS (10×), pH 7.4	Thermo Fisher Scientific	Cat#70011069
PBS (1×), pH 7.4	Thermo Fisher Scientific	Cat#10010023
Percoll	Sigma-Aldrich	Cat#17-0891-02
Prolong™ Gold antifade reagent with DAPI	Invitrogen	Cat#P36980
RPMI 1640 Medium	Thermo Fisher Scientific	Cat#11875093
Sodium azide, 5%	Fisher Scientific	Cat#71448-16
Triton X-100	American Bio	Cat#9002-93-1
UltraPure 0.5 M EDTA, pH 8.0	Life Technologies	Cat#15575020
Other		
Cover Glass Thickness 1 $^1/_2$ 22 × 22 mm	Corning	Cat#2850-22
Epredia™ Single Cytofunnel™ with White Filter Cards	Thermo Fisher Scientific	Cat#5991040
Epredia™ Cytoclip™ Stainless-Steel Slide Clip	Thermo Fisher Scientific	Cat#59910052
Glass Coplin Jar	Thermo Fisher Scientific	Cat#E94
Unifrost Plus Microscope Slides Adhesive, Treated Surfaces	Azer Scientific	Cat#EMS200G+
ImmEdge® Hydrophobic Barrier PAP Pen	Vector Laboratories	Cat#H-4000
Thermo Scientific™ Cytospin™ 4 Cytocentrifuge	Thermo Fisher Scientific	Cat# A78300003
Sorvall Legend T Benchtop Centrifuge Rotor: 75006445	Sorvall Legend	Cat#SO-LEGT
BD FACS Aria II sorter	BD Biosciences	N/A
Beckman Coulter Allegra 6 Centrifuge Rotor: GH-3.8 Swing Bucket Rotor	Beckman Coulter	Cat#BE-A6
Leica TCS SP8	Leica	N/A
Software and algorithms		
FlowJo 10	BD	http://flowjo.com

MATERIALS AND EQUIPMENT

Stock Percoll			
Reagent	Volume (1 gradient)	Volume (4 gradients)	Storage
Percoll	4.5 mL	18.0 mL	20°C–25°C
10× PBS	0.5 mL	2.0 mL	20°C–25°C
Total Volume	5.0 mL	20.0 mL	20°C–25°C

Note: Stock Percoll solution is used to make the 50% and 25% Percoll solutions.

50% Percoll solution			
Reagent	Volume (1 gradient)	Volume (4 gradients)	Storage
Stock Percoll	2.5 mL	10.0 mL	20°C–25°C
1× PBS	2.5 mL	10.0 mL	20°C–25°C

25% Percoll solution			
Reagent	Volume (1 gradient)	Volume (4 gradients)	Storage
Stock Percoll	1.65 mL	6.60 mL	20°C–25°C
RPMI 1640	4.95 mL	19.8 mL	20°C–25°C

⚠ CRITICAL: The preparation of the Percoll solutions should be made fresh on the same day of its use, since long-term storage of Percoll solutions may affect its pH.

Note: RPMI 1640 key resources table is used for the creation of 25% Percoll solution, and its pink color helps to visually distinguish the layers between the 50% and 25% Percoll. Investigators may substitute RPMI 1640 with 1× PBS with caution as the 50% Percoll layer and 25% Percoll layer may not be as easily discernible. We have cell viability of >95% when using RPMI 1640.

Flow cytometry staining (FACS) Buffer (500 mL)		
Reagent	Final concentration	Volume
10× PBS	1×	50 mL
5% Sodium Azide	0.5%	10 mL
0.5 M EDTA	1.0 mM	1 mL
HI-FBS	2%	10 mL
ddH_2O	N/A	429 mL
Total	N/A	500 mL

Note: Once FACS buffer is filtered and sterilized, it may be stored at 4°C for up to 6 months.

Alternative: 1× PBS can be used in place of 10× PBS.

