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Xiaoyan Cao, Ph.D.
Review Editor
JoVE
1 Alewife Center Suite 200 Cambridge MA 02140

Dear Dr Xiaoyan Cao,

Please find enclosed our invited article (manuscript and video), revised for a second time according to the requirements of the Editors, entitled: ***Murine Precision-Cut Liver Slices as an Ex Vivo Model of Liver Biology*** by *Pearen, Lim et. al.,* for consideration for publication in *JoVE*. The manuscript contains **red** highlighted text to indicate the final edits made to the original version.

Also enclosed is a point-by-point response addressing all of the Editorial comments, The revised video has not been uploaded to the *JoVE* manuscript submission website, rather, a high-resolution version of our revised video has been uploaded to the following site, as requested:
https://www.dropbox.com/request/2LWM9MUdVaaaqkn7mtvd?oref=e

We sincerely hope that you will now find this revised article and video suitable for publication in *JoVE* and look forward to your response.

Yours sincerely,

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TITLE:

Murine Precision-Cut Liver Slices as an Ex Vivo Model of Liver Biology

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KEYWORDS:

liver, slices, vibratome, ex vivo, hepatocytes, tissue, culture, stellate cells

SUMMARY:

This protocol provides a simple and reliable method for the production of viable precision-cut liver slices from mice. The ex vivo tissue samples can be maintained under laboratory tissue culture conditions for multiple days, providing a flexible model to examine liver pathobiology.

ABSTRACT:

Understanding the mechanisms of liver injury, hepatic fibrosis, and cirrhosis that underlie chronic liver diseases (i.e., viral hepatitis, non-alcoholic fatty liver disease, metabolic liver disease, and liver cancer) requires experimental manipulation of animal models and in vitro cell cultures. Both techniques have limitations, such as the requirement of large numbers of animals for in vivo manipulation. However, in vitro cell cultures do not reproduce the structure and function of the multicellular hepatic environment. The use of precision-cut liver slices is a technique in which uniform slices of viable mouse liver are maintained in laboratory tissue culture for experimental manipulation. This technique occupies an experimental niche that exists between animal studies and in vitro cell culture methods. The presented protocol describes a straightforward and reliable method to isolate and culture precision-cut liver slices from mice. As an application of this technique, ex vivo liver slices are treated with bile acids to simulate cholestatic liver injury and ultimately assess the mechanisms of hepatic fibrogenesis.

INTRODUCTION:

The pathogenesis of most chronic liver diseases (i.e., viral hepatitis, nonalcoholic steatohepatitis, cholestatic liver injury and liver cancer) involves complex interactions between multiple different liver cell types that drive inflammation, fibrogenesis, and cancer development[1,2]. To understand the molecular mechanisms underlying these chronic liver-based diseases, the interactions between multiple liver cell types must be investigated. While multiple hepatic cell lines (and more recently, organoids) can be cultured in vitro, these models do not accurately emulate the complex structure, function, and cellular diversity of the hepatic microenvironment[3]. Furthermore, cultured liver cells (in particular, transformed cell lines) may deviate from their original source biology. Animal models are used experimentally to investigate the interactions between multiple liver cell types. However, they may become significantly reduced in scope for experimental manipulation, due to significant off-target effects in extrahepatic organs (e.g., when testing potential therapeutics).

The use of precision-cut liver slices (PCLS) in tissue culture is an experimental technique first used in drug metabolism and toxicity studies, and it involves the cutting of viable, ultrathin (around 100−250 µm thick) liver slices. This permits the direct experimental manipulation of liver tissue ex vivo[4]. The technique bridges an experimental gap between in vivo animal studies and in vitro cell culture methods, overcoming many drawbacks of both methods (i.e., practical limits on the range of experiments that can be performed in whole animals as well as loss of structure/function and cellular diversity with in vitro cell culture methods).

Furthermore, PCLS vastly increases experimental capacity compared to whole animal studies. As one mouse can produce more than 48 liver slices, this also facilitates the use of both control and treatment groups from the same liver. In addition, the technique physically separates the liver tissue from other organ systems; therefore, it removes potential off-target effects that can occur in whole animals when testing the effects of exogenous stimuli.

In this protocol, PCLS are generated using a vibratome with a laterally vibrating blade. Other

studies have successfully used a Krumdieck tissue slicer, as described in Olinga and Schuppan[5]. In the vibratome, lateral vibration of the blade prevents tearing of the ultrathin tissue caused by shear stress, as the blade is pushed into the tissue. Both the vibratome and Krumdieck tissue slicer work effectively without structural embedding of liver tissue, which streamlines the slicing procedure. This technique can also be used to create PCLS from diseased livers, including those from mouse models of fibrosis/cirrhosis[6] and hepatic steatosis[7].

In addition to demonstrating the techniques required for preparation and tissue culture of PCLS, this report also examines the viability of these ex vivo tissues by measuring adenosine triphosphate (ATP) levels and examining tissue histology to assess necrosis and fibrosis. As a representative experimental procedure, PCLS are treated with pathophysiological concentrations of three different bile acids (glycocholic, taurocholic, and cholic acids) to simulate cholestatic liver injury. In the context of cholestatic liver injury, taurocholic acid in particular has been shown to be significantly increased in both the serum and bile of children with cystic fibrosis-associated liver disease[8].

