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Protocol for Primary Mouse Hepatocyte Isolation -- University of Minnesota TMCs

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Cellular Senescence Network (SenNet) Method Development Community

Allie Pybas

ABSTRACT

STAR Protocol for Primary Mouse Hepatocyte Isolation -

Protocol for primary mouse hepatocyte isolation_STARProtocols.pdf

Protocol used for isolating primary hepatocytes to be used in scRNAseq and bulk RNA seq.

ATTACHMENTS

863-2227.pdf

MATERIALS

KEY RESOURCES TABLE

	A	B	C
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
	Chemicals, Peptides, and Recombinant Proteins		
	HBSS with calcium, magnesium and phenol red	Biological industries	02-015-1A
	HBSS no calcium, no magnesium and no phenol red	Biological industries	02-018-1A
	EDTA (0.5 M)	Fisher bioreagents	BP2482-500
	HEPES (1 M)	Sigma-Aldrich	H0887-100ML
	Ketamine (Clorketam)	Vetoquinol	00 92297 43 082
	Xylazine (Sedaxylan)	EuroVet	SEDAXYLAN

Protocol status: Working
We use this protocol and it's working

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	A	B	C
	DMEM low glucose	Biological industries	01-050-1A
	Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium	Biological industries	02-023-1A
	Phosphate Buffered Saline (PBS) ×10	Hylabs	BP507/500D
	L-Glutamine Solution	Biological industries	03-020-1B
	Penicillin-Streptomycin Solution	Biological industries	03-031-1B
	William's E Medium, no glutamine	Gibco	12551-032
	Fetal Bovine Serum (FBS)	Biological industries	04-007-1A
	Collagen	Sigma-Aldrich	C3867-1VL
	Percoll	Santa Cruz biotechnologies	sc-500790A
	Trypan Blue Solution	Biological industries	03-102-1B
	Liberase™ TM Research Grade	Sigma-Aldrich	5.4E+09
	Experimental Models: Organisms/Strains		
	Mouse strain: C57BL/6JOlaHsd	Envigo	N/A
	Other		
	Insulin syringe	Becton Dickinson (BD)	BD 324912
	Cell strainer, 70 µm	Corning	CLS431751-50EA
	Cell lifter	Corning	CLS3008
	Peristaltic pump	Gilson	Miniplus 3 PVC tubing 2.06 mm diameter
	Mouse dissection tray	N/A	N/A
	Water bath	N/A	N/A
	Sterile 50 mL centrifuge tubes	Corning	430829
	Sterile 25 mL serological pipettes	Bio-SORFA	315100
	27 gauge needle	BD Microlance	302200
	70% ethanol	N/A	N/A
	Head wearing magnifier eye loupe (optional)	N/A	N/A

MATERIALS AND EQUIPMENT

Collagen Solution (50 mL)

	A	B	C
	Reagent	**Final Concentration**	**Stock Concentration**
	Collagen	0.01%; 0.1 µg/mL	100%; 1 mg/mL
	Sterile double deionized water (DDW)	-	-
	Total		

Note: Due to viscosity of collagen, we recommend doing a serial dilution (e.g., diluting collagen 1:100 and then diluting it again 1:100).

Anesthesia Mix

	A	B	C	D
	Reagent	**Final Concentration**	**Stock Concentration**	**Volume (µL)**
	PBS	N/A	N/A	40
	Ketamine	30 mg/mL	100 mg/mL	30
	Xylazine	6 mg/mL	20 mg/mL	30
	Total			100

Perfusion Buffer

Perfusion Buffer

	A	B	C	D
	Reagent	**Final Concentration**	**Stock Concentration**	**Volume (mL)**
	HBSS no Ca^{2+} no Mg^{2+} no phenol red	-	-	487
	EDTA	0.5 mM	0.5 M	0.5
	HEPES	25 mM	1 M	12.5
	Total			500

Notes:

- The final pH at 37°C should be 7.4
- EGTA can be used as alternative to EDTA. We have found no difference in yield

between the two chelating agents.

