A perfusion-independent high-throughput method to isolate liver sinusoidal endothelial cells

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Summary

Liver sinusoidal endothelial cells (LSECs) critically regulate homeostatic liver function and liver pathogenesis. However, the isolation of LSECs remains a major technological bottleneck in studying molecular mechanisms governing LSEC functions. Current techniques to isolate LSECs, relying on perfusion-dependent liver digestion, are cumbersome with limited throughput. We here describe a perfusion-independent high-throughput procedure to isolate LSECs with high purity. Indifferently from previous perfusion-independent approaches, we coarsely chopped liver tissue into 5 mm diameter fragments and incubated them in the digestion mix for 30 minutes with intermittent mixing with a 5 ml pipette. This led to the safeguarding of LSEC integrity and yielded 8.5 ± 1.0 million LSECs per liver, which is comparable to previously reported yields for perfusion-dependent protocols for isolating LSECs. Combining magnetic and fluorescence-activated cell sorting, LSECs from different zones of the hepatic sinusoid can now be isolated in high numbers in less than 2 hours for downstream applications including proteomics. These technical advancements reduce post-mortem changes in the LSEC state and aid in reliable investigation of LSEC functions.

Introduction

The liver is the metabolic powerhouse of our body. While hepatocytes serve as key functional units of the liver driving major metabolic processes, LSECs have emerged as critical modulators of hepatic function in the past decade[1]. LSECs are anatomically unique as they lack an organized basement membrane and harbor fenestrae to facilitate macromolecular transport to and from hepatocytes[2,3]. Further, LSECs secrete angiocrine signals to orchestrate hepatocyte functional zonation. In line, genetic ablation of Rspondin 3, a canonical Wnt activator primarily derived from LSECs, disrupted the metabolic zonation of hepatocytes along a liver sinusoid in adult mice[4]. Likewise, angiocrine signals WNT2 and WNT9B regulate the expansion of AXIN2+ hepatocytes to maintain hepatocyte turnover under homeostatic conditions[5,6]. Additionally, recent evidence suggests an age-related decline in LSEC function predisposes the liver towards a higher risk of developing steatosis[7]. Together, these studies highlight the gatekeeper functions of LSECs in maintaining liver health. Yet, these new findings have raised more questions about the functionality of LSECs and how can angiocrine signals be exploited as potential therapeutic targets to ameliorate liver pathologies.

Attributed to the discontinuous structure of sinusoidal vasculature, the isolation of healthy LSECs has been a challenging bottleneck for pursuing *ex vivo* functional analysis. Numerous LSEC isolation procedures have been described with the majority of those employing perfusion of the liver with tissue digestion buffer followed by a combination of magnetic and FACS-based enrichment of LSECs[8]. While perfusing the liver with digestion buffer allows isolating LSEC, it poses three major challenges – i) perfusing per se applies mechanical shear that potentially induces technical alterations in LSECs' molecular circuits, ii) it strongly limits the throughput as a scientist has to perfuse one mouse at a time, and iii) it requires additional equipment and training before a scientist can insert a cannula to perfuse the liver. LSEC isolation becomes even more challenging in mice with liver pathologies including fibrosis and cancer. The accumulation of excessive extracellular matrix requires longer perfusion times with digestion mix and negatively affects the yield of LSEC isolation.

To tackle these challenges and isolate LSECs in high purity, the present study describes a perfusion-independent protocol for LSEC isolation. By opting for a perfusion-independent procedure, we circumvented the need for specialized equipment and training as well as substantially improved the scale of LSEC isolation. Focusing on the first step of the procedure – the digestion of the liver tissue, we found that coarse (approximately 5 mm in diameter), instead of fine, chopping of the liver tissue safeguarded LSECs and enabled a yield that is comparable to previously reported yields from perfusion-dependent isolation protocols[8]. Further characterization revealed that our isolation procedure led to a uniform capturing of LSEC from all zones of the hepatic sinusoid, allowing for zone-specific isolations. Together, the newly established LSEC isolation protocol will serve as an important tool for enabling life scientists to study liver vasculature with minimal prior knowledge and training and propel further research efforts in understanding LSEC biology.

