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Isolation of primary mouse hepatocytes and non-parenchymal cells from a liver with precancerous lesions

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Summary/Abstract

In the early stages of liver carcinogenesis, rare hepatocytes and cholangiocytes are transformed into preneoplastic cells, which can progressively acquire a neoplastic phenotype, favoured by the failure of natural antitumor immunosurveillance. The detailed study of both hepatic parenchymal (e.g. hepatocytes) and non-parenchymal cells (NPCs), such as immune cells, could help understand the cellular microenvironment surrounding these pre-cancerous and neoplastic lesions.

Cultures of primary hepatocytes are of interest in various biomedical research disciplines, serving as an *ex vivo* model for liver physiology. Obtaining high viability and yield of primary mouse hepatocytes and other liver cell populations is technically challenging, thus limiting their use. In the first section of the current chapter, we introduce a protocol based on the two-step collagenase perfusion technique (by inferior vena cava) to isolate hepatocytes and, to a lower extent, NPCs and detailed the different considerations to take into account for a successful perfusion. The liver is washed by perfusion, hepatocytes are dissociated with collagenase, and different cell populations are separated by centrifugation. Various techniques have been described for the isolation of healthy and malignant hepatocytes; however, the viability and purity of the isolated cells is frequently not satisfactory. Here, we significantly optimized this protocol to reach improved yield and viability of the hepatocytes and concomitantly obtain preserved NPC populations of the liver.

Within NPCs, tissue-resident or recruited immune cells are essential actors regulating hepatocarcinogenesis. However, simultaneous isolation of hepatic leukocytes together with other cell types generally yields low immune cell

numbers hindering downstream application with these cells. In the second section of this chapter, as opposed to the first section primarily aiming to isolate hepatocytes, we present a tissue dissociation protocol adapted to efficiently recover leukocytes from non-perfused bulk (pre-)cancerous livers. This protocol has been optimized to be operator-friendly and fast compared to other liver processing methods, allowing easy simultaneous sample processing to retrieve hepatic (tumor-infiltrating) immune cells.

Key words

Primary mouse hepatocytes, isolation hepatocytes, non-parenchymal cells, preneoplastic hepatocytes, collagenase perfusion, isolation hepatic leukocytes, liver tumor-infiltrating immune cells

1. Introduction

Liver cancer is the second leading cause for cancer-related mortality worldwide, with hepatocellular carcinoma (HCC) accounting for 80–90% of primary liver cancers, while cholangiocarcinoma (CCA) accounts for 10–15% **(1, 2)**. Hepatic carcinogenesis is a complex and slow process that likely starts decades before the emergence of clinically detectable tumor mass and symptoms **(3)**. During the different stages of hepatocarcinogenesis, there is an accumulation of pathological, genetic and epigenetic, changes which, together with the failure of tissue immunosurveillance, facilitate cellular malignant transformation and tumour progression **(4–6)**. The formation of neoplastic lesions, like HCC or CCA from transformed hepatocytes or cholangiocytes, respectively, comes along with the establishment of a microenvironment which contributes to the survival and proliferation of the aberrant cells. Among the protumor mechanisms at work figure the exposure or release of immunosuppressive factors as well as the recruitment of immune cells endowed with regulatory functions **(7, 8)**. Thus, a complete overview of the (pre-)cancerous cell intrinsic mechanisms and their interaction with liver-resident and infiltrating immune cells is fundamental to understand hepatocarcinogenesis **(9)**.

The liver is mainly composed of hepatocytes (parenchymal cells), accounting for 60%–80% of its content, which are accompanied by other cell types (non-parenchymal cells, NPCs) like cholangiocytes (3%–5%), immune cells (12%–15%), stellate cells (5%–8%), and liver sinusoidal endothelial cells (20%); all important for hepatic immunological activities **(10)**. Isolation of both parenchymal and NPCs cells from pre-neoplastic and cancerous liver lesions in relevant pre-clinical models of HCC and CCA (such as the orthotopic mouse models of DEN-

CCl₄-induced HCC (**11**) and of oncogene-driven CCA (**12**) are essential tools to dissect the impact of these cell populations in the development and progression of liver cancers. Therefore, it is important to develop convenient methods to isolate both murine primary hepatocytes and NPCs, notably immune cells, while maintaining their function intact.

Regarding the isolation of epithelial liver cells, obtaining a highly pure and viable hepatocyte or cholangiocyte population is still challenging (**13**). Various protocols have been developed based on similar isolation strategies but slightly differing in reagents and steps, according to the different needs (**13–15**). Isolation protocols are generally composed of two parts: perfusion with different buffers (including enzymatic digestion with collagenase) and purification of desired cells. Although, the preparation of buffers and the isolation procedure take time, the main challenge of this technique is to perform the perfusion in a correct and efficient way. Certain surgical skills are necessary to perform this protocol and some practice necessary before being successful at obtaining high viability and yield of hepatocytes.

In the first section of this chapter we describe a method for the isolation and culture of the major cell types of a mouse liver: hepatocytes and NPCs. This detailed protocol employs perfusion of the liver with a collagenase-based enzyme buffer to effectively transform the intact liver into a single cell suspension. From this initial cell suspension, hepatocytes are isolated by specific centrifugation steps, yielding highly pure hepatocyte and NPCs suspensions. In this way, a large number of viable hepatocytes can be achieved from one mouse liver. Moreover, the viability of NPCs is high and the yield obtained from one mouse liver is acceptable for functional cell studies. Nevertheless, if specific populations of

NPCs are required, variations of this protocol should be used **(16–18)**, as presented in the second section of this chapter for leukocytes. The technique described here consists in the perfusion of a liver at early stages of tumor development using a peristaltic pump, followed by a Percoll gradient centrifugation protocol to capture lived hepatocytes. Separating live from dead cells with a Percoll step is also critical in obtaining healthy primary hepatocytes with a high viability.