Digestion Buffer

	A	B	C	D
	Reagent	**Final Concentration**	**Stock Concentration**	**Volume (mL)**
	HBSS with Ca^{2+}, Mg^{2+} and phenol red	-	-	487.5
	HEPES	25 mM	1 M	12.5
	Total			500

Note: The final pH at 37°C should be 7.4.

Maintenance Media

	A	B	C	D
	Reagent	**Final Concentration**	**Stock Concentration**	**Volume (mL)**
	Williams E media	-	-	490
	Glutamine	1%; 2 mM	100%; 200 mM	5
	Penicillin-Streptomycin Solution	1%	100%	5
		Pen-100 units/mL	Pen-10,000 units/mL	
		Strep-0.1 mg/mL	Strep- 10 mg/mL	
	Total			500

Note: Many protocols add dexamethasone, insulin, transferrin and selenium to maintenance media. We found that this is not needed in short-term culturing. Importantly, these reagents profoundly affect hepatocyte biology (Batista et al., 2019; Goldstein et al., 2013; Lin et al., 2007; Weiller et al., 2004) and thus may affect experiment outcome.

Plating Media

	A	B	C	D
	Reagent	**Final Concentration**	**Stock Concentration**	**Volume (mL)**
	DMEM low glucose	-	-	470
	FBS	5%	100%	25

	A	B	C	D
	Penicillin-Streptomycin Solution	1%	100%	5
		Pen-100 units/ mL	Pen-10,000 units/ mL	
		Strep-0.1 mg/ mL	Strep- 10 mg/ mL	
	Total			500

Liberase Stock Solution

	A	B	C
	Reagent	**Concentration**	**Amount**
	Liberase	1 mg/mL	50 mg
	Digestion buffer	-	50 mL
	Total		50 mL

Notes:

- The preparation of Liberase solution detailed here relates to preparation of stock concentration from powder. The stock is further diluted to a final concentration of 25 µg/mL during the procedure (step 5).
- We found that Liberase, a specific type of collagenase is significantly more reproducible than other commercial collagenases.
- Aliquot and store at -80°C. We have found that using aliquoted and frozen Liberase (with one or two freeze-thaw cycles) does not substantially affect enzyme activity (in contrast to other types of collagenase). Thus, there is no need to freshly prepare a Liberase solution.

Percoll Solution

	A	B	C	D
	Reagent	**Final Concentration**	**Stock Concentration**	**Volume (mL)**
	Percoll	90%	100%	9
	PBSX10	1×	10×	1
	Total			10

Note: Percoll solution should be prepared fresh during the procedure.

HBSS, calcium, magnesium **Sartorius Catalog #02-015-1A**

HBSS, no calcium, no magnesium, no phenol red **Sartorius Catalog #02-018-1A**

Ethylenediaminetetraacetic Acid (0.5M Solution/pH 8.0), Fisher BioReagents **Fisher Scientific Catalog #BP2482-500**

HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #Sigma H0887**

DMEM, low glucose, no glutamine **Sartorius Catalog #01-050-1A**

DPBS, no calcium, no magnesium **Sartorius Catalog #02-023-1A**

Phosphate Buffered Saline (PBS) x10 **Hylabs Catalog #BP507/500D**

L-Glutamine Solution **Sartorius Catalog #03-020-1B**

Penicillin-Streptomycin (10X) Solution **Sartorius Catalog #03-031-5B**

Gibco™ Williams E Medium, no glutamine **Fisher Scientific Catalog #12599059**

Certified Fetal Bovine Serum (FBS) **Sartorius Catalog #04-001-1A**

Collagen, Type I solution from rat tail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C3867-1VL**

Percoll®, 100 ml **Santa Cruz Biotechnology Catalog # sc-500790A**

Trypan Blue Solution **Sartorius Catalog #03-102-1B**

Liberase™ TM Research Grade **Merck MilliporeSigma (Sigma-Aldrich) Catalog #5401127001**

BD Veo™ Insulin Syringes with BD Ultra-Fine™ Needle 6mm x 31G 1mL/cc**Becton Dickinson (BD) Catalog #324912**

Corning® cell strainer **Corning Catalog #CLS431751-50EA**

Corning® cell lifter **Merck MilliporeSigma (Sigma-Aldrich) Catalog #CLS3008-100EA**

50 mL centrifuge tube **Corning Catalog #CLS430829**

BD Microlance™3 Needles **Becton Dickinson (BD) Catalog #302200**

BEFORE START INSTRUCTIONS

BEFORE YOU BEGIN

Note

Timing: 20 min. (plus incubation for 4–16 h)

Note: Steps 1–4 should be done in sterile conditions, in a biological hood (i.e., biosafety cabinet)

1. Prepare cell culture plates; 12-well, 6-well, 10 cm, and 15 cm plates are suitable for hepatocyte plating.

Note

Note: Lower diameter plates may be suitable but are less optimal due to decreased efficiency of cell dispersion across the well surface.