Liver progenitor cells have also been treated in vitro with taurocholic acid to simulate the elevated taurocholic acid levels observed in patients, and this treatment caused increased proliferation and differentiation of liver progenitor cells towards a biliary (cholangiocyte) phenotype[9]. Subsequently, PCLS were treated ex vivo with elevated levels of taurocholic acid, and increased cholangiocyte markers were observed. This supports the in vitro observation that taurocholic acid drives biliary proliferation and/or differentiation in the context of pediatric cystic fibrosis-associated liver disease[9].

PROTOCOL:

All animal experiments were performed in accordance with the Australian code for the care and use of animals for scientific purposes at QIMR Berghofer Medical Research Institute with approval from the institute animal ethics committee. Male C57BL/6 mice (15−20 weeks old) were obtained from the Animal Resources Centre, WA, Australia.

NOTE: All solutions, media, instruments, hardware, and tubes that contact the samples must be sterilized or thoroughly disinfected with a 70% ethanol solution and handled using sterile techniques to minimize the risk of culture contamination.

1. Setup of the vibratome

1.1. Prepare sterile Krebs-Henseleit buffer solution with 2 g/L glucose (**Table of Materials**). Check that the pH of the buffer is 7.4. If the pH is higher, saturate the buffer with carbogen (95% O_2 + 5% CO_2) or incubate within a 5% CO_2 tissue culture incubator to correct the bicarbonate buffering system. Refrigerate the sealed sterile buffer solution at 4 °C.

1.2. Insert the vibratome blade into the cutting arm. Make sure the blade is tightly fixed to the cutting arm, as vibrations can cause the blade to come loose.

1.3. Disinfect the blade and cutting arm with a 70% ethanol solution.

CAUTION: Avoid contact with the blade.

1.4. Disinfect the buffer tray with a 70% ethanol solution and wipe it with a sterile tissue.

1.5. Insert the buffer tray into the vibratome and tighten the mounting mechanism.

1.6. Set the cutting arm to the maximum available height.

1.7. Set the blade angle to 10° below horizontal, sloping downwards to the sample. Make sure that the blade angle is tightly fixed.

NOTE: The optimal blade angle may differ depending on the vibratome model and tissue characteristics.

1.8. Disinfect the specimen holder with a 70% ethanol solution.

1.9. Connect the cooling water for the Peltier thermoelectric cooler located under the buffer tray.

NOTE: Some vibratome models use an ice-bath for cooling instead.

1.10. Fix the water-out tube to a drain and turn on the water. Set the cooler to 4 °C. Run cooling water at a rate of >400 mL/min.

1.11. Fill the buffer tray almost to the top with sterile ice-cold Krebs-Henseleit buffer (with 2 g/L glucose). Leave additional Krebs-Henseleit buffer solution on ice.

2. Liver removal and preparation

2.1. Sterilize or disinfect all surgical instruments including curved forceps, flat square-tip forceps, tweezers, surgical clamps, and scissors using autoclaving.

2.2. Prior to liver removal, deeply anesthetize mice using an intraperitoneal injection of 100 mg/kg ketamine and 12.5 mg/kg xylazine.

NOTE: Other anesthetics may be used, or mice can be euthanized by CO_2 asphyxiation/cervical dislocation if the liver is quickly removed to prevent hypoxia-induced damage.

2.3. Disinfect skin surfaces on mice by wetting with a 70% ethanol solution. Secure mice on their backs with all extremities pinned to a dissecting board.

2.4. Make a vertical midline incision into the skin from the base of the abdominal cavity to just above the diaphragm.

NOTE: Incisions towards the extremities are made to facilitate the retraction of the skin.

2.5. Pull the skin back while cutting any connective tissue between the skin and abdominal cavity. Prevent hair from being transferred to the abdominal cavity as much as possible. Pin or clamp the skin away from the abdominal cavity.

2.6. Using clean forceps and scissors, open the abdominal cavity and lower thoracic cavity.

2.7. Removing the liver quickly without damaging the lobes

2.7.1. Using blunt tools (e.g., flat square-tip forceps), guide the upper liver lobes downwards towards the abdomen. Cut the connective tissue surrounding the upper liver lobes using scissors.

2.7.2. Guide the liver lobes upwards towards the diaphragm. Hold the central vascular bundle of the liver with forceps.

NOTE: It will not affect the procedure if the central vascular bundle is damaged.

2.7.3. Pull the central vascular bundle away from the mouse and cut the remaining connective tissue, blood vessels, etc. to remove the liver. Take care not to 1) damage the liver lobes and 2) cut into the gastrointestinal tract, as this may contaminate the liver with microorganisms.

2.8. Place the removed liver into a sterile 10 cm tissue culture dish half-filled with ice-cold Krebs-Henseleit buffer solution. Using a blunt instrument to guide the lobes, cut the center of the liver to divide it into separate lobes.

2.9. Select one liver lobe (use the largest first), and place it flat side down onto a new sterile 10 cm dish. Keep all other liver lobes in the dish of Krebs-Henseleit buffer solution stored on ice for subsequent use.