Buffers for cytocentrifugation

Coating slides Solution: 30% FBS in 1× PBS		
Reagent	Final concentration	Volume
HI-FBS	30%	3 mL
10× PBS	1×	1 mL
ddH_2O	N/A	6 mL
Total	N/A	10 mL

Note: We recommend making this on the day of the experiment, as mentioned in the before you begin section.

Note: Solution is good for 1 week if stored at 4°C.

Buffers for immunostaining

Fixative Solution: 4% Paraformaldehyde		
Reagent	Final concentration	Volume
16% PFA	4%	2.5 mL
10× PBS	1×	1 mL
ddH_2O	N/A	6.5
Total	N/A	10 mL

Note: We recommend making fixative solution fresh on the day of the experiment. The solution is good for 1 week if kept at 4°C.

⚠ CRITICAL: Paraformaldehyde (PFA) can be a toxic solution and irritant, and we recommend utilizing a chemical hood when handling PFA. PFA must be disposed as per institutional guidelines.

Quenching Solution: Ammonium Chloride NH_4Cl		
Reagent	Final concentration	Volume
NH_4Cl	50 mM	0.08 g
10× PBS	1×	3 mL
ddH_2O	N/A	27 mL
Total	N/A	30 mL

Note: We recommend making buffers on the day of the experiment.

Permeabilization Solution: 0.1% Triton		
Reagent	Final concentration	Volume
Triton X-100	0.1%	30 µL
10× PBS	1×	3 mL
ddH_2O	N/A	26.97 mL
Total	N/A	30 mL

Note: Triton X-100 is a viscous solution, and therefore it is advisable to make 10% Triton X-100 solution and then dilute further to 0.1%.

Blocking Solution: 10% FBS in 1× PBS		
Reagent	Final concentration	Volume
HI-FBS	10%	1 mL
10× PBS	1×	1 mL
ddH_2O	N/A	8 mL
Total	N/A	10 mL

Note: We recommend making blocking solution fresh each time on the morning of the experiment. It may be stored at 4°C up to 1 week.

STEP-BY-STEP METHOD DETAILS

Isolation of liver endothelial cell (LEC)-enriched non-parenchymal cell (NPC) population

Timing: 2.5 h for 4 mouse livers

The range for LEC-enriched NPCs isolated from a wild-type C57BL/6 mouse liver varies on average between 1.5–2.5 million cells, and $CD45^-CD31^+$ LECs accounts for approximately 15%–30% of isolated NPCs.

Note: Centrifuge at 4°C for all steps, except for gradient spin. To improve cell viability, keep cells on ice during all steps, except for the gradient spin.

1. Dissociate collagenase-digested mouse liver by gentle pipetting.
   a. Filter through a 40 µM filter into a 50 mL conical tube.
   b. Fill the 50 mL conical tube with RPMI 1640 media.
   c. Spin at 60 × *g* for 2 min at 4°C.

Note: Centrifuge [Sorvall Legend T Benchtop Centrifuge, with rotor: 75006445] is pre-cooled to 4°C. Alternative desk-top centrifuge with swinging bucket rotors may be utilized.

Note: The purpose of this spin is to pellet the hepatocytes. The supernatant will contain the liver NPCs, which include endothelial cells, hepatic stellate cells, Kupffer cells, and immune cells.