Perfusion techniques can be performed either on the entire liver *in vivo* **(13, 14, 19–21)** or dissected liver lobes **(22)**. The perfusion of dissected lobes is generally restricted to the left and medium lobes that are the most accessible. Even though perfusion of dissected liver lobes remains feasible, *in vivo* perfusion yields more viable primary hepatocytes due to reduced stress conditions. Indeed, in the latter process, hepatocytes bathe in a temperature and pH-controlled buffer which allows gentle tissue dissociation.

Most *in vivo* protocols utilize the two main vessels connecting the liver with other organs to insert the cannula for perfusion: inferior vena cava (IVC) **(13, 19, 20)** or portal vein **(21)**. The position of the portal vein is at a skewed angle and in direct contact with the hepatic lobe, which leads to difficulties in positioning and stabilizing the inserted cannula without damaging liver parenchyma. A suture is thus usually needed to tie the cannula in place with a surgical knot, which must be done very carefully as the cannula may move and also the portal vein is prone to damage. Generally, the diameter of the portal vein is also smaller than IVC, complicating the catheterization. In this sense, it has been reported that perfusion through IVC resulted in less viable primary hepatocytes than portal vein **(23)**. However, in our experience, the cannulation of IVC appears more convenient.

During this procedure, we also proposed as an optional step to surgically knotted the IVC to secure the cannula. In the present protocol, perfusion is proposed via IVC, and the isolated primary hepatocyte viability, measured using Trypan blue, varies between 80% and 95% depending on pre-existing damages of the liver perfused **(24–26)**. The estimated yield is ~30-60 million hepatocytes and ~1-5 million NPCs from one liver isolated from an 8 to 12-week-old mouse. Counting viability and cell number is mandatory after purification since the results of subsequent experiments will vary depending on cell confluence and cell death.

This two-step perfusion procedure consists in 1) flushing blood out of liver and “washing” the liver as well as to eliminating calcium with EGTA, and 2) dissociation with a collagenase solution that loosens attachment between cells by breaking down collagen within the extracellular matrix and releases liver cells. To achieve this, at least two buffers should be used, one to wash out the blood in the liver (Perfusion buffer) and one for digestion (Digestion buffer). In this protocol, we used a HBSS-based Perfusion buffer (with EGTA) and a Digestion buffer based on William’s Medium with added collagenase. Chelating calcium with EGTA facilitates loosening of cell-cell connections by perturbing calcium-dependent adhesion factors. The preparation of these buffers is based on commercially available solutions and does not vary in pH or components, from batch to batch, minimizing interfering variables.

In summary, this procedure can be completed in 3-4 hours, from dissecting mouse to plating isolated cell liver populations, thus providing a useful technique to primary hepatocyte-related studies. In order to test the efficiency, the present primary hepatocytes isolation protocol was performed on a oncogene-induced CCA model in early stages **(12)**. We performed the perfusion in a 16-week-old

female C57BL/6 at day 28 after delivery of the oncogene-encoding plasmids when typically, 5 to 10 (1-2 mm in size) persistent nodules are present in the liver at this time point. For other types of liver cancer such as HCC induced by Western Diet plus CCl₄ that induces NASH before tumor development, other conditions and variations of this protocol should be taken into account, such as the concentration of collagenase **(16, 25, 26)**.

While the abovementioned protocol is optimized for the recovery of a pure viable hepatocyte population, it does not always yield high cell numbers from the NPC fraction, in particular, of immune cells. Tissue-infiltrating and resident leukocytes mediate the immunosurveillance of pre-cancerous and neoplastic lesions and some rare but key cell populations such as dendritic cells can only be identified (e.g. by flow cytometry) when a high number of leukocyte is recovered. Such purposes may require not only the obtention of an abundant leukocytic fraction per animal but also the processing of multiple samples in parallel, a limitation of the protocol above presented. Thus, it is important to implement cell isolation methods allowing to recover high yields of viable leukocytes in an optimized time-frame manner.

In the second section of this chapter, we present a protocol to obtain a single cell suspension of cells isolated from a non-perfused whole (pre-)neoplastic liver. This single cell suspension is particularly enriched in leukocytes compared to other liver processing and dissociation methods, but still contains other cell types such as neoplastic cells and non-transformed cells like fibroblasts or endothelial cells. In brief, the protocol consists in a quick and easy step of liver collection, followed by tissue mechanical and enzymatical dissociation with the aid of scalpels and a commercially available enzymatic cocktail. This is complemented

by the *ex vivo* removal of erythrocytes with a red blood cell lysis buffer, avoiding the necessity for *in vivo* liver perfusion typically used to remove such cells. *In vivo* liver perfusion requires time and a well-trained person to perform it and constitutes a limiting point for simultaneous multiple sample analyses. After washing in PBS to remove remaining enzyme excess, cells are ready for downstream applications.

This protocol does not include any steps allowing the removal of cell debris and dead cells or other non-leukocyte cell types. Thus, it is optimized for downstream flow cytometry applications in which such cell types can be identified and disconsidered during post-acquisition analyses. If the objective is to obtain a pure viable leukocyte population, additional steps including removal of cell debris and dead cells (i.e. using adapted cell gradients or magnetic beads) and/or leukocyte enrichment (i.e. using magnetic beads) or cell sorting (by means of fluorescently-labeled antibodies) will be required.