Results

Step-by-step procedure for isolating liver sinusoidal endothelial cells

The whole procedure can be classified into three major steps (**Fig. 1**):

(i) Liver tissue processing to obtain single-cell suspension (~45 mins)

(ii) Enrichment of non-parenchymal cells (~25 mins)

(iii) Isolation of high-purity LSECs (~40-60 mins)

⚠ The time required for the isolation depends on the number of mice processed in parallel as well as the total cells sorted via FACS. Additionally, it would rely on FACS settings used for cell sorting. FACS sorting with a 70 µm nozzle allows for high-speed analysis with up to 15,000 events processed per second, while a 100 µm nozzle would allow cell sorting with a speed of up to 10,000 events per second. The selection of FACS settings would largely depend on the downstream application of isolated LSECs. The time indicated here is based on processing four mice in parallel and isolating 1 mio LSECs for gene expression analysis.

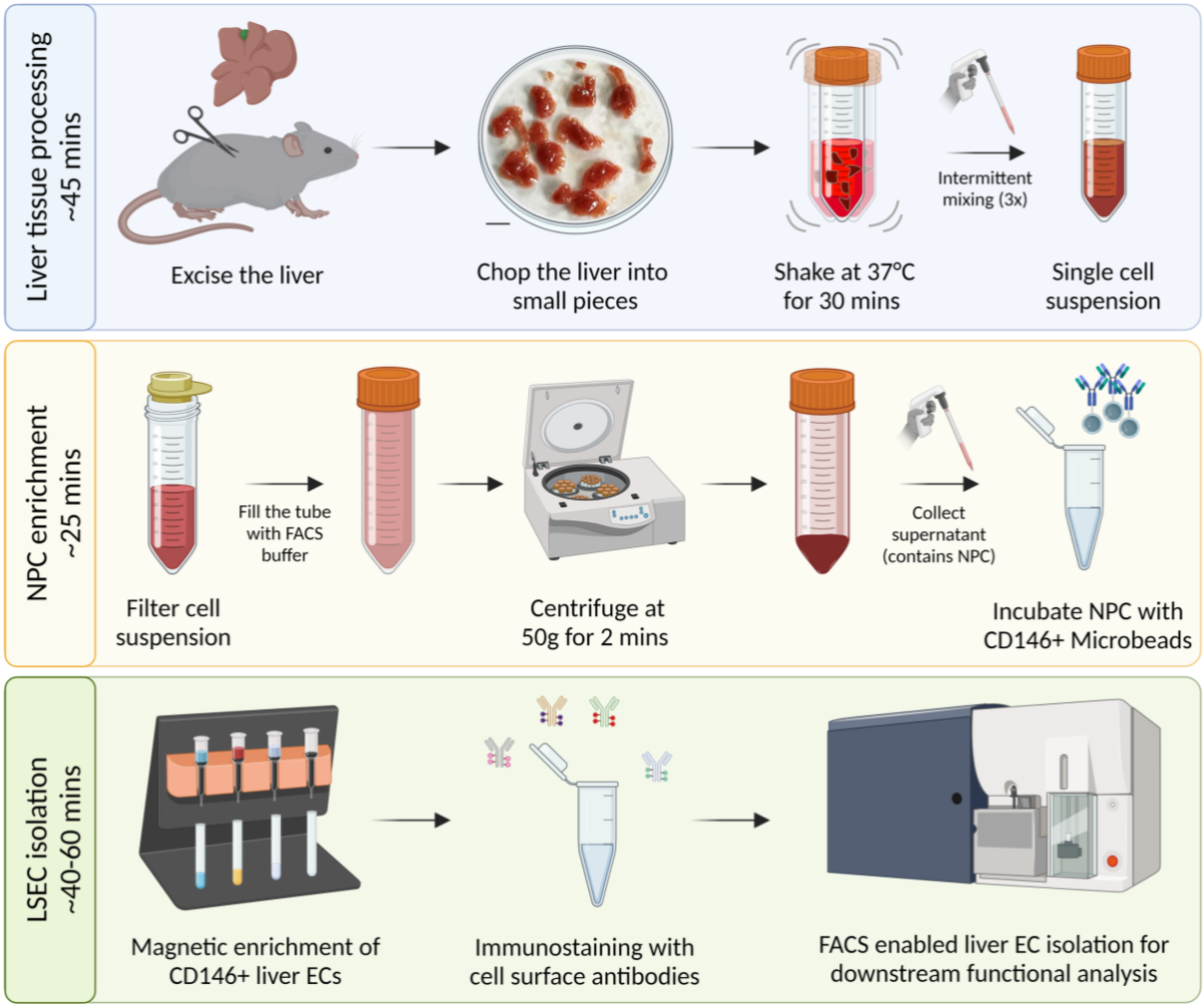

Figure 1. Step-by-step schematic depiction of the LSEC isolation protocol. Scale bar = 5 mm.

Step I - Liver tissue processing

1. Before sacrificing the mice, freshly prepare the digestion buffer and store it at 4°C till use.
   → 5 ml of digestion buffer is required to digest one adult mouse liver weighing ~1 g.
2. Excise the liver and transfer it into a 6 cm petri dish containing phosphate buffered saline (PBS) solution.
3. Swiftly wash the liver in the PBS solution and transfer it to the dry lid for chopping.
4. Use curved scissors to coarsely chop the liver into small pieces of around 5 mm in size.

△ Refer to the image of coarsely chopped liver fragments in **figure 1**. Too fine chopping of the liver tissue will result in a lower yield of LSECs.

5. Carefully transfer the liver pieces into a 15 ml conical tube and add 5 ml of the digestion buffer.