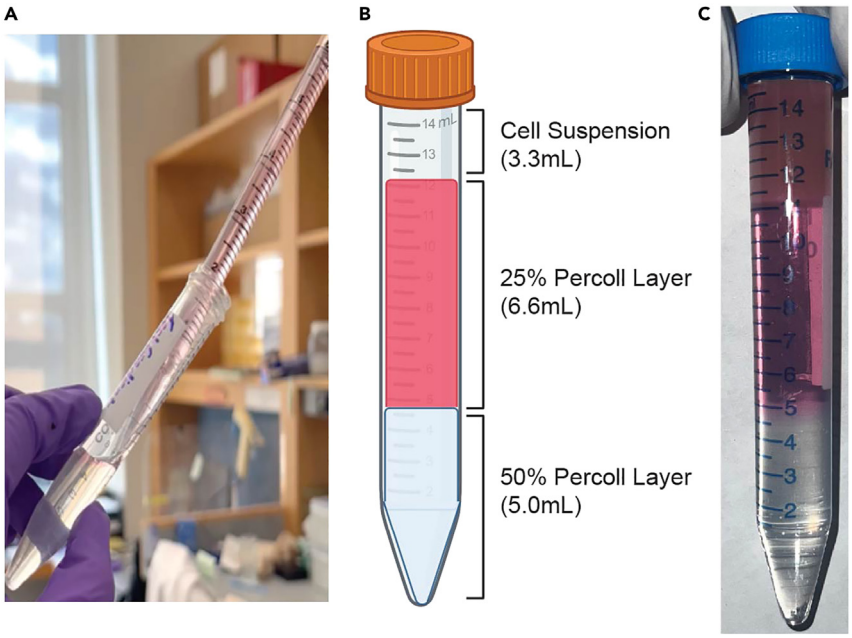

Figure 1. Schematic overview of Percoll gradient, and layering of Percoll gradient

(A) Methods video S1. Generation of the Percoll gradient by layering the 25% Percoll over the 50% Percoll layer, related to Step 5.

(B) Schematic representation of Percoll gradient, with cell suspension layer at the top, middle layer containing 25% Percoll solution. and bottom layer containing 50% Percoll solution.

(C) Photograph demonstrating a clear delineation of the Percoll layers.

2. Carefully collect the supernatant and transfer it to a new 50 mL conical tube using an automatic pipettor.

   Note: The supernatant volume varies between 40–45 mL.

   Note: After collecting the supernatant, the hepatocyte pellet can be either collected for downstream applications or discarded.

3. Repeat steps #1 and #2 at least three times to remove nearly all hepatocytes.
4. Centrifuge NPC-containing supernatant at 400 × *g* for 10 min at 4°C.
5. Create Percoll gradients by layering 25% Percoll solution over 50% Percoll solution, if not already done. See Figure 1, 'preparation 2. Percoll solutions and gradient' under 'before you begin' section and Methods video S1.
6. After the 10-min centrifugation in step #4, discard the supernatant.
7. Resuspend LEC-enriched pellet in 3.3 mL of RPMI 1640 by gentle pipetting.
8. Complete the gradient by carefully layering the 3.3 mL of resuspended cells in RPMI 1640 on top of the 25% Percoll layer (Figure 1).

   ⚠ CRITICAL: If the gradient is disrupted, the yield of enrichment for LECs will not be optimal. We recommend, once the gradients are created, to place the gradients in a tube rack. Furthermore, we recommend to not agitate the tubes until ready for use.

9. Centrifuge gradient at 900 × *g* for 20 min at RT (20–22°C). Set the brake to 0.

   Note: Centrifuge utilized for Percoll gradient step is Beckman Coulter Allegra 6 Centrifuge.