When such steps are not required, the cell isolation protocol can be completed in less than 2 hours and several samples can be processed simultaneously. We have applied this protocol to recover viable hepatic leukocytes from orthotopic mouse models of HCC (by DEN-CCl₄-induction) and CCA (by oncogene injection) from up to fifty bulk liver cell suspensions bearing pre-cancerous and neoplastic lesions simultaneously.

Overall, isolation of primary hepatocytes and NPCs is used for a wide variety of experiments on liver physio(patho)logy, such as *in vitro* experiments, *ex vivo* flow cytometry or single-cell RNA sequencing analysis. Since the techniques described here are performed in a pathological liver which already presents morphological, cellular and structural changes, the recovered cell populations

can be analysed directly after isolation. Research and therapeutic applications create a high demand for primary hepatocytes and NPCs. In the two protocols presented, efficiency and cell viability were set as a priority, with all reagents ready-to-use and available commercially and isolation steps reduced to the minimal number.

2. Materials

2.1. Reagents

1. 10X HBSS^{-/-} (without calcium and without magnesium)
2. 1 M HEPES, pH=7.4
3. 0.5M EGTA
4. 1X William's E Medium (without glutamine)
5. Collagenase from *Clostridium histolyticum* (Type IV, 0.5-5.0 FALGPA units/mg solid, ≥125 CDU/mg solid)
6. 1X PBS (without calcium and magnesium, pH=7.4)
7. 1 X DMEM/F12 (1:1) (with glutamine)
8. 1X RPMI Medium 1640 (with glutamine)
9. High-glucose DMEM medium
10. Penicillin/Streptomycin
11. Fetal Bovine Serum (FBS)
12. Glucose
13. Bovine Serum Albumin (BSA)
14. Percoll
15. NaCl
16. Trypan blue solution
17. 10X Red Blood Cell (RBC) lysis buffer
18. Mouse strain: C57BL/6J (from 6 weeks-old)

19. 70% EtOH
20. Isoflurane (gas anaesthesia system; according to the specific animal protocol at your institution and animal facility)
21. Aluminum foil
22. Liver dissociation kit (Miltenyi Biotec, Ref: 130-105-807)
23. Sterile deionized distilled water (ddH₂O)
24. Perfusion buffer: 50 mL 1X HBSS^{-/-} (without calcium and without magnesium), 0.5 mL 1M HEPES pH 7.4, 0.02 mL 0.5M EGTA
25. Digestion buffer: 25 mL William's E Medium, 0.25 mL 1M HEPES pH7.4, 7.5 mg collagenase from *Clostridium histolyticum* (Type IV, 0.5-5.0 FALGPA units/mg solid, ≥125 CDU/mg solid)
26. Attachment medium (AM): 45 mL DMEM/F12, 1 mL 1M HEPES pH7.4, 0.5 mL Penicillin/Streptomycin, 0.3 mL 1M Glucose, 5 mL FBS, 0.5 mL 20% P/V BSA
27. Stock Isotonic Percoll (SIP) solution: 10 mL Percoll, 1.25 mL 1.5M NaCl, 1.25 mL 10X HBSS^{-/-} (without calcium and without magnesium)

2.2. Disposables and equipment

1. 70 µm Cell strainer
2. 100 µm Cell strainer
3. Peristaltic pump
4. Peristaltic pump 2.06 mm tubing
5. Sterile Eppendorf and centrifuge tubes

6. Sterile serological pipettes and tips
7. Gel-loading pipet tip for Western Blot (cannula)
8. Scalpel non-sterile
9. Mouse dissection tray
10. Water bath
11. Centrifuge
12. Laminar flow hood
13. Sterilized scissors
14. Sterilized curve forceps
15. Mini clamp (Bulldog serrefines)
16. Butterfly probe (grooved director)
17. Heat lamp
18. Cotton buds
19. Surgery threat
20. Ø 100 mm Petri dishes
21. 6 well-plate (Corning)
22. 12 well-plate (Corning)
23. Bright-field microscope
24. Incubator
25. Isoflurane vaporizer
26. Tape/ scotch for animal experimentation purpose

27. Parafilm
28. Dissection Tray
29. Precision balance
30. Fridge
31. Ice
32. Mixing / Rocket platform
33. (GentleMACS™) C tubes (Miltenyi Biotec, Ref: 130-093-237)
34. (GentleMACS™) Octo Dissociator with Heaters (Miltenyi Biotec, Ref: 130-096-427)
35. Sterilized surgical tools for tissue harvesting: two scissors and two forceps (named 1 and 2)
36. Sterilized surgical tools for tissue mechanical dissociation: scissors, scalpels, forceps
37. Cotton patches
38. Support for tissue harvesting

3. Methods

3.1. Isolation of primary mouse hepatocytes and NPCs by liver perfusion

3.1.1. Preparation of buffers_ Time: 30 min

1. Weigh 7.5 mg of collagenase in a sterile 50 mL centrifuge tube, protect from light and keep at -20°C until the moment of perfusion with Digestion buffer (see **Note 1**).
2. Prepare working solutions: Perfusion buffer, Digestion buffer (without adding the collagenase) and maintenance medium, here called Attachment medium (AM) (see **Notes 2, 3, 4 and 5**).
3. Pre-warm Perfusion buffer and Digestion buffer (without collagenase) at 40°C. Keep AM solution in ice.