2. Cover the bottom of the plates/wells with 0.01% rat-tail collagen solution.
3. Incubate for 4–16 h at 37 °C under sterile conditions (e.g., in a humidified

CO_2 incubator).

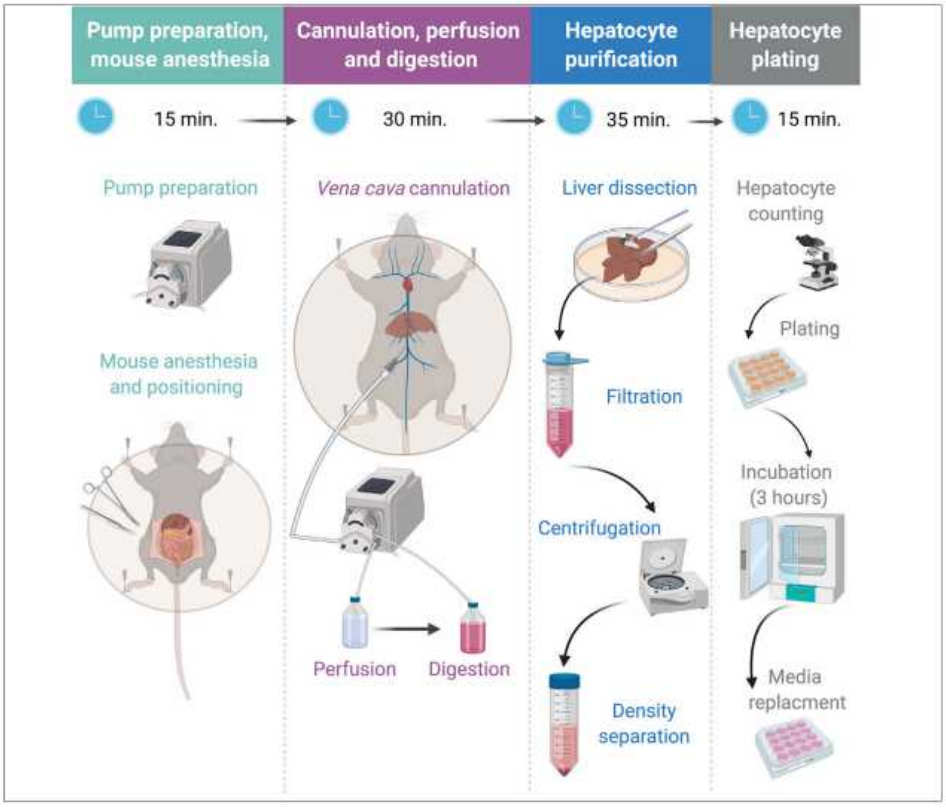
4. Wash with PBS, aspirate PBS.

Note

Note: Collagen-coated plates can be prepared days in advance provided they are stored under sterile conditions.

