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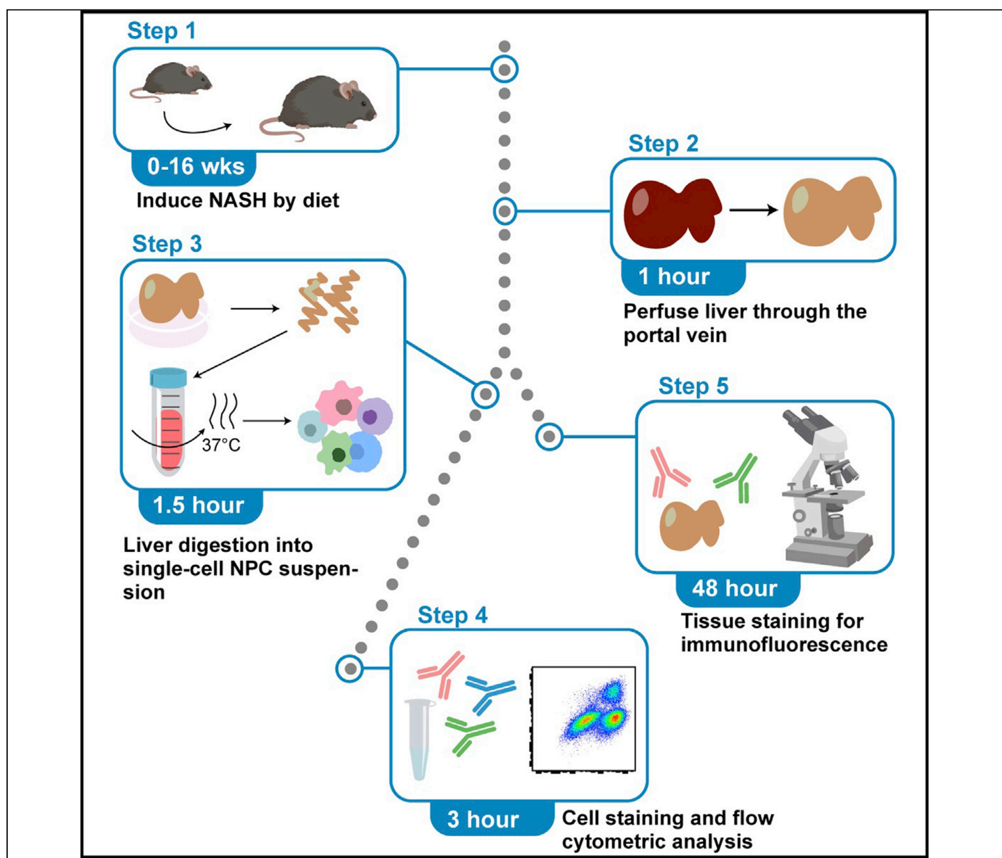
Protocol

Comprehensive analysis of liver macrophage composition by flow cytometry and immunofluorescence in murine NASH



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Highlights

Protocol for the isolation and analysis of hepatic macrophages in fatty liver disease

Review of the primary macrophage subsets present in NASH

Outline of macrophage analysis by flow cytometry and tissue imaging

Description of a reproducible and efficient portal vein cannulation technique

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Protocol

Comprehensive analysis of liver macrophage composition by flow cytometry and immunofluorescence in murine NASH

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SUMMARY

Recently, it has become evident that macrophage diversity increases in the liver during the pathogenesis of non-alcoholic steatohepatitis (NASH). Here, we provide a detailed protocol for the analysis of liver macrophage subsets in mice with non-alcoholic fatty liver disease (NAFLD) and early NASH using flow cytometry and immunofluorescence (IF). These methods can be used to assess the composition and localization of macrophage subsets during NASH.
For complete details on the use and execution of this protocol, please refer to Daemen et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for isolation of liver macrophages of mice with established NASH. However, we have also used this protocol for isolation and analysis of liver macrophages of healthy, standard diet-fed mice and mice from a variety of genetic backgrounds. As antibodies to CCR2 and CX3CR1 do not work well for flow cytometry following liver digestion the use of *Ccr2*-GFP or *Cx3cr1*-GFP reporter mice can be employed to identify *Ccr2/Cx3cr1*-high liver macrophages. Thus, we will describe here the use of this protocol for both *Ccr2/Cx3cr1*-GFP and wild-type mice.

Induction of diet-induced NASH

Timing: 0–16 weeks

1. Co-house same sex mice in specific pathogen free animal facilities with regular drinking water. If comparing wild type (WT) to knockout (KO) or transgenic mice it is critical to ensure mixing of genotypes in cages. Littermate controls should also be utilized.
2. Induce NASH in 8-week-old mice by replacing regular chow with a NASH diet, i.e., the fructose, cholesterol, palmitate (FPC) diet or a diet with similar composition (i.e., AMLN), for at least 16 weeks. Provide fresh NASH chow weekly. Although female or male mice can be used the NASH phenotypes are more severe in male mice.

Note: Duration of feeding may need to be optimized depending on the experimental question, mouse strain and specific NASH diet.

