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Corresponding Author:	Samar Ibrahim Mayo Clinic Rochester: Mayo Clinic Minnesota Rochester, MN UNITED STATES
Corresponding Author's Institution:	Mayo Clinic Rochester: Mayo Clinic Minnesota
Corresponding Author E-Mail:	Ibrahim.Samar@mayo.edu
Order of Authors:	Samar Ibrahim
	Qianqian Guo
	Kunimaro Furuta
	Ahmed Aly
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TITLE:

Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

AUTHORS AND AFFILIATIONS:

Qianqian Guo[1], Kunimaro Furuta[1], Ahmed Aly[1], Samar H. Ibrahim[1,2]

[1]Division of Gastroenterology & Hepatology, Mayo Clinic, Rochester, Minnesota, USA
[2]Division of Pediatric Gastroenterology, Mayo Clinic, Rochester, Minnesota, USA

Email addresses of co-authors:
Qianqian Guo (guo.qianqian@mayo.edu)
Kunimaro Furuta (furuta.kunimaro@mayo.edu)
Ahmed Aly (aly.ahmed@mayo.edu)
Samar H. Ibrahim (ibrahim.samar@mayo.edu)

Corresponding author:
Samar H. Ibrahim (ibrahim.samar@mayo.edu)

KEYWORDS:

liver sinusoidal endothelial cells (LSECs), collagenase perfusion, magnetic beads positive selection, flow cytometry, immunohistochemistry, scanning electron microscopy

SUMMARY:

Here we outline and demonstrate a protocol for primary mouse liver sinusoidal endothelial cell (LSEC) isolation. The protocol is based on liver collagenase perfusion, nonparenchymal cell purification by low-speed centrifugation, and CD146 magnetic bead selection. We also phenotype and characterize these isolated LSECs using flow cytometry and scanning electron microscopy.

ABSTRACT:

Liver sinusoidal endothelial cells (LSECs) are specialized endothelial cells located at the interface between the circulation and the liver parenchyma. LSECs have a distinct morphology characterized by the presence of fenestrae and the absence of basement membrane. LSECs play enthential roles in many pathological disorders in the liver, including metabolic dysregulation, inflammation, fibrosis, angiogenesis, and carcinogenesis. However, little has been published about the isolation and characterization of the LSECs. Here, this protocol discusses the isolation of LSEC from both healthy and nonalcoholic fatty liver disease (NAFLD) mice. The protocol is based on collagenase perfusion of the mouse liver and magnetic beads positive selection of nonparenchymal cells to purify LSECs. This study characterizes LSECs using specific markers by flow cytometry and identifies the characteristic phenotypic features by scanning electron microscopy. LSECs isolated following this protocol can be used for functional studies, including adhesion and permeability assays, as well as downstream studies for a particular pathway of interest. In addition, these LSECs can be pooled or used individually, allowing multi-omics data generation including RNA-seq bulk or single cell, proteomic or phospho-proteomics, and Assay

for Transposase-Accessible Chromatin using sequencing (ATAC-seq), among others. This protocol will be useful for investigators studying LSECs' communication with other liver cells in health and disease and allow an in-depth understanding of the role of LSECs in the pathogenic mechanisms of acute and chronic liver injury.

INTRODUCTION:

Liver sinusoidal endothelial cells (LSECs) line the hepatic sinusoid walls and are the most abundant nonparenchymal cells in the liver[1]. LSECs are distinguished from other capillary endothelial cells elsewhere in the body by the presence of fenestrae and the lack of a classical basement membrane or a diaphragm[2,3]. Hence, the LSECs possess distinctive phenotypic and structural characteristics that enhance their permeability and endocytic capacity to eliminate a variety of circulating macromolecules, including lipids and lipoproteins. LSECs play a pivotal role in the crosstalk between parenchymal and nonparenchymal cells, such as stellate cells and immune cells. LSECs are key in maintaining liver homeostasis by keeping the stellate cells and Kupffer cells in a quiescent status[4]. LSECs modulate the composition of hepatic immune cells populations by mediating adhesion and trans-endothelial migration of circulating leukocytes[5,6]. During acute and chronic liver injury[7], including ischemia-reperfusion injury (IRI)[8], nonalcoholic steatohepatitis (NASH)[9], and hepatocellular carcinoma (HCC), LSECs undergo phenotypic changes known as capillarization characterized by defenestration and formation of basement membrane[10]. These phenotypic changes in LSECs are associated with LSECs dysfunction and the acquisition of pro-thrombotic, pro-inflammatory, and profibrogenic properties.

Several methods for the isolation of LSECs from mouse liver have been developed[11]. Some techniques depend on separating nonparenchymal and parenchymal cells followed by density gradient centrifugation to purify the LSECs from nonparenchymal fractions. The limitation of this method is the presence of contaminating macrophages in the final steps of LSECs isolation, which could affect the purity of the isolated LSECs[12]. This protocol is based on collagenase perfusion of the mouse liver and $CD146^+$ magnetic beads positive selection of nonparenchymal cells to purify LSECs. LSECs isolated using this method show high purity and preserved morphology and viability. These LSECs are optimum for functional studies, including permeability and adhesion assay, as well as downstream studies for pathways of interest. Moreover, with the growing interest in generating big datasets in both clinical research and discovery science, these high-quality LSECs isolated from both healthy and diseased livers with nonalcoholic steatohepatitis (NASH) or other conditions can be pooled or used individually, allowing multi-omics data generation and comparison between health and disease[13,14]. In addition, the isolated LSECs can be employed to develop two-dimensional as well as three-dimensional in vitro models like organoids to decipher the activated signaling pathway in LSECs and their intercellular communication with other liver cells under different noxious stimuli and in response to various therapeutic interventions.

PROTOCOL:

Animal protocols were conducted as approved by the Institutional Animal Care and Use Committee (IACUC) of Mayo Clinic. Eight-week-old C57BL/6J male mice were purchased from

Jackson Laboratory. Mice were housed in a temperature-controlled 12:12-h light-dark cycle facility with free access to diet.

1. Preparation of collagen-coated culture dish or plate

1.1. To make 50 mL of 0.02 mol/L acetic acid, add 0.6 mL of glacial acetic acid to 49.4 mL of H_2O.

1.2. Make 50 µg/mL collagen type I in 0.02 mol/L acetic acid. Dilution depends on the concentration of the lot.

1.3. Coat 10 cm culture dishes with 3 mL of collagen solution. Incubate at room temperature (RT) for 1 h.

NOTE: When using a different dish or plate for culture, the volume of coating solution needs to be adjusted based on culture are, generally use 6–10 µg/cm^2.

1.4. Remove excess fluid from the coated surface, and wash with Phosphate-buffered saline (PBS) 3 times. Allow the dishes to air dry.

1.5. If the collagen solution is not sterile, sterilize the collagen-coated dishes by exposure to ultraviolet (UV) light for 10 min in a sterile tissue culture hood.

2. Equipment setup

2.1. Set up the heated and humidified recirculating perfusion apparatus as shown in **Figure 1B**.

2.2. Rinse the perfusion system using 10% bleach for 5 min followed by sterile water for another 10 min.

2.3. Drain the rinsing liquid as much as possible before perfusing the collagenase solution.

2.4. Infuse collagenase solution through the perfusion system (as shown in **Figure 1B**) to prewarm it at 37 °C. Set the pump rate at speed 1 as shown on the speed control dial (equal to 40 mL/min).

2.5. Keep this speed the same throughout the whole procedure. Reuse the collagenase solution run in a closed circuit during the day of the LSEC isolation.

NOTE: The pump speed is fixed at 1 to avoid mechanical pressure that could perturb LSECs biological condition.

3. Surgical procedure

3.1. Weigh the mouse.

3.2. Inject 90 mg/kg body weight of ketamine and 10 mg/kg bodyweight of xylazine intraperitoneally (IP).

3.3. Once the mouse is nonreactive to painful stimuli, secure the mouse to the surgical surface, as shown in **Figure 1B**.

3.4. Spray the mouse abdomen with 70% ethanol. Using surgical scissors, make an incision of ~5 cm long starting from the lower part of the abdomen up to the xiphoid process.

3.5. Next, make two lateral cuts using small iris scissors on each side of the abdomen to fully expose the abdominal organs.

3.6. Place the sheath of the intravenous (IV) catheter under the animal's back to lift and level the abdomen.

3.7. Using a regular curved dressing forceps, gently pull the intestines and the stomach off to the left of the animal.

3.8. Place a 5-0 surgical suture under the inferior vena cava (IVC) just below the exposed left kidney. Tie a loose hitch in the suture.

