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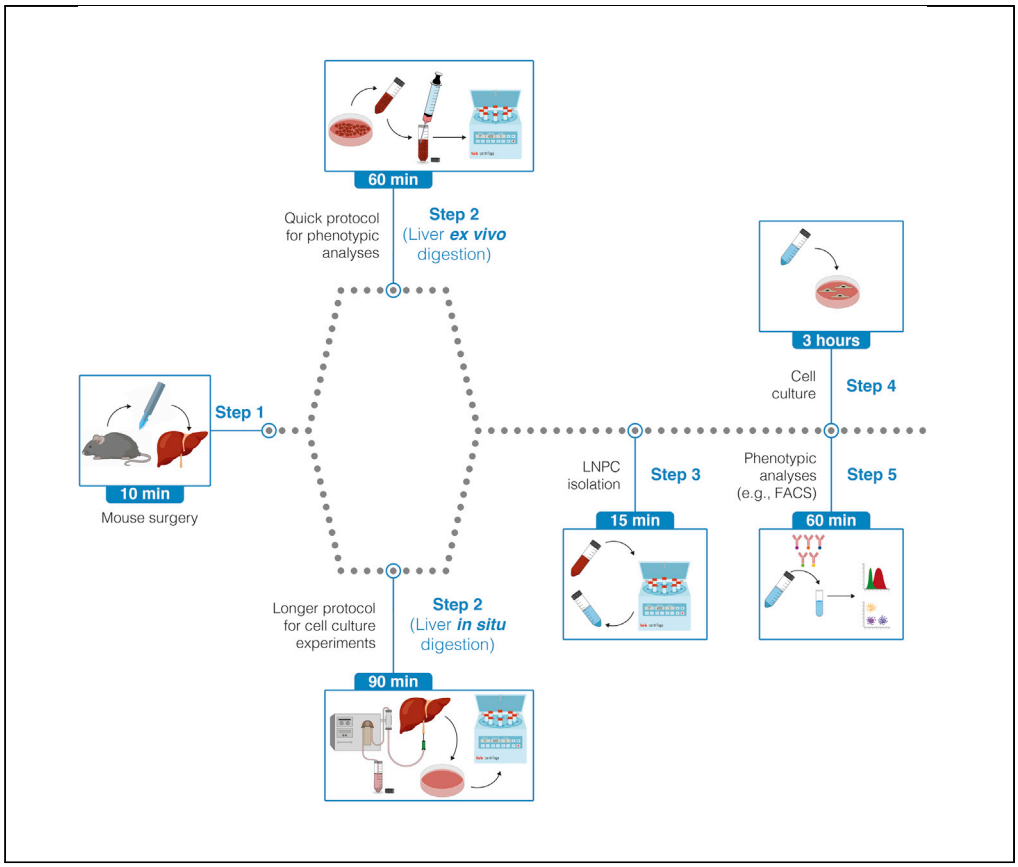
Isolation of mouse Kupffer cells for phenotypic and functional studies



Here, we provide detailed protocols for the isolation of mouse Kupffer cells – the liver-resident macrophages – for phenotypic (e.g., via flow cytometry, mass cytometry or RNA-sequencing) analyses or for functional experiments involving cell culture. The procedures presented can be adapted for the isolation of other hepatic cell populations.

Francesco Andreata, Camille Blériot, Pietro Di Lucia, ..., Mirela Kuka, Florent Ginhoux, Matteo Iannacone

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Highlights

Protocol for Kupffer cell (KC) isolation

Suitable for the simultaneous isolation of other hepatic cell populations

Isolated KCs are suitable for phenotypic and functional analyses

We provide critical tips for cell processing and FACS-based sorting

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Isolation of mouse Kupffer cells for phenotypic and functional studies

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SUMMARY

Here, we provide detailed protocols for the isolation of mouse Kupffer cells – the liver-resident macrophages – for phenotypic (e.g., via flow cytometry, mass cytometry, or RNA-sequencing) analyses or for functional experiments involving cell culture. The procedures presented can be adapted for the isolation of other hepatic cell populations.

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

BEFORE YOU BEGIN

In addition to its metabolic functions, the liver is endowed with unique immunological features (Bénéchet et al., 2019; Ficht and Iannacone, 2020; Iannacone and Guidotti, 2021). It is comprised by parenchymal cells (the hepatocytes) as well as non-parenchymal cells (LNPCs). Among the latter, Kupffer cells (KC) reside within liver sinusoids and represent the most abundant resident macrophage population of the organism. KCs have long been known for their scavenger and phagocytic functions but can also present antigens to $CD8^+$ T cells and promote either tolerance or effector differentiation. Efficient, reproducible methods for the isolation of Kupffer cells suitable for phenotypical and functional analyses are hence of paramount importance in order to study the biology of liver-resident macrophages.