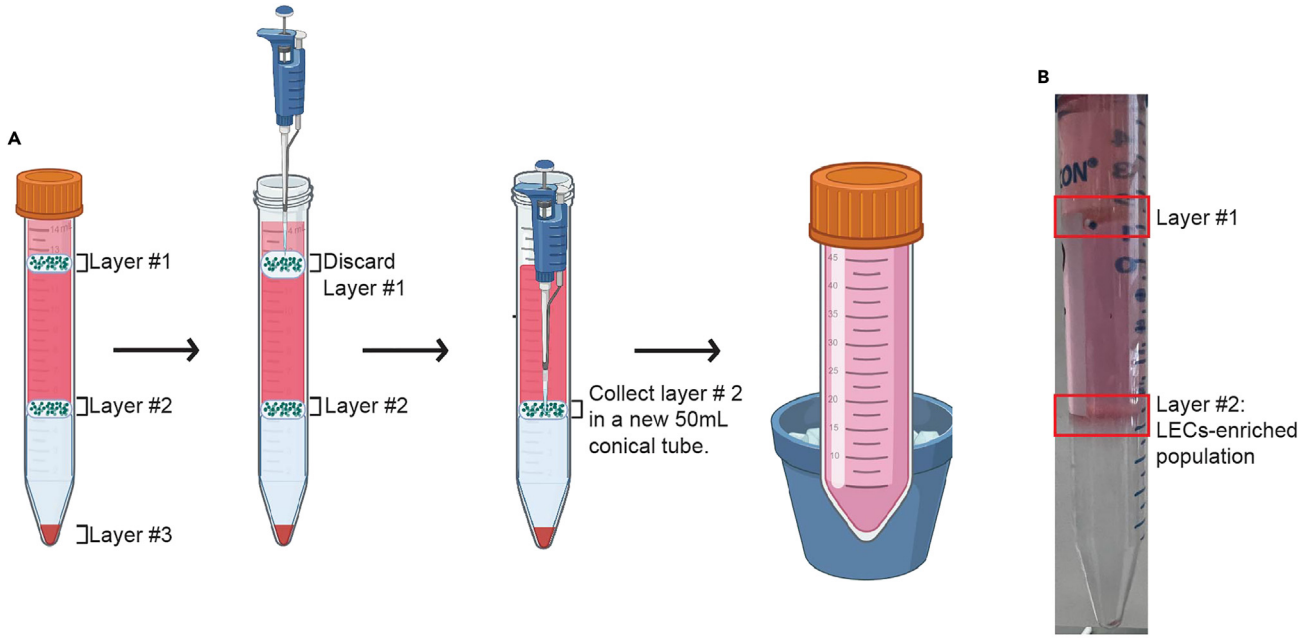

Figure 2. Schematic overview of identification and collection of LEC-enriched population upon Percoll gradient

(A) Layer #1 (top layer) contains hepatic stellate cell- enriched population and is initially removed and discarded. Layer #2 contains LEC-enriched NPCs, and is subsequently collected into a 50 mL conical tube for further centrifugation.

(B) Photograph of tube post- gradient centrifugation with clear delineation of LEC enrichment layer (Layer #2).

This centrifuge is set at RT (20–22°C) key resources table.

⚠ CRITICAL: If brake is not set to 0, the gradient will be disrupted, and the LEC-enriched population will not be easily identified.

10. After the gradient spin, the interface between the middle and bottom layer contains the LEC-enriched population depicted as Layer #2 (Figure 2). Carefully remove and discard Layer #1 (Figure 2).

Note: Layer #1 contains hepatic stellate cells-enriched population, which can be used for downstream analysis.

11. Collect Layer #2 into a new 50 mL conical tube on ice. This layer contains LECs, Kupffer cells and other immune cells. (Figure 2).
12. Add RPMI 1640 to fill up the 50 mL conical tube containing the collected LEC-enriched NPCs (Figure 2).
13. Spin at 550 × *g* for 10 min at 4°C.

Note: See troubleshooting 1.

14. Discard supernatant and minimize any disruption of the pellet.

Note: This pellet contains LEC-enriched NPCs, which also includes Kupffer cells and other immune cells. This step aims to wash the cells from the remaining Percoll.

15. Resuspend pellet in 1 mL of RPMI 1640 for counting. Cells can be counted using a hemocytometer or an automated cell counter.

Note: The average range for LEC-enriched NPCs isolated from an adult wild-type mouse liver is 1.5–2.5 million cells.

Liver endothelial cell (LEC) immunostaining and fluorescence-assisted cell sorting (FACS) protocol

Timing: Total time: 1–3 h (dependent on how many samples used for FACS)

Timing: 1 h (1 h for up to 10 experimental samples) (for immunostaining)

Timing: ~ 30 min per sample (FACS)

Prior to beginning FACS protocol, investigators should have pre-made FACS buffer ready to be used. The recipe for the FACS buffer is in materials and equipment section. After isolation of LEC-enriched population, LEC populations can be purified by performing FACS.

16. Pipette 1 million cells from each sample into one well of a 96-well U bottom plate. Create wells for unstained and single-color controls.