3.9. Place another 5-0 surgical suture around the hepatic portal vein (PV) just above the splenic vein branching point off the hepatic PV. Tie a loose hitch in the suture.

3.10. Using the PV suture as tension, insert the 20 G IV catheter in the hepatic PV 1 cm below where it branches to the right and left hepatic PV.

3.11. Slide the catheter up the vein but keep it below the branching area. Allow the blood to travel down the catheter until it begins to drip out.

3.12. With some of the Buffer A (**Table 1**) in an intravenous (IV) bottle positioned above the surgical area, use an IV line, and attach it to the catheter. Flush the liver with this solution while avoiding air entry into the system.

3.13. Tie off the suture around the inferior vena cava below the kidney. This will allow the liver to retro perfuse.

3.14. Cut the IVC below the suture to allow the animal to bleed out.

NOTE: This step must be performed quickly to avoid congestion of the liver.

3.15. Secure the IV catheter with the PV suture.

3.16. Once perfusing, cut away the stomach, intestines, spleen, and other entrails attached to the liver.

3.17. Cut away the diaphragm and the major vessels from the thoracic cavity. Remove the liver from the animal and place it on the perfusion tray.

3.18. Carefully remove the IV line and hook up the collagenase solution in the recirculating chamber.

NOTE: Steps 3.18–3.20 need to be done within 5 min, so the liver will not be perfused too long with Buffer A. Be very careful not to allow any air bubbles into the liver.

3.19. Allow the liver to perfuse until the capsule becomes mottled and appears mushy (10–15 min or more. Period varies depending on the different lots of collagenase).

3.20. Once digested, remove the liver from the chamber and place it in a 10 cm Petri dish with about 20 mL of serum-free Dulbecco's Modified Eagle Medium (DMEM).

3.21. Gently pick apart the liver with a couple of pipette tips and discard the biliary tree.

3.22. Filter the liver suspension through a 70 µm cell strainer into a 50 mL conical tube.

3.23. Centrifuge the cell suspension at 50 x *g* for 2 min at RT. Collect the supernatant containing nonparenchymal hepatic cells.

NOTE: Hepatocytes are precipitated in the pellet, which can be further purified using gradient centrifugation as previously described[15].

4. Separation of nonparenchymal hepatic cells and LSECs purification

NOTE: Purify the $CD146^+$ LSECs using the immunomagnetic beads, following the manufacture's instructions.

4.1. Centrifuge the supernatant at 300 x *g* for 5 min at 4 °C.

4.2. Collect the cell pellet and resuspend in 1 mL of isolation buffer (**Table 1**), determine the cell number using an automatic cell counter following the manufacturer's instructions.

4.3. Centrifuge the cell suspension at 300 x *g* for 10 min at 4 °C, aspirate the supernatant completely.

4.4. Resuspend the pellet with 90 µL of isolation buffer per 10^7 total cells.

4.5. Add 10 µL of CD146 microBeads per 10^7 total cells. Mix well and incubate for 15 min at 4 °C.

4.6. To wash the cells, add 1–2 mL of isolation buffer per 10^7 cells, centrifuge at 300 x g for 10 min, and then aspirate the supernatant completely.

4.7. Resuspend up to 10^9 cells in 500 µL of isolation buffer (**Table 1**).

NOTE: For higher cell numbers, scale up buffer volume accordingly.

4.8. Prepare the separation column, rinse it with 3 mL of isolation buffer.

4.9. Apply the cell suspension onto the column stacked with 70 µm pre-separation filters.

4.10. Wash the column with the 3 mL of isolation buffer 3 times.

NOTE: When washing the column, the isolation buffer should be added as soon as the column reservoir is empty.

4.11. Remove the column from the separator and place it on a 15 mL centrifuge tube. Pipette 5 mL of isolation buffer onto the column.

4.12. To recover the magnetic beads labeled cells, firmly push the plunger into the column to flush out the cells.

4.13. Centrifuge at 300 x *g* for 5 min at 4 °C. LSECs are ready for microscopic examination and downstream analysis.

5. LSECs immunophenotyping and purity assessment by flow cytometry

5.1. Determine the cell numbers of isolated LSECs using an automatic cell counter following the manufacturer's instructions.

5.2. Centrifuge the cells at 300 x *g* for 5 min, aspirate the supernatant completely.

5.3. Take 1 x 10^6 cells/tube and resuspend with 90 µL of staining buffer (**Table 1**).

5.4. Add 10 µL/tube of mouse FcR block and 1 µL of viability dye, incubate at 4 °C for 10 min.

5.5. Stain the cells with a combination of CD45, CD146, and stabilin-2 antibodies diluted at 1:50 with staining buffer. Incubate at 4 °C for 20 min.

5.6. Wash the cells with 5 mL of staining buffer and centrifuge at 300 x *g* for 10 min, then aspirate the supernatant completely.

5.7. Resuspend the cells with 300 µL of staining buffer and run through the flow cytometer.

6. LSECs culture and examination

6.1. Seed 1 x 10^6 cells/well in a 6-well plate and culture it with endothelial cells growth medium consisting of 5% fetal bovine serum (FBS), 1% endothelial cells growth supplement, and 1% primocin solution.

6.2. Examine the isolated LSECs by light microscopy. Acquire bright-field images with a 10x magnification.

7. **LSECs morphology and fenestrae examination by scanning electron microscopy**

7.1. Pre-coat the cell culture inserts (3 µm pore size) with collagen solution.

7.2. Culture isolated LSECs (120,000 cells) on the insert with endothelial cells growth medium in a 24-well plate at 37 °C warm and humidified atmosphere. Allow cells to settle down and adhere for 2 h.

7.3. For cell fixation, add an equivalent volume of prewarmed at 37 °C Trump's fixative to the cell culture media.

7.4. After incubation for 10 min, replace 50% diluted Trump's fixative with undiluted Trump's fixative.

7.5. Fix the cells in Trump fixative for 2 h, then incubate for 1 h in 1% osmium tetroxides.

7.6. Proceed with samples dehydration, drying in a critical point drying device, mounting, sputter coating, and examination using a scanning electron microscope.

REPRESENTATIVE RESULTS:

Experimental schematics and equipment set up:

In this protocol, mouse liver was digested using a closed perfusion circuit, then nonparenchymal cells and hepatocytes were separated by low-speed centrifugation at 50 x *g* for 2 min. Primary LSECs were isolated using CD146 magnetic beads selection from the nonparenchymal fraction. The experimental schematics are shown in **Figure 1A**. The cannula was placed through the PV while the inferior vena cava was tied up to ensure unidirectional perfusion of collagenase through the liver (**Figure 1B**). The in-house perfusion chamber is equipped with a heating and humidification system to ensure a 37–40 °C warm and humidified apparatus air. The perfused collagenase circulates through a closed system and can be reused for two or three mice during the isolation day to make the isolation more cost-effective.

Assessment of isolated LSECs purity and surface markers by flow cytometry:
The purity of the isolated LSECs was assessed by employing well-characterized specific LSEC surface markers CD146 and Stabilin-2[16–18]. Stained cells were analyzed on a flow cytometer; data were analyzed using FlowJo software.

LSEC population was gated (**Figure 2A**) and analyzed singlets for the following gating strategy; viable cells were defined using viability dye staining before labeling cells with specific markers (**Figure 2B**). Then $CD45^-$ population was gated to exclude any immune cells contamination. The isolated LSECs reached 94.8% viability (**Figure 2C**). The percentage of $CD45^-$ cells was 89.7% (**Figure 2D**), and 92.3% of the cells were CD146 and Stabilin-2 double-positive ($CD45^-$ $CD146^+$stabilin-2^+) LSECs (**Figure 2E**).

Morphology of the isolated LSECs:
The isolated LSECs were seeded in a 6-well plate at 1 x 10^6 cells/well, cultured in a complete growth medium, and examined by light microscopy after 6 h of culture (**Figure 3A**). LSECs were plated on a 3 µm pore-sized culture insert to visualize the LSECs fenestrae by scanning electron microscope (SEM). After fixation and processing, the fenestrae were identified, as shown in **Figure 3B**. As previously reported, LSECs lose their fenestrae in culture overnight[19]; hence, it is recommended to process the cells as soon as possible after the isolation for downstream studies to avoid dedifferentiation *in vitro*.