This STAR protocol provides two distinct methods for KC isolation: a quick, scalable procedure for phenotypic analyses (method #1, steps 1 through 26), or a longer, more laborious preparation best suited for *in vitro* functional studies (method #2, steps 27 through 48).

KCs isolated with either methods are suitable for the desired downstream application, and we provide detailed information and suggestions on how to perform cell culture (steps 49 through 53) as well as flow cytometry analysis and cell sorting (steps 54 through 63).

Prepare the reagents for liver digestion, related to method #1

Prepare the *ex vivo* digestion medium

Timing: 15 min

1. Prepare a solution of plain RPMI supplemented with 0.2 mg/mL of collagenase, 5 units/mL of Deoxyribonuclease I and 10% FBS.
2. Prewarm the solution at 37°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
InVivoMAb anti-mouse CD16/CD32 antibody	Bio X Cell	Bio X Cell Cat# BE0307; RRID:AB_2736987
Brilliant Violet 605(TM) anti-mouse CD31 antibody	BioLegend	BioLegend Cat# 102427; RRID: AB_2563982
Brilliant Violet 650(TM) anti-mouse/human CD11b antibody	BioLegend	BioLegend Cat# 101239; RRID: AB_11125575
FITC anti-Mouse CD45	BioLegend	BioLegend Cat# 103108; RRID:AB_312973
PE anti-mouse ESAM antibody	BioLegend	BioLegend Cat# 136203, RRID: AB_1953300
PE-CF594 Rat Anti-Mouse Ly-6G	BD Biosciences	BD Biosciences Cat# 562700; RRID: AB_2737730
PE-CF594 Rat Anti-Mouse CD49b	BD Biosciences	BD Biosciences Cat# 562453; RRID: AB_11153857
PE-CF594 Rat Anti-Mouse CD19	BD Biosciences	BD Biosciences Cat# 562291; RRID: AB_11154223
PE-CF594 anti-Mouse CD3e	BD Biosciences	BD Biosciences Cat# 562286; RRID: AB_11153307
PerCP/Cyanine5.5 anti-mouse I-A/I-E	BioLegend	BioLegend Cat# 107626; RRID: AB_2191071
PE/Cyanine7 anti-mouse Tim-4	BioLegend	BioLegend Cat# 130010; RRID: AB_2565719
APC anti-mouse CD206 (MMR) antibody	BioLegend	BioLegend Cat# 141708; RRID: AB_10900231
APC/Cyanine7 anti-mouse F4/80	BioLegend	BioLegend Cat# 123117; RRID: AB_893489
Chemicals, peptides, and recombinant proteins		
DAPI	Thermo Fisher Scientific	Thermo Fisher Scientific Cat# D1306; RRID: AB_2629482
ViaKrome 808	Beckman	Beckman Cat# C36628
Brilliant stain buffer	BD Biosciences	BD Biosciences Cat# 659611; RRID: AB_2870505
Heparin sodium salt	Sigma Aldrich	Sigma Aldrich Cat# H4784
Liver Perfusion Medium	Thermo Fisher Scientific	Thermo Fisher Scientific Cat# 17701038
Liver Digest Medium	Thermo Fisher Scientific	Thermo Fisher Scientific Cat# 17703034
Hepatocyte Wash Medium	Thermo Fisher Scientific	Thermo Fisher Scientific Cat# 17704024
Deoxyribonuclease I from bovine pancreas	Sigma Aldrich	Sigma Aldrich Cat# D4263
Collagenase from *Clostridium histolyticum*	Merck Life	Merck Life Cat# C5138
Trypan Blue Solution	Sigma Aldrich	Sigma Aldrich Cat# T8154
Roswell Park Memorial Institute (RPMI) medium 1640	Gibco	Gibco Cat# 61870-010

