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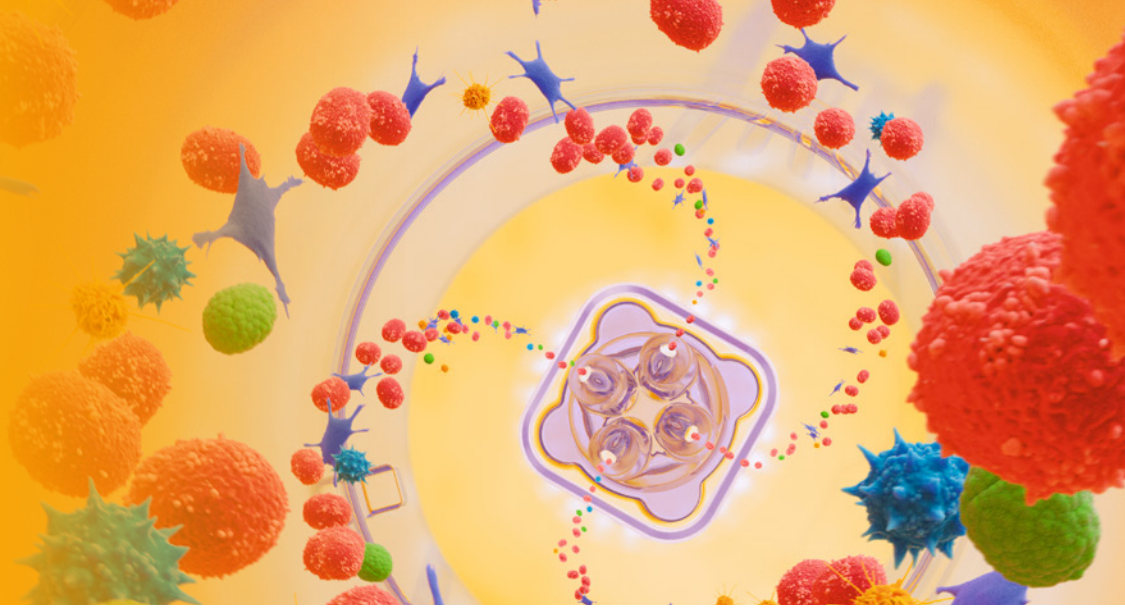

# Isolation of hepatocytes and non-parenchymal cells (NPCs) from fatty liver mouse model

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## Background

Hepatocytes, the major functional cells of the liver, play a crucial role in metabolism, detoxification, and protein synthesis. Traditional perfusion techniques, while effective in isolating hepatocytes, present several challenges. These methods are often time-consuming and require extensive expertise and manual handling, which can result in inconsistent cell yield and viability. These challenges can be particularly pronounced when working with diseased liver tissue, where fat, fibrosis, and altered vascular architecture complicate perfusion and hepatocyte isolation.

The gentleMACS™ Perfusion Technology addresses these challenges by providing a streamlined and automated solution for liver perfusion and hepatocyte isolation. This innovative system minimizes manual handling, reduces processing time, allows working on samples in parallel, and improves reproducibility. It also ensures consistently high yields of intact hepatocytes from healthy and diseased rodent livers.

In this study, a mouse model of cirrhotic liver was developed that can be effectively used to study metabolic dysfunction-associated steatohepatitis (MASH). This model is useful for investigating the pathophysiology and potential treatments of liver diseases associated with metabolic dysfunction. The gentleMACS Perfusion Technology was used to successfully isolate hepatocytes and NPCs from mouse cirrhotic livers.

This application note highlights how the gentleMACS Platform enables efficient liver perfusion, isolating viable cells while preserving cell integrity for reliable downstream applications in liver research.

