We present a protocol for isolating hepatocytes from mice with established non-alcoholic fatty liver disease. This protocol consists of liver perfusion using a peristaltic pump, followed by a modified 25% and 90% Percoll gradient centrifugation protocol to capture lipid-laden hepatocytes that are usually lost using traditional isolation protocols. This protocol enables simultaneous isolation of normal and lipid-filled hepatocytes. Lipid-filled hepatocytes can be used in cell culture systems to study drug metabolism, hepatotoxicity, or glucose and lipid metabolism.
Protocol
Isolation, culture, and functional analysis of hepatocytes from mice with fatty liver disease

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SUMMARY
We present a protocol for isolating hepatocytes from mice with established non-alcoholic fatty liver disease. This protocol consists of liver perfusion using a peristaltic pump, followed by a modified 25% and 90% Percoll gradient centrifugation protocol to capture lipid-laden hepatocytes that are usually lost using traditional isolation protocols. This protocol enables simultaneous isolation of normal and lipid-filled hepatocytes. Lipid-filled hepatocytes can be used in cell culture systems to study drug metabolism, hepatotoxicity, or glucose and lipid metabolism.

For complete details on the use and execution of this protocol, please refer to Sharabi et al. (2017) and Kegel et al. (2016).

BEFORE YOU BEGIN
This protocol will isolate viable hepatocytes from healthy murine livers, as well as from mice with established non-alcoholic steatohepatitis. This protocol has been modified based on previous work to enable isolation of lipid-filled hepatocytes (Berry and Friend, 1969; Kreamer et al., 1986; Pertoft, 2000; Gonçalves et al., 2007; Li et al., 2010; Kegel et al., 2016; Sharabi et al., 2017).

Note: This protocol can be used to isolate hepatocytes from mice with different genetic backgrounds and mutations to facilitate the investigation of gene function in liver disease.

Induction of diet-induced NASH in mice

© Timing: 1–10 weeks

1. All animal procedures need to be approved by the Institutional Animal Care and Use Committee. All experiments in this protocol were approved by the Stanford Animal Care and Use Committee protocol #32982.
2. House 6–8 week old male or female C57BL/6 mice in a cage of 4–5 mice per cage in a temperature-controlled (20°C–22°C) room on a 12-h light/dark cycle.
3. Feed mice with either a chow diet or with a diet to induce non-alcoholic steatohepatitis, hereafter referred to as NASH diet (Research Diets). The NASH diet consists of 40% kcal fat (palm oil), 20% kcal fructose, and 2% cholesterol and has previously been shown to induce clinical, biochemical, and histological features of fatty liver disease in animals (Radhakrishnan et al., 2020).
4. Hepatic lipid accumulation can be seen at the cellular level after a week of feeding, while whole-body changes such as body weight and blood glucose are typically seen starting at 6 weeks on
this diet. Measure body weights and non-fasted glucose levels using a glucometer weekly. At 6 weeks and beyond, NASH diet-fed mice are expected to demonstrate significantly higher body weights, higher blood glucose levels, and enlarged and pale livers (Figure 1) compared to chow-fed mice.

Note: C57BL/6 mice are suitable for inducing NASH with this diet, but other strains such as 129Sv can be used as well, but the exact time of feeding might have to be optimized (Fengler et al., 2016).

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins |        |            |
| HBSS buffer         | Gibco  | Cat# 14175-095 |
| Potassium chloride  | Sigma  | Cat# P5405 |
| Sodium bicarbonate  | Sigma  | Cat# S6297 |
| UltraPure 0.5 M EDTA, pH 8.0 | Invitrogen | Cat# 15575-038 |
| Williams E media    | Quality Biological | Cat# 112-033-101 |
| Corning regular fetal bovine serum | Corning | Cat# 35-010-CV |
| DMEM high glucose   | Sigma  | Cat# D6429 |
| HEPES buffered saline | Sigma-Aldrich | Cat# 51558 |
| Sodium pyruvate     | Thermo Fisher | Cat# 11360070 |
| Dexamethasone       | Sigma  | Cat# D1756 |
| Insulin, human recombinant | Millipore Sigma | Cat# 91077C |
| Penicillin/streptomycin | Gibco | Cat# 15140-122 |
| Collagenase IV      | Sigma  | Cat# C5138 |
| PBS                 | Gibco  | Cat# 10010-023 |
| 10× PBS             | Gibco  | Cat# 70011044 |
| Percoll             | Sigma  | Cat# P1644 |