6. Incubate the samples at 37°C in a pre-heated incubation shaker at 180 rotations per minute for 30 minutes.
7. During the digestion, perform intermittent mixing of samples with a 5 ml serological pipette every 10 minutes.

△ This protocol was established using 5 ml serological pipettes with an opening mouth diameter of roughly 2.5 mm. Using a serological pipette with a small opening diameter might negatively affect the LSEC yield. We advise against using pipette tip or syringe-needle combination for mixing due to high shear.

△ After 30 minutes of digestion, there might still be remaining liver pieces, yet proceed with the next step. Elongating the digestion step might negatively affect the health of isolated LSECs.

Step II – Enrichment of non-parenchymal cells (NPCs)

8. Transfer the digested cell suspension with a 5 ml serological pipette into a 50 ml conical tube through a 100 µm cell strainer.
9. In the case of a blocked cell strainer, use the plunger of a 5 ml syringe to press the remaining liver pieces through the cell strainer.
10. Wash the plunger and the cell strainer with 5 ml of FACS buffer.
11. Fill the conical tube with FACS buffer up to the mark of 50 ml.
12. Centrifuge the tube at 50g, 4°C for 2 minutes.
13. The pellet primarily contains hepatocytes, while the supernatant is enriched for NPCs. Carefully collect the supernatant with a 25 ml serological pipette and transfer it into a new 50 ml conical tube.
14. Centrifuge the tube at 450g, 4°C for 5 minutes and discard the supernatant.
15. The resulting pellet consists of NPCs. Resuspend the pellet in 200 µl of CD146-selection buffer and incubate at 4°C for 20 minutes.
16. During the incubation time, prepare for the magnetic enrichment.
    a. Place the LS columns in the QuadroMACS™ magnetic separator rack.
    b. Equilibrate each column by adding 5 ml of FACS buffer.