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3. Monitor body weight and record at least every 4 weeks.
4. If needed, perform glucose and insulin tolerance tests 1–2 weeks prior to sacrifice.
5. Sacrifice animals and harvest liver at the end of 16 weeks NASH diet.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal CD11b-APC-Cy7 (clone M1/70) - 1:100	BioLegend	Cat# 101226
Rat monoclonal CD45-BUV395 (clone 30-F11)-1:100	BD Biosciences	Cat# 564279
Rat monoclonal F4/80-AF647 (clone BM8)- 1:100	BioLegend	Cat# 123122
Rat monoclonal Ly6C-FITC (clone RB6-8C5)- 1:100	BioLegend	Cat# 108406
Rat monoclonal MHCII-BV605 (clone M5/114.15.2) – 1:300	BioLegend	Cat# 107639
Rat monoclonal TIM4-BV421 (clone 21H12)- 1:500	BD Biosciences	Cat# 742773
Rat monoclonal VSIG4-PeCy7 (clone NLA14)- 1:200	Invitrogen-Thermo Fisher	Cat# 25-5752-82
Rat monoclonal CLEC2-PE (clone 17D9) – 1:100	BioLegend	Cat# 146104
Armenian Hamster monoclonal CD11c-BV711 (clone N418)- 1:100	BioLegend	Cat# 117349
Chicken polyclonal GFP – unconjugated – 1:500	Abcam	Cat# ab13970
Rat monoclonal F4/80 - unconjugated (clone BM8) – 1:200	Invitrogen-Thermo Fisher	Cat# 13-4801-85
Rat monoclonal CD63 - unconjugated (clone NGV-4) – 1:25	BioLegend	Cat# 143902
Rabbit monoclonal Gpnmb - unconjugated (clone EPR18226-147) 1:50	Abcam	Cat# 188222
Donkey anti-chicken AF488 – 1:500	Jackson ImmunoResearch	Cat# 703-545-155
Goat polyclonal anti-CLEC4F-unconjugated – 1:100	R&D Systems	Cat# AF2784
Donkey anti-rat AF594 – 1:200	Invitrogen-Thermo Fisher	Cat# A21209
Donkey anti-rabbit AF647 – 1:500	Jackson ImmunoResearch	Cat# 711-605-152
Goat anti-rabbit AF594 – 1:500	Jackson ImmunoResearch	Cat# 111-585-003
Donkey anti-goat AF647 – 1:500	Jackson ImmunoResearch	Cat# 705-605-003
Chemicals, peptides, and recombinant proteins		
Collagenase A	Sigma	Cat# C5138
DNase I	Sigma	Cat# 10104159001
DMEM	Sigma	Cat# D5671
L-glutamine	Sigma	Cat# G7513
Penicillin-Streptomycin (P/S)	Gibco	Cat# 15140-122
Fetal Bovine Serum (FBS)	Sigma	Cat# F2442
Sodium Pyruvate	Corning	Cat# 25-000-CI
Bovine Serum Albumin (BSA)	Lampire Biological Laboratories	Cat # 7500804
EDTA	Corning	Cat# 46-034-CI
ACK lysing buffer	Gibco	Cat# A10492-01
Zombie Aqua	BioLegend	Cat# 423101
Fc Block	BD Biosciences	Cat# 553143
10% Neutral buffered formalin	Sigma	Cat# HT5011-1CS
Sucrose	IBI Scientific	Cat# 57-50-1
Tissue-Tek O.C.T.	Sakura	Cat# 4583

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Triton X-100	Sigma	Cat# X100
Hoechst 33342	Thermo Fisher	Cat# 62249
ProLong Gold Antifade reagent	Thermo Fisher	Cat# P36934
Phosphate buffered saline (PBS)	Sigma	Cat# P3813
Experimental models: organisms/strains		
Mouse C57BL/6j	Jackson Laboratory	Cat# 000664
Mouse *Ccr2*-GFP	Dr. Kory Lavine	N/A
Software and algorithms		
FlowJo v10.7.1	FlowJo	https://www.flowjo.com/
Becton Dickinson X20 Flow Cytometer	BD Biosciences	https://www.bdbiosciences.com/en-us/applications/research-applications/multicolor-flow-cytometry
Other		
NASH diet	Envigo	Cat# TD.190142
Fisherbrand Variable-Flow Peristaltic Pump	Fisher Scientific	Cat# 13-876-2
IV Catheter 24 *g* × $^{3}/_{4}$''	Terumo	Cat# SROX2419V
Ethilon Nylon Suture 4-0	Ethicon	Cat# 662G
Extension tubing set 42''	Baxter	ACT5612
Cotton tips	N/A	N/A
Petridish 100 mm Not TC-treated	Corning	Cat# 351029
Personna Single Edge Razor blades	N/A	94-120
70 μm Nylon Cell strainer	Corning	Cat# 431751
TPP PES 0.22 μm filter media bottle (500 mL)	MilliporeSigma	Cat# Z760900

MATERIALS AND EQUIPMENT

Collagenase A stock

Add 13.3 mL DMEM to 1 *g* collagenase A powder in the supplied vial to achieve concentration of 75 mg/mL. Vortex to mix and incubate at 37°C water bath for 30–40 min to promote dissolution. Store as 1 mL aliquots for up to 12 months at −20°C and avoid repeated freeze/thaw cycles.

DNaseI stock

Add 2 mL DI water to reconstitute 100 mg DNaseI powder in the supplied vial. Transfer solution to new 50 mL conical tube. Rinse the vial with 2 mL of DI water and transfer the rinse to the same conical tube. Add 16 mL DI water to the conical tube to achieve 5 mg/mL stock concentration. Vortex to mix and store as 1 mL aliquots for up to 12 months at −20°C. Avoid repeated freeze/thaw cycles as this will reduce enzyme activity.