(Continued on next page)
### Materials and Equipment

Prepare all buffers and media according to the tables and keep on ice until use. Refer to the Key Resources Table and Materials and Equipment for a complete list of materials, equipment, and recipes.

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**Critical:** Prepare the digestion buffer the day of isolation. Avoid repeated freeze-thaws of the digestion buffer. Pre-warm the perfusion and digestion buffer to 42°C before starting the isolation procedure. Keep the plating media on ice.

#### Perfusion buffer

| Reagent               | Stock concentration | Final concentration | Amount |
|-----------------------|---------------------|---------------------|--------|
| HBSS                  | 1 x                 | 1 x                 | 500 mL |
| KCl                   | w/v                 | 0.4 g/L             | 0.2 g  |
| Sodium Bicarbonate    | w/v                 | 2.1 g/L             | 1.05 g |
| EDTA                  | n/a                 | 0.2 g/L             | 0.1 g  |
| Total                 |                     |                     | 500 mL |
Adjust pH to 7.4, sterilize using a 0.22 μm filter, store at 4°C for up to 2 months. Adjust the pH to 7.4 after the solution has reached 42°C if needed.

### Digestion buffer

| Reagent                      | Stock concentration | Final concentration | Amount |
|------------------------------|---------------------|---------------------|--------|
| DMEM high glucose            | 1×                  | 1×                  | 89 mL  |
| Penicillin/Streptomycin      | 100×                | 1% (v/v)            | 1 mL   |
| Collagenase IV               | n/a                 | 1 mg/mL             | 100 mg |
| HEPES                        | 10 g/L              | 10 μg/mL            | 100 μL |
| FBS                          | n/a                 | 10% (v/v)           | 10 mL  |
| **Total**                    |                     |                     | 100 mL |

Adjust pH to 7.4, always prepare freshly, do not store.

### 90% Percoll media

| Reagent                      | Stock concentration | Final concentration | Amount |
|------------------------------|---------------------|---------------------|--------|
| Percoll                      | n/a                 | n/a                 | 9 mL   |
| 10× PBS                      | 10×                 | 1×                  | 1 mL   |
| Resuspended hepatocytes      |                     |                     | 10 mL  |
| **Total**                    |                     |                     | 20 mL  |

Prepare at 20°C–24°C in a sterile environment, always prepare freshly, do not store.

### 25% Percoll media

| Reagent                      | Stock concentration | Final concentration | Amount |
|------------------------------|---------------------|---------------------|--------|
| Percoll                      | n/a                 | n/a                 | 2.5 mL |
| 10× PBS                      | 10×                 | 1×                  | 1 mL   |
| ddH2O                        | n/a                 | n/a                 | 6.5 mL |
| Resuspended hepatocytes      |                     |                     | 10 mL  |
| **Total**                    |                     |                     | 20 mL  |

Prepare at 20°C–24°C in a sterile environment, always prepare freshly, do not store.

### Plating media

| Reagent                      | Stock concentration | Final concentration | Amount |
|------------------------------|---------------------|---------------------|--------|
| Williams E media             | 1×                  | 1×                  | 425 mL |
| Glutamax                     | 200 mM              | 2 mM                | 5 mL   |
| Penicillin/Streptomycin      | 50×                 | 2% (v/v)            | 10 mL  |
| Sodium Pyruvate              | 100 mM              | 2 mM                | 10 mL  |
| Dexamethasone                | 1 mM                | 1 μM                | 500 μL |
| Insulin                      | 1 mM                | 0.1 μM              | 50 μL  |
| FBS                          | n/a                 | 10% (v/v)           | 50 mL  |
| **Total**                    |                     |                     | 500 mL |

Prepare in a sterile environment, adjust pH to 7.4, store at 4°C for up to 1 week.
Prepare in sterile environment, adjust pH to 7.4, store at 4°C for up to 1 week.