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Complement inactivated Fetal Bovine Serum (FBS)	Corning	Corning Cat# 35-079-CV
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Gibco Cat# 15140122
Phosphate Buffered Saline (PBS) 1×, pH 7.4	Gibco	Gibco Cat# 10010023
Phosphate Buffered Saline (PBS) 10×, pH 7.4	Gibco	Gibco Cat# 70011044
70% Ethanol (v/v)	Sigma Aldrich	Sigma Aldrich Cat# 51976-500ML-F
2,2,2-tribromoethanol	Sigma Aldrich	Sigma Aldrich Cat# T48402
2-methyl-2-butanol	Sigma Aldrich	Sigma Aldrich Cat# 240486
Ammonium chloride (NH_4Cl)	Merk	Merk Cat# 254134
Potassium bicarbonate ($KHCO_3$)	Merk	Merk Cat# 237205
Na_2EDTA	Merk	Merk Cat# E5134
NaN_3	Merk	Merk Cat# S2002
Percoll	Sigma Aldrich	Sigma Aldrich Cat# P4937
Experimental models: organisms/strains		
Mouse: C57BL/6	Charles River	C57BL/6 colony
Software and algorithms		
FlowJo V10	FlowJo	https://www.flowjo.com/
Other		
FACS CANTO II	BD Bioscience	N/A
CytoFLEX LX	Beckman Coulter	N/A
FACSAria Fusion	BD Bioscience	N/A
Centrifuge 5810/5810R	Eppendorf	N/A
Peristaltic pump PLP 380	Behr Labor	Behr labor Cat# B00454739
Neubauer chamber	Blaubrand	Blaubrand Cat# 717805
Student Adson Surgical forceps	Fine Science	Finescience Cat# 91106-12
Student fine scissors	Fine Science	Finescience Cat# 91460-11
6-well culture plate flat bottom	Corning	Corning Cat# 3516
Petri dishes, polystyrene (60 mm × 15 mm)	Sigma Aldrich	Sigma Aldrich Cat# P5481
10 mL syringe	Pic Solution	Picsolution Cat# 02076120090300
Hypodermic needle 18G	Pic Solution	Picsolution Cat# 02070520300800
Falcon Cell Strainers 70 μm	Falcon	Falcon Cat# 352350
5 mL polystyrene round bottom FACS tubes	Falcon	Falcon Cat# 51976
15 mL tubes	Falcon	Falcon Cat# 352096
50 mL tubes	Falcon	Falcon Cat# 352070

MATERIALS AND EQUIPMENT

FACS Buffer		
Reagent	Final concentration	Amount
FBS	1%	5 mL
Na_2EDTA (0.5 M)	2 mM	2 mL
NaN_3 (10% wt/v)	0.05%	2.50 mL
PBS	n/a	490.5 mL
Total	**n/a**	**500 mL**
[Store at 4°C up to one month]		

Alternatives: 1% FBS can be substituted with 1% BSA.

Note: For cell sorting experiments that involve *in vitro* culture, FACS buffer should be prepared under sterile conditions, filtered through 0.2 μm filter and should not contain NaN_3.

ACK Buffer		
Reagent	Final concentration	Amount
NH_4Cl	0.15 M	8.02 g
$KHCO_3$	10 mM	1 g
Na_2 EDTA (0.5M)	0.1 mM	200 μL
double-distilled H_2O (ddH_2O)	n/a	1 L
Total	**n/a**	**1 L**

Note: Adjust pH to 7.2 and filter through 0.2 μm filter. Keep it sterile at 20°C–25°C.

Avertin		
Reagent	Final concentration	Amount
2,2,2-tribromoethanol	45 mM	2.5 g
2-methyl-2-butanol	2.5%	5 mL
ddH_2O	n/a	195 mL
Total	**n/a**	**200 mL**

Note: Dissolve 2.5 g of tribromoethanol into 5 mL of 2-methyl-2-butanol by warming to 55°C with gentle swirling. Add slowly (dropwise) the 5 mL of dissolved tribromoethanol into 200 mL of sterile ddH_2O. Filter through 0.2 μm filter and store at 4°C for up to 4 months.

Percoll 36%		
Reagent	Final concentration	Amount
Percoll (100%)	36%	7.2 mL
PBS 10×	1×	800 μL
RPMI	n/a	12 mL
Total	**n/a**	**20 mL**

Note: Mix 7.2 mL of Percoll (100%) with 0.8 mL of PBS (10×) to achieve a Iso-osmolar Percoll solution. Then, mix this solution with 12 mL of plain RPMI to achieve a final Percoll density of 36%.

STEP-BY-STEP METHOD DETAILS

Harvesting the liver for *ex vivo* LNPCs isolation (method #1)

Timing: 10 min

1. Euthanize the mouse according to the authorized ethical guidelines.
2. Sanitize the mouse abdomen surface with 70% ethanol (Figure 1A).
3. Cut through the peritoneum (Figure 1B), by first performing a vertical cut from the lower end of the abdomen to the ribcage, and then performing horizontal cuts at the mid-section of the abdomen to both sides until the organs of the mouse are exposed. Pay attention not to damage any organs and gently expose the liver.
4. Push the intestine right-hand sideward by using the blunt end of a forceps to get access to the liver (Figure 1C).
5. Load a 10 mL syringe with 10 mL of PBS and a 27G needle.
6. Carefully insert the needle in the inferior vena cava and perfuse 2 mL until the organ starts to swell (Figure 1D).
7. Cut the portal vein with scissors, paying attention not to damage the liver (Figure 1E).
8. Complete the perfusion until 10 mL of PBS have been injected in about 2 min (Figures 1F and 1G).