FIGURE AND TABLE LEGENDS:
Figure 1: Experimental schematics and equipment set up. (**A**) Experimental schematics were designed using Bio render. (**B**) Equipment setup and process. The liver was harvested and perfused in a closed collagenase perfusion apparatus. The liver was then transferred into a dish and dissociated. The liver suspension was collected. The nonparenchymal cell fraction was separated by centrifugation at 50 x *g* for 2 min. LSECs were purified using magnetic beads.

Figure 2: Assessment of isolated LSECs purity and surface markers by flow cytometry. Data analysis and gating strategy as shown, (**A**) gated LSECs and (**B**) singlets based on forward scatter (FSC)/side scatter (SSC). Isolated LSECs were stained with a viability dye and a combination of CD45, CD146, and Stabilin-2 antibodies. Singlets were gated and analyzed, (**C**) viable LSECs were gated on (**D**) $CD45^-$ population and analyzed for (**E**) CD146 and Stabilin-2 expression. All gates were determined by fluorescence minus one (FMO).

Figure 3: Morphology of isolated LSECs. (**A**) Bright field picture of cultured LSECs. Isolated LSECs were cultured in the complete growth medium for 6 h and examined by light microscopy (Scale bar 100 µm). (**B**) LSEC was examined by scanning electron microscope (SEM). White arrows indicate LSEC fenestrae (Scale bar, left panel 5 µm, right panel 1 µm).

Table 1: Buffers recipe. The table comprises the composition of the buffers and solutions used in this study.

DISCUSSION:

In the current manuscript, we describe a protocol for LSEC isolation from mouse liver consisting of two-step collagenase perfusion and subsequent magnetic-activated cell sorting (MACS). This protocol consists of the following three steps: (1) Perfusion through the PV with a calcium-free buffer followed by a collagenase-containing buffer to achieve liver cell dispersion; (2) Exclusion of hepatocytes with low-speed centrifugation; and (3) MACS-based positive selection of LSECs from nonparenchymal cells (NPCs) using anti-CD146 magnetic beads. The whole procedure could be completed within 3 h. Furthermore, the cost for this procedure, including all the consumable supplies, is around 150 USD per mouse, suggesting that this method of LSEC isolation is overall efficient and cost-effective. We use steps (1) and (2) for isolation of primary mouse hepatocytes as well, which is beyond the scope of the current manuscript.

There are a few critical steps in portal cannulation and liver perfusion: (i) Catheter positioning: the catheter tip should be placed no less than 3 mm distal to the PV bifurcation of an adult mouse, which is usually identified in the hilum of the liver. Deep placement of the catheter tip in the PV will compromise the perfusion of some lobes of the liver. Poor perfusion of a lobe manifests as an absence of a color change of the lobe and can be rectified if noticed immediately by a slight repositioning of the catheter into the distal PV. This maneuver could possibly optimize lobe perfusion. (ii) Air bubbles: the infusion route between the pump and the PV should be kept completely free from air bubbles. Minute air entry into the PV can cause an air embolism, which results in incomplete liver perfusion. To eliminate air in the catheter after removing the inner needle, Buffer A is injected with a syringe proximal to the bubble to expel any air before connecting the infusion line to the catheter. (iii) Collagenase strength: To maintain collagenase activity during the perfusion, it is essential to assure that Buffer B infused into the PV has the precise concentration of collagenase and pH and is kept at approximately 37 °C. As shown in **Figure 1**, a customized chamber is used to keep the whole perfusion system in a 37 °C warm and humidified atmosphere; in addition, the oxygen supply is maintained during collagenase perfusion to achieve complete digestion. An alternative option includes a water bath to pre-warm the collagenase solution[20], and an open collagenase perfusion system in situ where the collagenase solution drains out of the liver through the cut IVC[21].

Although the protocol uses relatively bold catheters (20 G) to secure an adequate perfusion flow, a thinner catheter will also work well based on previous reports[22–24]. However, it is sometimes difficult to successfully cannulate the PV especially when mice are used for specific disease models or if they have PV anomalies. If PV cannulation fails, mechanical and enzymatic liver digestion using an enzyme kit and gentle tissue dissociator is an alternative method for steps (1) and (2) to obtain nonparenchymal cell (NPC) suspension. We have confirmed that the cell yield and viability in this alternative method of dissociation without liver perfusion was comparable to the original method (data not shown). Portal vein perfusion for liver digestion has been employed by others and us[13,16,20]. Furthermore, this method avoids the theoretical risk of LSECs phenotypic or functional alterations induced by mechanical force during liver digestion. Therefore, the use of the PV perfusion-based method depicted in this protocol is so far highly recommended.

Here, the optimum LSEC yield and purity when employing the current protocol are shown. We

obtained approximately 2–5 x 10^6 cells per mouse from both healthy mice and mice with diet-induced NASH using the current protocol. As for purity, the positivity of the same surface marker used for immunomagnetic separation (CD146) supports the accuracy of our isolation technique. In addition, there are several well recognized LSEC surface markers, such as lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1), CD32b, and Stabilin-2. Controversies still exist around these markers; for example, i) LYVE-1 is present also in lymphatic endothelial cells, and ii) LSECs in a periportal area lack CD32b expression[18]. Thus, to examine the purity of these isolated LSECs with flow cytometry, Stabilin-2 was employed as an established specific LSEC marker in addition to CD146. Using this methodology, we confirmed that the isolated LSECs had >94% viability and >90% purity (**Figure 2**). The LSEC-specific phenotype of the isolated cells was further confirmed using SEM, where most of the cells have fenestrae (**Figure 3B**), the characteristic morphological feature of LSECs.

Among the LSECs isolation methodologies, immunomagnetic beads selection is the technique used the most to isolate the LSECs from the NPC fraction[24–27]. In contrast, other methods include centrifugal elutriation and selective adhesion-based separation[22,28,29]. The non-immunomagnetic methods have been shown to deliver high yields of LSECs (approximately 9 x 10^6 cells per mouse)[11]. Hence, a relatively lower yield of isolated LSEC could be a limitation of the current protocol. On the other hand, the non-immunomagnetic selection-based methods require high technical expertise and sometimes show inconsistent results on cell yields and purities[11], while the immunomagnetic selection-based method holds the advantage of consistent yield and purity over other existing methods. Moreover, for immunomagnetic beads selection-based cell isolation, the specificity of cell surface markers used is always a matter of concern. For example, CD31, which has been used previously as a marker for immunomagnetic LSEC separation, is dominantly expressed on the PV endothelium and capillarized LSECs[3]. Although CD146 has been reported to be expressed also on natural killer cells and hepatic stellate cells[30,31],the high purity of LSECs isolated using this protocol mitigates concerns regarding the specificity of the endothelial marker CD146. Comparison of the pros and cons of various methodologies for LSEC isolation is discussed elsewhere[11,18,32]. Comparison of cell yields and purities between other methods and this method is beyond the scope of the current manuscript and warrants future investigations.

In conclusion, we present a primary mouse LSEC isolation protocol based on one of the most widely used and accepted approaches. Isolated LSECs display high purity and preserved function. Moreover, this protocol is efficient and applicable to the healthy mouse liver as well as the liver from different disease mouse models. High-quality LSECs from these mice can be employed for multiomics data set generation[13,14]. Thus, this approach facilitates the identification of molecular mechanisms regulating the function of LSEC and improves our understanding of their roles in intercellular communication in the liver in health and disease.

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DISCLOSURES:

All authors have no conflicts to disclose.