STEP-BY-STEP METHOD DETAILS

This protocol is aimed at isolating hepatocytes from mouse liver. Following anesthesia, the vena cava is cannulated and the liver is perfused to chelate calcium and wash out blood. Then, collagenase is perfused to the liver in order to dissociate extracellular matrix. Finally, the liver is dissected and hepatocytes are purified by density-based separation. This protocol presents several advances over similar protocols (Berry and Friend, 1969; Casciano, 2000; Klaunig et al., 1981; Li et al., 2010; Renton et al., 1978; Seglen, 1976; Severgnini et al., 2012). The main improvements of this protocol are better reproducibility, shortened duration, reduced technical challenge, increased yield and higher viability. These are achieved by several steps we altered or optimized. For example: (a) We found that retrograde perfusion through the vena cava permits easier cannulation as opposed to portal vein cannulation. (b) Periodical clamping of the portal vein provides a visible checkpoint for proper perfusion and greatly facilitates efficient washing and digestion. (c) The type of collagenase used (Liberase) shows quicker digestion and better reproducibility compared to other collagenases. (d) Percoll-based density separation results in a population of purified hepatocytes of high viability. Some of these protocol improvements were already implemented in our previous publications (Goldstein et al., 2017a; Goldstein et al., 2017b) where we isolated hepatocytes for experiments
demanding a high yield of cells (such as chromatin immunoprecipitation sequencing – ChIP-seq).



Pump Preparation and Mouse Anesthesia

1

Note

Timing: 15–20 min

Here, the pump is washed and primed with perfusion buffer. The mouse is anesthetized and positioned on the dissection tray.

This section is shown in Methods Video S1.

Warm water bath to 42 °C.

2 Place perfusion buffer in the water bath.

3 Prepare the peristaltic pump:

3.1 Run 70% ethanol through the tubing.

3.2 Run air through the tubing for 30–60 s.

3.3 Wipe the end of the tubing with a paper towel.

3.4 Connect 27-gauge needle to the outlet end of the tubing using a luer lock (Figure 1).

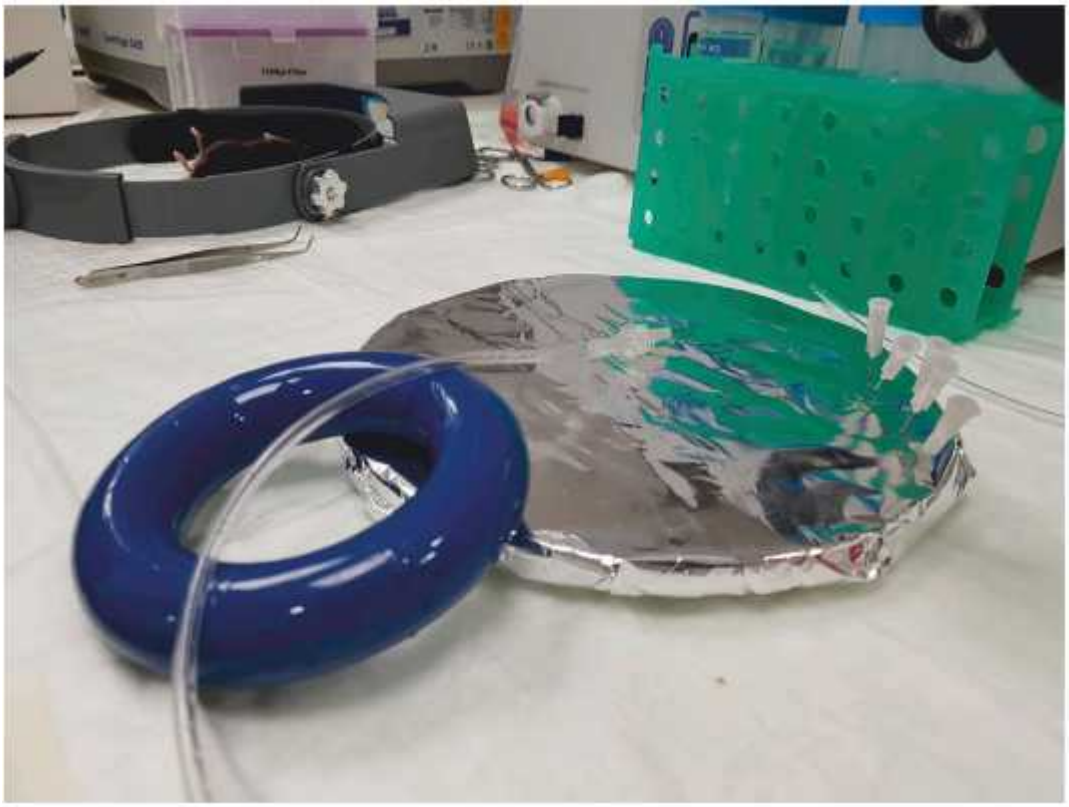


Figure 1. Needle and Tubing Preparation.
The needle is connected to the outlet end of the tubing by using a luer lock. The tubing

and needle fixture is supported by a heavy object (here a media bottle ring weight) to elevate it from the dissection tray. This elevation facilitates proper cannulation.