3.1.2. Isolation of cell populations from liver

3.1.2.1. Before perfusion_ Time: 20 min

(see **Notes 6 and 7**)

1. Properly arrange all instrumentations and solutions that are used during the experimentation prior to procedure. Setup of the peristaltic pump with the proper tubing (adapted for this specific procedure) (see **Notes 8 and 9**) (**Figure 1**).
2. For mouse anaesthesia, an isoflurane chamber with nose cone attachment is needed. Anesthetize the mouse by inhalation of 1.5-3 vol% isoflurane in 100% oxygen at a flow rate of 1 L/min. The depth of anesthetization is sufficient when the following vital criteria are reached: regular spontaneous

breathing, no reflex after setting of pain stimuli between toes, and no response to pain (see **Note 10**).

3. During the complete experimentation, keep the animal with the legs fix with stripes of tape.
4. Clean abdominal area fur with 70% EtOH.

3.1.2.2. Perfusion_ Time: 30 min

1. Grab the skin with a pair of straight blunt forceps and perform a midline abdominal incision of the skin (below the thoracic cavity). Do not penetrate into the peritoneal cavity.
2. Gently free the skin from the peritoneum. Grab the peritoneum lifting straight upward and open the peritoneal cavity with a midline abdominal incision, ensuring to see the tip of the scissors in order not to injure underlying organs. Incision in the peritoneum should be performed along the linea alba (midlines white fascia) (see **Note 11**) (**Figure 2a**).
3. Gently pull out the intestines and all the peritoneal organs with a saline-moistened cotton tip (see **Note 12**). Expose the inferior vena cava and the portal vein (**Figure 2b**).
4. Isolate the inferior vena cava from the connective tissue with the help of curved forceps and cotton tips. Once the vein is isolated from its connective tissue, the forceps can be used as a guide to place a butterfly probe (grooved director) under the vein; this probe allows to pass a surgical thread under the vein and present a surgical knot. The probe can then be used as a guide to cannulate the vein (see **Notes 13** and **14**).

5. Catheterize the IVC with Perfusion buffer, maintaining a flow (<0.2 mL/min or <0.3 rpm) with the peristaltic pump. For catheterization, use a cannula (see **Notes 15, 16 and 17**).
6. Close the surgical thread to hold the cannula in place. Once the cannula is inside the IVC, the portal vein should start to inflate meaning Perfusion buffer is indeed flowing through the liver.
7. Cut portal vein to release excess blood.
8. Open the diaphragm and clamp the superior vena cava (SVC) (see **Notes 18 and 19**).
9. Infuse with Perfusion buffer (approx. 8 mL) at 2 mL/min. The buffer is kept in the thermostated bath at 40°C during perfusion. Use a heating lamp to maintain the temperature of the liver at approximately 37°C. During this step, dissolve the collagenase (7.5 mg kept at -20°C) in 25 mL of Digestion Buffer (kept in the thermostated bath) (see **Notes 20 and 21**) (**Figure 2c and 2d**). Perfusion time of liver “washing” should be 3 min approximately.
10. Change the infusion tubing into the warm Digestion buffer (25 mL). Perfuse at 3.5 mL/min until the volume of 25 mL is finished without allowing air to enter. Perfusion time should be 7 min approximately (see **Note 22**).
11. Take out the cannula and then remove gently the liver of the abdominal cavity in one piece. Using forceps, grab the liver hilum (central connective tissue between the lobes) and slightly lift upwards, cut all the connections of the liver to other organs and finally remove the gallbladder (see **Note 23**).

3.1.2.3. After perfusion: Hepatocyte isolation_ Time: 90 min

1. Place the liver on a 100 mm-diameter plate with 10 mL of cold AM and gently disaggregate it with the bottom part of two blue tips (see **Note 24**).
2. Filter the single cell suspension with a 70 μm strainer in a 50 mL centrifuge tube. Use a 10 mL serological pipette. Add another 5 mL of cold AM to rinse the $\text{\O}100$ mm plate and strainer.
3. Centrifuge at 500 rpm (30 *g*) for 5 min at 4°C.
4. Collect the supernatant (SN) containing the NPCs (see Section 3.1.2.4) or discard it. Resuspend (gently) the pellet in 12.5 mL of cold AM. From this step on, the work must be done under laminar flow if the cells are destined to *in vitro* culture.
5. Prepare a Stock Isotonic Percoll (SIP) solution containing (for 1 liver): 10 mL Percoll + 1.25 mL 10X HBSS + 1.25 mL 1.5M NaCl.
6. Gently add the 12.5 mL of hepatocytes to a 50 mL centrifuge tube with SIP solution slowly: 12.5 mL of hepatocytes already resuspended in AM over the 12.5 mL of SIP. Invert the tubes 5 times to ensure homogenization (see **Note 25**).
7. Centrifuge at 1000 rpm (218 *g*) for 10 min at 4°C.
8. Discard SN (aspiration with the pump), wash cells 3 times in 10 mL of cold AM repeating centrifugation for 5 min at 500 rpm (30 *g*) at 4°C (see **Note 26**).
9. Resuspend the cells in 5 mL cold AM and keep the suspension at 4°C.
10. Count cells and viability in a Neubauer chamber (25 μL cell suspension + 50 μL AM + 25 μL Trypan Blue).

11. Proceed with the experiments or, for *in vitro* culture, plate 500000 cells/well in 6 well-plates. Use AM (room temperature or at 37°C) for seeding. Allow cells to attach for 3-4 h at 37°C. Carefully change medium of cells (AM) and incubate overnight at 37°C in a humidified CO₂ incubator before proceeding with treatments (see **Note 27**).