2.10. Trim around 10% of the material from the tallest edge of the liver lobe while lying flat.

NOTE: Trimming is important, as this tissue edge will contact the cutting blade first; therefore, a tissue edge that is relatively perpendicular to the cutting blade will prevent tissue compression that can occur because of shallow angles in the uncut liver lobes.

2.11. Trim the other three tissue edges.

NOTE: This removes some of the fibrous Glisson’s capsule and will make separation of the tissue slices easier.

2.12. Mounting the liver lobe on the specimen holder

2.12.1. Place a thin layer of cyanoacrylate glue (superglue or medical/veterinary grade cyanoacrylate glue) on the specimen holder towards the front, sized slightly larger than the trimmed liver lobe.

NOTE: Check that the cyanoacrylate glue does not contain non-cyanoacrylate additives.

2.12.2. Gently pick up the liver lobe with sterile forceps using a corner away from the cutting edge, then pat dry any residual buffer from the flat side of the liver lobe using sterile absorbent material.

2.12.3. Place the large flat side of the liver lobe on the cyanoacrylate glue patch with the largest edge facing towards the front. Allow it to cure in the air for 1−2 min.

NOTE: Cyanoacrylate adhesives cure quickly (~2 min) in response to residual moisture and tissue proteins, and they will firmly attach the tissue to the specimen holder.

3. Production of liver slices

3.1. Place the specimen holder with the attached liver lobe into the vibratome buffer tray. Make sure the liver lobe is fully covered by buffer.

NOTE: If needed, top up the level of ice-cold Krebs-Henseleit buffer solution. If the buffer level obscures the cutting process, increase or decrease the level.

3.2. Set the cutting speed.

NOTE: This may need to be empirically determined depending on the vibratome model and blade characteristics. As a starting guide, use a speed of 57 Hz/3,420 rpm. The vibratome speed can be measured using an audio spectrum analyzer application on a smartphone.

3.3. Lower the cutting blade until it is located just above the liver lobe using the height dial. Run the vibrating cutting arm over the sample.

3.4. Return the blade back using the crank handle in reverse. Activate the vibrations while returning the blade. After the blade has returned and is not above the sample, lower the blade height by 250 µm.

3.5. Repeat the cutting process until the top of the tissue is removed. Discard the first one or two slices, as these will contain Glisson's capsule and therefore do not contain much functional liver tissue compared to other slices.

3.6. To cut liver slices, slowly advance the vibrating blade into the tissue by turning the handle. Use a small paintbrush (disinfected with a 70% ethanol solution) to gently guide the tissue during the cutting process.

3.7. Use the paintbrush to lift the cut tissue slices and place the tissue slice into a sterile tube of ice-cold Krebs-Henseleit buffer. When not in use, keep this tube on ice between slices.

NOTE: Sometimes, the tissue does tear during the cutting process, but for most purposes the tissue is still usable.

3.8. Repeat the cutting process until the base of the liver lobe is reached. Discard any liver slices that have visible cured glue.

NOTE: The cured glue is visible as white areas on the tissue slices. A typical liver lobe will only have a useable thickness of around 2 mm. Therefore, only around eight 250 µm liver slices are produced from each lobe.

3.9. Cut the tissue slices until near the cyanoacrylate glue. Do not cut into the glue.

3.10. Repeat the whole process with other liver lobes until the required number of tissue slices is obtained.

3.11. To clean the cured cyanoacrylate glue and tissue from specimen holder, either use a blade to scrape the cured glue/tissue mixture off, or soften and clean the mixture with acetone or dimethyl sulfoxide.

4. Tissue culture

NOTE: All tissue culture work must be performed in a sterile laminar flow hood.

4.1. In a tissue culture laminar flow hood, pipette 1 mL of William's E medium containing 2.0 g/L glucose, 10% fetal bovine serum (FBS), 2 mM L-glutamine supplement, 100 U/mL penicillin, and 100 µg/mL streptomycin into 12 well tissue culture plates.

NOTE: Other antibiotics and antifungal agents may also be added. Some studies have used additives in the tissue incubation medium (i.e., dexamethasone and insulin[10]), but these can interfere with cell signaling.

4.2. Place the tube containing liver slices from ice into the tissue culture hood. To remove the tissue slices, gently swirl the mixture and gently tip into a sterile 10 cm dish while swirling.

4.3. Using sterile sharp-point forceps and a scalpel, cut the tissue slices into roughly uniform sizes (e.g., 15 mm^2).

NOTE: This step is performed to ensure a consistent surface area. Because mouse liver lobes are significantly different in size and some tissue slices will tear, this will produce a range of PCLS with varying surface areas. The final surface area sizes of the PCLS depend on how much material is needed in subsequent analysis applications and how many replicates are needed. Cutting large PCLS into smaller multiple pieces of the same approximate size will innately allow an increased number of experimental replicates.

4.4. Using sterile forceps or a small paintbrush, transfer the cut tissue slices to the wells of a 12 well plate containing 1 mL of medium.