## Material and methods

### Mouse preparation

In this study, the stelic animal model (STAM) was used as a model that shows the same disease progression as human MASH or hepatocellular carcinoma (HCC) liver. To generate the STAM mouse model, two-day-old male C57BL/6 mice were given a single dose of streptozotocin (200 µg/per mouse) to reduce the ability to secrete insulin. At four weeks of age, the mice were fed a high-fat diet (HFD32, High Fat Diet32, CLEA Japan) to simulate a phenotype similar to late-stage type 2 diabetes, leading to conditions such as fatty liver, MASH, fibrosis, and ultimately HCC. At 16 weeks of age, STAM mice were sacrificed and the left lateral liver lobe was resected for *ex vivo* perfusion using the gentleMACS Octo Dissociator with Heaters in combination with gentleMACS Perfusers and Perfusion Sleeves, while the rest of the liver remained intact to be used for immunohistochemical staining (fig. 1).

### *Ex vivo* liver perfusion

The left lateral lobe of the liver was resected from STAM mice and perfused using the gentleMACS Perfusion Technology, which includes the gentleMACS Perfuser, Liver Perfusion Kit, and Perfusion Sleeves, according to the protocol with minor adjustments in subsequent procedures:

- For STAM mice, at step 6 of the liver perfusion kit protocol, program 37C_r_LIPK_1 was used, which provides a longer enzymatic digestion time compared to the standard mouse protocol (12.5 min instead of 10 min).
- At step 31 of the protocol, after tissue dissociation and hepatocyte release, the samples were centrifuged twice at 30×g for 5 minutes each at 4 °C.

After centrifugation, the hepatocytes formed a pellet, and the supernatant was subsequently collected to isolate NPCs, such as liver sinusoidal endothelial cells (LSECs).

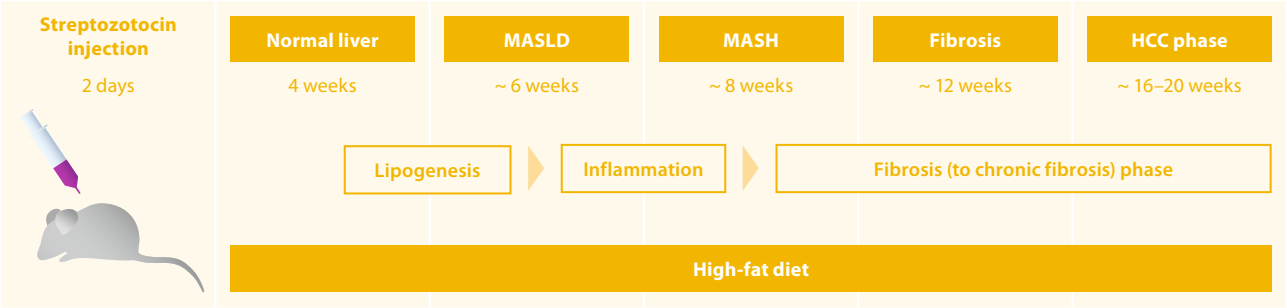


**Figure 1: The progression of normal liver to the hepatocellular carcinoma HCC stage.**[1] Metabolic dysfunction-associated steatotic liver disease (MASLD); metabolic dysfunction-associated steatohepatitis (MASH), hepatocellular carcinoma (HCC). Age of the mouse is indicated in days and weeks.

### Cell isolation

**Hepatocytes:** Hepatocytes were sedimented by low-spin centrifugation, and yield and viability were measured using a hemocytometer and trypan blue staining (table 1). Hepatocytes were cultured in 10% FBS/DMEM + Pen-Strep medium on collagen type I–coated 12- and 24-well plates (IWAKI®). Cells were washed 12 hours after seeding to remove non-attached dead cells. Hepatocyte characteristics and specific markers were confirmed by brightfield microscopy and immunofluorescence staining using anti-albumin antibody (1:50, Proteintech®) and anti-E-cadherin antibody (1:40, R&D Systems®) to stain albumin and E-cadherin, respectively (fig. 2).

**LSECs:** Following low-spin centrifugation, the supernatant containing NPCs was analyzed using the MACSQuant® Analyzer 16 Flow Cytometer. Cells were labeled using fluorochrome-conjugated monoclonal antibodies, anti-mouse: CD45-VioBlue® (REA737) and CD31-PE-Vio® 770 (REA784), to measure viable LSECs. To enrich LSECs, flow cytometry was followed by magnetic cell labeling to deplete $CD45^+$ cells and isolate $CD146^+$ (LSECs) cells using the autoMACS® Neo Separator. Yield and viability of the enriched LSECs were determined by propidium iodide (PI) staining and analyzed by MACSQuant Analyzer 16 (fig. 3).