Zombie Aqua stock

Prepare according to manufacturer's instruction. Briefly, reconstitute 1 vial of lyophilized reagent with 100 μL DMSO (included in the kit). Vortex to mix. Store as 10 μL aliquots up to 6 months at −20°C and protect from light.

30% Sucrose solution

Dissolve 150 *g* of sucrose in 200 mL of autoclaved PBS with a magnetic stir bar. Once dissolved, add more PBS until it reaches the 500 mL line. Aliquot into 50 mL conical tubes for working solutions. Store at 20°C–24°C for up to 6 months.

Complete DMEM

Reagent	Final concentration	Amount
DMEM	n/a	435 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Heat-inactivated FBS	10% (v/v)	50 mL
P/S (10 ,000U/mL)	1% (v/v)	5 mL
Sodium pyruvate (100 mM)	1% (v/v)	5 mL
L-glutamine solution (100 mM)	1% (v/v)	5 mL
Total		**500 mL**

Prepare in a sterile environment and filter through 0.22 μm PES membrane. Store at 4°C, in the dark, for up to 6 months.

Digestion Buffer (for ~1–2 *g* of liver)

Reagent	Final concentration	Amount
DMEM	n/a	15 mL
Collagenase A (75 mg/mL)	0.75mg/mL	150 μL
DNaseI (5mg/mL)	50μg/mL	150 μL
Total		**15 mL**

Always prepare fresh digestion buffer on the day of harvest. Digestion buffer can be kept at room temperature during the harvest (1–2 h).

FACS buffer

Reagent	Final concentration	Amount
PBS	n/a	498 mL
BSA	n/a	2.5 *g*
0.5 M EDTA	2 mM	2 mL
Total		**500 mL**

Prepare in a sterile environment and filter through 0.22 μm PES membrane. Store at 4°C for up to 6 months.

Blocking buffer for immunofluorescence

Reagent	Final concentration	Amount
PBS	n/a	50 mL
BSA	1%	0.5 *g*
Triton ×100	0.3% (v/v)	150 μL
Total		**50 mL**

Always prepare freshly on the day of staining for optimal results. Store at 4°C for up to 48 h if needed for next-day secondary antibody staining.

Zombie Aqua

Reagent	Final concentration	Amount
Zombie Aqua stock	1:250	0.4 μL
PBS	n/a	100 μL
Total		**100 μL**

Prepare fresh for staining. Protect from light and keep on ice.

Fc Block

Reagent	Final concentration	Amount
Fc Block	1:10	1 μL
FACS buffer	n/a	9 μL
Total		**10 μL**

Prepare fresh for staining. Keep on ice.

Flow cytometry antibody cocktail		
Reagent	Final concentration	Amount
FACS buffer	n/a	100 µL
Conjugated antibodies	1:100–1:500	0.2–1 µL
Total		100 µL

Prepare fresh for staining. Protect from light and keep on ice.

STEP-BY-STEP METHOD DETAILS

Assemble perfusion pump and harvest table

Timing: 10 min

1. Cover the surface of a plastic tray with a sheet of absorbent bench paper and then layer with two paper towels.
2. Assemble tools including: surgical scissor, tweezer, suture, catheter, 2 cotton tips, razor blade, petri dishes, a stack of paper towels, pump, tubing for the pump, a bottle of PBS, a beaker, 70% ethanol, and biohazard bags for waste and carcass disposal.
3. Attach the tubing to the peristaltic pump. Place the inlet end of the tubing into the bottle of PBS and the outlet end in a beaker. Start the pump until the air is cleared from the line and the entire tubing is filled with PBS (Figure 1A).

Set up reagents for harvest

Timing: 10–30 min

4. Prepare complete DMEM and FACS buffer (as described in materials and equipment).
5. Prepare and label 3 sets of 50 mL conical tubes for each liver sample.
6. Prepare digestion buffer (as described in materials and equipment) in the first set of 50 conical tubes.
7. If collecting liver samples for IF, prepare 500 µL formalin solution in 1.5 mL Eppendorf tubes.

Perfusion of mouse liver

Timing: 15 min/mouse

Proper perfusion of the liver is essential for maximum yield of liver macrophages for downstream analyses including, but not limited to, flow cytometry and immunofluorescence.

8. Euthanize the mouse using carbon dioxide and subsequently perform cervical dislocation.
9. Weigh and record the body weight of the mouse using a balance.
10. Place the mouse in a prone position and sterilize the abdomen with 70% ethanol.
11. Use a tweezer to lift up the skin near the genitals and make an incision with scissors. Pull the skin up to expose the body cavity membrane.
12. Carefully cut open the peritoneal cavity membrane and move the small intestines aside to fully expose the liver.
13. Locate the portal vein underneath the liver. Place a suture underneath the vein and secure a half-knot (Figure 1A).
14. Cannulate the portal vein with the catheter until its tip reaches the branching point of the vein and passes the half-knotted suture. Tighten the knot to secure the catheter and remove the needle. A successful cannulation will result in a flash of blood into the catheter (Figure 1B; Methods video S1).