Note: Volume to pipette for 1 million cells will depend on total cell counting done on step #15. We recommend to resuspend 1 million cells in around 200 μL of FACS buffer.

Note: Keep cells and plate on ice. This protocol is optimized for 1 million cells per well.

17. Spin plate at 300 × *g* for 2 min at 4°C. Discard supernatant by flicking the plate.
18. Prepare Fc Block working solution by diluting 1:100 in FACS buffer. FACS buffer recipe is in materials and equipment section.
19. Resuspend cells in 25 μL of Fc Block working solution (step#18). Pipette up and down several times.
20. Incubate on ice for at least 10 min.

Note: Given the presence of Fc receptors on LECs, and to minimize unspecific antibody binding, we recommend incubating samples with Fc Block for 10 min. There is no significant difference between blocking for 10 min or up to an hour.

21. While cells are incubating on ice, prepare dilutions of single-color controls and antibody master mix.
    a. Single color controls: Each antibody will have its own single-color control. Cover diluted single-color control with aluminum foil as conjugated fluorescent antibodies are light-sensitive.
    b. Master mix: For experimental samples with multiple antibodies, dilute antibodies anti-mouse CD31 (1:100), and anti-mouse CD45 (1:100) in FACS buffer. For specific antibodies used please see key resources table. Cover tube with aluminum foil, as conjugated fluorescent antibodies are sensitive to light.

Note: Single color controls are utilized in FACS sorting to reveal the spectral overlap between different fluorophores and will allow the user to compensate for this overlap.

22. Add 150 μL of FACS buffer to each well to wash Fc Block.
23. Spin plate at 300 × *g* for 2 min at 4°C.
24. Discard supernatant by flicking the plate.
25. Resuspend pelleted cells in 25 μL of antibody mix. For unstained sample, and DAPI control sample suspend in 25 μL FACS buffer.

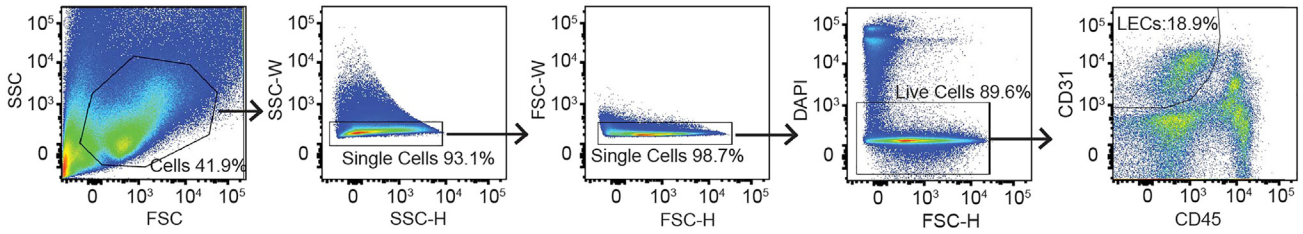

Figure 3. Flow cytometry gating strategy for identification of LECs

LEC-enriched NPCs are stained and gated on DAPI$^-$, CD45$^-$ and CD31$^+$ cells, identifying liver endothelial cells.

⚠ CRITICAL: To ensure proper staining, pipette up and down minimum 5 to 6 times to ensure cells are evenly stained.

26. Incubate for 20 min on ice in the dark (container should be covered with lid or aluminum foil).
27. Add 150 μL of FACS buffer to each well to wash the cells. Spin plate at 300 × g for 2 min at 4°C.
28. Discard supernatant by flicking the plate.
29. Resuspend cells in 200 μL FACS buffer, and transfer to a 5 mL polystyrene round-bottom tube.
30. Add 1 μL of DAPI (1:100 dilution) to DAPI-single color control well and experimental samples approximately 5 min prior to FACS.

Note: DAPI dye penetrates quickly into the membranes of dead or dying cells and therefore we recommend adding it only approximately 5 min prior to FACS.