3.1.2.4. After perfusion: Non-parenchymal cells isolation_ Time: 40 min

1. Collect SN for NPCs from *step 4* of hepatocyte isolation (Section 3.1.2.3).
2. Centrifuge at 500 rpm (30 g) for 5 min at 4°C to get rid of hepatocytes contamination.
3. Collect again the SN for the NPCs.
4. Centrifuge the SN with NPCs at 1500 rpm (500 g) for 5 min at 4°C.
5. Remove SN where hepatocytes will remain.
6. Add 5 mL of RBC Lysis buffer 1X to lyse the erythrocytes and incubate for 5 min at room temperature.
7. Centrifuge at 1500 rpm (500 g) 5 min at 4°C.
8. Wash 2 times with 5 mL of RPMI + 10% FBS.
9. Centrifuge at 1500 rpm (500 g) 5 min at 4°C.
10. Resuspend in 1 mL (depending on the size of the pellet) of RPMI + 10% FBS.
For *in vitro* culture: count cells and proceed to seeding (all NPCs from one liver could be seeded in 1 or 2 wells of a 12 well-plate) (see **Note 28**).

3.2. Generation of leukocyte-rich single cell suspensions from non-perfused whole livers

(see **Notes 29, 30** and **31**)

3.2.1. Reagent and material preparation_ Time: 15 min

Day -1

The following materials can be prepared the day before the experiment to reduce as much as possible the time of liver collection, processing and dissociation, preserving this way cell viability, especially when multiple samples are processed in parallel.

1. Prepare sterilized surgical tools.
2. Prepare GentleMACS™ C tubes for liver collection: Label C tubes and add 3 mL of DMEM medium. Medium-containing tubes can be stored at 4°C until use.
3. Prepare 50 mL tubes for liver processing. For this, label tubes, remove cap and place a cell strainer of 100 µm on top. Keep all the tube caps for later use. Cover with aluminum foil until use to prevent contamination.
4. Prepare 15 mL tubes for liver processing (see **Note 32**). For this label tubes, remove cap and place a cell strainer of 70 µm on top. Keep all the tube caps for later use. Until then, cover with aluminum foil to prevent contamination.

Day 0

The following buffer can be prepared before starting the liver collection.

5. Prepare liver dissociation mix following the manufacturer's instructions (i.e. Miltenyi Biotec). Briefly, first dissolve the following enzymes (see **Note 33**):

- Enzyme D: Dissolve powder in 3 mL of DMEM medium. Place the tube for 5-10 min on a mixing platform to reach complete solubilization. Do not pipette or vortex.
 - Enzyme R: Dissolve powder in 2.7 mL of DMEM medium.
 - Enzyme A: Dissolve powder in 1 mL of DMEM medium.
6. Then, prepare the liver dissociation mix. Consider 5 mL of mix per dissociated liver. Liver dissociation mix (5000 μ L): 4700 μ L DMEM, 200 μ L Enzyme D, 100 μ L Enzyme R, 20 μ L Enzyme A (see **Note 34**). For optimal liver dissociation, pre-warm the liver dissociation mix at 37°C in a waterbath or incubator until use.

3.2.2. Liver collection_ Time: 2-5 min per sample

1. Euthanize mouse (see **Note 35**).
2. After vital signs vanished, fix the animal in an appropriate support using tape to retain extremities. Wet abdominal fur using 70% EtOH and cotton patches. Using forceps and scissors, perform laparotomy, opening the peritoneum in “V” shape in order to expose the abdominal cavity and fully visualize the liver. Retrieve the skin above the abdomen if necessary (see **Note 36**).
3. To visualize the portal vein and hepatic vein and artery, first delicately move the lateral and medium liver lobes up with a clean forceps (see **Note 37**). Then, separate liver from other abdominal organs by slightly pushing abdominal organs down with forceps. Using clean scissors, cut the portal vein and hepatic vein and artery to separate the liver from the abdominal organs. If necessary, separate or cut other liver connections with supportive tissue. After this, liver will be completely isolated from other organs.

4. Transfer the isolated liver to a Petri dish containing 1X PBS. Remove the bile vesicle and wash the liver. Weight the liver to adjust, if necessary, the volume of liver dissociation mix (see **Note 38**). Then transfer to C tubes placed on ice (**Figure 3**).

3.2.3. Liver processing and dissociation into a single cell suspension_

Time: 1h30 per sample (but simultaneous sample isolation is possible)

1. Remove 1.5 mL of medium from C tubes. Then cut liver into small pieces (around 1 mm²) by either using scissors directly inside the C tube or else by transferring the liver and medium to a petri dish and cut with scalpels or scissors and forceps. For the latter, put back the processed liver in the C tube.
2. Add 5 mL of pre-warmed liver dissociation mix to each C tube. Close the tube (until hearing the cap click) and turn it upside down so that all the liver cell suspension ends up on the cap of the C tube. Then place it in the gentleMACS™ Octo Dissociator with heaters (see **Note 39**) (**Figure 3**).
3. On the gentleMACS™ Octo Dissociator, launch the following programs (last software update in June 2021):