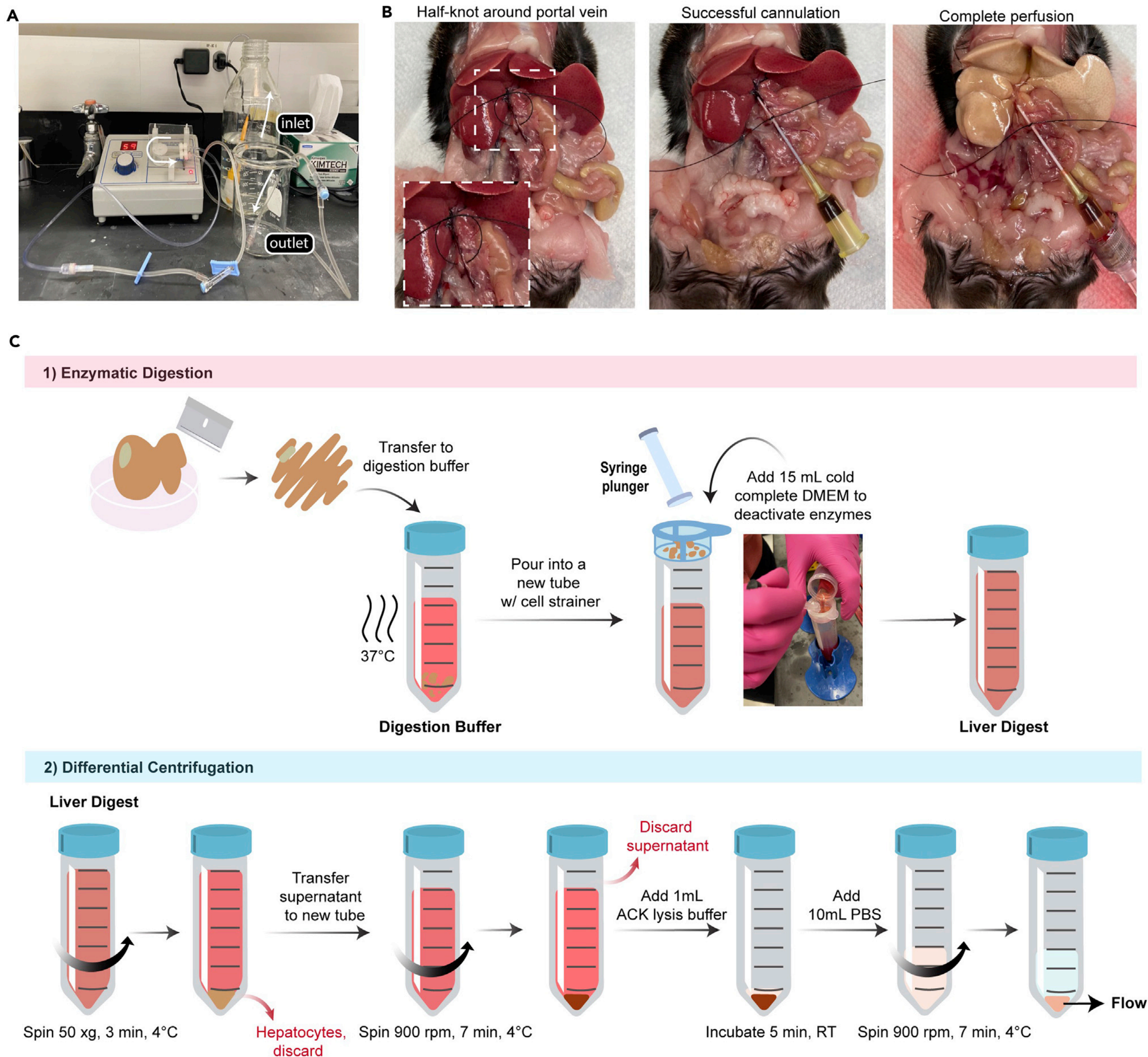

Figure 1. Illustrations for murine portal vein cannulation and subsequent processing into single-cell suspension for flow cytometry

(A) Set up of the variable-flow peristaltic pump for liver perfusion. The inlet of the tubing is placed in a bottle of autoclaved PBS with the outlet end of the tubing placed in a beaker until the catheter is attached and inserted into the portal vein. Arrows indicate the flow of PBS from inlet to outlet.

(B) Step-by-step procedure (left to right) for securing a half-knot around the portal vein, identifying the "flash" for successful portal cannulation, and completing liver perfusion.

(C) Schematic diagrams of enzymatic digestion and centrifugation protocols required to isolate non-parenchymal cells (including macrophages) from the liver.

Optional: Blood can be collected at this point from portal vein and/or IVC using 1mL syringe with a 21G × 1 $^{1}/_{2}$ needle. Washing the tube with EDTA can be used to prevent clotting in the tube.

15. Perfuse the portal vein with PBS using the peristaltic pump at a rate of 5 mL/min. After attaching the tubing onto the catheter immediately cut the inferior vena cava (IVC) below the liver to release blood and PBS from the liver. The liver should start to appear pale and beige in color.

⚠ CRITICAL: Start the flow of PBS prior to inserting the tubing onto the catheter to prevent the introduction of air bubbles into the liver vasculature.

16. Increase the flow rate to 10 mL/min. Apply gentle pressure on the IVC periodically and massage the liver with cotton tipped wood applicator to promote clearance of sinusoidal blood. Perfuse for a minimum of 3 min, or until the liver is completely pale (Figure 1A). The total perfusion volume of PBS should be about 30 mL.
17. Remove catheter, suture, and gallbladder from the liver. Resect the liver from the body cavity (Figure 1A).
18. Weigh and record total liver weight.
19. Resect specific lobes of the liver for downstream analyses: for example, left medial and lateral lobe for flow cytometry and right lateral lobe for immunofluorescence. For flow cytometry between 0.5- 1 gm of tissue is recommended. (Further processing for immunofluorescence: see section Immunofluorescence of liver tissue)

Optional: Depending on experimental question, the remaining liver tissue can be processed for other analyses including, but not limited to, histology, RNA, protein, or triglyceride quantification. For histology, submerge tissue into 500–750 μL formalin and incubate at 4°C for 24 h. Transfer tissue into 70% ethanol for subsequent paraffin embedding. For RNA, protein and triglyceride quantification, flash freeze tissue in liquid nitrogen and transfer to −80°C.