After enrichment, $1\times10^6$ LSECs per well were seeded in 6-well plates coated with collagen type I, using EGM™-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit™ (Lonza®) with 5% FBS.

Following culture, the functionality of LSECs was confirmed by DiI-conjugated acetylated low-density lipoprotein (DiI-ac-LDL) staining. Cells were incubated with 10 mg/ml DiI-ac-LDL (Invitrogen™) for 4 hours after 2 hours of serum starvation. After incubation and washing, cells were examined by fluorescence microscopy (fig. 4).

### Histological staining

Hematoxylin and eosin (HE), and Sirius red staining were performed on the remaining liver tissue samples to confirm the pathological changes of the MASH liver (fig. 5).

## Results

### Hepatocyte yield and viability

The pellet containing the hepatocytes was gently resuspended, and the yield and viability of the hepatocytes were quantified microscopically (table 1).

| Description | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Left lateral liver lobe weight (mg) | 400 | 390 | 340 |
| Yield (hepatocytes/ left lateral liver lobe) | $8\times10^6$ | $6.3\times10^6$ | $8\times10^6$ |
| Yield (hepatocytes/g liver) | $2\times10^7$ | $1.62\times10^7$ | $2.35\times10^7$ |
| Viability (%) | 96 | 98 | 95 |

**Table 1:** Hepatocyte yield is given as total yield per left lateral lobe and per gram (g) of mouse liver. Cell viability was determined using hematocytometer and trypan blue staining (n = 3, male STAM mice, 16 weeks).

Hepatocytes were cultured and immunofluorescent staining was performed after 24 hours to show the characteristic shape and markers of hepatocytes (fig. 2).

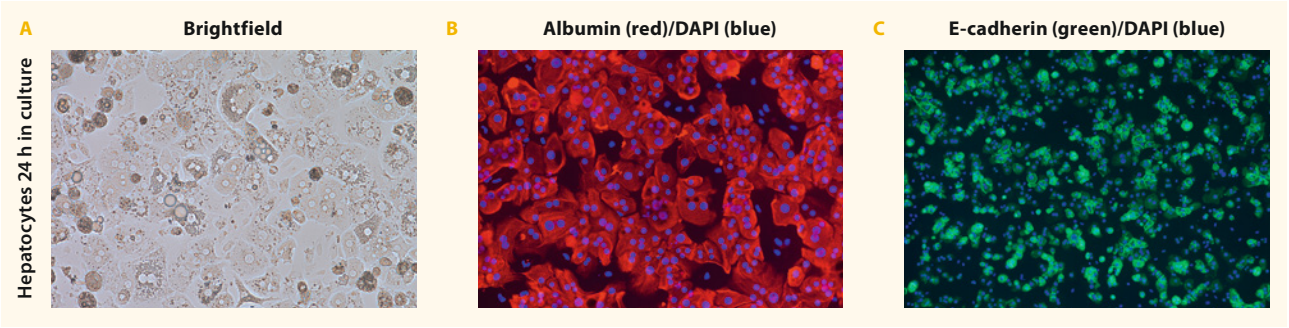


**Figure 2:** 24 hours after culturing the hepatocytes, cells were observed using (A) brightfield microscopy, showing their characteristic hexagonal shape and binucleated morphology. Immunofluorescence staining further demonstrated that hepatocytes isolated using the gentleMACS Perfusion Technology expressed typical parenchymal markers, in particular (B) albumin and (C) E-cadherin. Nuclei were stained with DAPI (blue).