Note: After FACS staining, cells are now ready to either be analyzed by flow cytometry or to be sorted for collection of pure LEC populations. LECs can be sorted on DAPI$^-$ (live cells), CD45$^-$ (hematopoietic marker), and CD31$^+$ (endothelial marker). Cell sorting was conducted on BD FACS Aria II platform and analysis of cells were conducted on FlowJo software. Alternative FACS sorters are Sony SH800, or BD FACS Aria III platform. Sorting instructions are beyond the scope of this protocol. A helpful guideline about how to operate FACS machines can be found in.[6] Please refer to Figure 3 for gating strategy for isolating DAPI$^-$CD45$^-$CD31$^+$ LECs FACS.

Note: A successful sorting experiment can yield on average ~190,000–300,000 DAPI$^-$, CD45$^-$, CD31$^+$ LECs from a sort of 20–30 min per tube. For FACS, a 100 μm nozzle is recommended to minimize LEC stress and death.

Note: See troubleshooting 2.

LEC-cytocentrifugation and immunostaining protocol

Timing: ~ 2 days

To perform immunofluorescence/confocal microscopy of sorted DAPI$^-$CD45$^-$CD31$^+$ LECs, cytocentrifugation was performed to mount cells onto slides. The purpose of cytocentrifugation is to place a monolayer of cells onto a circular area on a slide. This allows cells to be kept intact for further examination of cellular and subcellular proteins and structures.

Note: All cytocentrifugation buffers and recipes are outlined in materials and equipment section. We recommend making buffers on the day of the experiment and prior to starting this module of the protocol.

⚠ CRITICAL: Please see before you begin section regarding slide preparation prior to LEC-cytocentrifugation protocol.

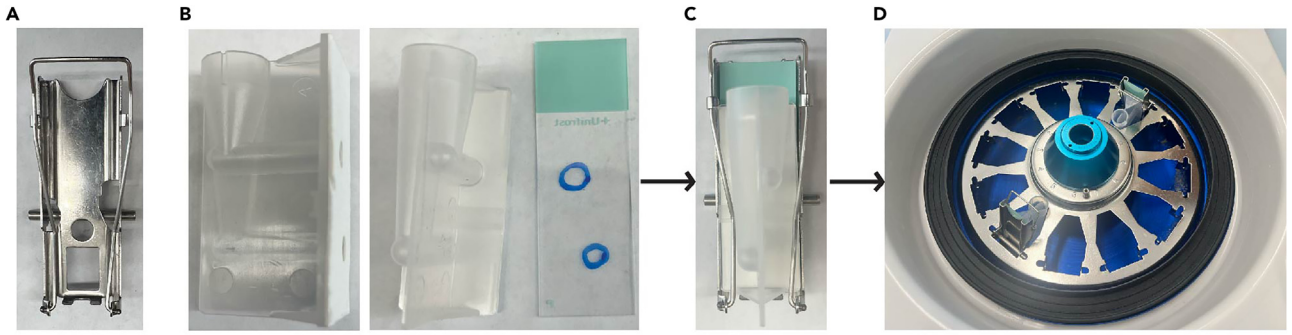

Figure 4. Preparation of a slide in cytospin holder for cytocentrifugation using Cytospin™ 4 centrifuge

(A) Photograph of Epredia™ Cytoclip™ Stainless-Steel Slide Clip (key resources table).

(B) Epredia™ Single Cytofunnel™ with White Filter Cards side and top views, next to microscopic slide with marked sharpie circles of where cells will be cytospin onto the slide. [Related to Step 33].

(C) Cytofunnel with white filter cards facing the microscope slide inserted in the slide clip.

(D) Placement of the cytofunnel with filter cards plus slide inserted in slide clip into the cytospin holder for running in a Cytospin™ 4 centrifuge.

31. Spin sorted LECs at 550 × *g* for 7 min at 4°C to pellet these cells of interest.

⚠ CRITICAL: The above speed and time has been optimized for sorted LECs. Higher speeds than the one listed above may lead to cell death or altered morphology.