Step III – Isolation of high-purity LSECs

17. Wash the cells with 5 ml of FACS buffer and centrifuge at 450g, 4°C for 5 minutes. Subsequently, discard the supernatant.
18. Resuspend the cell pellet in 1 ml of FACS buffer and apply it to an equilibrated LS column through a 100 µm cell strainer.
19. Wash the filter with 1 ml of FACS buffer.
20. Allow the NPC suspension to pass through the LS column.
21. Wash the LS column by applying 9 ml of FACS buffer.
22. Remove the LS column from the magnet and place it onto a fresh 15 ml conical tube.
23. Add 5 ml of FACS buffer to the LS column. Elute the cells by purging the column with the supplied plunger.
24. Centrifuge the elute at 450g, 4°C for 5 minutes and discard the supernatant.
25. Resuspend the pellet in 200 µl of the staining mix and transfer it to a 1.5 ml tube.
26. Incubate the cell suspension at 4°C for 20 minutes, protected from the light.
27. Wash the cells with 1 ml of FACS buffer and centrifuge at 450g, 4°C for 5 minutes.
28. Carefully remove the supernatant and resuspend the pellet in 200 µl of FACS buffer.
29. Transfer the sample into a FACS tube through the 35 µm cell strainer in the lid.
30. Store filtered samples in FACS tubes on ice and proceed with FACS-based cell sorting.
    a. Before analyzing a sample, add dead cell exclusion dye (FxCycle Violet stain, 1 µl per sample).
    b. Sort viable CD31+CD146+ cells using the FACS gating strategy shown in **figure 2A**.
    c. Reanalyze a small portion of FACS-sorted LSECs to evaluate the purity of sorted cells.
    d. Depending on the downstream application, process the FACS-sorted LSECs.

⚠ We recommend always plotting a histogram of CD117/Kit as a control for successful isolation of LSECs from all regions of a hepatic sinusoid. A successful LSEC isolation will result in a plateau-like distribution in the histogram for CD117 staining. Refer to the **figure 3A**.

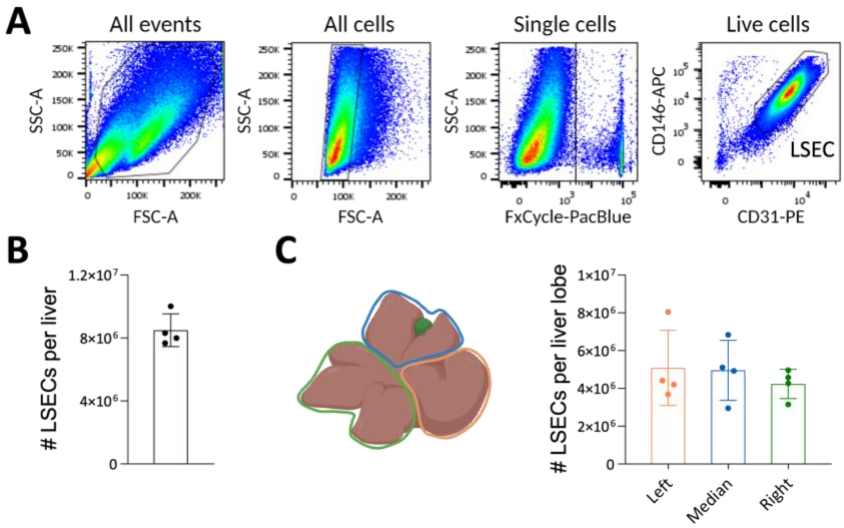

Figure 2. (**A**) Sequential gating strategy to identify and sort CD31+CD146+ LSECs. (**B**) The dot plot shows the total count of LSECs calculated per liver sample [mean ± SD, n = 4 mice]. (**C, left**) The liver was divided into three parts as shown in the picture. (**C, right**) Each part was processed to isolate LSECs. The dot plot shows the total count of LSECs isolated from each part of the liver [mean ± SD, n = 4 mice].