Note: It is a lab-dependent choice to decide which lobe to use for specific analysis, but it is encouraged to maintain consistency across different experimental groups and different experiments.

Enzymatic digestion and dissociation of liver tissue into single-cell suspension

Timing: 1.5 h

This step describes the process of collagenase A digestion and differential centrifugation of liver tissue to prepare single-cell suspension for flow cytometry (Figure 1B).

Note: The cell suspension obtained after this step can also be used for alternative purposes, for example flow sorting, single-RNA sequencing, and/or *ex vivo* function assays.

20. Weigh and record the liver tissue dedicated for flow cytometry.
21. Place the liver in a petri dish and mince finely with a razor blade.
22. Transfer the minced liver into a prepared digestion buffer and keep on ice until the end of the tissue harvest.
23. At the end of the harvest, warm up all samples in a 37°C water bath for 5 min.
24. Place the liver mixture on a shaker at 37°C with rotation for 30 min to digest. Vortex mixture after 15 min.
25. Pre-wet 70 μm cell strainers placed onto new 50 mL falcon tubes with 5 mL cold complete DMEM.
26. Transfer the digested liver mixture onto the cell strainers to allow pass-through of cells into the new 50 mL tubes.
27. To inactive enzymatic activity, add 15 mL cold complete DMEM to the original tubes and carefully pour the rinse onto the cell strainers, while mashing any undigested liver pieces using the end of a syringe plunger.
28. Centrifuge the strained cell suspension at 50 × *g* for 3 mins at 4°C to initially separate hepatocytes (pellet) and non-parenchymal cells (NPCs) (supernatant).
29. Carefully pour the supernatant containing NPCs into a new 50 mL falcon tube. Discard pellet containing hepatocytes. Of note, ~10% of KCs will be lost in the hepatocyte pellet.

Note: Hepatocyte pellet may be loose, use caution while pouring or use a pipette.

Optional: Hepatocytes in the pellets can also be processed separately if of interest.

30. Centrifuge the NPC suspension at 163 *g* for 7 min at 4°C to pellet the NPCs. The supernatant can be discarded.
31. Resuspend the NPC pellet with 1 mL ACK lysis buffer and incubate for 5 min, at 20°C to lyse red blood cells. Add 10 mL PBS to wash.
32. Centrifuge cell suspension at 163 *g* for 7 min at 4°C to re-pellet the NPCs. At this point the pellet should be pale and free of red blood cells.
33. Resuspend the cells in 1 mL PBS and transfer to 1.5 mL Eppendorf tubes. Keep on ice.

Pause point: Samples can be kept on ice for 1–2 h until ready to proceed to the next step. If pause, resuspend cells in 1 mL FACS buffer instead of PBS.

Staining for flow cytometry

Timing: 2 h

This step details the method of staining liver NPCs for identifying macrophage populations (Kupffer cells (KCs), monocyte-KCs, hepatic lipid-associated macrophages, and monocytes) by flow cytometry. Key markers include CD45, CD11b, F4/80, TIM4, VSIG4, CLEC2, Ly6C and MHCII. For this protocol, the antibody panel in Table 1 will be used as an example.

Optional: Depending on experimental question, additional markers may be added, for example CD11c for dendritic cells, Ly6G for neutrophils, and/or Siglec F for eosinophils.

Note: This staining protocol utilizes Zombie Aqua to assess live/dead status of cells prior to staining for surface markers, hence requiring cell suspension to be free of BSA in step 36 Alternatively, DAPI can also be used to identify live cells. For DAPI staining, resuspend stained-cells in 1× DAPI solution in FACS buffer (according to manufacturer instruction) prior to analyzing samples on the cytometer.

34. Pellet the cells using a bench-top centrifuge at 650 × *g* for 3.5 min at 4°C.
35. If cells are in buffers containing BSA, wash off and exchange with PBS.
36. Resuspend pellet with 100 μL Zombie Aqua solution at 1:250 dilution in PBS for live/dead stain. Incubate on ice and in the dark for 15 min.
37. Wash the cells by adding 700 μL of FACS buffer to cell suspension. Pellet the cells at 650 × *g* for 3.5 min at 4°C. Aspirate the supernatant.
38. Resuspend the pellet with 10 μL Fc block solution. Incubate on ice and in the dark for 5 min.

Table 1. Example staining panel for analysis by BD X20 flow cytometer

Reagent	Final concentration
CD45 - BUV395	1:100
CD11b - APC-Cy7	1:100
F4/80 - AF647	1:100
CLEC2 - PE	1:100
TIM4 - BV421	1:500
VSIG4 - PE-Cy7	1:200
Ly6C - FITC	1:100
MHC II - BV605	1:300
CD11 c - BV711	1:100
Zombie Aqua	1:250

For exact antibodies, see key resources table.

39. Add 90 μL of antibody cocktail to the cell suspension. Resuspend and incubate on ice and in the dark for at least 45 min (Table 1).
40. Wash the cells by adding 700 μL FACS buffer to the cell suspension. Pellet the cells at 650 × *g* for 3.5 min at 4°C. Aspirate the supernatant.
41. Resuspend the cells in 300 μL FACS buffer.
42. Filter the cell suspension through a 70-μm cell strainer into 5 mL polystyrene round-bottom flow tube and keep on ice in the dark. The samples are now ready to be analyzed on a flow cytometer.