Program	Number of rounds
m_liver_03	1
37C_m_LIDK_1	1
m_liver_04	1

4. After dissociation, filter liver suspension through 100 μm cell strainer on top of a 50 mL tube. Add 5 mL of DMEM medium on top of the filter to wash it. Then discard the filter and close the tube.
5. Centrifuge for 5 min at 300 g at 4°C (see **Note 40**).
6. In the meantime, prepare 1X Red Blood Cell (RBC) lysis buffer by diluting 10X RBC lysis buffer in sterile deionized distilled water (ddH₂O). *Example for 10 mL = 1 mL 10X RBC lysis buffer + 9 mL ddH₂O.*
7. Discard supernatant by aspiration and resuspend the cell pellet in 5mL of 1X RBC lysis buffer to remove erythrocytes (**Figure 3**).
8. Incubate at room temperature for 7 min with 1X RBC lysis buffer (see **Note 41**).
9. Add 5 mL of PBS to each tube.
10. Centrifuge for 5 min at 400 g at 4°C.
11. Discard supernatant by aspiration.
12. Add 10 mL of PBS to wash the cell pellet. Close the tube and mix well by shaking until the pellet is fully resuspended. If needed, resuspend it by pipetting (see **Note 42**) (**Figure 3**).
13. Pass the cell suspension through a 70 μm cell strainer on top of a 15 mL tube. Then discard the filter and close the tube.
14. Centrifuge for 5 min at 400 g at 4°C.
15. Discard supernatant by aspiration.
16. Resuspend cell pellet according to downstream applications. Pipette gently to guarantee sample homogeneity. Some examples of applications include:
 - Cell surface and intracellular staining for flow cytometry.
 - Cell culture (see **Note 43**).

- Single-cell RNA sequencing analyses (see **Note 43**).

4. Notes

1. Excessive collagenase will lead to overdigestion and should be avoided to prevent cell death. If overdigestion is observed, the concentration of collagenase in the Digestion buffer should be reduced.
2. Perfusion buffer, Digestion buffer (without collagenase), AM and collagenase (7,5 mg aliquot kept at -20°C) can be prepared the day before the perfusion and kept at 4°C.
3. Stock Isotonic Percoll (SIP) solution should be prepared extemporaneously, before the Percoll gradient.
4. Maintain sterile conditions during this procedure, mainly if the final goal is to culture the isolated cells. It is better to prepare the buffers and, if possible, conduct the perfusion and following steps under a laminar flow (specially once the liver is removed from the peritoneal cavity).
5. Many protocols add dexamethasone, insulin or non-essential aminoacids to maintenance medium. These reagents are not necessary for short-term experiments and may affect hepatocyte physiology.
6. Wear latex gloves, face mask, surgical gown and hair cap to protect the human operator and keep the perfusion field reasonably aseptic.
7. Experiments must comply with institutional and ethics regulations concerning the use of animals for research purposes.
8. We recommend before all isolations to rinse the tubing pump with 70% EtOH and then with 1X PBS or 1X HBSS. Finally, rinse the tubing with warm Perfusion buffer and let the solution circulate the system for some minutes before cannulation. Verify that there is no air inside the tubing.

9. The cannula is connected to the outlet end of the tubing and fix with parafilm.
The tubing and cannula fixture can be supported by an object to elevate it from the dissection tray. This elevation facilitates proper cannulation and the object allows then to fix the tubing with a stripe of tape.
10. Other anaesthetics can be used, such as ketamine/xylazine mixture.
11. While cutting to the sides to completely open the peritoneal cavity, be aware of the presence of small veins in the peritoneum and try to elude them to avoid unnecessary bleeding before the perfusion.
12. Do not injure any organ or cause any stress, the physiology of the animal must remain intact until the perfusion is completed.
13. Slowly try to separate the inferior vein cava from the fat with the help of the cotton tips, curved forceps can be used at both sides of the vein to separate it from connective tissue. Be careful not to put pressure or touch the vein as this may tear apart the vein and cause the liver to bleed out.
14. Some perfusions by IVC are done without the isolation of the vein. Nevertheless, we prefer to isolate the vein before inserting the cannula since it helps to visualize the vein and increases the chance of successful cannulation.
15. The cannula used in this protocol is a gel-loading pipet tip for Western Blot, with a bevel made with a scalpel. A mice catheter or needle (27-gauge) can also be used. In our experience, the cannula used in this protocol enter the IVC without any problem and does not tear apart the vein.

16. At the moment of catheterization, the pump speed should be set to the minimum. Once the cannula is inside the vein, the peristaltic pump speed can be set up to the standard flow rate for perfusion.
17. Insert the cannula above the kidney parallel to the inferior cava vein, before reaching the hepatic hilum.
18. We close the system by clamping the SVC in order not to have reflux of the solutions perfused, the buffers and excess blood can exit from the portal vein.
19. Steps 5 to 8 should be done as fast as possible to avoid disturbing the liver and have an homogenous perfusion. Once the SVC is clamped, the isoflurane mask can be removed and the isoflurane chamber switched off.
20. At this moment, if the catheterization and perfusion are properly performed, the liver should lose its typical red colour and become pale, and slightly swollen. If the portal vein exit is blocked for a few seconds with a cotton tip, the liver should swell and then relax. Sometimes, the IVC is cannulated but the liver does not change color. This may be because the cannula punctures the IVC twice, entering and exiting the vein; in this case, try to pull the cannula back in the vein without removing it completely. It is important that all the hepatic lobes become washed and change color to ensure a 100% perfusion of the hepatic tissue before introducing the Digestion buffer.
21. The temperature of the buffers and conditions under which the surgery is performed determine how well primary hepatocytes will survive the isolation. Low temperature may cause increased death of hepatocytes by cold shock and reduce isolation yield. Besides, the collagenase within the Digestion Buffer needs an optimal temperature for maximal enzymatic activity. The

chosen water bath temperature may be adjusted considering the time that the buffers take to flow through perfusion tubing until it reaches the liver (length of the perfusion tube), the lab conditions, the environment temperature, and the type of Peristaltic Pump. Also, the distance between the heating lamp and the liver must be taken into account in order to maintain optimal temperature conditions.