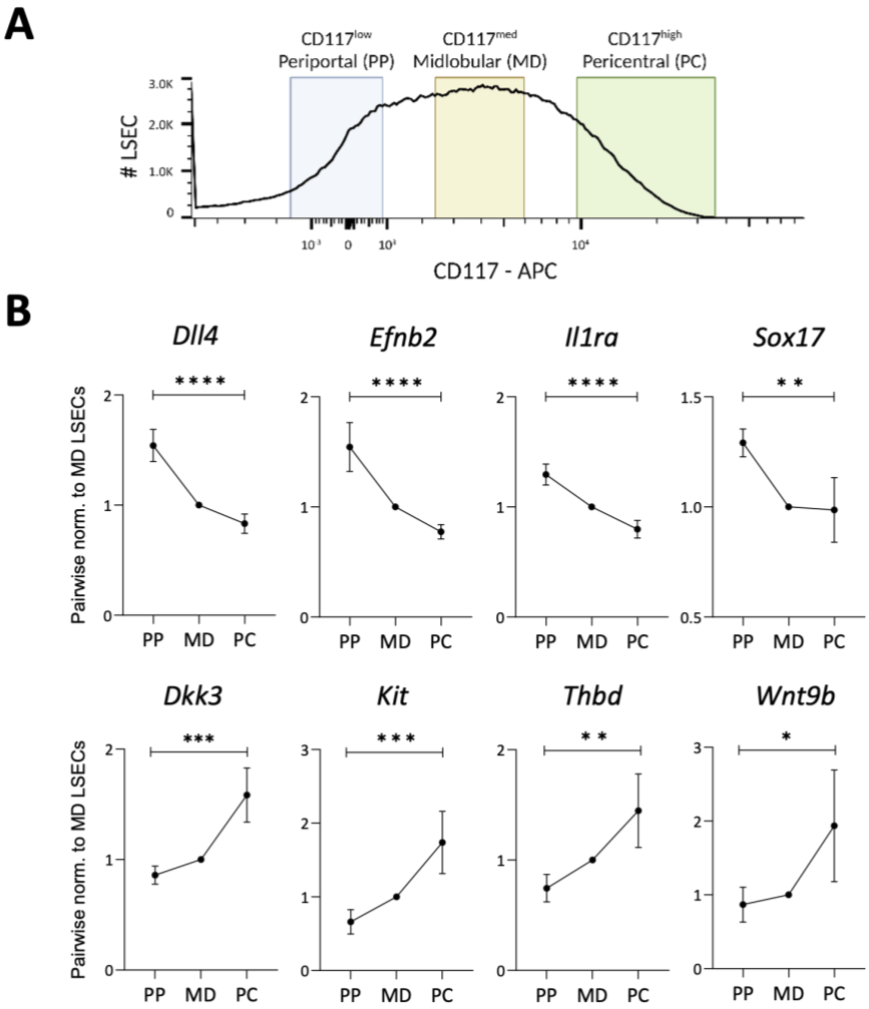

Figure 3. (**A**) CD117-based stratification to undertake hepatic zone-specific LSEC isolation. (**B**) Gene expression analysis for known periportal (top) and pericentral (bottom) zone-specific LSEC genes [mean ± SD, n = 4 mice]. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (one-way *ANOVA* test).

Application of the established method for isolating LSECs

Employing the above-described perfusion-independent procedure, we reliably isolated an average of 8.5 X 10^6 LSECs from the liver tissue of 10-12 weeks old C57BL/6NRj mouse (**Fig. 2B**). Next, we compared whether our protocol could uniformly isolate LSECs from all lobes of the liver tissue. To this end, the liver tissue was segregated into three parts – left lobe, median lobes and right lobes, each part constituting nearly one-third of the total liver mass (**Fig. 2C**). Thereafter, LSECs were isolated from each of these liver parts using our protocol. We could successfully isolate LSECS from all three parts of the liver, with nearly 4 X 10^6 LSECs from each of the segregated liver tissues (**Fig. 2C**). Surprisingly, these data hint towards a yield of approximately 12 X 10^6 LSECs from the whole liver, potentially suggesting that our initial isolation with the whole liver is rather an underestimation of the total number of LSECs that could be recovered with the newly established protocol. Perhaps the CD146 magnetic beads used for the initial enrichment of LSECs were saturated while using NPCs from the whole liver tissue.

Considering LSECs constitute a rather heterogeneous cell population with their function displaying a zonation along the hepatic sinusoid, we next wanted to investigate whether our protocol is capturing LSECs from all different regions of the hepatic sinusoid. To achieve this, CD31+CD146+ LSECs were sequentially analyzed for the surface abundance of CD117 (*Kit*). In line with previous publication[9], CD117 staining on LSECs manifested a plateau-like shape (**Fig. 3A**), suggesting that our protocol was able to capture LSECs from all zones of the hepatic sinusoid. To verify the spatial origin of LSECs, we stratified and isolated LSECs based on the mean fluorescence intensity of CD117 – periportal (PP, $CD117^{low}$), midlobular (MD, $CD117^{med}$), and pericentral (PC, $CD117^{high}$). Gene expression analyses corroborated the origin of sorted LSECs with periportal LSECs enriched for *Dll4, Efnb2, Il1a,* and *Sox17* whereas pericentral LSECs enriched for *Dkk3, Kit, Thbd,* and *Wnt9b* (**Fig. 3B**). Together, these data substantiate that our protocol can successfully capture LSECs from all zones of the hepatic sinusoid and present a strategy for isolating zone-specific LSECs for downstream functional analysis.