Immunofluorescence of liver tissue

Timing: 1–2 days

This step will explain immunofluorescence staining of macrophage markers in liver tissue. This can be used for analysis of identified NASH-associated macrophage markers, i.e., TREM2, Gpnmb and CD63, that have been proven difficult to analyze via flow cytometry (Daemen et al., 2021; Remmerie et al., 2020). Additional information that can be obtained is localization of macrophage subsets in the tissue as well as their proximity to other parenchymal and non-parenchymal cells.

43. Following perfusion, harvest liver lobe and place the tissue into a tube containing formalin. It is critical to ensure the tissue is submerged in formalin, which typically requires a volume of 500–700 μL of fixative. We typically use the right medial liver lobe for IF. Keep on ice until all tissue is harvested.

    CRITICAL: To maintain tissue architecture of the liver, immediate incubation in fixative following tissue isolation is essential.

44. Incubate at 4°C for 24 h.
45. Transfer liver lobe to 750 μL 30% sucrose in PBS at 4°C for 24 h. A minimum incubation of 24 h is required, but incubation may be extended up to 72 h.
46. Submerge and freeze liver lobe in OCT in mold and store at −80°C. Ideally, the liver will be sectioned in the horizontal plane and therefore the smooth dome shaped portion of the liver lobe should be placed face down in the OCT media.
47. Cut 8 μM tissue sections using a cryostat on glass slides. Tissue sections can be stored at −80°C for several months.
48. Prepare 50 mL blocking buffer fresh on the day of staining.
49. Retrieve frozen slides from −80°C and let them air dry for 15–20 min at 20°C.

    CRITICAL: Prevent drying of the tissue sections at any step during the rest of the staining protocol.

50. Incubate slides in PBS for 5 min in glass chamber, followed by 45 min in blocking buffer.
51. Prepare primary antibody, 50 μL per section, in blocking buffer.
52. Carefully extract slides from glass chamber and dry slides around the tissue sections with Kimwipe. Encircle sections with using a hydrophobic pen and add 50 μL primary antibody solution on each section.
53. Keep slides in a container on wet paper towels 16–24 h at 4°C.
54. Incubate slides in PBS for 5 min in glass chamber to wash and perform this step three times.
55. Carefully extract slides from glass chamber and dry slides around the tissue sections with Kimwipe. Re-apply hydrophobic barrier with pen if necessary.
56. Prepare secondary antibody solution and add 50 μL per section. Incubate for 1 h at room temperature in the dark.
57. Incubate slides in PBS for 5 min in glass chamber to wash, 2 times.
58. Prepare 50 mL Hoechst dye (1:25000) and add solution to slides in glass chamber. The nuclear dye should be prepared fresh for each experiment.

59. Retrieve slides from glass chamber, dry carefully, and apply a small drop of prolong gold anti-fade reagent. Place cover slip on top and let slides dry for minimum of 1 h in dark.
60. Imaging can be done with light or confocal microscopy. For optimal result, image within one week of staining.

EXPECTED OUTCOMES

The estimated yield per gram of liver tissue of CD45-positive cells and total F4/80^{hi} macrophages can vary from 0.5–1.5 and 0.1–0.4 million cells, respectively. Exact cell number is dependent on many factors including perfusion quality and variation in digestion efficiency. The gating strategy described below may be used to identify the liver macrophage subsets previously described in NASH, but alternative gating strategies have been utilized by other labs (Remmerie et al., 2020; Tran et al., 2020). When using this protocol, it is expected that ~ 90% of the CD45^{pos} cells will be viable by live-dead staining. Although this can be somewhat variable, if the viability is below 70% this may indicate a problem with the isolation protocol. All liver macrophages are identified as F4/80^{hi}, CD11b^{int} (Figure 2A) and consist of the sinusoidal macrophages as well as a small number of liver capsular cells. The sinusoidal macrophages are composed of embryonically derived resident Kupffer cells (KCs), which can be identified by high expression of TIM4 (Figure 2B) and recruited monocyte-derived macrophages (MdMs) which are almost exclusively TIM4^{lo}, although a subset of mo-KCs can begin to express TIM4 after several weeks in the liver (Figure 2B)(Scott et al., 2016). Typically, we have found the percentage of TIM4^{lo} MdMs correlates with NASH severity. MdMs can be further subdivided into the monocyte-derived Kupffer cells (mo-KCs) which are TIM4^{lo}, VSIG4^{hi} (or CLEC4F^{hi}), and lipid-associated macrophages (LAMs), which are TIM4^{lo}, VSIG4^{lo} (Figure 2C)(Daemen et al., 2021). LAMs are likely the equivalent of the previously described NASH-associated macrophages (NAMs) as these populations both exhibit high expression of the markers TREM2, CD9, CD63 and Gpnmb (Xiong et al., 2019). The use of *Ccr2*-GFP or *Cx3cr1*-GFP reporter mice allows for further identification of *Ccr2*^{hi}/*Cx3cr1*^{hi} LAMs (C-LAMs) (Figure 2C)(Daemen et al., 2021). Several recent papers have also demonstrated CLEC2 as a marker of sinusoidal liver macrophages (Seidman et al., 2020). CLEC2 is highly expressed by most liver macrophages except for a small subpopulation of MdMs, which are high in *Ccr2* and *Cx3cr1*. These cells are found in the C-LAM gate by flow cytometry (Figure 2D). This CLEC2^{lo} population may include liver capsular cells and/or an early precursor of LAMs, but additional studies will be necessary to delineate the trajectory of these macrophages.