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29 Smedsrød, B., Pertoft, H., Eggertsen, G., Sundström, C. Functional and morphological characterization of cultures of Kupffer cells and liver endothelial cells prepared by means of density separation in Percoll, and selective substrate adherence. *Cell and Tissue Research.* **241** (3), 639–649 (1985).
30 Despoix, N. et al. Mouse CD146/MCAM is a marker of natural killer cell maturation. *European Journal of Immunology.* **38** (10), 2855–2864 (2008).
31 Strauss, O., Phillips, A., Ruggiero, K., Bartlett, A., Dunbar, P. R. Immunofluorescence identifies distinct subsets of endothelial cells in the human liver. *Scientific Reports.* **7** 44356 (2017).
32 Halpern, K. B. et al. Paired-cell sequencing enables spatial gene expression mapping of

527 liver endothelial cells. *Nature Biotechnology.* **36** (10), 962–970 (2018).
528

Figure 1

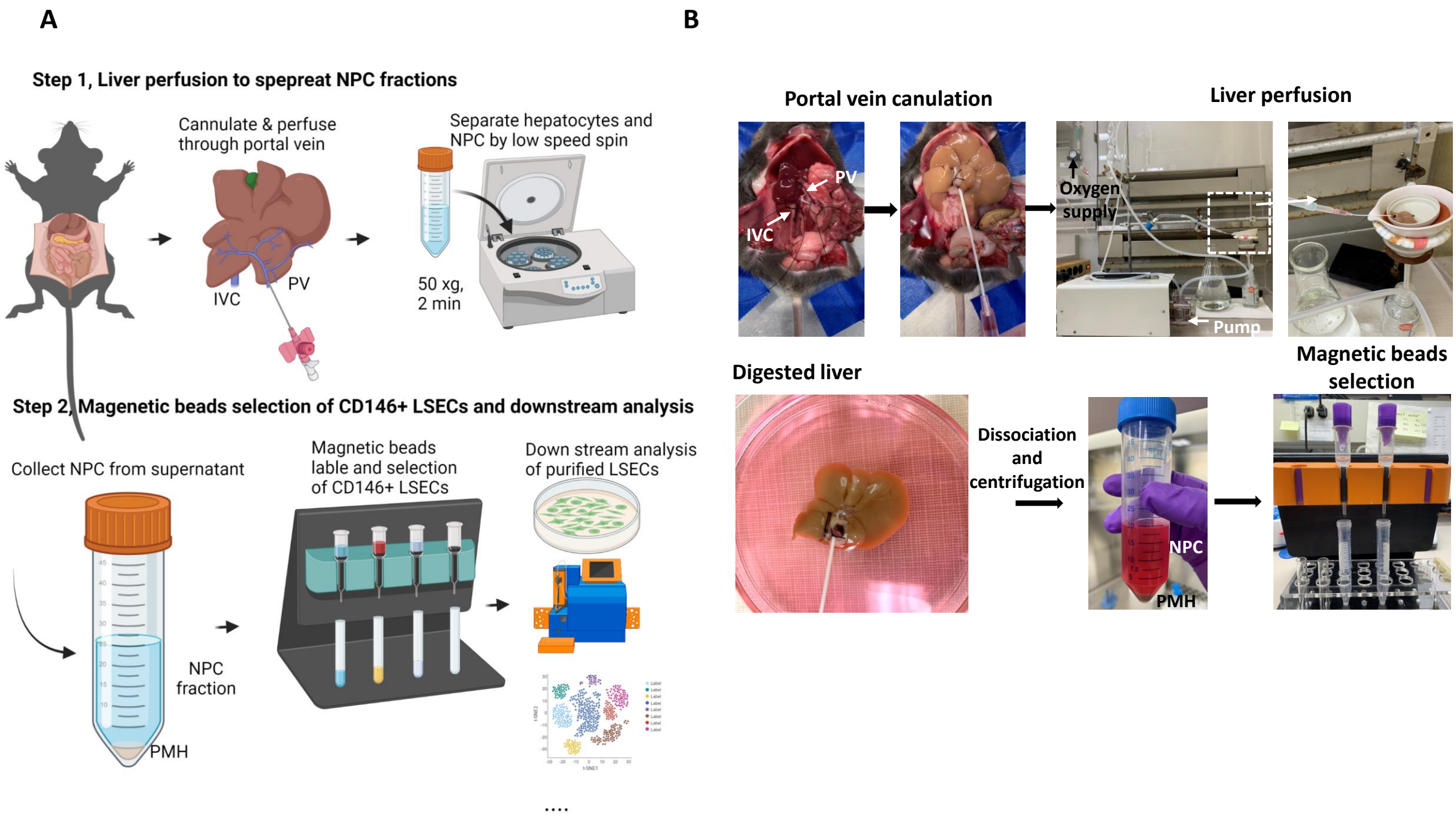


Figure 2

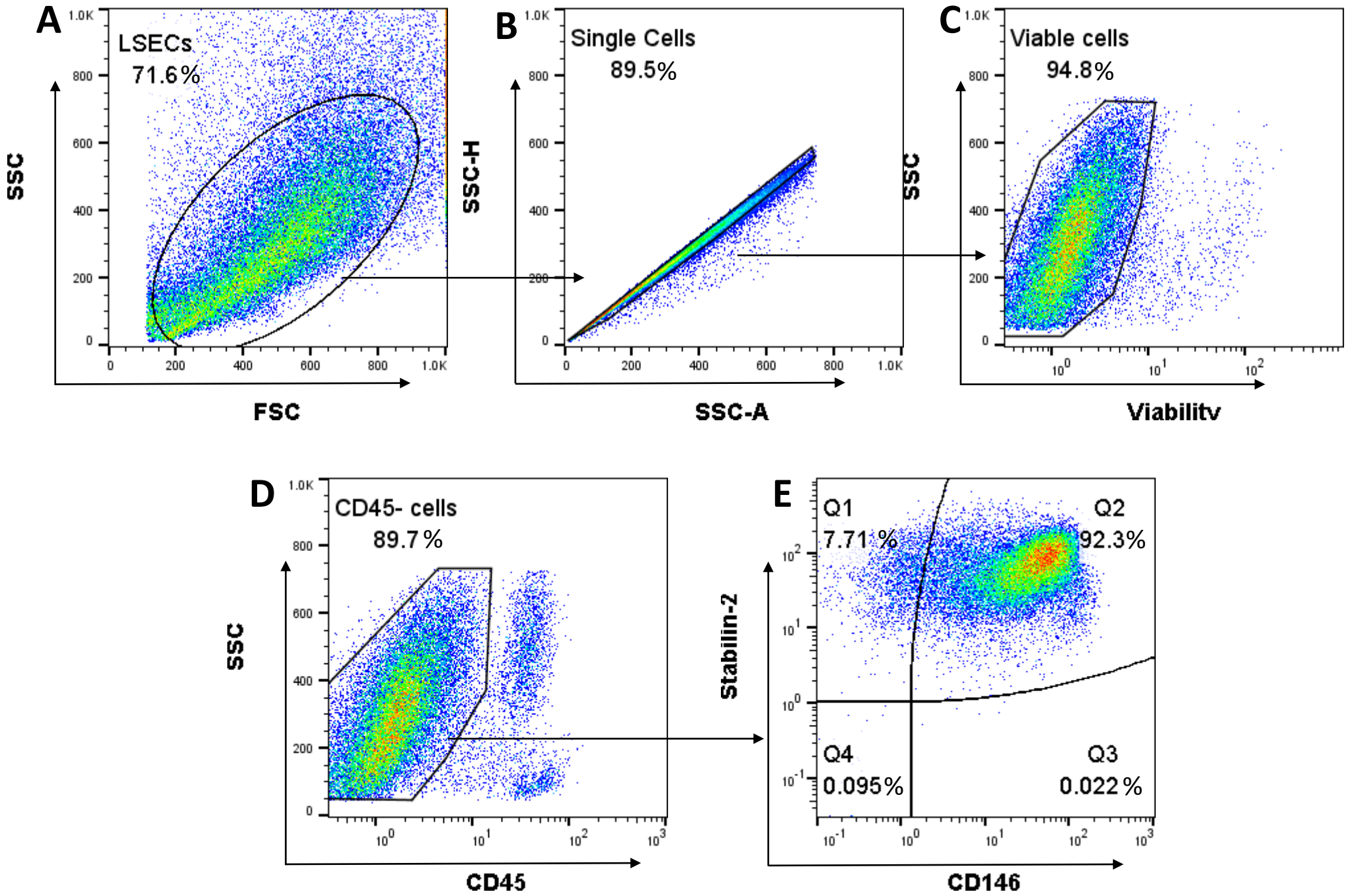
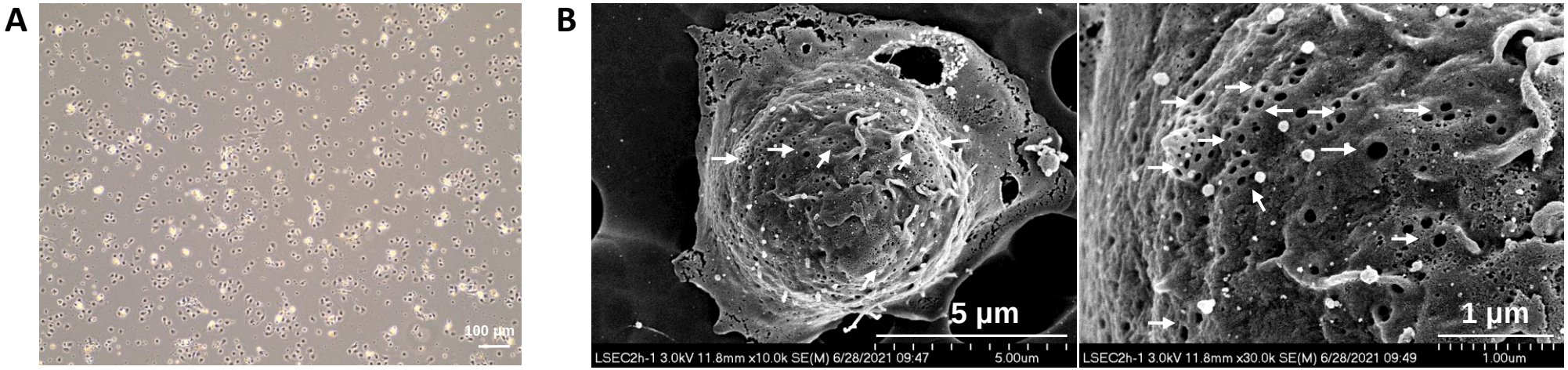