**STEP-BY-STEP METHOD DETAILS**

**Day of isolation: preparation of buffers and setup of perfusion pump**

© Timing: 1.5 h

This step includes the preparation of buffers and preparation of the perfusion pump. The perfusion system includes a water bath and a peristaltic pump with adjustable speed. The required volumes for hepatocyte isolation from one mouse are described in the table.

| Maintenance media                  | Stock concentration | Final concentration | Amount |
|------------------------------------|---------------------|---------------------|--------|
| Williams E media                   | 1×                  | 1×                  | 475 mL |
| Glutamax                           | 200 mM              | 2 mM                | 5 mL   |
| Penicillin/Streptomycin            | 50×                 | 2% (v/v)            | 10 mL  |
| Sodium Pyruvate                    | 100 mM              | 2 mM                | 10 mL  |
| Dexamethasone                      | 1 mM                | 0.1 μM              | 50 μL  |
| Insulin                            | 1 μM                | 0.1 nM              | 50 μL  |
| BSA                                | n/a                 | 0.2% (w/v)          | 1 g    |
| Total                              |                     |                     | 500 mL |

Prepare in sterile environment, adjust pH to 7.4, store at 4°C for up to 1 week.

**One digestion reaction**

| Buffers/Media | Needed for | Total amount |
|---------------|------------|--------------|
| Perfusion Buffer | Liver Perfusion | ~ 40 mL |
| Digestion Buffer | Liver Perfusion | ~ 30 mL |
| Plating Media | Liver Perfusion Hepatocyte isolation Plating | ~ 320 mL |
| 90% Percoll Media | Hepatocyte isolation | 20 mL |
| 25% Percoll Media | Hepatocyte isolation | 20 mL |

1. Prepare the perfusion buffer, digestion buffer, plating media, Percoll media, and maintenance media prior to the isolation. Place perfusion buffer and digestion buffer sufficient for one digestion reaction in two separate 50 mL tubes in a 42°C water bath for at least 30 min prior to the isolation. 40 mL perfusion buffer and 30 mL digestion buffer are sufficient for isolation of hepatocytes from one mouse.

2. Set up the perfusion pump by mounting the peristaltic pump with a long 1/32” ID × 3/32” OD silicone tubing immersed in a water bath. The tubing will be connected to the pump with a 3/32” Polypropylene coupler (Figure 2A).

3. Place the inlet tubing into a 50 mL tube with perfusion buffer in the 42°C water bath.

4. Set the perfusion pump speed at 1–2 mL/min. Note that the settings will vary depending on the instrument used (Figure 2B).

5. Place the surgical table at an angle in a waterproof container in close proximity to the perfusion pump.

6. Assemble the outlet catheter by attaching a 25G 7/8 needle to the outlet tubing (Figure 2C).

7. Rinse the pump with 10 mL 70% EtOH and 10 mL water.

8. Run the pump with perfusion buffer for 5 min to ensure an even flow rate.
CRITICAL: Prepare the digestion buffer 1 h prior to the isolation and place in a 42°C water bath. The water bath should not be above 42°C as that can inactivate the collagenase in the digestion buffer. The hepatocyte culture media can be made in advance and kept in 4°C for up to a month.

△ CRITICAL: The exit temperature (the fluid that will enter into the inferior vena cava) should be ∼37°C. The temperature of the solution gradually cools as it is passing through the tubing. By maintaining the buffers in the water bath at 42°C, the exit temperature will be ∼37°C after passing through the perfusion tubing. The length of tubing and the flow rate will affect this temperature loss, so it is advised to measure that the exit temperature is ∼37°C before starting the perfusion procedure.