Liver Ly6C^{hi} monocytes can be found within the CD11b^{hi}, F4/80^{int} population and can be identified by gating for Ly6C^{hi}, MHCII^{lo}, and SSC^{lo} (Figure 2E). Alternatively, antibodies to Ly6G and SiglecF can be used here to gate on neutrophils and eosinophils. When using *Ccr2*-GFP or *Cx3cr1*-GFP reporter mice, classical monocytes can also be identified as Ly6C^{hi}, *Ccr2*^{hi}/*Cx3cr1*^{hi} (Figure 2E).

Importantly, in the flow cytometric analysis, LAMs are currently identified by the absence of KC surface markers (TIM4, VSIG4), because flow cytometry of known LAM markers, i.e., TREM2, Gpnmb, and CD63, after tissue digestion has been challenging. Therefore, the presence and number of these cells also be assessed by immunofluorescence and confocal imaging. With this approach, co-staining of CD63 or Gpnmb with CLEC4F and *Ccr2*-GFP allows for simultaneous visualization and separation of LAMs, KCs/mo-KCs and C-LAMs, respectively (Figures 2F–2G). TIM4 staining can also be used to aid in the identification of Em-KCs vs. mo-KCs.

QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify the flow cytometry absolute cell number should be corrected for the amount of liver tissue initially digested. Given the day-to-day variation in efficiency of cell isolation, same day comparison is recommended when analyzing parallel experimental groups. Quantification of immunofluorescence can be done using software such as ImageJ and can be expressed as number of cells per field of view. Quantification of total fluorescent area could also be performed, but importantly this does not correct for changes in macrophage size which may vary with disease state.

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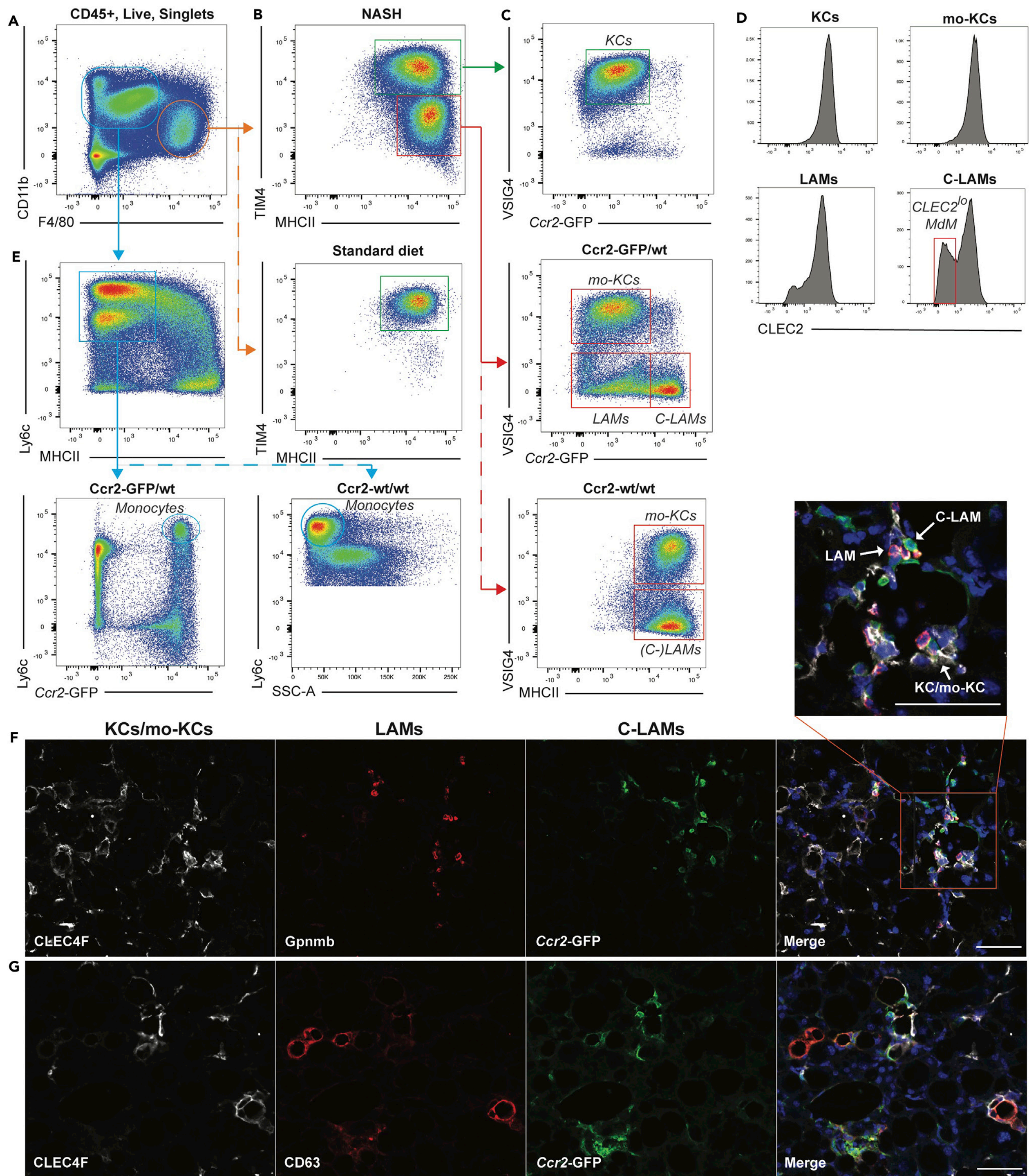

Figure 2. Example flow plots and immunofluorescence images to identify macrophage sub-populations in the NASH liver

(A) After gating for CD45-positive, live and singlet cells, liver macrophages can be identified as F4/80^{hi}, CD11b^{int} cells (orange circle).