Materials and Methods

Mice

C57BL/6NRj mice were purchased from Janvier Laboratories. 10- to 12-weeks old mice were used in this study unless otherwise indicated. All mice were housed on a 12-hour light/12-hour dark cycle with free access to food and drinking water in specific pathogen-free animal facilities. All animal experiments were per the governmental (Regierungspräsidium Karlsruhe, Germany) and institutional (Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany) guidelines for the care and ethical use of laboratory animals.

Liver tissue digestion and subsequent enrichment of LSECs

C57BL/6NRj mice were euthanized by cervical dislocation, and liver tissues were excised for isolating LSECs. The step-by-step method for isolating LSECs is described in the results section. Here, we describe all materials used and the composition of various buffers.

Coarsely chopped liver fragments from each mouse were digested in 5 ml liberase-based digestion mix (5 ml of DMEM [Thermo Fisher Scientific, Cat #61965059] + 1 mg Liberase™ Research Grade [Roche, Cat #5401127001] + 0.1 μg DNAse I [Sigma-Aldrich, Cat #10104159001]).

For magnetic enrichment of LSECs, NPCs from each liver were incubated in 200 μl of CD146-selection mix (175 μl FACS buffer + 25 μl of CD146 microbeads [Milentyi Biotec, Cat #130-092-007]). FACS buffer used for preparing the selection mix and washing the LS columns [Milentyi Biotec, Cat #130-042-401] contains 3% FCS in PBS solution.

Immunofluorescence staining and flow cytometry

Subsequent to magnetic selection, enriched LSECs were stained for various surface markers [CD31 (MEC 13.3), CD146 (ME9F1), and CD117 (2B8)]. All antibodies were purchased from BioLegend. Dead cells were excluded by FxCycle Violet staining [Thermo Fisher Scientific, Cat #F10347]. Stained cells were analyzed on either BD FACS Melody™ or BD Aria II cell sorting platforms. Total LSECs per liver were calculated using CountBright plus counting beads [Thermo Fisher Scientific, Cat #C36995]. Frequencies of individual cell populations were quantified using FlowJo software.

Gene expression analysis

Total RNA from FACS-sorted LSECs was isolated using PicoPure™ RNA isolation kit [Thermo Fisher Scientific, Cat #KIT0204]. Total RNA was transcribed into complementary DNA using a QuantiTect reverse transcription kit [Qiagen, Cat #205313]. Quantitative PCRs (qPCRs) were performed with TaqMan fast advanced master mix [Thermo Fisher Scientific, Cat #4444557]. TaqMan primers (*Actb*, Mm00607939_s1; *Dll4*, Mm00444619_m1; *Dkk3*, Mm00443800_m1;

Efnb2, Mm00438670_m1; *Il1a*, Mm00439620_m1; *Kit*, Mm00445212_m1; *Sox17*, Mm00488363_m1; *Thbd*, Mm00437014_s1; and *Wnt9b*, Mm00457102_m1) were ordered from Thermo Fisher Scientific. Gene expression was calculated based on the ΔΔCt relative quantification method. mRNA abundances were normalized to *Actb* expression as indicated.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10 (GraphPad Software). Data are expressed as means ± SD. Used statistical tests are indicated in corresponding figure legends. A P value of less than 0.05 was considered statistically significant.

Discussion

Revisiting every step of the liver sinusoidal endothelial cell isolation procedure, the present study established a perfusion-independent high-throughput method to isolate ultrapure LSECs from the mouse liver. Combining our newly established liver digestion procedure with magnetic and FACS-based purification steps, ultrapure (>98%) LSECs were retrieved in a yield comparable to existing perfusion-dependent methods. Circumventing the need for perfusion means that the current protocol (i) offers scalability, allowing researchers to perform parallel LSEC isolation from relatively large cohorts of mice, (ii) bypasses the need for substantial hands-on training to insert a cannula for perfusing the liver, and (iii) minimizes the alterations in molecular circuits in isolated LSECs driven by perfusion-induced shear stress.