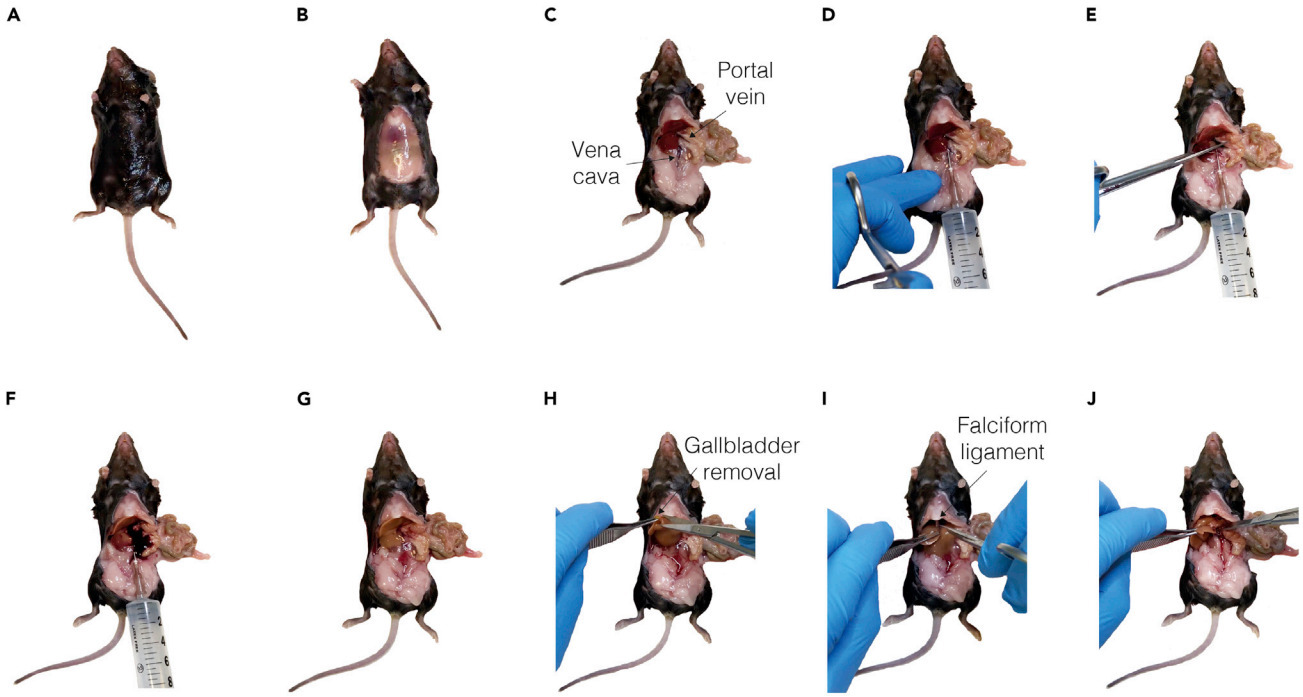

Figure 1. Surgical preparation and harvesting of the liver

(A and B) (A) Euthanized mouse is sanitized with 70% ethanol and the peritoneal cavity is exposed (B).

(C) The intestine is pushed sideward in order to expose the portal vein and the vena cava.

(D and E) (D) Liver is perfused through the vena cava. After 2 mL of perfusion the liver vasculature becomes engorged (E); at this moment the portal vein is cut.

(F and G)(F) Liver is perfused with 10 mL of PBS and the organ clears immediately turning into a light brown color (G).

(H–J) (H) The gallbladder is removed, and the hepatic ligaments are cut (I) before the complete harvest of the liver (J).

9. Remove the gallbladder (Figure 1H) and cut the liver ligaments connecting the liver to surrounding tissues (Figures 1I and 1J).
10. Collect the liver in ice-cold plain RPMI until next step.

Note: the following protocol has been adapted from (Blériot et al., 2020) and optimized for the digestion of half a liver. However, the volume of the digestion medium can be adjusted according to the amount of liver to be processed. If KC isolation is performed to be followed by a cell culture experiment, both mouse surgery and organ processing should be performed under sterile conditions (e.g., under a cell culture hood).

Processing the liver for *ex vivo* LNPCs isolation

Timing: 60 min

11. Transfer the liver to a 60 mm Petri dish and use blunt-end scissors to mince it in 1–2 mm^3 small pieces.
12. Transfer the liver pieces in a 50 mL Falcon tube containing 10 mL of pre-warmed *ex vivo* digestion medium (see above).
13. Incubate at 37°C for 30 min.
14. Vortex the sample every 10 min at 2000 rpm.
15. After incubation, gently homogenize the digested liver pieces by forcing them 7–8 times into a 10 mL syringe loaded with a 18G needle.
16. Filter the homogenized liver solution through a 70 μm cell strainer in a new 50 mL Falcon tube and wash the filter with 10 mL of plain RPMI.