Liver perfusion and enzymatic dissociation of liver cells

© Timing: 0.5–1 h

This step will perfuse the liver followed by enzymatical digestion using collagenase IV to dissociate the liver cells into a single-cell suspension.

9. Euthanize the mouse by CO₂ asphyxiation and clean the abdominal area with 70% EtOH.
10. Make an incision in the abdominal area to expose the liver, inferior vena cava, and portal vein.
11. Place the catheter into inferior vena cava (Figure 3A).
12. Start the perfusion pump with perfusion buffer at a rate of 2 mL/min. Ensure the correct flow rate by measuring the outlet volume as a fraction of time.
13. After 2 min, cut the hepatic portal vein to clear the liver from blood.
14. Continue the perfusion for an additional 10 min. This step will use 25 mL perfusion buffer in total. Successful perfusion will result in a pale, enlarged liver (Figure 3B).
15. When 2–5 mL is left of the perfusion buffer, add the digestion buffer to the 50 mL tube without stopping the pump.

16. Digest for 10–20 min at 2 mL/min. This will use 20–30 mL digestion buffer in total.

17. When visible tissue dissociation is seen, carefully remove the liver including the gall bladder and placenta completely (Figure 3C).

18. Add 10 mL cold plating medium to the dish. Without puncturing the gall bladder, dissociate the liver cells by gently swirling the liver in cold plating media. Use a pipette with a 1,000 μL tip to generate a homogenous cell suspension (Figure 3D).

19. Transfer the cell suspension to a 70 μm strainer placed on a 50 mL tube on ice (Figure 3E). Wash the plate with an additional 10 mL plating medium and transfer to the 50 mL tube.

**CRITICAL:** The exact time for optimal liver digestion will vary slightly between mice. Signs of underdigestion include inability to mechanically dissociate the liver into a single-cell suspension. Signs of overdigestion include complete digestion of the liver into a homogenous solution before transfer to the petri dish. Digestion is considered successful when the liver can readily be transferred to the petri dish, and the cell solution is homogenous without visible liver pieces after mechanical dissociation and can easily be passed through the 70 μm strainer.

**Single-cell hepatocyte isolation using Percoll gradient centrifugation**

© Timing: 4–6 h
This step will separate viable hepatocytes with different sizes of lipids from dead hepatocytes and other non-parenchymal cell types in the cell suspension using Percoll gradient centrifugation. The Percoll media is composed of colloidal silica and has been extensively used for the isolation of cells and organelles (Kreamer et al., 1986; Pertof, 2000). The density of the Percoll solution is dependent on its concentration, the total g-force, and the centrifugation time, which will affect the gradient formation. Depending on the requirements, conditions such as centrifugal force, centrifugation time, and Percoll density gradient composition can be modified.

20. Centrifuge the strained cells at 50 × g for 5 min at 20°C–24°C to separate hepatocytes from non-parenchymal cells. Hepatocytes will pellet at 50 × g.
21. Discard the supernatant.
22. Resuspend the pellet in 20 mL plating media and wash the cells twice at 50 × g for 5 min.
23. Resuspend the pellet in 40 mL plating media. Divide the solution into four tubes with 10 mL solution in each tube.
24. Prepare two sets of Percoll/hepatocyte mix for a total of 4 tubes with either 25% or 90% Percoll by adding 10 mL of 20°C–24°C 90% Percoll media to two of the tubes and 25% Percoll media to the remaining tubes. The final volume of the Percoll/hepatocyte mix will be 20 mL.
25. Centrifuge one set of tubes (25% and 90% Percoll/hepatocytes) at 100 × g and the other set of tubes (25% and 90% Percoll/hepatocytes) at 1,000 × g. Centrifuging cells at 1,000 × g is predicted to sediment cells with higher lipid content compared with 100 × g. This step will isolate viable non-lipid-filled and lipid-filled hepatocytes (Figure 3F).
26. Resuspend the pellets in 20 mL plating media and spin at 50 × g for 4 min twice to remove the Percoll media.
27. Resuspend the final hepatocyte pellets in 1 mL media and measure cell number and viability using trypan blue and a manual or automatic hemocytometer.
28. Seed cells into collagen coated plates. Plate 4 × 10^5 viable cells in 6-well plates or 2 × 10^5 cells in 12-well plates and place plates in a 37°C incubator with 5% CO₂ for 3–4 h.
29. After 3–4 h, wash once with PBS to remove dead cells, replace with maintenance medium (Figure 3G). Experiments can start the same or next day.