Figure 3



KRH stock solution (10x)	
Reagents	Concentration
NaCl	1.15 M
HEPES (free acid)	0.2 M
KCl	0.05 M
KH_2PO_4	0.01 M

Buffer A	
Reagents	Concentration
KRH solution, PH=7.4	1x
EGTA	0.5 mM
Adjust PH to 7.4, filter through a 0.2 μm filter before use. Stored at room temperature for up to 6 months.	

Buffer B	
Reagents	Concentration
KRH solution, PH=7.4	1x
$CaCl_2$	1 mM
Adjust PH to 7.4, filter through a 0.2 μm filter before use. Stored at room temperature for up to 6 months.	

Collagenase solution	
Reagents	Concentration
Buffer B, PH=7.4	125 mL
Collagenase	35–40 mg, add BSA up to 100 mg

Percoll solution	
Reagents	Concentration
Percoll	22.5 mL
PBS (10x), PH=7.4	2.5 mL

Isoaltion/Staining Buffer	
Reagents	Concentration
MACS rinsing buffer	1250 mL
BSA stock	125 mL

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Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

RESPONSES TO EDITORIAL COMMENTS

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you for your thorough proofreading of our manuscript and kind suggestion. We revised the manuscript to our best knowledge and included all the details that are recommended by editor and reviewers.

2. Please shorten your title to "Isolation and characterization of mouse primary liver sinusoidal endothelial cells for downstream analyses".

Response: The title has been changed to "Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells".

3. Please rephrase the Summary to include between 10-50 words.

Response: We rephrased the summary as follows to better fit within the word limit:

SUMMARY:

Here we outline and demonstrate our protocol of primary mouse liver sinusoidal endothelial cell (LSEC) isolation. The protocol is based on liver collagenase perfusion, non-parenchymal cell purification by low-speed centrifugation and CD146 magnetic bead selection. We also phenotype and characterize these isolated LSECs using flow cytometry and scanning electron microscopy.

3. Please define all abbreviations before use. For example: PBS, IV, PV, MACS, etc.

Response: Thank you for bringing this to our attention. All the abbreviations were defined the first used in the manuscript and figures and figure legends.

4. Please revise the following lines to avoid overlap with previously published work: 53-54, 126-127, 187-205, 214-215.

Response: We carefully checked the results of iThenticate screening attached to the editor's comment and edited the manuscript accordingly.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove such symbols and names like MACS™ Dissociator, MACS Smart Strainer;

Percoll (sigma); MacsQunt, Miltenyi, Germany; Millicell, Millipore, PITP01250; InVivoGen, San Diego, CA; Hitachi S-4700 microscope (Hitachi Inc, Pleasanton); (#130-105-807, Miltenyi Biotec, Bielefeld, Germany; gentleMACS™ Dissociator (# 130-093-235) etc.
Obviously, if you have optimized your protocol with certain instruments/reagents/software, you will need to mention them, but include just the bare minimum information (e.g., name of product) in the manuscript and come up with a generic term to refer to the product (include this in the comments column in the Table of Materials) after the first mention so that you don't keep repeating commercial terms throughout the paper.

Response: All the description containing commercial languages have been replaced with brief name of the products followed by merchandise details. In addition, all the metrials used in the manuscript were also included in Table 2.

6. Protocol section only contains numbered action steps in IMPERATIVE TENSE which describes how to perform the technique being presented. You can add NOTES after steps to draw the reader's attention to a safety or other precaution, which need not be in imperative tense. In this regard, please consider providing reaction set-ups and solution compositions as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

Response: Thank you for the advice. The solution section is removed from the revised manuscript as Table 1 includes all the recipes of the solutions used in the protocol.

7. Please move materials (lines 90–101) to Table of Materials.

Response: Both material and reagents' section were removed from the revised manuscript and components were added to Table 2.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Response: Numbering adjusted as recommended

9. Please add more details to your protocol steps. Please include volume and concentrations of solutions, buffers, reagents and size of dishes (line 124) used in the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: Thank you for the drawing our attention to this important point. We added the required details throughout the whole protocol. We specified the tissue culture materials and the volume of buffer used for each step when applicable.

10. Line 121: Please use SI unit denotation for all units throughout the manuscript: L, mL, µL, cm, kg, etc. Hours, minute, and seconds can be written as h, min, s, respectively.

Response: All the units used in the protocol have been edited carefully to comply with the SI unit denotation.

11. Line 122: Please use the symbol µ instead of u.

Response: We replaced as recommended.

12. Line 131: Please include a space between all numbers and the corresponding unit throughout the manuscript and in the figures: 5 min, 50 x g, 37 °C, etc.

Response: We edited the manuscript accordingly.

13. Line 140: Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia. Do you shave the surgical area prior to incision? Please mention.

Response: Since this procedure is an end point surgery, the mouse bleeds out to death soon after the liver is perfused and removed for collagenase perfusion. The whole surgery takes 15 min, the IACUC protocol does not include the usage of vet ointment. We do not shave the surgical area as we open the mouse abdominal skin first then cut the peritoneum laterally open.

14. Line 144: Please mention the length of the incisions.

Response: This step is changed to line 159 in the revised manuscript and edited as follows:

Spray mouse abdomen with 70% ethanol. Using surgical scissors make an incision of approximately about 5 cm long starting from the lower part of the abdomen up to the xyphoid process.

15. Line 148- 149: Were forceps used to pull these organs? If yes, please specify. Please include

the suture size and refer to the Table of Materials for suture information (after moving information to the table).

Response: This step is changed to line 128~135 in the revised manuscript and edited as follows:

> *Next, make two lateral cuts using small iris scissors on each sides of the abdomen to fully expose the abdominal organs.*
> *Place the sheath of the intravenous (IV) catheter under the back of the animal to lift, and level the abdomen.*
> *Using 4-inch regular curved dressing forceps gently pull the intestines and the stomach off to the left of the animal.*
> *Place a 3.0 surgical suture under the inferior vena cava (IVC) just below the exposed left kidney. Tie a loose hitch in the suture.*
> *Place another 3.0 surgical suture around the hepatic portal vein just above the splenic vein branching point off the hepatic portal vein. Tie a loose hitch in the suture.*

16. Line 171: Please discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

Response: Since this is an end point surgery, the mouse bleeds out to death when liver is perfused and moved out for collagenase perfusion. No post-surgical treatment is needed.

17. Line 172: Please mention the rate at which the collagenase solution is perfused into the liver.

Response: The pump rate is set at speed 1 as shown on the speed control dial. It is equal to 40 mL/min.

18. Line 186, 208: How was the cell number determined? Please also mention in notes after these steps what can be expected.

Response: The cell number was counted using automatic cell counter. The product information has been included in the material Table 2.

19. Line 212: Please include antibody dilutions.

Response: This step is changed to line 203 in the revised manuscript and edited as follows:

Stain cells with a combination of CD45, CD146 and Stabilin-2 antibodies diluted at 1:50 with staining buffer. Incubate in 4 °C for 20 min.

20. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Formatted and highlighted as recommended.