17. Centrifuge at 50 rcf for 3 min.
18. Recover the aqueous phase and transfer it in a new 50 mL Falcon tube. Discard the pellet containing dead hepatocytes and debris.
19. Centrifuge at 400 rcf for 5 min.
20. After this centrifugation step the pellet contains target LNPCs. Discard the top aqueous phase.
21. Lyse red blood cells resuspending the cell pellet with 2 mL of ACK and incubate for 30 s at 20°C–25°C.
22. Add 20 mL of plain RPMI to restore osmolarity and centrifuge at 400 rcf for 5 min.
23. Resuspend the cell sediment in 10 mL of ice-cold plain RPMI. From now on all the steps are done at 4°C.
24. Filter cell suspension through a 70 μm cell strainer in a new 50 mL Falcon tube.
25. Mix an aliquot of the cell suspension cell suspension 1:2 in trypan blue and determine cell count of viable cells with a Neubauer chamber.
26. Proceed with the desired downstream application (steps 49 through 53 for cell culture or steps 54 through 63 for flow cytometry and cell sorting).

⚠ CRITICAL: cell sediment in step 20 is loose, pay attention if a vacuum aspiration system is used to discard the supernatant.

Optional: at steps 20–21 there might be clogs in the cell pellet, cut the tip of a 1 mL pipette to facilitate the resuspension process.

In situ liver digestion for the isolation of hepatocytes and LNPCs (method #2)

Timing: 90 min

The following step-by-step section describe an alternative, longer but more gentle protocol for the isolation of LNPCs. This method also allows the simultaneous recovery of hepatocytes and replaces steps 1 through 28.

27. Prewarm the Liver perfusion medium and liver digestion medium in the water bath at 37°C (see Figure 2 for pump configuration).
28. Prime and wash the pump tubes with prewarmed HBSS. Pump flux during the procedure should be set at 5 mL/min.
29. Inject the mouse intravenously with 200 μL of heparin solution (100 Units in PBS) to avoid coagulation issues that can potentially interfere with the perfusion and with the digestion.
30. Anesthetize the mouse with Avertin (600 μL for a mouse of 25 grams) at 37°C intraperitoneally.
31. Spread the mouse with 70% EtOH and cut through the peritoneum, by first performing a vertical cut from the lower end of the abdomen to the ribcage, and then performing horizontal cuts at the mid-section of the abdomen to both sides und the organs of the mouse are exposed.
32. Gently push the intestine right-hand sideward by using the blunt end of a forceps to get access to the liver.
33. Canulate the inferior vena Cava (25G needle).

Optional: Place an open silk suture around inferior vena Cava to help the canula to be in place.

34. Start the perfusion with Liver Perfusion Medium for 3 min after severing the portal vein. This medium is meant to clean the liver from blood and to start the loosening of cell-to-cell contacts.
35. Perfuse with warm Liver digestion medium for 12 min and pay attention that no air bubbles should be introduced during the entire process. After 8 min, start checking the liver consistency with a wet cotton applicator. Digested liver loses elasticity and assumes a reticulated appearance.
36. Once the liver is completely digested, remove the gallbladder, and cut the liver ligaments connecting the liver to the surrounding tissue.

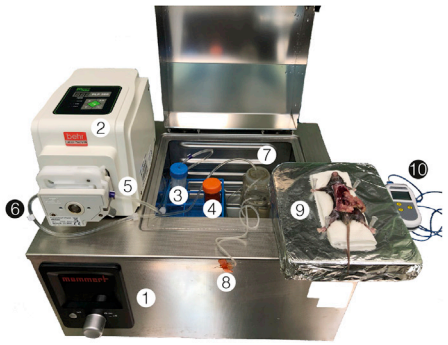

Figure 2. Configuration of the equipment for the in situ digestion of the liver

37. Transfer liver into a 60 mm Petri dish with Hepatocyte Wash Medium and carefully decapsulate the organ using surgical forceps (for more information see the online Methods video S1).

38. Gently shake the liver while holding it with a tweezer. This procedure allows digested cells (Hepatocytes and LNPCs) to be released in the Hepatocyte wash medium (online Methods video S1).
39. Transfer the obtained cell suspension to a 50 mL falcon tube and reach 50 mL of volume with the Hepatocyte Wash Medium.
40. Centrifuge at 20 rcf for 3 min.

Note that at the end of each 20 rcf centrifugation the pellet is enriched by Hepatocytes, while soluble fraction by LNPCs.

41. At this step the pellet is composed mainly by Hepatocytes (HC) and the soluble fraction by LNPCs.
42. Repeat steps 40 to 41.
43. Collect separately HC and LNPC and resuspend each fraction in separate 15 mL tubes containing 10 mL of 36% Percoll solution and gently mix.
44. Centrifuge 2000 rpm for 20 min without brake.

Note: the purpose of the Percoll separation is to get rid of cellular and tissue debris to obtain a clearer cell preparation.