32. Resuspend sorted LECs in adequate volume aiming for a final concentration of 50,000–70,000 cells per 100 μL.

⚠ CRITICAL: This recommended concentration was optimized for adequate LEC density on the slide.

33. Prior to loading the cells, circle bottom of slide with a sharpie or hydrophobic pen as the circle is where the cells will be found after they are spun onto the slide (see Figure 4).

⚠ CRITICAL: Drawing a circle where the cells will be placed on the slide will facilitate to quickly locate the cells for imaging studies (Figure 4).

Note: The following steps for centrifugation utilized the Thermo Scientific™ Cytospin™ 4 Cytocentrifuge.

34. Place cytology funnel (cytofunnel) against pre-coated slide (Figure 4).
35. Gently pipette the cell suspension of 100 μL into the cytofunnel after both the funnel and slide are secured with the cytoclip, and insert into metal cytocentrifugation apparatus (Figure 4D).
36. Centrifuge at 800 RPM for 3 min.
37. Carefully retrieve the slide from the cytocentrifuge by lifting the slide straight up. Avoid touching any surfaces while lifting slide from centrifuge.

⚠ CRITICAL: When retrieving the slide, lift slide straight up to avoid scraping the cells.

Note: See troubleshooting 3.

38. Place slides in a humidifier chamber and proceed with fixation, permeabilization, and staining.

Note: Humidifier chambers can be easily made from empty pipette boxes, by filling the box up to 1/3 with water, and slides are placed above where pipette tips were previously stored.

⚠ CRITICAL: The steps regarding fixation, permeabilization, and staining depend on antibodies of interest. Optimization may be required for each antibody of interest for the steps mentioned below.

Note: See troubleshooting 4.

39. Fix with 4% PFA (50–100 µL /slide) at RT (20–22°C) for 15 min.

Note: Depending on the antibodies used, alternative fixatives might be required.

40. Wash slides 3× with 1× PBS for 5 min in Coplin jars key resources table. After each 5-min wash, remove excess fluid.

Note: Paper towel or kim-wipe may be used to remove excess fluid; however, great care must take place to not disturb the sample/cells on the slide.

41. Quench the aldehydes with 50 mM ammonium chloride solution at RT (20–22°C) for 10 min.

Note: Quenching the aldehydes reduces fluorescence emission and quenches aldehydes, resulting in decreased background noise at the time of imaging.[7]

42. Wash slides 3× with 1× PBS for 5 min in Coplin jars. After each 5-min wash, remove excess fluid.
43. Permeabilize slides with 0.1% Triton in 1× PBS at RT (20–22°C) for 15 min.

Note: Investigators may choose to use alternative permeabilization solutions based on the fixative and antibodies used.

44. Wash slides 3× with 1× PBS for 5 min in Coplin jars. After each 5-min wash, remove excess fluid.
45. Block in 10% FBS in 1× PBS at RT (20–22°C) for 1 h.
46. Wash slides 3× with 1× PBS for 5 min in Coplin jars. After each 5-min wash, remove excess fluid.
47. Dilute primary antibody in blocking solution. Depending on antibody, consider incubation 8–12 h at 4°C or for 2 h at RT (20–22°C).
48. Wash slides 3× with 1× PBS for 5 min in Coplin jars. After each 5-min wash, remove excess fluid.
49. Dilute secondary antibody in blocking solution to desired concentration. For most antibodies, staining for 1 h at RT (20–22°C) with aluminum foil cover over humidifying chamber should be sufficient.
50. Wash slides 3× with 1× PBS for 5 min in Coplin jars. After each 5-min wash, remove excess fluid.

⚠ CRITICAL: During these washes place aluminum foil around Coplin jars as to not expose slides to light as the cells are now stained with fluorescent antibodies.

51. Mount slides by placing one drop of Fluoromount-G™ Mounting Medium with DAPI key resources table where the cells are located on the slide and cover it with a cover slip.

Note: Alternative mounting media may be utilized, such as VectaShield Antifade with DAPI.