Tissue processing: Focusing particularly on the liver digestion step, we optimized the protocol to maximize tissue digestion along with safeguarding the health of LSECs. Surprisingly, chopping the liver tissue into coarse pieces (around 5 mm in diameter) instead of fine chopping was important to prevent the loss of LSECs and obtain uniform results. Furthermore, intermittent mixing with a 5 ml serological pipette during tissue digestion, instead of a pipette or a combination of needle-syringe, led to a high recovery of healthy LSECs. These two alterations in the processing of liver tissues yielded a nearly 5-fold higher number of LSECs as compared to previous perfusion-independent isolation approaches[10].

Digestion buffer: In the present study, we used research-grade liberase enzyme for digesting the liver tissue. This was primarily driven by the user experience as liberase offered very high consistency in terms of tissue digestion among various lots. In contrast, the efficacy of collagenase and dispase varied depending on their lots, often requiring different concentrations of the enzymes to be used for tissue processing. Yet, all collagenase-based enzymatic digestion mixtures should work with the current protocol. We have recommended the liberase-based digestion buffer largely due to our own experiences.

Isolating other hepatic cells: While the present study optimized the isolation of LSECs from the liver tissue, we can envision a rather broad application of the current protocol for the isolation of various hepatic cells. For example, in the step with a 50g centrifuge during NPC enrichment, the cell pellet largely consists of hepatocytes which can be directly used for various hepatocyte-based assays. Given that hepatocytes manifest a functional zonation, the isolated hepatocytes can be FACS sorted into zone-specific hepatocytes[11]. Likewise, the total NPC fraction can be used to isolate tissue infiltrating immune cells and hepatic stellate cells. While immune cells can be purified employing either specific cell surface markers-based FACS strategy or selective adhesion for Kupffer cells[12,13], NPCs can be processed through a Nycodenz gradient to enrich stellate cells as described previously[14]. Together, by achieving a uniform digestion of the liver tissue in a perfusion-independent manner, we believe that we overcame the critical bottleneck, and our established protocol can be applied to potentially isolate all hepatic cell types.

Zone-specific LSEC isolation: Addressing the functional heterogeneity in LSECs across the hepatic sinusoid, we tested the surface presentation of CD117 (Kit) among CD31+CD146+

LSECs. Indeed, our isolation protocol successfully captured LSECs from all regions of the hepatic sinusoid as demonstrated by the expected trend of the expression of periportal and pericentral specific genes. These periportal and pericentral marker genes were selected based on published single-cell RNA sequencing study of the liver vasculature[15]. Building on these findings, our method can be employed to isolate hepatic zone-specific LSECs in high quantities. In our analysis, we stratified LSECs based on the mean fluorescence intensity of CD117. Based on this parameter, the lowest 15% of LSECs were sorted as $CD117^{low}$, and the highest 15% of LSECs were categorized as $CD117^{high}$. This would imply that quantitatively, we could isolate around 1.25 million LSECs each from the periportal and pericentral regions of the hepatic sinusoid. The high quantity of LSECs isolated from different hepatic zones will enable future studies to undertake functional analyses such as proteomics and even observe post-translational modifications in zonated LSECs.

In conclusion, the method described in the present study should facilitate high-throughput isolation of ultrapure LSECs. The perfusion-independent nature of the procedure should ensure high reproducibility of experiments and favor a broad application of the isolation method in laboratories that classically have little liver expertise. The current experiments demonstrate our continuous efforts to simplify the isolation protocol for different hepatic cells to better understand cellular and molecular crosstalk within the liver microenvironment.

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Author contributions: ABEG, YM, TB, and MS conceived and designed the study. ABEG, YM, TB, MW, SK, SG, PSRK and MS performed experiments. FP and MH provided technical support. ABEG, YM, TB, and MS analyzed and interpreted data. MS supervised the project. ABEG, YM, and MS wrote the manuscript. All authors discussed the results and commented on the manuscript.

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