45. At the end of the centrifugation, discard the Percoll soluble fraction (containing cellular debris) and resuspend each cell pellet in the appropriate medium (LNPC fraction should be suspended in RPMI, hepatocytes in hepatocyte medium).
46. Filter LNPCs fraction through a 40 μm cell strainer.

Optional: lyse red blood cells with 2 mL of ACK for 30 seconds at 25°C, centrifuge and resuspend the cell pellet in 10 mL of RPMI.

47. Mix an aliquot of each cell suspension 1:2 in trypan blue and determine cell count of viable cells with a Neubauer chamber.
48. Proceed with the desired downstream application (step from 49 to 53 for cell culture or step from 54 to 66 for flow cytometry and cell sorting).

⚠ CRITICAL: make sure that the tube carrying the digestion medium to the liver is at 37°C when reaches the vena cava. If the solution is at lower temperature, enzymatic activity might be suboptimal.

Note: Hepatocytes are extremely fragile cells and should be maintained at 37°C during the entire procedure to maximize cell vitality.

Isolating KCs by cell adhesion

Timing: 3 h

49. Prepare the culture medium supplementing plain RPMI with 10% FBS and 100 U/mL Penicillin/Streptomycin.
50. Resuspend LNPCs to a density of 1–3×10^7/mL in culture medium.
51. Plate 1×10^7 LNPCs per well in a 6-well culture plate.
52. Incubate for 2 h at 37°C with 5% CO_2.
53. Remove cell debris and non-adherent cells by gently washing adherent cells with cold PBS.

Note: more than 95% of adhering cells are Kupffer cells (Li et al., 2014). To maintain Kupffer cell differentiation, add 50 ng/mL of recombinant M-CSF (R&D system cat #416-ML) in the culture medium.

Staining KCs from isolated LNPCs for FACS sorting

Timing: 2 h

54. Transfer LNPCs in FACS tubes at 5×10^7/mL.

Note that in order to sort 10^6 KCs, a good starting point is 2×10^7 of total LNPCs.

55. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
56. Incubate with 200 µL of anti-CD16/CD32 (5 µg/mL in FACS buffer) for 15 min at 4°C.
57. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
58. Incubate with 300 µL of antibody mix for 40 min at 4°C.
59. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
60. Incubate with 200 µL of DAPI (5 µg/mL in FACS buffer) for 5 min at 4°C.
61. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
62. Resuspend in FACS buffer at 5×10^6 cells per mL.
63. Sort target cells at 4°C using a 100 µm nozzle.

⚠ CRITICAL: sheath pressure should not exceed 20 psi to preserve cell viability.

Recommended: To enhance viability of sorted cells, collection FACS tubes pre-coated with 5% BSA or 5% FBS for 14–16 h at 4°C to neutralize the electrostatic charges of polystyrene tubes should be used. Otherwise, polypropylene tubes can be used since have neutral charge.

Note: If cells are sorted for functional downstream applications (e.g., cell culture), do not supplement FACS buffer with NaN_3. To further improve cell viability, collection media should contain at least 10% FBS.

Antibody mix

Antigen	Fluorophore	Clone	Final concentration (µg/mL)
CD31	BV605	390	5
CD11b	BV650	M1/70	1
CD45	FITC	30F11	2
I-A/I-E (MHCII)	PerCP-Cy5.5	M5/114.15.2	1
ESAM	PE	1G8/EASAM	1

(*Continued on next page*)

Continued

Antigen	Fluorophore	Clone	Final concentration (μg/mL)
CD3 (lineage)	PE-CF594	UCHT1	3
CD19 (lineage)	PE-CF594	1D3	1
Ly6G (lineage)	PE-CF594	1A8	1
CD49b (Lineage)	PE-CF594	DX5	2
TIM-4	PE-Cy7	RMT4-54	1
CD206	APC	C068C2	2
F4/80	APC-Cy7	BM8	3

Optional: brilliant stain buffer can be used in place of FACS buffer to minimize Brilliant Violet dyes staining artifacts.

Alternatives: fixable Live/Dead (L/D) dyes can be used to gate live cells instead of DAPI. In this case, at the end of LNPC isolation procedure, cells must be washed in plain PBS and Live/Dead staining should be performed at 20°C–25°C for 20 minutes.

Note: sessile LNPCs (e.g., KCs and Liver Sinusoidal Endothelial cells - LSECs), once extracted from the liver during the digestion process, are slightly auto fluorescent and have more proteins on their membrane surface. For this reason, since fixable dyes stain amine reactive groups, the use of Live/Dead might be suboptimal in separating dead cells. DAPI – being impermeable to the membrane of live cells – is more appropriate when possible (see Figure 3).