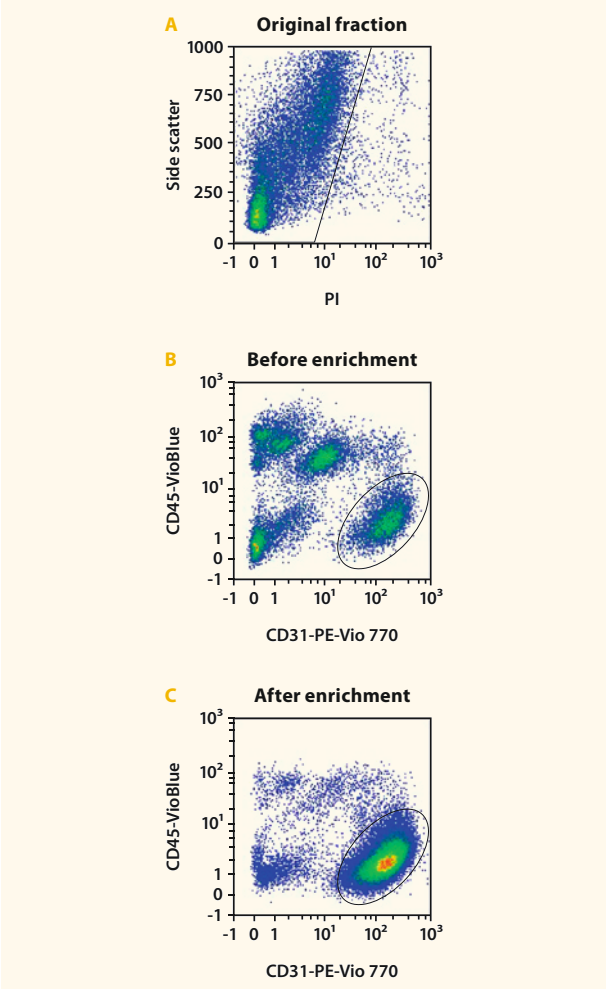


**Figure 3:** Gating strategy for the identification of LSECs. (A) Dead cells were excluded from the analysis based on scatter signals and PI fluorescence. The number of NPCs amounted to $4.7\times10^6$ cells. (B) Cells were plotted based on CD45 and CD31 expression to identify LSECs as $CD45^-$ $CD31^+$ population. (C) Following enrichment of the LSCEs ($CD\ 146^+$) using magnetic cell labeling, the purity of the $CD31^+$ $CD45^-$ cells was increased from 29% to 92%.

## LSECs

To isolate LSECs, one of the samples was further processed. For this purpose, the supernatant containing NPCs was analyzed by flow cytometry using the MACSQuant Analyzer 16. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence. Flow cytometry analysis revealed a total of $4.7\times10^6$ NPCs per left lateral liver lobe ($1.38\times10^7$ cells per gram liver) with 97% viability.
To identify LSECs and exclude other cell types, specific markers were used. CD31-PE-Vio 770 was used to positively identify LSECs as endothelial cells, while CD45-VioBlue was used to exclude non-LSEC cells, such as leukocytes. This analysis yielded approximately $1.3\times10^6$ LSECs.

Subsequently, LSECs were enriched using $CD45^+$ and $CD146^+$ MicroBeads. The autoMACS Neo Separator was used for the positive selection of $CD146^+$ cells (LSECs) and depletion of $CD45^+$ cells (leukocytes). Flow cytometry analysis showed that the purity of the LSECs increased from 29% to 92% after enrichment (fig. 3).

To confirm the characteristics and functionality of the isolated LSECs after *ex vivo* perfusion, Dil-ac-LDL staining was performed on cultured cells 44 hours after enrichment. The results showed successful uptake of Dil-ac-LDL by the LSECs, consistent with their typical phenotype. This finding confirmed the preservation of LSEC-specific characteristics after exposure to a fatty acid–rich environment (fig. 4).

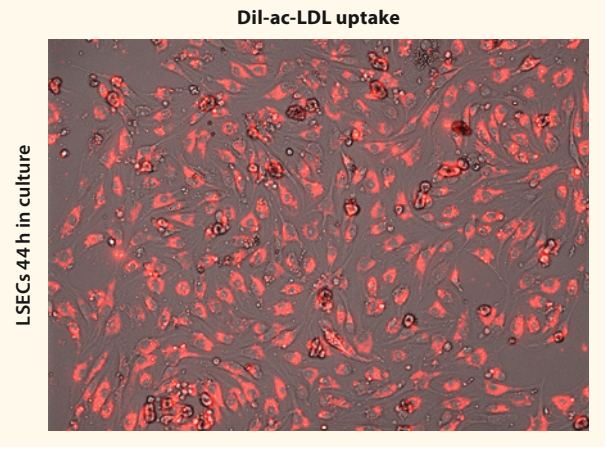


**Figure 4:** Cultured LSECs after 44 hours, demonstrating successful internalization of Dil-ac-LDL. Red fluorescence indicates the uptake of Dil-ac-LDL, confirming the functional activity of the cultured LSECs.