NOTE: Take care to confirm the consistency of thickness and shape of tissue slices. Sections that are darker than average suggest the tissue thickness is larger and need to be discarded. Lighter slices suggest the presence of cured cyanoacrylate glue and need to be discarded.

4.5. Incubate the liver slices at 37 °C and 5% CO_2 under 95% humidity.

NOTE: Other groups have used methods to enhance oxygen delivery to PCLS, which appear to prolong tissue survival time[6,10-13] (see discussion section).

4.6. On the following day, change the culture medium using a manual pipette (rather than suction) to prevent the loss of tissue through suction errors. Subsequently, change the medium on the PCLS daily. The PCLS are now ready for experimental use.

5. Example application of PCLS

5.1. At 16 h post-generation, treat PCLS with three different bile acids: glycocholic acid (GCA), taurocholic acid (TCA), and cholic acid (CA) (as sodium salts, all at a concentration of 150 µM) for 2 days.

5.2. After 2 days of bile acid treatment, extract mRNA by placing tissue slices in ice-cold RNA lysis buffer and quickly homogenize using beads with a bead homogenizer (**Table of Materials**).

5.3. Purify the RNA using an RNA isolation kit (**Table of Materials**) and create cDNA from the purified RNA.

5.4. Perform quantitative polymerase chain reaction (**Table of Materials**) using specific primers (**Table 1**) on the cDNA to examine gene expression.

REPRESENTATIVE RESULTS:

To determine the cell viability of PCLS over time, tissue ATP levels were measured. ATP levels are typically proportional to viability. PCLS (around 15 mm^2 in area) were cultured in normal William's E medium with 10% FBS, then at specific timepoints, liver slices were removed from tissue culture and homogenized with both ATP and protein (for normalization) concentrations (**Table of Materials**) being measured (**Figure 1A**). For biochemical assays like this, normalization is

important, as the cut liver slices are not necessarily identical in dimensions. ATP levels (relative to protein) were suppressed immediately post-isolation and after 1 h (**Figure 1A**), suggesting short-term metabolic stress from the cooling and cutting procedures. However, ATP levels recovered by 3 h. ATP levels remained elevated at up to 5 days of tissue culture, indicating no significant decrease in viability. Hematoxylin and eosin (H&E) staining of liver slices suggested that limited tissue necrosis (characterized by nuclear pleomorphism) occurred in culture from around days 2 and 3. Tissue necrosis levels progressed to severe by day 5 (**Figure 1B**). Considering these morphological data, taken together with the ATP results, it is recommended to use this experimental tissue model for up to 3 days.

PCLS also displayed increasing collagen accumulation at later culture timepoints, as shown by thickening of Picro-sirius red-stained collagen fibers at day 5 (**Figure 2**). This thickening of collagen fibers suggests that spontaneous fibrogenic processes are active in PCLS obtained using this method. This process appears independent of PCLS isolation methodology with the thickening of collagen fibers[6] and profibrogenic gene expression[14] occurring from both vibratome and Krumdieck tissue slicers, respectively, over time. The development of these spontaneous fibrogenic processes needs be taken into account when interpreting PCLS biology, particularly with experiments associated with fibrotic processes.

We have previously shown the significant induction of cholangiocyte-specific gene connexin 43 (*Cx43*) and secretion of glutamyl transpeptidase (*Ggt1*) protein by TCA in PCLS[9]. The expression of cholangiocyte-specific genes relative to housekeeping controls (glyceraldehyde 3-phosphate dehydrogenase [*Gapdh*] and hypoxanthine phosphoribosyltransferase 1 [*Hprt1*]) in PCLS treated with TCA, GCA, or CA were examined by qPCR using specific primers. Consistent with a previous report, a significant induction in the expression of cholangiocyte-specific genes cytokeratin 19 (*CK19*; **Figure 3A**) and connexin 43 (*Cx43*; **Figure 3B**) were observed by both GCA and TCA. *Ggt1* expression was increased by both bile acids; although, this did not reach statistical significance, possibly due to experimental variation (**Figure 3C**). The expression of CA was unaffected by any bile acid. The induction of cholangiocyte-specific genes suggests that GCA may also be involved in cholestatic liver injury, as previously reported for TCA[8,9].

FIGURE AND TABLE LEGENDS:

Figure 1: Tissue viability of precision-cut liver slices (**PCLS**)**.** (**A**) ATP and protein levels in PCLS were measured immediately (T + 0) and at 1 h, 3 h, 6 h, and 1−5 days post-isolation. (**B**) To examine cell morphology, PCLS were fixed, paraffin-embedded, sectioned, and stained with H&E immediately (day 0) and at 1, 2, and 5 days post-isolation. A Kruskal-Wallis test with Dunn's multiple comparisons test was performed relative to T + 0. All data are represented as mean ± SEM (n = 2−3 mice) with *$p < 0.05$. The scale bars represent 100 μm.

Figure 2: Assessment of collagen deposition in PCLS. PCLS were fixed, paraffin-embedded, sectioned, and stained with Picro-Sirius Red to visualize collagen fibers at days 0, 1, 2, and 5 post-isolation. The scale bars represent 200 μm.