**Note:** Keep the Percoll medium at 20°C–24°C to maintain the pH between 8.5–9.5.

**Note:** Depending on the degree of hepatic steatosis, differences in cell numbers from the different Percoll gradient fractions can vary between isolations. When isolating cells from NASH mice, the largest number of hepatocytes will be collected from the 25% Percoll density gradient, while the lowest number of hepatocytes will be obtained from the 90% Percoll density gradient.

**Optional:** If complete removal of erythrocytes is desired, spin the hepatocyte pellet at 20 × g for 3 min after step 20 and remove the supernatant.

**Optional:** If non-parenchymal cells are desired, collect the supernatant from step 21 and spin the supernatant solution at 650 × g to pellet non-parenchymal cells such as stellate cells, endothelial cells, and Kupffer cells.

**Optional:** If the cells will be analyzed with single-cell RNA sequencing or RNA sequencing, continue directly from step 27 without plating the cells.

**Pause Point:** It is necessary to complete the whole procedure without pause to maintain cell viability.
Functional characterization of hepatocytes from NASH mice

© Timing: 2–4 h

This step will functionally characterize the hepatocytes using oil red O staining. Alternative analyses include drug testing, lipogenesis, and gene expression analyses. The protocol here uses hepatocytes plated in a 12-well plate.

Oil red O working solution (sufficient for one 12 well plate)

| Reagent   | Stock concentration | Final concentration | Amount |
|-----------|---------------------|---------------------|--------|
| Oil red O | n/a                 | n/a                 | 4.2 mL |
| ddH2O     | n/a                 | n/a                 | 2.8 mL |
| Total     |                     |                     | 7 mL   |

Prepare the solution 10 min before use.

30. Prepare 7 mL oil red O Solution by mixing oil red O with H2O at a 3:2 ratio. 7 mL is sufficient for one 12-well plate when using 500 μL per well.
31. Gently wash the cells twice with 1 mL PBS.
32. Add 0.5 mL per well of 3% Formalin diluted in PBS and incubate for 0.5–1 h at 20°C–24°C.
33. Discard the formalin and wash the cells twice with 1 mL ddH2O.
34. Add 0.5 mL 60% isopropanol and incubate for 5 min.
35. Discard the isopropanol and apply the oil red O Working Solution. Rotate the plate to ensure even distribution and incubate for 10–20 min at 20°C–24°C.
36. Discard the oil red O solution and wash the cells 2–5 times with ddH2O until clear.
37. Add 1 mL ddH2O and view red lipid droplets under a microscope using a 40× objective.

Optional: For nuclei staining, add Hematoxylin after step 36 and incubate for 1 minute. After discarding Hematoxylin, wash the cells 2–5 times with ddH2O until clear. Nuclei should appear blue under the microscope.

Note: Stained cells can be stored in 4°C for up to 1 week. Keep the cells immersed in water to avoid drying.

△ CRITICAL: Do not store the mixed oil red O solution for more than 2 h. Reusing the oil red O Working Solution is not recommended as it can result in poor staining quality.

EXPECTED OUTCOMES

The estimated yield is ~1–2 million hepatocytes from one liver isolated from 6–9 weeks diet-induced NASH with a viability ranging from 50%–90% (Figure 4A). This can be compared to the yield from one mouse liver isolated from chow-fed mice which is ~5–20 million cells. Typically, shorter time on NASH diet will result in a higher cell number and viability. Furthermore, with less severe liver steatosis, a higher number of hepatocytes will be obtained from the 90% Percoll gradient, while more severe steatosis will result in a larger fraction of hepatocytes isolated from the 25% Percoll gradient.