22. The time of perfusion with the Digestion buffer will depend on the specific collagenase activity (units/mg). Collagenase is a calcium-dependent enzyme and therefore calcium is included in the Digestion buffer. Since collagenase activity varies between lots, we recommend optimization when using a new lot. Therefore, careful observation of the timing of digestion is essential, as this otherwise can result in either incomplete digestion or overdigestion.

23. To clean the tubing, rinse it with 70% EtOH.

24. If the liver does not disaggregate properly, it means that the digestion was insufficient. On the other side, if it disaggregates too easily it tends to indicate overdigestion.

25. The volume of the density gradient is optimized for one murine liver per gradient.

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

27. While most protocols require pre-coating of plates with collagen or gelatine for better attachment of primary hepatocytes, in the present protocol we found that plate coating was not necessary for certain plates (e.g., Corning).

28. After 30 minutes, transfer the medium from the plated well to a clean one, and add 1mL of RPMI + 10% FBS to the first empty well where the Kupffer cells will remain, while the hepatic stellate cells will remain in the second well. Since the yield of NPCs is not high, pooling livers is recommended to increase cell number.
29. Due to the use of the mouse liver dissociation kit (Miltenyi Biotec, Ref: 130-105-807) this protocol is optimized for the recovery of immune cells from liver for downstream purposes (flow cytometry, cell culture, etc.) If other cell populations need to be recovered (e.g. hepatocytes, fibroblasts), we recommend adapting filter size to your desired population and evaluate impact of processing on cell viability of non-immune cell populations.
30. If the final purpose requires cell sterility (i.e. for cell culture), perform all steps under sterile conditions (i.e. appropriate cell culture hood, sterile or filtered reagents).
31. All along the protocol, if temperature is not specified, products are used at room temperature (i.e. 16°C -23°C).
32. If a small cell pellet is expected, we recommend the use of 15 mL tubes to facilitate cell pellet visualization. If a large cell pellet is observed, 50 mL tubes can be used.
33. Dissolved enzymes are stable up to 6 months after aliquoting when stored at -20C.
34. The following recipe is recommended for one mouse with a liver weight ranging between 750 and 1200 mg.
35. We performed euthanasia by cervical dislocation of conscious non-anesthetized mice following the institutional and ethics regulation concerning

the use of animals for experimental purpose. Alternatively, mice can be euthanized under anesthesia (i.e. using a chamber with isoflurane at 2.5-3%).

36. The use of two pairs of forceps and scissors, one for the fur and skin and one for liver collection is recommended to avoid liver contamination.

37. During all the liver collection, avoid pressing the liver with forceps to reduce potential liver damage.

38. Knowing the liver weight may be necessary for some downstream applications (i.e. normalization of flow cytometry data).

39. If the gentleMACS™ Octo Dissociator with heaters is not available, dissociation can be performed in gentleMACS™ Octo Dissociator without heaters by replacing program “37C_m_LIDK_1” step by a 30 minutes incubation at 37°C under continuous rotation of the liver suspension, as indicated in the manufacturer’s instructions.

40. Setting temperature at 4°C is recommended to preserve cell viability, but all centrifuge steps can alternatively be performed at room temperature.

41. If after 7 min erythrocytes are still visible (red pellet), prolong incubation up to a total time of 10 min.

42. We prefer to avoid pellet pipetting to reduce the risk of cell sticking and loss to the tips.

43. To this end, if a pure viable leukocyte population is desired, we recommend to proceed to additional steps including removal of cell debris and dead cells (i.e. using adapted cell gradients or magnetic beads) and/or leukocyte enrichment (i.e. using magnetic beads) or cell sorting (by means of fluorochrome-labeled antibodies).

Conflicts of interest

GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Sotio, Tollys, Vascage and Vasculox/Tioma. GK has been consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders.

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Figure legends

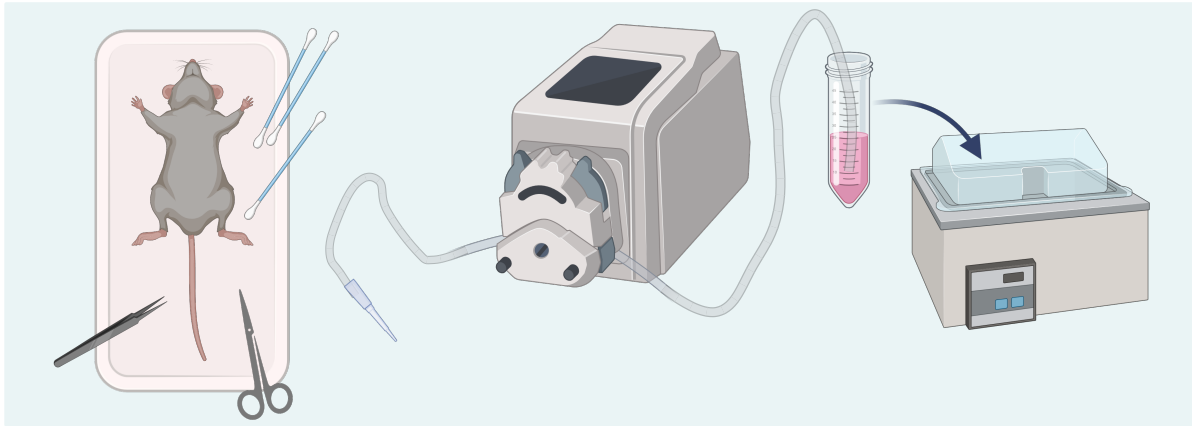
Figure 1. Graphical abstract of the procedure for the isolation of hepatocytes and NPCs by IVC perfusion of livers with (pre-)cancerous lesions.