4 Prime the tubing with warm perfusion buffer (pump speed 3 mL /min).

Note

Notes:
- Purge some perfusion buffer to wash residual ethanol.
- Avoid bubbles in the tubing.
- The optimal pump speed varies greatly depending on the tubing diameter, needle gauge and other specifications. In this protocol, pump speed was optimized to the detailed tubing specifications and needle gauge (see Key Resources Table).

5 Prepare two 50 mL tubes (labeled "DB-chilled" and "DB-Liberase"), each with 10 mL digestion buffer. Keep the tube labeled "DB-chilled" On ice . Add 250 µL Liberase solution to the tube labeled "DB-Liberase" and warm it in the water bath.

6 Anesthetize mouse by intraperitoneal injection of anesthesia mix (3.75 µL /g body weight. Final concentration for ketamine = 112.5 mg /kg; for xylazine = 22.5 mg /kg).

Note

Notes:
- Make sure the mouse is completely anesthetized by the pedal reflex.
- Mice should be 8–10 weeks old, younger mice have smaller veins, older mice have more fat lining the vena cava. Both these attributes make cannulation more challenging.

7 Place mouse on the edge of the dissection tray and secure limbs using needles (Figure 2).

Note

Note: The head should be protruding outside the tray. This facilitates easier cannulation in later steps.

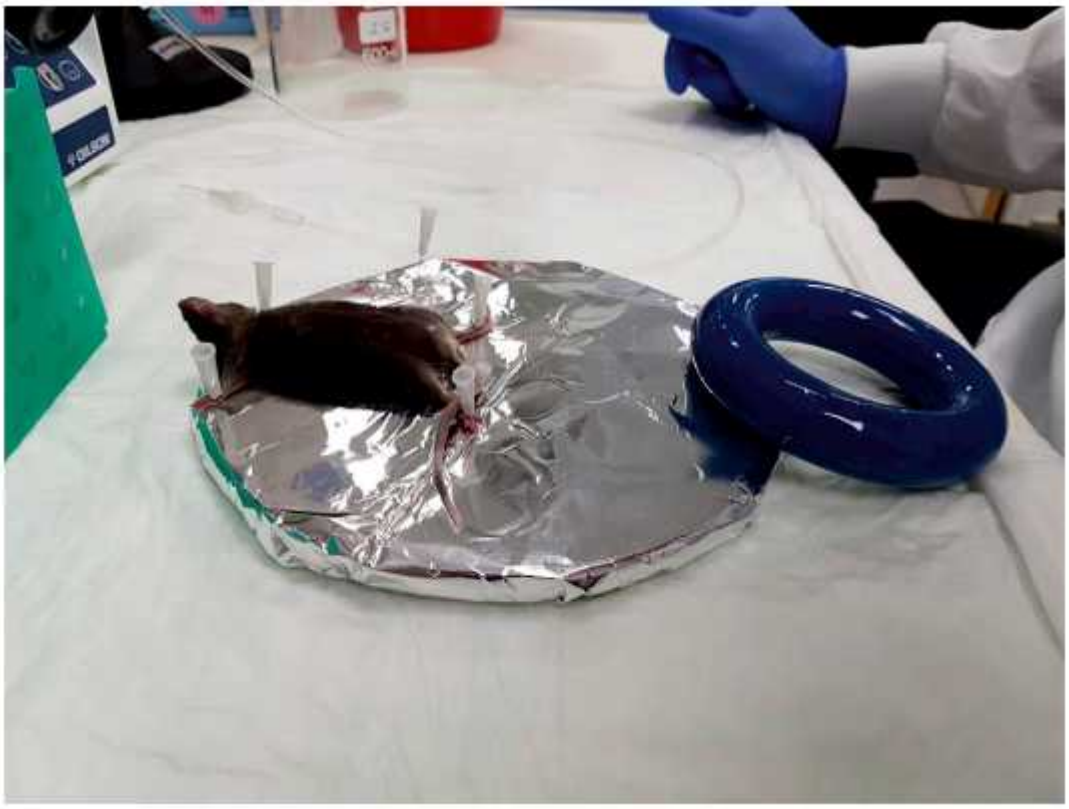


Figure 2. Mouse Positioning on the Dissection Tray.
The anaesthetized mouse is placed on the edge of the dissection tray with its head protruding outside and its limbs secured with needles. Securing limbs with tape is not recommended because the area will be soaked with liquid during procedure, potentially loosening adhesion.