21. As we are a methods journal, please ADD to the Discussion along with citations:
a) Any limitations of the technique
b) The significance with respect to existing methods

Response: Thank you for mentioning this important point. In the revised manuscript line 341~347 we added both a) the limitation and b) the significance with respect to existing methods as follows:

a) The non-immunomagnetic methods have been shown to deliver high yields of LSECs (approximately $9x10^6$ cells per mouse) [11]. Hence, a relatively lower yield of isolated LSEC could be a limitation of our current protocol.

b) On the other hand the non-immunomagnetic selection-based methods require high technical expertise and sometimes show inconsistent results on cell yields and purities[11], while our immunomagnetic selection-based method holds the advantage of consistent yield and purity over other existing methods.

22. Please combine Tables 1–6 and include all recipes in one table called something like Recipes of solutions used in the LSEC isolation protocol.

Response: Table 1~6 have been combined as Table 1 and named Buffer recipe in the revised manuscript.

23. Please remove corresponding author details from acknowledgements section.

Response: This description has been removed as recommended.

24. Please ensure that the references are numbered in the order of appearance/citation and appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

Response: Edited as recommended

25. Please add more details about the equipment and software used in the Table of Materials and sort alphabetically by the name of the material. This table should serve as a handy reference to users to know what is required to follow this protocol.

Response: All the materials, reagents, equipment and software have been included in the revised Table 2.

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Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

RESPONSES TO COMMENTS BY REVIEWER #1

We thank the reviewer for his/her thoughtful and constructive examination of our manuscript. We have addressed all the reviewer's concerns in detail. Our responses to the reviewer's comments are as follows:

1. Please give more detail of the source in the table of materials, for example, state and country.

Response, A new column with details of each material used in the manuscript has been added to the table as recommended and showed as follow:

Name of Material / Equipment	Company	Catalog Number	State/Country
2.0-inch 20G Intra Venous (IV) catheter	Terumo	SR-OX2051CA	SOmerset, NJ, USA
2~3-inch perfuion tray with a hole in the center*			
405/520 viability dye	Miltenyi	130-110-205	Bergisch Gladbach, Germany
4-inch regular curved dressing forceps	Fisher Brand	FS16-100-110	
5-0 Perma-Hand silk suture	Ethicon	A182H	Raritan, NJ, USA
Anti-stabilin-2 (Mouse) mAb-Alexa FluorÃ 488	MBL International	D317-A48	Woburn, MA, USA
BSA stock (Miltenyi, cat#)	Miltenyi	130-091-376	Bergisch Gladbach, Germany
CD146 (LSEC) Antibody, anti-mouse	Miltenyi	130-118-407	Bergisch Gladbach, Germany
CD146 (LSEC) MicroBeads, mouse	Miltenyi	130-092-007	Bergisch Gladbach, Germany
CD45-VioGreen	Miltenyi	130-110-803	Bergisch Gladbach, Germany
Collagen type I	Corning	354236	Corning, NY, USA
Collagenase II	Gibco	17101-015	Waltham, MA, USA
Endothelial cells growth medium	ScienCell Research Laboratories	211-500	Carlsbad, CA, USA
FcR blocking reagent, mouse	Miltenyi	130-092-575	Bergisch Gladbach, Germany
FlowJo software, version 10.6	Becton, Dickinson and Company		
Hardened Fine scissors	F.S.T	14091-11	Foster city, CA, USA
Heated (37 °C) and humidified recirculating perfusion apparatus equipped with Oxygen injection at a rate of 10psi.*			
Hitachi S 4700 scanning electron microscope	Hitachi Inc	SEM096	Pleasanton, CA, USA
LS columns	Miltenyi	130-042-401	Bergisch Gladbach, Germany
MACS pre-separation filters (70 µm)	Miltenyi	130-095-823	Bergisch Gladbach, Germany
MACS rinsing buffer	Miltenyi	130-091-222	Bergisch Gladbach, Germany
MACS Smart Strainer (70 µm)	Miltenyi	130-098-462	Bergisch Gladbach, Germany
MACSQunt flow cytometer	Miltenyi		Bergisch Gladbach, Germany
Millicell Cell Culture Insert	Millipore Sigma	PITP01250	Burlington, MA, USA
Nexcelom cell counter	Nexcelom bioscience	Cellometer Auto T4 Plus	Lawrence, MA, USA
Percoll	GE Healthcare	17-0891-01	Chicago, IL, USA
Surgical scissors	F.S.T	14001-12	Foster city, CA, USA
Very small curved dressing forceps	F.S.T	11063-07	Foster city, CA, USA
* Items are customize made in house			

2. It is better to give a number for "Table os materials".

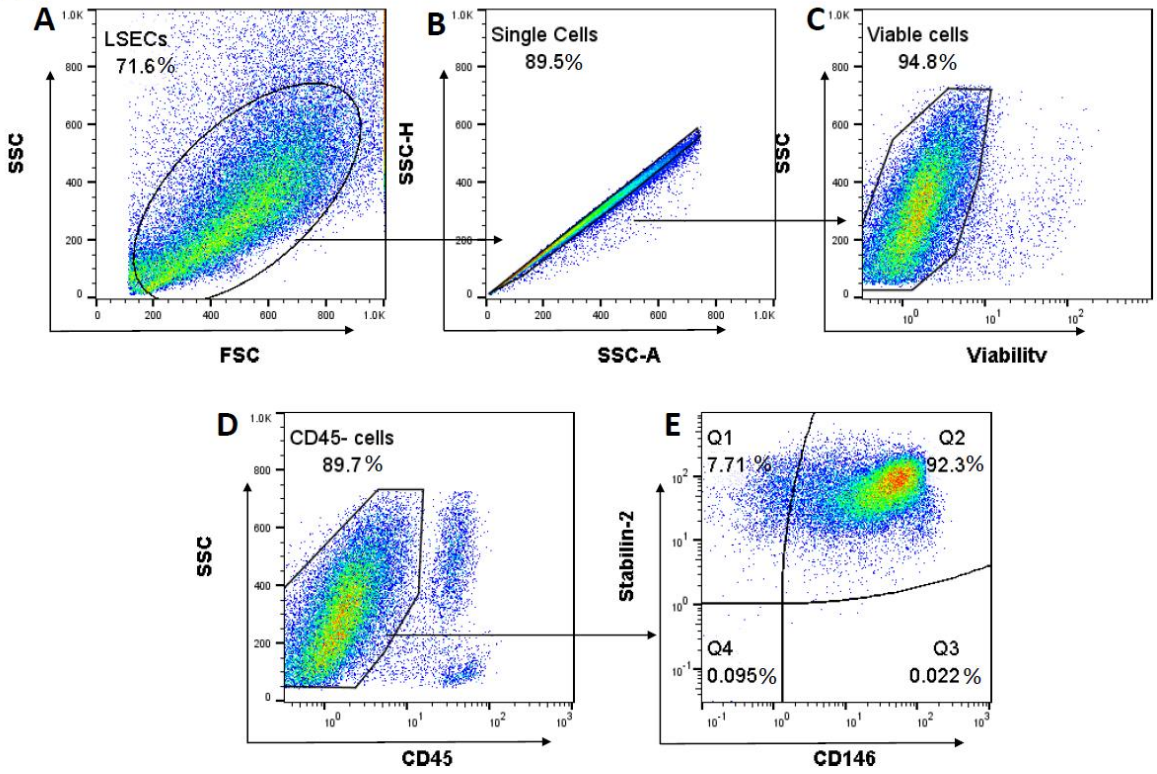
Response, Table named as Table 2 Table of materials, is now updated in the manuscript and the table title accordingly.

3. Figure 2 The words in the figure are a little smaller. Furthermore, please add % after 94.4% and the others.

Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

Response: The flow cytometry data was re-analyzed and fonts in the figure were edited as recommended and included bellow:

Figure 2



The description of this part of the experiment in the results section was edited accordingly,

Line 246~251,

We gated LSEC population (Figure 2A) and analyzed singlets for the following gating strategy, viable cells were defined using viability dye staining before labeling cells with specific markers (Figure 2B). Then CD45- population was gated to exclude any immune cells contamination. The isolated LSECs reached 94.8 % viability (Figure 2C) The percentage of CD45- cells was 89.7 % (Figure 2D), and 92.3 % of the cells were CD146 and stabilin-2 double positive (CD45-CD146+stabilin-2+) LSECs (Figure 2E).

The figure legend for Figure 2 was edited as follows,

Figure 2. Assessment of isolated LSECs purity and surface markers by flow cytometry. Data analysis and gating strategy as shown, gated LSECs (A) and singlets (B) based on FSC/SSC. Isolated LSECs were stained with viability dye and a combination of CD45, CD146 and Stabilin-2 antibodies. Singlets were gated and analyzed, viable LSECs (C) were gated on

$CD45^-$ population (D) and analyzed for (E) CD146 and Stabilin-2 expression. All gates were determined by fluorescence minus one (FMO).