(B) In a standard diet fed mouse almost all liver macrophages are resident TIM4^{hi} Kupffer cells (KCs) (green box). In contrast, the NASH liver also contains significant numbers of recruited TIM4^{lo} monocyte-derived macrophages (MdMs) (red box).

(C) TIM4^{lo} macrophages can be further subdivided into VSIG4^{hi} monocyte-KCs (Mo-KCs) and VSIG4^{lo} lipid-associated macrophages (LAMs). Using *Ccr2-* or *Cx3cr1*-GFP reporter mice also facilitates the identification of *Ccr2/Cx3cr1*-GFP-positive LAMs (C-LAMs).

Figure 2. *Continued*

(D) Although most liver macrophages are CLEC2^{hi}, there is a CLEC2^{lo} subpopulation can be identified within the TIM4^{lo}, VSIG4^{lo} MdMs. These CLEC2^{lo} macrophages express high levels of *Ccr2/Cx3cr1*.

(E) Monocytes can be identified by subsequent gating for CD11b^{hi}, MHCII^{lo}, Ly6C^{hi}, and low side scatter area (SSC-A). They can also be identified as *Ccr2/Cx3cr1*^{hi} cells within the CD11b^{hi}, MHCII^{lo}, Ly6C^{hi} gate when using reporter mice.

(F and G) With immunofluorescence imaging LAMs can be identified using antibodies against Gpnmb or CD63 (red) and monocytes/C-LAMs by *Ccr2*-GFP (green). CLEC4F staining (white) allows for the identification of KCs as well as mo-KCs. A magnification image is shown to illustrate identification of macrophage subsets in the liver tissue. Nuclei are presented in blue. Scale bar is 50 μM.

LIMITATIONS

The described protocol can be used for both standard diet healthy livers and NASH livers. However, when using healthy livers NPC pellet is larger when compared to the same tissue volume of a NASH liver. This can result in slow and difficult flow cytometry as the number of macrophages per given volume is reduced. When regularly using standard diet controls, the use of a percoll gradient may be preferred for better purification of macrophages, although this dramatically lowers the yield (Lynch et al., 2018). Fibrosis of the liver can also result in more difficult perfusion, digestion, and extraction of liver macrophages. Consequently, this may result in lower macrophage yield and/or favor extraction of specific macrophage populations, thereby giving distorted results on liver macrophage composition. Confirmation of flow cytometry results with immunofluorescence is therefore recommended. As an alternative direct perfusion of the liver with collagenase improves liver digestion and macrophage yield, however, this excludes the use of any tissue for immunofluorescence, histology, RNA, and protein analysis.

TROUBLESHOOTING

Problem 1

Unsuccessful cannulation of portal vein (step 14)

Potential solution

Cannulation of the IVC for perfusion is a possible alternative, though this often results in lower quality perfusion and subsequently lower cell yield. It is advised to practice portal vein cannulation until achieving reliable perfusion quality. Please see video file of portal vein cannulation (Methods video S1).

Problem 2

Low yield of liver macrophages (step 42)

Potential solution

A low yield can result from low perfusion quality or incomplete digestion of the liver. Ensure the liver is properly perfused until a pale and beige appearance. In addition, ensure that the digestion buffer with collagenase is prepared fresh and the samples are heated to 37°C before digestion on the shaker.

If a higher yield of macrophages is required, for example for flow sorting and/or plating of the macrophages, direct perfusion of the liver with collagenase coupled with digestion on the shaker can be considered. However, as described before this excluded use of liver tissue for other analyses.

Problem 3

Kupffer cell autofluorescence (step 42)

Potential solution

KCs are extremely vacuolar macrophages and as such have significant autofluorescence in the green channel. Therefore, it is critical to include appropriate isotype control antibodies for the green channel to ensure that the positive signal is real. Similarly, when using GFP-reporter mice it is important to include some NTG littermate controls to confirm the findings, see figure 4 of the Daemen et al. manuscript (Daemen et al., 2021).

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Problem 4

Low Kupffer cell viability (step 42)

Potential solution

As mentioned above, it is expected that CD45 positive cells will have a viability of ~ 90% using this protocol and gating strategy. If the viability is below 70% this may indicate a problem with the isolation protocol. The most likely steps that could lower cell viability are the collagenase digestion at 37 deg and/or the red cell lysis step. If either of these steps is allowed to continue beyond the recommended time, then immune cell viability can be compromised. It is also important to keep samples on ice with FACS buffer, rather than PBS to maximize cell viability.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joel D. Schilling (schillij@wustl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate a new dataset or code for analysis.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100511.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.D., M.M.C., and J.D.S.; methodology, S.D., M.M.C., and J.D.S.; investigation, S.D., M.M.C., and J.D.S.; writing-original draft, S.D., M.M.C., and J.D.S.; writing-review & editing, S.D., M.M.C., and J.D.S.; and funding Acquisition, J.D.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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