Figure 3: Bile acids induce cholangiocyte-specific gene expression. At 16 h post-isolation,

medium on PCLS was changed, and new medium was added along with 150 µM glycocholic (GCA), taurocholic (TCA), or cholic (CA) acids (sodium salts). PCLS were harvested after 2 days, and the expression of cytokeratin 19 (*CK19*; **A**), connexin 43 (*Cx43*; **B**), and γ-glutamyl transpeptidase 1 (*Ggt1*; **C**) was examined relative to the geometric mean of *Gapdh* and *Hprt1*. Dunnett's multiple comparisons test was performed relative to untreated control tissue slices (n = 15). All data are represented as mean ± SEM (n = 4 mice, triplicate slices; *p < 0.05, **p < 0.01).

Table 1: qPCR primers.

DISCUSSION:

The protocol demonstrates the application of murine PCLS isolation and tissue culture, and the procedures are designed to assess both viability and utility as well as examine impacts of exogenous mediators of liver pathobiology using biochemical assays, histology, and qPCR. The experimental utility of PCLS tissue culture in rodents and humans has been demonstrated in a wide range of applications, including experimental investigations in microRNA[15]/RNA[9]/protein expression[16], metabolism[17], viral infection dynamics[10,18], infection signaling[19], tumor invasion[12], toxicity studies[4,13,15,20], DNA damage studies[21], cell biology[14], and secretion studies[9].

While ATP levels indicate cellular viability in PCLS up to 5 days in culture, H&E staining suggest that severe necrosis was occurring by 5 days in culture. The main factor that appears to limit the viability of PCLS is oxygen availability[6,11]. Several studies have included enhanced oxygen availability and consequently increased the viability time of PCLS in tissue culture. Methods used to increase oxygen availability include the incubation of tissue in oxygen-rich atmospheres[10,11], use of the oxygen carrier perfusion perfluorodecalin[12], shaking/rolling the culture during incubation[6,10,13], and tissue culture plates designed to maximize oxygenation[6].

Recently, an innovative air-liquid interface tissue culture system has been described with functional utility stated at longer than 7 days in culture[14]. This protocol uses ambient atmospheric oxygen in a normal tissue culture incubator. The method allows PCLS to be accessible to laboratories that do not have highly specialized, custom equipment to safely enhance oxygen delivery. However, if such methods to enhance oxygen delivery are available, they may improve long-term PCLS viability in culture.

The accumulation of collagen in PCLS after day 3 in culture suggests that spontaneous fibrogenic processes are active within this model. Spontaneous fibrosis has also been observed previously in PCLS[6,14,22-24] and is possibly mediated by damage-associated molecular patterns (DAMPs) released by the cutting process or tissue necrosis. DAMPs act as signaling molecules that subsequently activate pro-fibrotic signaling pathways[25,26]. Furthermore, spontaneous fibrosis in PCLS could be mediated by chemokines released from the activation of stellate cells and/or Kupffer cells (i.e., transforming growth factor beta [TGF-β]). In this context, a recent article by Bigaeva et al.[24] suggests that spontaneous fibrosis in PCLS is in part mediated by TGF-β signaling, as incubation of PCLS with TGF-β inhibitor Galunisertib inhibited gene expression changes associated with spontaneous hepatic fibrosis. Another possible mechanism is that the slicing procedure initially induces cell proliferation signaling pathways and entry of hepatocytes into the

cell cycle; however, this mechanism fails and results in cell cycle arrest in mid-G1 phase[27]. Cell cycle arrest is associated with hepatic fibrosis rather than hepatic regeneration[28].

In the representative experimental procedure, PCLS are treated with three different bile acids (GCA, TCA, and CA) for 2 days to simulate cholestatic liver injury. Two of the bile acids (GCA and TCA) induce significant expression of *CK19* and *Cx43*, which are genes associated with cholangiocyte function. This suggests the expansion or differentiation of cells towards a cholangiocyte lineage in PCLS treated with these bile acids. This is consistent with our previous work showing a similar effect using TCA[9] on PCLS. Furthermore, it is also consistent with in vivo observations that show feeding taurocholate to rats increases cholangiocyte numbers[29]. The treatment of liver tissue slices with bile acids simulates hepatic cholestasis, and it is speculated that the increase in the expression of genes associated with cholangiocyte function is an attempt by the liver tissue to create additional bile ductules to increase bile secretion. Given the specific induction of these genes is produced by the conjugated bile acids (GCA and TCA) but not the unconjugated CA, it is suspected that these effects are mediated by the sphingosine-1-phosphate receptor 2, a cell surface receptor with preferential activation by conjugated bile acids[30].