4. There are some spelling errors, for example "w/o" in line 108, "os" in Table os materials.

Response: Thank you for bringing this spelling errors to our attention. We edited all the errors to the best of our knowledge. Line 108 is removed as all the materials ans reagents recipes moved to Table 1.

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Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

RESPONSES TO COMMENTS BY REVIEWER #2

We thank the reviewer for his/her thoughtful and constructive examination of our manuscript. We have addressed all the reviewer's concerns in detail. Our responses to the reviewer's comments are as follows:

RESPONSES TO COMMENTS BY REVIEWER #2

We thank the reviewer for his/her thoughtful and constructive examination of our manuscript. We have addressed all the reviewer's concerns in detail. Our responses to the reviewer's comments are as follows:

Manuscript Summary:
Efficient isolation protocol for liver sinusoidal endothelial cells (LSECs) is helpful to move the field forward. Guo et al. described a LSEC isolation protocol using collagenase perfusion and CD146 magnetic beads selection. Overall, this is a well-written protocol. However, the method described does not seem to be very different from those previously published by other investigators (refs #12). The difference should be mentioned. Suggestions below may help to make this protocol more attractive to the liver research community.

Major Concerns:
1. Addition of LSEC isolation method from mice with liver disease such as NAFLD will be informative. In fact, the authors included "NAFLD" in the keyword list.

Response: Thank you for bringing up this key point, we do use this protocol to isolate LSECs from NASH livers and do the downstream analysis and comparison with LSECs from healthy liver. We agree it will be informative for the readers to widely apply this protocol to their studies. We edited and discussed this point in the revised manuscript as follows:

Line 35~36,

Here, we discuss our protocol of LSEC isolation from both healthy and nonalcoholic fatty liver disease (NAFLD) mice.

Line 76~79,

Moreover, with the growing interest in generating big datasets in both clinical research and discovery science, these high quality LSECs isolated from both healthy and diseased liver with nonalcoholic steatohepatitis (NASH) or other conditions can be pooled or used individually allowing multi-omics data generation and comparison between health and disease [13,14].

Line 325~327,

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Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

We obtained approximately 2~5x10^6 cells per mouse from both healthy mice and mice with diet-induced NASH using our current protocol.

Line 360~361,

Moreover, this protocol is efficient and applicable to the liver disease animal models with NASH for big data set generation. [13,14]

2. It is unclear what is the major difference, compared to the published protocols by other investigators, as mentioned above.

Response, we summarized three main advantages using our protocol for LSEC isolation. First, the liver perfusion is conducted in the closed, humidified 37 °C chamber with oxygen supply, this device provides the best environment for collagenase digestion. In addition, the perfusion system is closed allowing recovery and reuse of the collagenase solution on multiple mice on the day of the isolation adding a cost-effective advantage. Second, we used positive magnetic beads selection to purify LSECs instead of centrifugal elutriation or selective adhesion-based separation. Immunomagnetic bead selection provides high-throughput isolation of LSECs with high purity and requires less technical expertise. Third, we used CD146 magnetic beads to select CD146-positive LSECs from non-parenchymal cells. As recently reported, CD146 is a more specific and mature LSEC surface marker. When co-stained with another specific LSEC marker Stabilin-2, we obtained >90% purity of isolated LSECs. We edited the discussion section of the revised manuscript as follows,

Line 344~353,

On the other hand, the non-immunomagnetic selection-based methods require high technical expertise and sometimes show inconsistent results on cell yields and purities [11]*. On the other hand, our immunomagnetic selection-based method holds the advantage of consistent yield and purity over other existing methods. Moreover, for immunomagnetic beads selection-based cell isolation, the specificity of cell surface markers used is always a matter of concern. For example, CD31, which has been used previously as a marker for immunomagnetic LSEC separation, is dominantly expressed on the PV endothelium and capillarized LSECs*[3]*. Although CD146 has been reported to be expressed also on natural killer cells and hepatic stellate cells*[30,31]*,the high purity of LSECs isolated using our protocol mitigates concerns regarding the specificity of the endothelial marker CD146.*

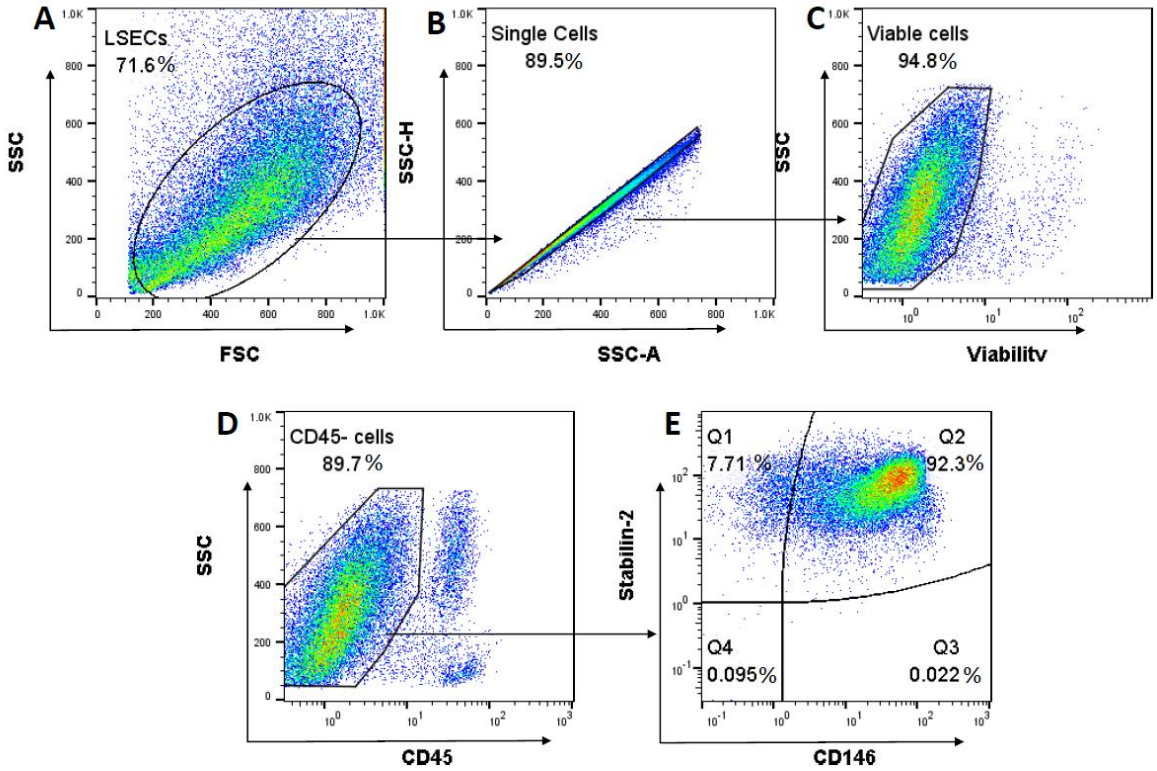
3. Purity of LSECs in the final step should checked also by including other liver cell markers. LSECs often attach to other liver cells (Halpern et al., Nature Biotechnology, 36: 962-970, 2018). Thus, it is possible that Stabilin-2 positive cells may be attached to hepatocytes or other liver cells.

Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

Response: There are a couple of key steps to prevent the LSECs from attaching to the other liver cells during the purification. First, the buffer used in the magnetic bead selection steps was ice-cold during the magnetic separation. Second, the CD146 bead-labeled NPCs were loaded through 70 μm pre-separation filter before the cells go through the magnetic column. And third, the LSEC phenotype analyzed by flow cytometry was gated on single cells. We included the detailed gating strategy for flow cytometry shown in Figure 2. All LSECs were gated by singlets and analyzed the viable cells for $CD45^-$ cell population, and $CD146^+Stabilin\text{-}2^+$ cells were analyzed to show the purity of the isolated LSECs.

The edited Figure 2 shown as follows,

Figure 2



The description of this part of the experiment in the results section was edited accordingly,

Line 246~251,

We gated LSEC population (Figure 2A) and analyzed singlets for the following gating strategy, viable cells were defined using viability dye staining before labeling cells with specific markers (Figure 2B). Then $CD45^-$ population was gated to exclude any immune cells contamination. The isolated LSECs reached 94.8% viability (Figure 2C). The percentage of $CD45^-$ cells was 89.7% (Figure 2D), and 92.3 % of the cells were CD146 and stabilin-2 double positive ($CD45^-CD146^+stabilin\text{-}2^+$) LSECs (Figure 2E).

Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

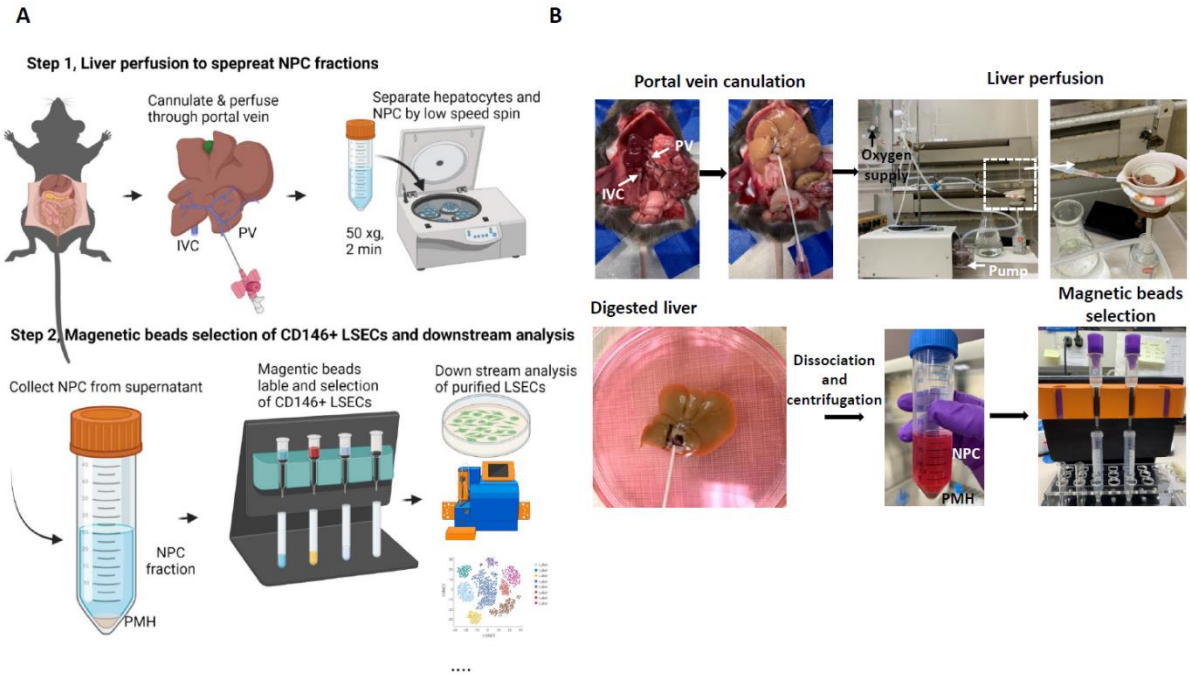
The figure legend for figure 2 was edited as follows:

Figure 2. Assessment of isolated LSECs purity and surface markers by flow cytometry. Data analysis and gating strategy as shown, gated LSECs (A) and singlets (B) based on FSC/SSC. Isolated LSECs were stained with viability dye and a combination of CD45, CD146 and Stabilin-2 antibodies. Singlets were gated and analyzed, viable LSECs (C) were gated on CD45- population (D) and analyzed for (E) CD146 and Stabilin-2 expression. All gates were determined by fluorescence minus one (FMO).

Minor Concerns:
1. The illustrated flowchart needs additional labeling, particularly in Figure 1A, Step 2.

Response: We reedited the flowchart in Figure 1, included more detailed labeling of each step as follows:

Figure 1



2. While the flowchart with illustrations is helpful, an addition of simple flowchart that summarizes an entire isolation method would be useful.

Response: Thank you for this thoughtful suggestion. Adding a flowchart illustration is not possible, for length and space limitation. However, we clearly covered the step by step approach in the protocol to compensate for that.

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Isolation and Characterization of Mouse Primary Liver Sinusoidal Endothelial Cells

RESPONSES TO COMMENTS BY REVIEWER #3

We thank the reviewer for his/her thoughtful and constructive examination of our manuscript. We have addressed all the reviewer's concerns in detail. Our responses to the reviewer's comments are as follows:

Reviewer #3:
Manuscript Summary:
The authors describe a protocol for the isolation of primary LSECs from mouse liver, based on magnetic bead purification. There is a requirement for highly purified and viable LSECs to facilitate further studies into LSEC biology, so this description in a video format is likely useful. The methodology described is fair, and explanations adequate. Together with a video demonstration, it is likely to lead to the suggested outcomes.

Major Concerns:
No major issues.

Minor Concerns:
1) Suggest removing NAFLD as a keyword as this is not relevant to the protocol

Response: We have removed NAFLD as a key word as suggested.

2) The authors define LSECs are CD45-/CD146+/Stabilin-2+. I note that there are now several other markers of LSECs used, and this should at least be mentioned in the discussion. The markers include Lyve-1 and CD32b.

Response:

2) Thank you for bringing up these important points. We edited the discussion section and mentioned the other LSEC markers as follows:

Line 327~334

As for purity, the positivity of the same surface marker used for immunomagnetic separation (CD146) support the accuracy of our isolation technique. In addition, there are several well recognized LSEC surface markers, such as lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1), CD32b, and stabilin-2. Controversies still exist around these markers; for example, i) LYVE-1 is present also in lymphatic endothelial cells, and ii) LSECs in a periportal area lack CD32b expression[18]. Thus, to examine the purity of these isolated LSECs with flow cytometry, we employed stabilin-2 as an established specific LSEC marker in addition to CD146.

3) Are LSECs isolated in this manner plateable (beyond the 2-hour time-point) used for SEM? If so this description (and a figure demonstrating their endothelial morphology) would be helpful. The major issue in LSEC studies is not necessarily the isolation, but the ability to obtain sufficient numbers of healthy cells that can be maintained in culture.

Response:

The LSECs obtained using the current protocol maintain their viability and morphology for hours to days of culture. However, as primary mouse LSECs show remarkable decrease in their fenestrae after 2 days of normal 2-D culture, we usually plate these cells soon after the isolation and complete the in vitro experiments using these cells within 24 hours. We included representative pictures of SEM for LSECs examined two hours after the isolation. Based on the reviewer's comment, we included a brightfield picture of isolated LSECs from healthy mouse cultured for 6 hours (Figure 3A) in the revised manuscript as shown below:

Line 252~254,

Morphology of the isolated LSECs. The isolated LSECs were seeded in a 6-well plate at 1x10^6 cells/well and cultured in complete growth medium (ScienCell Research Laboratories, Carlsbad, CA, USA), and examined by light microscopy after 6 hours of culture (Figure 3A).

Figure 3

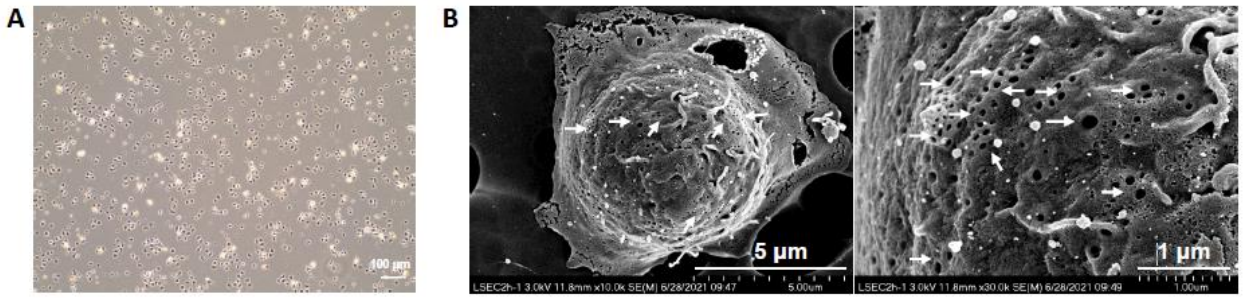

Figure 3. Morphology of isolated LSECs. A, Bright field picture of cultured LSECs. Isolated LSECs were cultured in the complete growth medium for 6 hours and examined by light microscopy. (Scale bar 100 µm), B, LSEC was examined by scanning electron microscope (SEM). White arrows indicate LSEC fenestrae (Scale bar, left panel 5 µm, right panel 1 µm).