### Tissue validation

While the left lateral liver lobe was used for tissue perfusion, the remaining liver was left intact and used for histological staining to validate the transition from normal liver to MASH state. HE staining showed accumulation of fat droplets (steatosis) in the cytoplasm of hepatocytes (fig. 5A). Sirius red staining revealed mild fibrosis extending from around the central vein to the surrounding hepatocytes, giving a histological appearance similar to human MASH (fig. 5B). These findings confirmed that STAM mice showed fatty liver pathology and can be used as a model to study fatty liver disease.

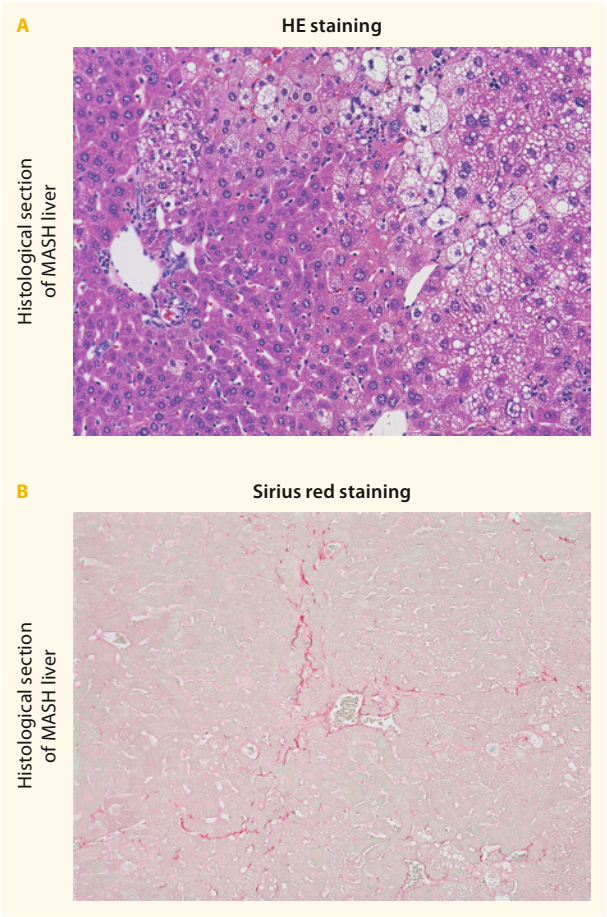


**Figure 5:** (A) HE staining was used to examine the overall tissue morphology and cellular details, confirming hepatic steatosis (accumulation of fat droplets within hepatocytes) and inflammatory changes. (B) Sirius red staining was used specifically to visualize collagen fibers within the liver samples.

## Conclusion

Miltenyi Biotec has developed a reliable and automated method using the gentleMACS Perfusion Technology to isolate high-quality parenchymal and NPCs from resected liver samples. Several publications have demonstrated the successful use of this technology to isolate liver cells from healthy rodent livers.[2–6] In this study, using STAM mice as a model of MASH liver, the gentleMACS Perfusion Technology has proven highly effective in isolating intact and functional hepatocytes as well as LSECs (as a representative subset of NPCs) from fatty liver samples, achieving both high cell yield and viability.

The gentleMACS Perfusion Technology provides an innovative method for *ex vivo* perfusion of a single liver lobe while allowing the same animal to be used for additional experimental procedures. This technology supports comprehensive research by enabling parallel studies, including immunohistochemistry, biochemical assays, and molecular analyses on different tissues or organs within the same animal.

In summary, the automated gentleMACS Perfusion Technology increases experimental efficiency by facilitating multiple analyses and providing consistent and reliable data.

## References


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