EXPECTED OUTCOMES

Usually, this protocol allows to recover 2–3*10^7 of total LNPCs and about 1.5–2*10^6 KCs from one healthy liver of a 7–9 week-old mouse. Considering the LNPC fraction, we do not find major differences in terms of absolute numbers when comparing the *ex vivo* and *in situ* digestion protocols.

LIMITATIONS

The *ex vivo* digestion combined with the mechanical dissociation of the liver has been designed to obtain reproducible whole LNPC preparations in a reasonable amount of time. This protocol is easily scalable, allowing the isolation of LNPC from several liver samples at the same time. The viability of recovered cells is greater than 94%, which makes the single cell suspensions obtained with this procedure suitable for functional and phenotypical analysis by multicolor flow cytometry, cell sorting and RNAseq (see Figure 4 for a suggested flow cytometry data analysis).

Although this protocol gives highly reproducible preparations, it is worth noting that the execution of critical steps (e.g., the homogenization step with the 18G syringe) should be performed consistently to minimize inter-user variability.

However, because of the mechanical force being used, the quick LNPC preparation does not permit the isolation of hepatocytes and enables only the partial recovery of other non-parenchymal cells like Hepatic Stellate Cells (HSC). For this purpose, the *in situ* intravenous injection of the enzymatic medium has the main advantage to completely digest the liver tissue without the need for mechanical disruption (Guidotti et al., 2015). This passage is essential to preserve hepatocytes integrity since those cells are extremely fragile and are lost during the quick *ex vivo* procedure.

In situ digestion might be preferred if hepatocytes or stellate cells must be isolated contextually and/or if a gentler preparation is demanded by the experimental design. Here, the main limitation is represented by the need of dedicated equipment (e.g., the peristaltic pump) and by the laboriousness of the *in situ* digestion itself, which makes this protocol not suitable for the processing of a large quantity of samples.

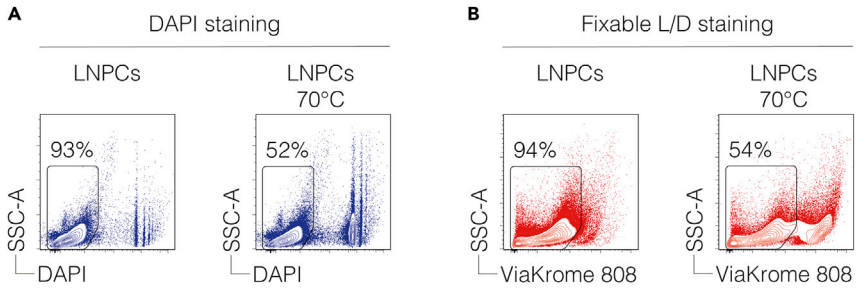

Figure 3. Different strategies to gate live cells

Representative dot plots of LNPC preparation in which live cells have been gated with DAPI staining (A) or with fixable Live/Dead staining (B). In separate samples, LNPCs have been mixed in 1:1 ratio with LNPCs placed at 70°C for 5 min to show actual dead cell population.

TROUBLESHOOTING

Problem 1

Liver perfusion during the *ex vivo* protocol at step 6 does not efficiently remove blood from the liver.

Potential solution

Failure in performing an effective liver perfusion leads to an irreversible contamination of LNPC preparation with non-resident, blood-borne cells. Therefore, this step is critical since it can be a potential source of sample-to-sample variability in term of LNPC composition and preparation.

To reach 100% success in the liver perfusion, insert the 27G needle of a PBS-charged 10 mL syringe in the inferior vena cava paying particular attention to be inside the vasculature. Start injecting 1–2 mL of PBS into the vena cava without cutting the portal vein at this step. Only once the liver vasculature becomes engorged, cut the portal vein with surgical scissors. This will make the hepatic perfusion more effective and the liver will clear immediately turning into a light brown color. At this point, complete the perfusion with the remaining PBS.

For more details, please refer to Figure 1.

Problem 2

Liver *in situ* digestion is suboptimal (step 36).

Potential solution

Enzymes need a controlled, stable temperature of about 37.5°C to perform efficiently. For this reason, it is mandatory that the output pipe carrying the digestion mix reaches the vena cava at the correct temperature (see Figure 2). To avoid unwarranted cooling of the digestion mix during its way from the thermostatic bath to the vena cava, one strategy might be to rise the temperature of the water bath up to 39°C–40°C. However, consider that higher temperatures can damage the enzymatic activity.

If necessary, shortening the output pipe tube might also prevent excessive dissipation of the heat. Alternatively, place the output pipe in the water bath again in order to re-warm the buffer (see Figure 2, label #7).

Checking the temperature of the digestion mix at the end of the tube with a digital thermometer is also recommended.

Problem 3

After the *ex vivo* digestion, at step 15, liver pieces are still too big and cannot pass through the 18G needle for the homogenization step.