8 Wet the fur thoroughly with 70% ethanol.

9 Make a “U”-shaped incision through the skin, secure the skin near the head using a needle (Figure 3).

Figure 3. Incision and Preparing for Cannulation.
The mouse fur and skin are cut in a "U" shape, the mouse skin is placed and secured near the head with a needle.

10 Move the intestine to the right to reveal the portal vein and vena cava (Figure 4). Place a stable and heavy object adjacent to the mouse hind legs to support the tubing such that it is slightly higher than the mouse, lay the tubing and the needle on the object (Figure 1). The edge of the needle should rest on the vena cava in a flat angle (i.e., parallel to the vein, Figure 5).

Note

Note: Make sure that the tubing and needle do not move or change angle when you let go.

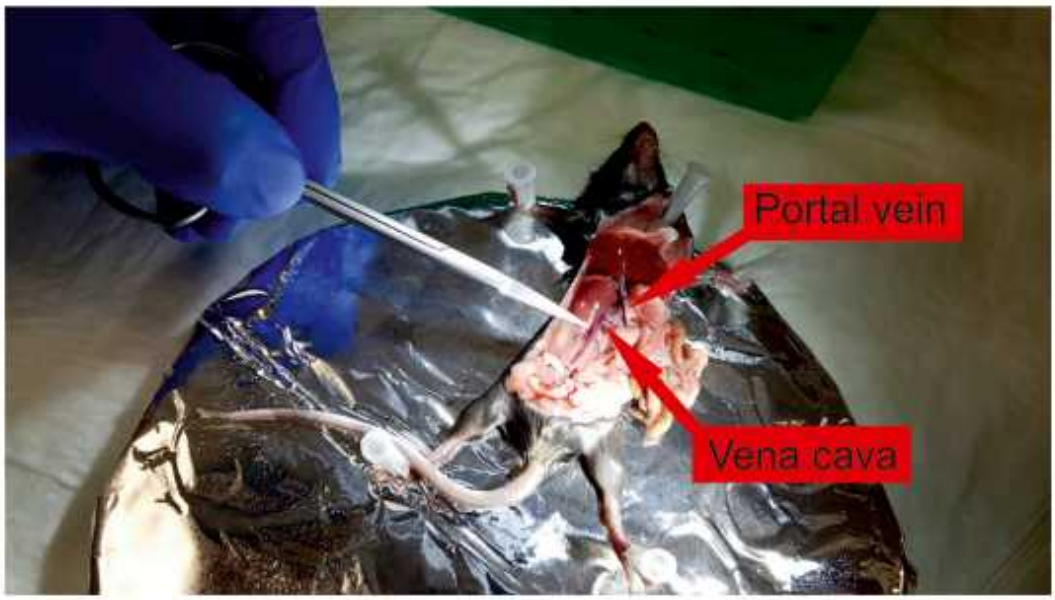


Figure 4. Portal Vein and Vena Cava exposure.
Mouse intestine and the rest of the viscera are moved to the right. Both the portal vein and vena cava are revealed.

Figure 5. Cannulation of the Vena Cava.
The tip of the needle is inserted (bevel side up, in an almost flat angle) into the vena cava above the kidney. The tubing rests on the elevating object without manual support.

Cannulation and Perfusion

30s

11

Note

Timing: 10–15 min

Here, the inferior vena cava is cannulated and the liver is perfused to wash out blood and circulating cells from the liver as well as to eliminate calcium via EDTA. Chelating calcium with EDTA facilitates loosening of cell-cell connections by perturbing calcium-dependent adhesion factors. This step, initially suggested by (Seglen, 1976) serves as a preparative step for liver digestion in the next section. The original protocol was developed for rat and therefore, perfusing the liver through the portal vein was amenable. We found that in mice, retrograde perfusion via the inferior vena cava is significantly less challenging and therefore more reproducible.