One key limitation of the application of PCLS is individual replicate variability. Since the liver is not homogenous and there is variability in production, liver slices tend to have larger biological variation than cell culture experimentation. In experimental studies with few variables, such as the representative procedure demonstrated above, this is overcome by using an increased number of replicates. However, this may become an issue for larger screening studies. In summary, the described protocol is a straightforward and reliable method to study aspects of liver pathobiology ex vivo, requiring little specialized equipment except for the vibratome.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

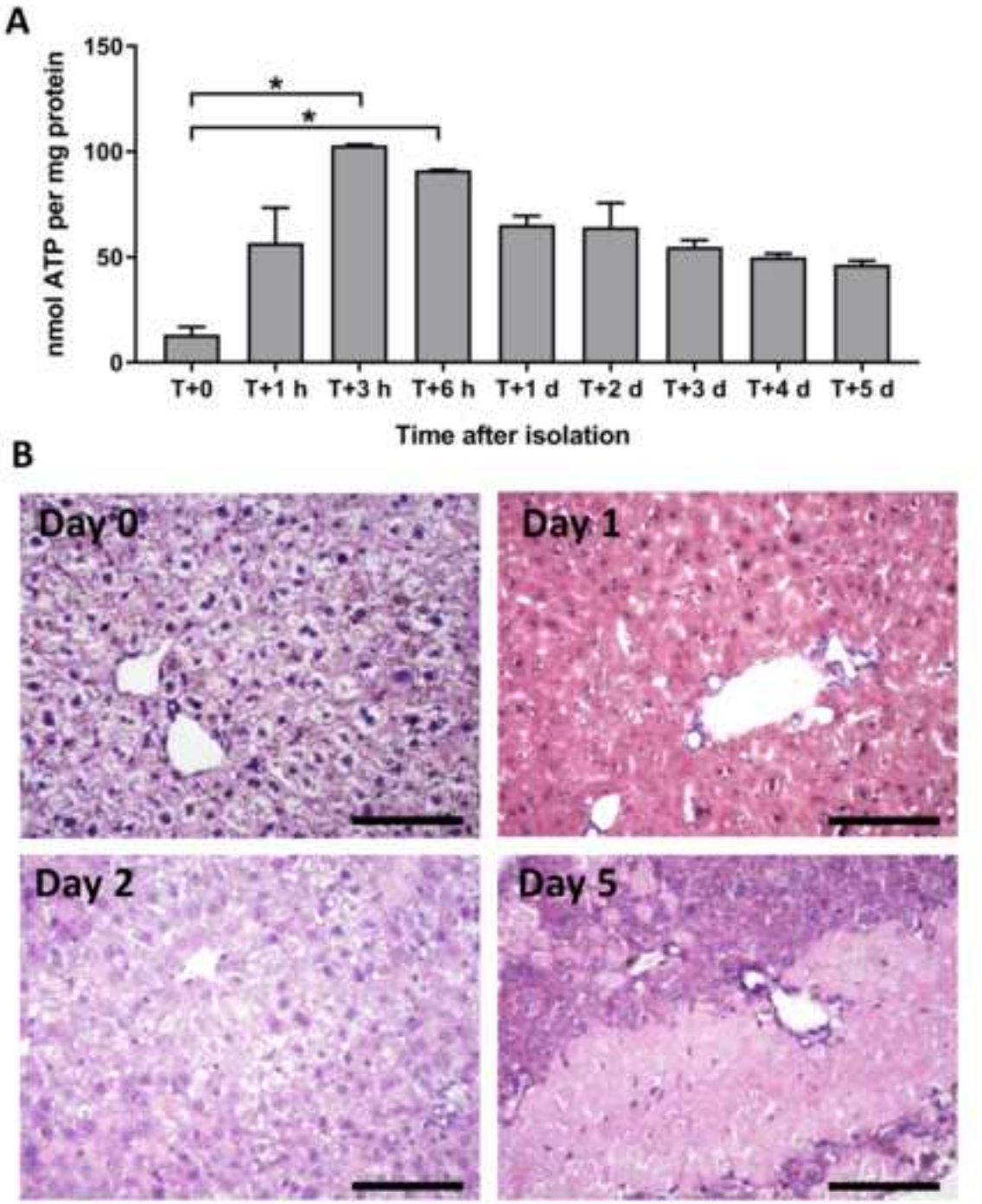

Figure 2

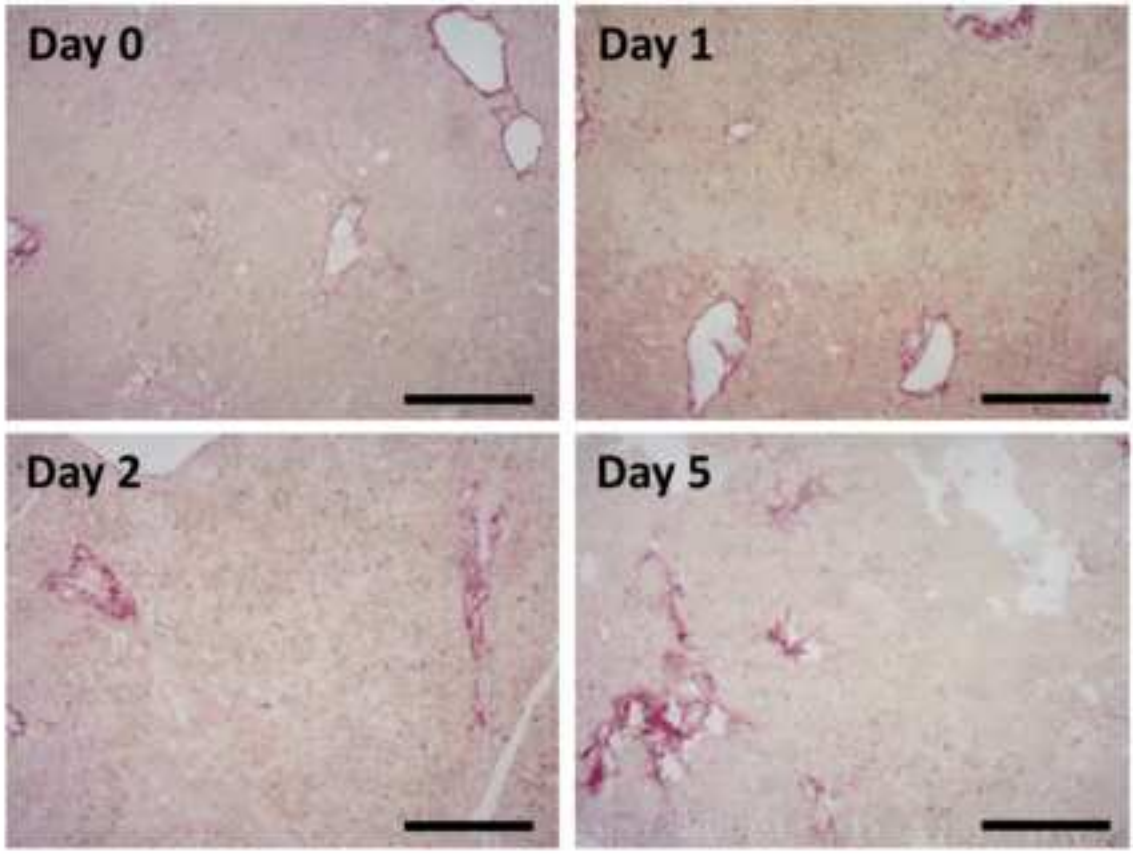

Figure 3

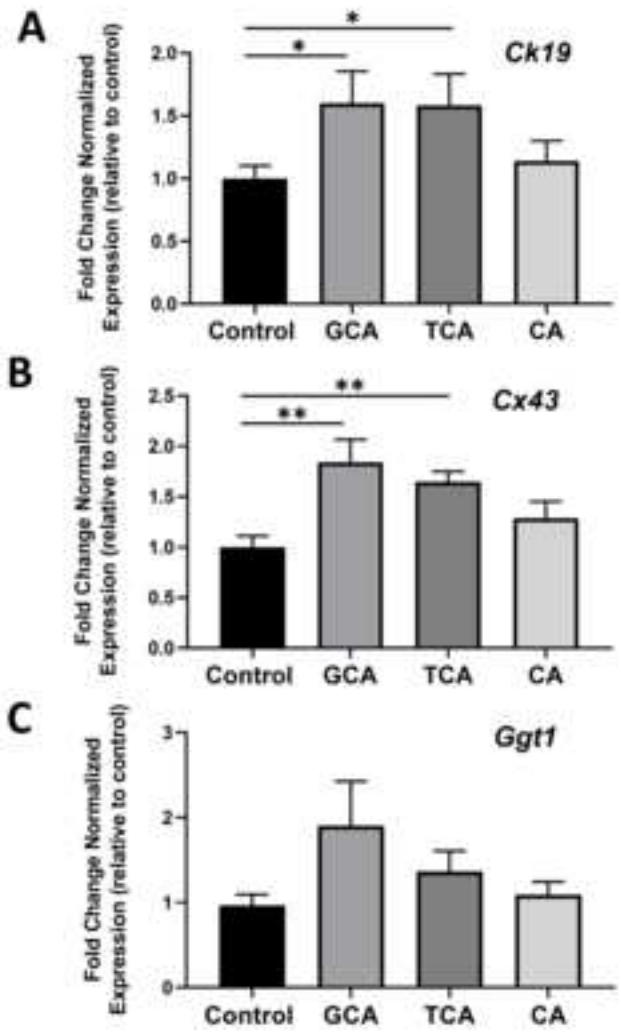

Table 1

Gene	NCBI accession	Forward	Reverse
Cx43	NM_010288	5′-AGCTAGGCGGCAAAAGTAGG-3′	5′-ACTCACTCATGTATACAGAACCAT-3′
Ck19	NM_008471	5′-GTCGAGGGAGGGGTTAGAGT-3′	5′-CCATCTGAGCTACCAGCGAG-3′
Ggt1	NM_008116	5′-CAGCACCACAGGAAAAGTTGAG-3′	5′-ACGGATTTCACCAGGGACAG-3′
Itgb4	NM_001005608	5′-GACCTATGAAGAAGGTGCTC-3′	5′-GGCTCAGATGCGTGCCATAG-3′
Hprt1	NM_013556	5′-CAAACTTTGCTTTCCCTGGT-3′	5′-TCTGGCCTGTATCCAACACTTC-3′
Hnf4α	NM_008261	5′-TGATAACCACGCTACTTGCCT-3′	5′-AGCCTACTTCTGAATGTTTGGTGT-3′
Gapdh	NM_008084	5′-AACTTTGGCATTGTGGAAGG-3′	5′-GGATGCAGGGATGATGTTCT-3′