⚠ CRITICAL: It is critical to avoid air bubbles near the cells when placing cover slips.

Note: Clear nail polish may be utilized to seal the edges of the coverslip to prevent displacement of the coverslip during imaging.

Note: Slides should be imaged as soon as the coverslips are dried. However, they are good for one week if kept at 4°C and protected from light. Samples were imaged on Leica TCS SP8 and examples of LEC images can be found in.[1]

EXPECTED OUTCOMES

The protocol described here provides a LEC-enriched NPC population from digested mouse liver(s), which will be an excellent starting material for flow cytometry and FACS. Successful completion of this protocol will yield on average 1.5–2.5 million cells of LEC-enriched NPCs, and ~190,000–300,000 sorted $DAPI^{-}CD45^{-}CD31^{+}$ LECs per adult wild-type mouse. Subsequently, sorted cells can be used for single-cell RNA sequencing analysis or imaging using confocal microscopy upon mounting of these cells on slides in accord to the optimized cytospin protocol outlined here.

LIMITATIONS

First, a limitation of this protocol is that the isolated LEC-enriched population contains several other liver cell populations, such as immune cells. To overcome this limitation and to attain a pure LEC population, we recommend FACS sorting of cells positive for endothelial cell marker CD31 and negative for hematopoietic marker CD45. Second, the isolation of LEC- enriched population yields on average 1.5–2.5 million cells of enriched LECs per mouse. After FACS, the number of sorted LECs isolated from an adult wild-type mouse liver varies between ~190,000 and 300,000 cells. The low cell number can be limiting for some downstream applications. To overcome this second limitation, pooling of cells from several mice may be required. Third, this protocol enriches for all LEC types. Notably, 80% of LECs are liver sinusoidal endothelial cells (LSECs). The field is still lacking specific antibodies and protocols which selectively identify and therefore distinguish LSECs, macrovascular venous ECs, such as portal vein and central vein ECs, macrovascular arterial ECs, and lymphatic ECs.[8,9]

TROUBLESHOOTING

Problem 1

No LEC layer present after gradient centrifugation [Steps 10 and 11].

Potential solution

There are two potential solutions to this issue: 1) ensure that the centrifuge break was set at "0"; and 2) prior to adding cells, ensure there was a clear layer between the 25% and 50% layer of the Percoll gradient. For further assistance on how to create the layers, please refer to Methods video S1.

Problem 2

During FACS analysis/sorting, no $CD45^{-}CD31^{+}$ LEC population was detected. [Note: FACS Staining]

Potential solution

Consider to conducting a trial on visualizing the cells on flow cytometry to ensure adequate cell-surface marker staining. In this instance, we would encourage investigators to ensure that they have their LEC-enriched NPCs in a single cell suspension, at ~1 million cells per well, and pipet 5 to 6 times as needed for proper staining.

Problem 3

When handling the slides from cytospin, the slide was scraped [Step 37].

Potential solution

Unfortunately, in this instance there is no easy fix, and we would recommend repeating the protocol as most likely the cells have been scraped off. To avoid scraping we recommend to lift the slide straight up in one single lifting motion.

Problem 4

At the time of visualizing the slides, no signal is detected. [expected outcomes]

Potential solution

The protocol listed above may need to be optimized for different antibodies. Nonetheless, positive control(s) should be utilized for microscopy, such as using antibodies for proteins that are expressed

in LECs: anti-CD31 antibody. In addition, doing a secondary antibody-alone control will ensure specificity of the primary antibody.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sílvia Vilarinho, (silvia.vilarinho@yale.edu).

Materials availability

This study did not generate new materials.

Data and code availability

This protocol did not generate new data or employ custom code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102480.

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AUTHOR CONTRIBUTIONS

S.C., K.D.F., and J.B. routinely performed all or some components of the protocol, and all authors participated in development, troubleshooting, and/or optimization of some or all portions of the protocol. S.C., K.D.F., and S.V. wrote the first draft, and all authors critically read and agreed with the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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