This section is shown in Methods Video S2.

Turn on the pump and let the warm perfusion buffer reach the needle (the buffer within the tubing has already cooled down to 20-25 °C).

Note

Note: Before proceeding, make sure you clearly see the portal vein and vena cava.

12

While buffer is running through the needle, insert the needle into the vena cava above the kidney (Figure 5).

Note

CRITICAL: After 1–2 s, you should see white spots forming in the liver and/or expansion/ swelling of the portal vein , this means perfusion buffer is indeed flowing through the liver (**Troubleshooting 1**).

Note: Insert the needle in a flat angle relative to the vein.

13

Immediately upon appearance of white spots and/or portal vein swelling (occurs 2–3 s after cannulation), cut the portal vein with scissors. The liver should clear of blood instantly; you'll observe plenty of blood rushing out of the portal vein and the liver will turn yellow-white within a few seconds.

14 Clamp the portal vein with forceps for 7–10 s (Figures 6A and 6B). Make sure no fluid is passing through.

Note

CRITICAL: Make sure the liver swells during clamp and then relaxes upon release of clamp (**Troubleshooting 2**).

Note: Clamping serves two purposes: (a) allowing perfusion buffer to reach all liver vasculature. (b) a visible checkpoint (liver swelling and relaxation) validating liver is indeed perfused. This is important because in some cases the liver seems white only due to passive clearing of blood from the severed portal vein and not from active perfusion.

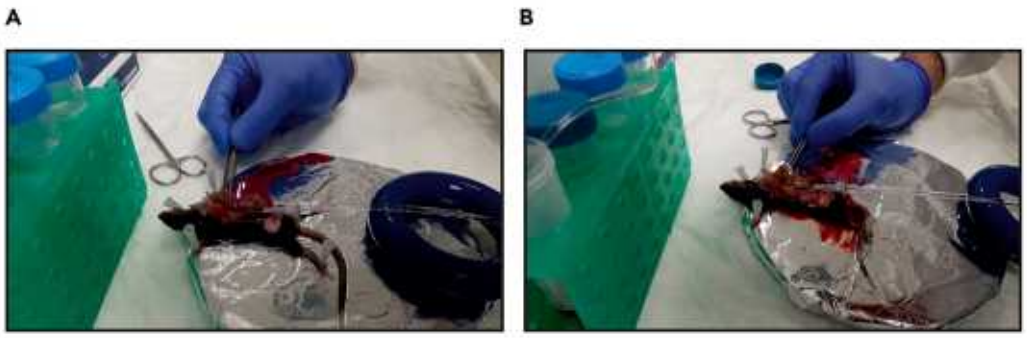


Figure 6. Clamping the Portal Vein.
(A and B) The portal vein is clamped with forceps for 7–10 s to stop fluid exiting the liver. Liver swells upon clamping (compare A to B).

15 After 00:00:30 perform a second clamp, make sure you observe liver swelling and relaxation. If 30s liver is completely washed of blood, move to the next section.

Digestion

16

Note

Timing: 10–15 min

Here, Collagenase (Liberase) is perfused to the liver in order to digest collagen in the extracellular matrix, thereby facilitating cell dispersion (originally proposed by Berry and Friend, (1969)). Collagenase is a calcium-dependent enzyme and therefore calcium is included in the digestion buffer.

This section is shown in Methods Videos S3 and S4.

Stop the pump and quickly transfer the inlet tubing from perfusion buffer to the pre-warmed "DB-Liberase" tube. Quickly turn the pump back on.

Note

Note: Make sure the tubing reaches the bottom of the 50 mL tube and make sure no bubbles got in the tubing (**Troubleshooting 3**).

17 Clamp the liver one more time, prior to digestion buffer arriving to the liver. Make sure the liver swells and relaxes.

18 While digestion buffer is perfused into the liver, clamp the portal vein every minute but no more than 3–4 times.

Note

Notes:

- When clamping while digestion buffer is perfused, the liver swells to maximal volume in the first clamp and does not relax. This is normal.
- Digestion buffer contains phenol red. This facilitates visualization of when the liver is perfused with digestion buffer rather than perfusion buffer (which does not contain phenol red).