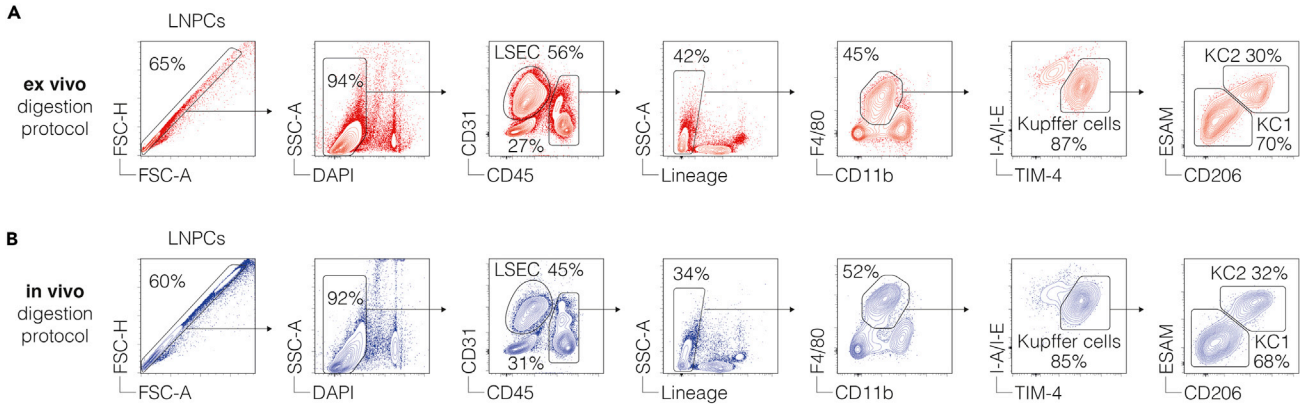

Figure 4. Gating strategy for Kupffer cell and LSEC identification in LNPC samples isolated from C57BL/6 mice

Representative dot plots of LNPC preparation obtained with the quick ex vivo digestion protocol (A) or with the in situ digestion protocol (B). Single, live cells can be divided in LSEC ($CD45^-$, $CD31^+$) and in hepatic leukocytes (which are $CD45^+$). Once discarded the lineage positive cells ($CD3^+$, $CD19^+$, $CD49^+$, $Ly6G^+$), the $F4/80^+CD11b^{int}$ macrophage population is pre-gated to further distinguish capsular macrophages ($TIM4^-$, $I\text{-}A/I\text{-}E^+$) from Kupffer cells ($TIM4^+$, $I\text{-}A/I\text{-}E^{int}$). Kupffer cells (KCs) can be divided in KC1 ($CD206^-$, $ESAM^-$) and in KC2 ($CD206^+$, $ESAM^+$) subpopulations.

Potential solution

Make sure to cut the liver pieces as small as possible before starting the enzymatic digestion. At the end of the mechanical disruption the liver should have a pulp consistency.

Alternatively, if liver pieces are still too big, use a serological 10 mL pipette and flush up and down for about 10 times before homogenizing with the syringe.

Please note that the homogenization through the 18G needle is another critical step and should be done consistently (e.g., same number of homogenizations for each sample) in order to minimize sample-to-sample variations.

Problem 4

The recovery of LNPC is low (step 25).

Potential solution

If cell recovery is below $2*10^7$ LNPC per liver, make sure that the enzymatic digestion has been performed for the correct amount of time (e.g., 30 min) at the correct temperature (e.g., 37°C). Alternatively, the homogenization step might not have been performed properly. In the latter case, make sure to move the plunger up and down for at least 7–10 times. At the end of this step no more liver pieces should be evident in the cell suspension. Moreover, after the filtration of the homogenized liver solution, limited debris should be present on the surface of the cell strainer.

Problem 5

The vitality of LNPC is below 90% (step 25).

Potential solution

Possible cell-damaging steps are represented by the liver homogenization and by the red blood cell lysis. Avoid excessive (e.g., more than 15) homogenizations through the 18G needle and be gentle in pushing/pulling the plunger in order to avoid incorporating air bubbles.

Otherwise, do not exceed 60 s incubation with the ACK solution.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol did not generate data sets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100831.

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AUTHOR CONTRIBUTIONS

All authors contributed to setting up or optimizing the protocols and in writing the manuscript.

DECLARATION OF INTERESTS

M.I. participates in advisory boards/consultancies for Gilead Sciences, Roche, Third Rock Ventures, Amgen, Allovir. M.I. is an inventor on patents filed, owned, and managed by San Raffaele Scientific Institute, Vita-Salute San Raffaele University and Telethon Foundation on technology related to work discussed in this manuscript (WO2020/016434, WO2020/016427, WO2020/030781, WO2020/234483, EU patent applications n. 19211249.8 and n 20156716.1, and UK patent application n. 1907493.9). F.G. is a member of the Immunity advisory board.

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