A frequently observed outcome from a hepatocyte isolation from different Percoll fractions is shown in the graph expressed as viable/dead cells from each Percoll fraction (Figure 4B). Accordingly, measuring the lipid content by oil red O staining shows increased lipid staining in the 25% Percoll gradient compared to 90%, demonstrating successful isolation of lipid-deficient and lipid-filled hepatocytes (Figure 4C).
LIMITATIONS

One distinct disadvantage of this method is the yield of viable lipid-filled hepatocytes that can be obtained in comparison to a typical hepatocyte isolation from healthy livers. Thus, the degree of NASH-induction might have to be optimized to achieve the highest yield while inducing a sufficient degree of steatosis. Additionally, cells from NASH mice are more sensitive to overdigestion. Therefore, careful observation of the timing of digestion is essential, as this otherwise can result in large variability between preparations.

Another disadvantage of culturing primary hepatocytes is the short time frame by which experiments have to be concluded (Shulman and Nahmias, 2013). Typically, primary hepatocytes will lose their
functional features within 24–48 h after plating, leading to a dramatic drop in typical hepatocyte genes such as albumin, while fibroblast markers such as afp are increased, indicating a loss of cell characteristics. This limits the number and types of downstream functional assays that can be performed. However, multiple types of analyses can be performed within this time frame which might have useful applications, such as drug metabolism assays, hepatotoxicity assays, or glucose and lipid metabolic assays. Alternatively, for long-term functional analyses, our isolation method could be used in conjunction with the 5C small molecule inhibitor cocktail for the preservation of hepatocyte-specific functions and differentiation (Xiang et al., 2019).

TROUBLESHOOTING

Problem 1
Failure to cannulate inferior vena cava (step 11).

Potential solution
If the catheter insertion into inferior vena cava is unsuccessful, the catheter can be inserted into the hepatic portal vein. Proceed with the perfusion according to the protocol, but instead cut the inferior vena cava to release excess blood.

Problem 2
Low cell viability or small cell pellets (step 27).

Potential solution
Low cell viability or small cell pellets can be due to either incomplete digestion or overdigestion.

Poor perfusion can result in incomplete digestion and low recovery of cells. This will lead to the inability to mechanically dissociate the liver into a single-cell suspension. To solve this problem, ensure that the perfusion using perfusion buffer results in a pale, slightly swollen liver before introducing the digestion buffer. Excessive perfusion and digestion will lead to overdigestion and should be avoided to prevent cell death. If overdigestion is seen, lower the speed of perfusion and digestion from 2 mL/min to 1 mL/min.

The time of digestion will depend on the specific collagenase activity in the digestion buffer. Due to lot-to-lot variability in collagenase activity, optimization is warranted when using a new lot.

Problem 3
Low cell recovery during Percoll gradient cell isolation even after successful perfusion (step 25).

Potential solution
This problem might occur when the temperature of the Percoll solution is incorrect or when too many cells are added to the gradient. Keep the Percoll medium at 20°C–24°C to maintain accurate density and to keep the pH between 8.5–9.5. The volume of the density gradient is optimized for one murine liver per gradient. If more cells are needed, several livers can be pooled together. If pooling livers from several mice, increase the volume of media and gradient media accordingly.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Katrin J. Svensson (katrinjs@stanford.edu).

Materials availability
This study did not generate any unique materials or reagents.
Data and code availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
Conceptualization, Y.J. and K.J.S.; Investigation, Y.J., M.Z., and K.J.S.; Writing – Original Draft, Y.J. and K.J.S.; Writing – Review & Editing, Y.J., M.Z., and K.J.S.; Funding Acquisition, K.J.S.; Supervision, K.J.S.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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