Figure 2. Step-by-step IVC perfusion. **(a)** Abdominal cavity exposure. **(b)** IVC exposure and visualization. **(c)** IVC cannulation and SVC clamp. **(d)** Visualization of fluids exiting through portal vein.

Figure 3. Summary of the main steps required for the generation of a leukocyte-rich single cell suspensions of leukocytes from non-perfused whole (pre-)neoplastic livers.

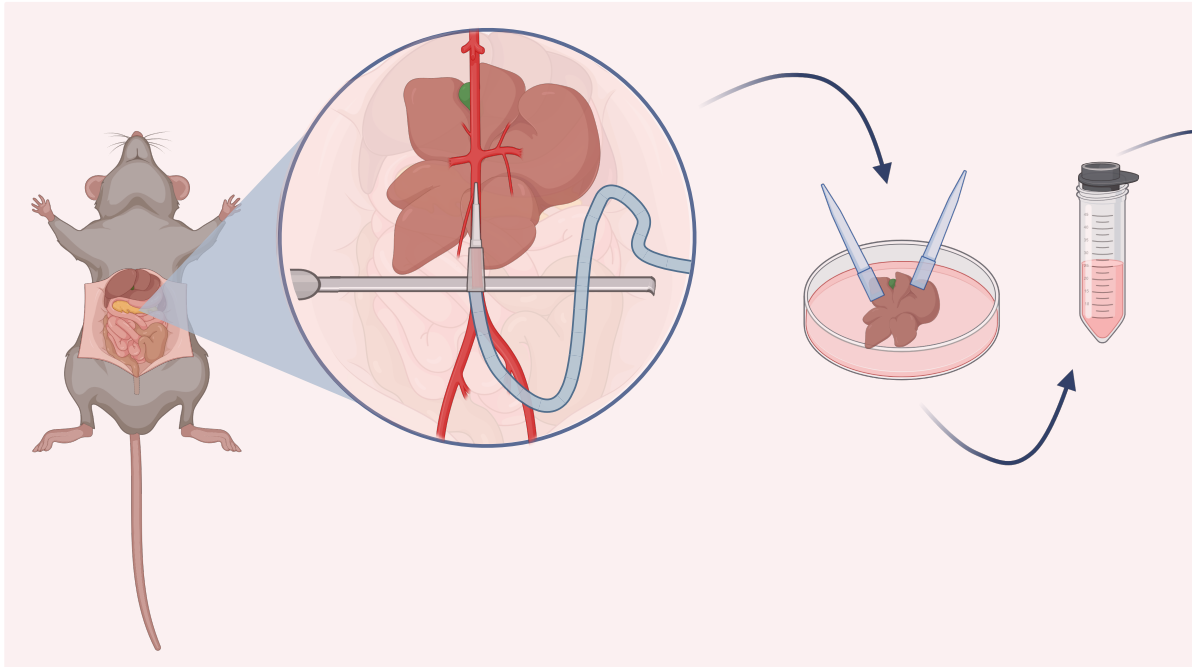
Before perfusion

Buffer preparation and workspace setup

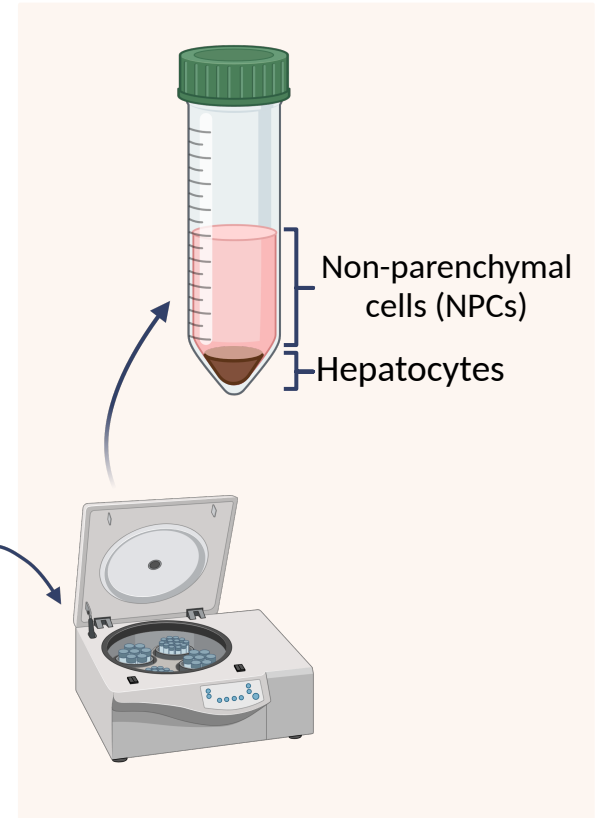


Perfusion

Cannulation and liver disgregation



Isolation & Purification





*Whole
non-perfused
liver*



Mechanical processing

**Mechanical +
enzymatic
dissociation**

Cell filtration

**Red
blood
cell
removal**

Cell filtration

Wash

**Bulk liver
single cell
suspension
(leukocyte-
rich)**

Total time
(min)

0

45

50

65

80