19 Remove the needle before air gets into the liver.

20 Dissect out the liver gently (it is now very flimsy and frail): using forceps, grab the central connective

tissue between the lobes and slightly lift upwards, using it as an anchor point. Cut all the connections of the liver to other organs, remove the gall bladder, place the liver in the tube labeled “DB-chilled” and put it back On ice .

Note

Note: If needed, the liver can stay On ice for 30–40 min.

21 Clean the pump with ethanol (see step 3).

Hepatocyte Purification

14m

22

Note

Timing: 30–40 min

Here, liver cells are released into suspension and viable hepatocytes are separated from dead hepatocytes and non-hepatocyte cells.

Steps 22–24 in this section are shown in Methods Video S5.

Notes:
- These steps should be done in sterile conditions, in a biological hood (i.e., biosafety cabinet).
- Use only 25 mL serological pipettes. Smaller bore pipettes may reduce hepatocyte viability.

Transfer liver and media to a 10 cm plate (not pre-coated with collagen).

23 Rupture liver sack with fine tip forceps in a few locations along the liver surface and gently release cells using a cell lifter.

Note

Notes:
- Tilt the plate to have the liver submerged in the media to improve releasing of cells.
- The liver should tear apart easily. Do not cut the liver to pieces, leave it whole.

24 Filter 5 mL of suspension through a 70 µm cell strainer into a 50 mL tube. Repeat with a new filter and a new tube for the remaining 5 mL.

25 Add another 10 mL of cold plating media to rinse plate, add 5 mL of it to each filter.

26 Spin at 50 x g, 4°C, 00:02:00 (Figure 7A). 2m

Note

Notes:

- Centrifuge with low acceleration and low brake to minimize trauma to hepatocytes.
- Hepatocytes are denser than other liver cells. Due to low centrifugation force, only hepatocytes are pelleted while other cells are left in the supernatant.

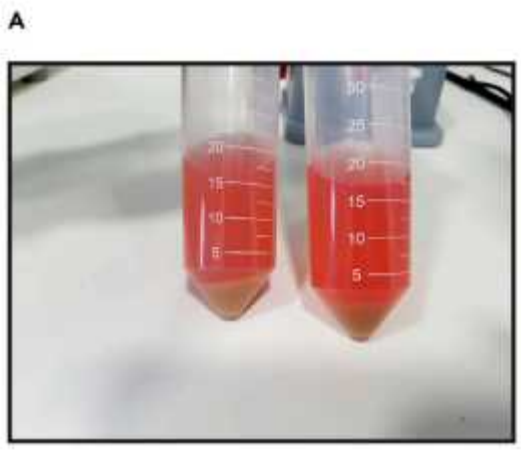


Figure 7. Hepatocyte Purification.
(A) Cells from the dissected liver are released into suspension and centrifuged. The pellet contains hepatocytes and the rest of the cells are left in the supernatant.

27 While the samples are spinning, prepare fresh Percoll solution from pre-chilled ingredients.

Note

Note: Percoll is used for density separation of the viable hepatocytes from dead hepatocytes and cell debris.

28 Aspirate most supernatant, leave ~ 1 mL and resuspend the cells by swirling the tube.

29 Add 10 mL plating media and resuspend by gentle swirling.

30 Add 10 mL Percoll solution and mix thoroughly by inverting the tube several times.

31 Spin at 200 x g, 4°C, 00:10:00 (Figure 7B). 10m

Note

Note: Centrifuge with low acceleration and low brake to minimize trauma to hepatocytes.

Figure 7. Hepatocyte Purification.
(B) The hepatocyte pellet (A) is re-suspended with Percoll solution and centrifuged again. The pellet contains viable hepatocytes while dead cells and debris are left in the supernatant.

32 Aspirate most supernatant, leave ~ 1 mL and resuspend the cells by swirling the tube.

Note

Note: The pellet contains live purified hepatocytes.

33 Aspirate supernatant, add 20 mL Williams E media

34 Suspension stored on ice and submitted for single cell capture using 10x Chromium platform