Name of Material/ Equipment	Company
10 cm Petri Dish	GREINER
12 Well Tissue Culture Plate Flat Bottom	Greiner Bio-one
70% Ethanol Solution (made with AR Grade)	Chem-Supply Pty Ltd
Acetone	Chem-Supply Pty Ltd
Cholic acid	Sigma-Aldrich
Cyanoacrylate Super Glue	Parfix, DuluxGroup (Australia)
Disposable Single Edge Safety Razor Blades	Mixed
Dissection Board	Made in-house
Fetal Bovine Serum	GE Healthcare Australia Pty Ltd
Forceps sharp point 130 mm long	ThermoFisher Scientific
Forma Steri-Cycle CO2 Incubator	ThermoFisher Scientific
Glutamine	Life Technologies Australia Pty Ltd
Glycocholic acid hydrate	Sigma-Aldrich
ISOLATE II RNA Mini Kit	Bioline (Aust) Pty Ltd
Ketamine 50 ml	Provet
Krebs-Henseleit Buffer with Added Glucose 2000 mg/L	Sigma-Aldrich
Laminar Flow Hood	
NanoDrop 2000/2000c Spectrophotometers	ThermoFisher Scientific
Penicillin-Streptomycin, Liq 100 ml	Life Technologies Australia Pty Ltd
Picro Sirius Red	ABCAM Australia Pty Ltd
Pipette Tips Abt 1000 µl Filter Interpath	Interpath
Pipette Tips Abt 10 µl Filter Interpath	Interpath
Pipette Tips Abt 200 µl Filter Interpath	Interpath
Pipette Tips Abt 20 µl Filter Interpath	Interpath
Precellys Homogeniser	Bertin Instruments
Protractor	Generic
Quantstudio 5 QPCR Fixed 384 Block	Applied Biosystems/ ThermoFisher Scientific
Scalpel Blade	Mixed
Scalpel Blade Holder	Mixed
SensiFAST cDNA Synthesis Kit	Bioline (Aust) PTY LTD
Small Paintbrush with Plastic Handle	Mixed
Square-Head Foreceps	Mixed

Sterile 50 ml Plastic Tubes	Corning Falcon
Surgical Clamps	Mixed
Surgical Forceps	Mixed
Surgical Pins	Mixed
Surgical Scissors	Mixed
Taurochoic acid	Sigma-Aldrich
Vibratome SYS-NVSLM1 Motorized Vibroslice	World Precision Instruments
Williams Medium E	Life Technologies Australia Pty Ltd
Xylazine 100 mg/mL 50 mL	Provet

Catalog Number	Comments/Description
664160	Sterile Dish
665180	
EA043-20L-P	Disinfection solution
AA008-2.5L	
C1129-100G	
	Other brands should work
	Sterile material over polystyrene
SH30084.02	
MET2115-130	
371	
25030081	
G2878-100G	
BIO-52073	
KETAI1	
K3753	Can also be made in house
	Hepa air filtration
15140-122	
ab246832	
24800	
24300	
24700	
24500	
P000669-PR240-A	
	To measure blade angle
	Plastic handle resists ethanol

352098

T-4009-5G
SYS-NVSLM1 With thermoelectric cooling
12551032 2.0 g/l glucose
XYLAZ4

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1. *Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Response: The formatted manuscript has been proofread and some minor textural changes have been added as "track changes" to the manuscript document. Between lines 102-108 we have edited the description of our previous study where we used this specific technique. This was edited to better differentiate the representative work in the current manuscript versus our previously published work and to provide a stronger contextual foundation for material that is discussed in the discussion. We believe the manuscript reads better now between lines 102-108.

2. *Please make the following changes to the video.*

a. Audio Quality: @10:44 There is an audio glitch in ""H&E staining was used..."" on the word ""used"", there is some distortion. Please re-record this line.

Response: This audio error has now been fixed.

b. Please remove all live audio from the video unless it is necessary for it to be heard (the vibratome sounds can stay). Most laboratory background noise recorded during live action shots can be removed, and all ""dead areas"" (where no speaking is happening for more than a couple seconds) in the narration track can be trimmed as well. This doesn't mean trimming out breaths between words, just the prolonged sections where the narration track is still active but no speaking is heard.

Response: We have removed all of the laboratory noise apart from the sounds of vibratome and homogeniser when in use. We have also decreased the volume of these instruments when in use to make the narration track clearer. In the narration track we have removed the low level microphone noise in the "dead areas" between speaking.

c. Text style: Change the title to "Murine Precision-Cut Liver Slices as an Ex Vivo Model of Liver Biology" as shown in the manuscript text.

Response: The title has been changed in the video. The author affiliation addresses have also been updated to reflect the address formatting changes that were made on the manuscript text.

@1:25: Please change the text to "Setup of the Vibratome".

Response: This text has been changed in the video. @1:23

@9:28: Please include a space between the number and its temperature unit (37 °C).

Response: This text formatting has been changed in the video. @9:28

d. Please submit a high resolution version of your revised video (up to 2 GB) here: https://www.dropbox.com/request/2LWM9MUdVaaaqkn7mtvd?oref=e

Response: The revised high resolution video has been uploaded with the name "Pearen et al JoVE60992 revised.mp4"

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