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TITLE:

Isolation and 3D Collagen Sandwich Culture of Primary Mouse Hepatocytes to Study the Role of Cytoskeleton in Bile Canalicular Formation In Vitro

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KEYWORDS:

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SUMMARY

Presented here is a protocol for the isolation of mouse hepatocytes from adult mouse livers using a modified collagenase perfusion technique. Also described is the long-term culture of hepatocytes in a 3D collagen sandwich setting as well as immunolabeling of the cytoskeletal components to study bile canalicular formation and its response to treatment.

ABSTRACT:

Hepatocytes are the central cells of the liver and are responsible for its metabolic function. As such, they form a uniquely polarized epithelium, in which two or more hepatocytes contribute apical membranes to form a bile canalicular network through which bile is secreted. Hepatocyte polarization is essential for correct canalicular formation and depends on interactions between the hepatocyte cytoskeleton, cell-cell contacts, and the extracellular matrix. In vitro studies of hepatocyte cytoskeleton involvement in canaliculi formation and its response to pathological situations are handicapped by the lack of cell culture, which aims to closely resemble the canaliculi network structure in vivo. Described here is a protocol for the isolation of mouse hepatocytes from the adult mouse liver using a modified collagenase perfusion technique. Also described is the production of culture in a 3D collagen sandwich setting, which is used for immunolabeling of cytoskeletal components to study bile canalicular formation and its response to treatments in vitro. It is shown that hepatocyte 3D collagen sandwich cultures respond to treatments with toxins (ethanol) or actin cytoskeleton altering drugs (e.g., blebbistatin) and serve as a valuable tool for in vitro studies of bile canaliculi formation and

function.

INTRODUCTION:

Hepatocytes, the central cellular structures of the liver that are responsible for its metabolic functions, are uniquely polarized epithelial cells. Their polarization, occurring in mammals shortly after birth, results in formation of the biliary canalicular network and is essential for proper bile secretion. Apical membranes of hepatocytes collectively form bile canaliculi, whereas basal membranes remain in contact with the endothelium of sinusoids. The loss of hepatocyte polarization leads to redistribution of bile transporters and results in pathological processes connected with bile retention in the liver (i.e., cholestasis).

The establishment and maintenance of hepatocyte polarization and the development of bile canaliculi entail complex mechanisms. These underlying processes depend on collective interplay among the hepatocyte cytoskeleton, cell-cell contacts, and interactions with the extracellular matrix[1]. The hepatocyte cytoskeleton consists of all three filament networks, the actin cytoskeleton, microtubules, and intermediate filaments, which provide structural support for canalicular formation. The differential role of cytoskeletal components in the regeneration and maintenance of bile canalicular networks has been previously illustrated in vitro in 3D collagen-sandwich hepatocyte cultures[2].

Actin microfilaments and microtubules are important during the initial stages of hepatocyte membrane polarization at the sites of canaliculus generation[2]. The actin cytoskeleton establishes the structure and function of bile canaliculi, forming membrane-associated microfilaments and the circumferential ring, thus supporting the canalicular architecture and inserting the actin cytoskeleton into tight and adherens junctions[3]. The ring of keratin intermediate filaments outside the actin cytoskeleton further stabilizes the canalicular structure[3].

The importance of proteins in hepatocyte junctional complexes in the organization of bile canaliculi architecture has been well-documented in several knock-out mouse models, which show distorted canaliculi in mice lacking both tight and/or adherens junctional proteins[4-6]. The deletion of the adherens junction protein α-catenin has been shown to lead to disorganization of the hepatocyte actin cytoskeleton, dilatation of bile canalicular lumens, leaky tight junctions, and effectively to a cholestatic phenotype[4]. Moreover, in vitro studies have shown the importance of adherens junction components E-cadherin and β-catenin in remodeling of the hepatocellular apical lumen and protein trafficking[7].

Strikingly, ablation of the cytoskeleton crosslinking protein plectin, which is the major keratin organizer, has revealed phenotypes comparable to those linked to the actin cytoskeleton[8]. This suggests a critical role of keratin intermediate filaments in supporting of the canalicular structure. In vitro studies utilizing 3D hepatocyte collagen sandwiches have also shown the importance of the AMP-activated protein kinase and its upstream activator LKB1 in bile canalicular network formation[9]. These findings were then further confirmed by subsequent in

vivo studies[10,11]. Thus, it has become clear that in vitro studies are necessary to further the understanding of signaling processes involved in the establishment of hepatocyte polarization, proper canalicular network formation, and bile secretion.

A major challenge in studying processes connected with bile canalicular formation and its response to pathological situations in vitro is using a method that closely resembles the situation in vivo[12]. Freshly isolated primary hepatocytes are not polarized; thus, they lose their function, morphology, and functional bile canaliculi in 2D culture conditions (e.g., changes in gene regulation, polarization, and de-differentiation[13-15]). Despite this fact, freshly isolated hepatocytes most closely reflect the nature of the liver in vivo, unlike liver-derived cell lines[16]. Even though they have been used in the past, immortalized cell lines do not exert the epithelial-like characteristic morphology of hepatocytes, and the bile canalicular lumens formed by these cells resemble liver canaliculi poorly[7]. Recently, 3D cultures of primary hepatocytes, from both mice and rats, have become a useful tool to investigate processes involved in bile canalicular network formation in vitro[9]. Primary hepatocytes cultured between two layers of collagen (referred to as a 3D collagen sandwich culture) can repolarize in several days. Because of high technical demands required when culturing mouse hepatocytes in 3D collagen sandwiches, here we present a complex protocol to isolate, to cultivate, and to immunolabel mouse hepatocytes embedded in 3D collagen sandwiches in order to characterize the involvement of cytoskeletal components during bile canalicular formation.

PROTOCOL:

All animal experiments were performed in accordance with European Directive 2010/63/EU and they were approved by the Czech Central Commission for Animal Welfare.

1. Materials

1.1. House animals under specific pathogen-free conditions according to the guidelines of the Federation for Laboratory Animal Science Associations with free access to regular chow and drinking water. House animals under a 12 h/12 h dark/light cycle. For hepatocyte isolation and culture, use 8–12 week-old animals.

1.2. Stock solutions A and B

1.2.1. Prepare stock solution A and stock solution B according to **Table 1** and **Table 2**, respectively, in advance. Dissolve all components in 1 L of distilled H_2O (dH_2O).

1.2.2. Adjust the pH of solutions to 7.2 and filter the solutions through a 0.2 µm filter. Both solutions can be stored at 4 °C for up to 6 months.

1.3. Stock solution C

1.3.1. Prepare solution C on the day of primary hepatocyte isolation.

1.3.2. Add all components according to **Table 3,** fill to a 50 mL total volume with dH_2O, and dissolve. Adjust the pH to 7.3.

1.3.3. Aliquot 50 mL of the solution in 50 mL tubes. Use one tube per animal. Place all required aliquots in a pre-warmed water bath (37 °C).

1.4. Stock solution D

1.4.1. Prepare solution D on the day of primary hepatocyte isolation.

1.4.2. Add all components according to **Table 4**, fill to a 30 mL total volume with dH_2O, and dissolve. Adjust the pH to 7.3.

1.4.3. Aliquot 30 mL of the solution in 50 mL tubes. Add collagenase I (5 mg/30 mL) into solution D. Use one aliquot per animal. Place all aliquots in a pre-warmed water bath (37 °C).

1.5. Stock solution E

1.5.1. Prepare solution E on the day of primary hepatocyte isolation.

1.5.2. Add all components according to **Table 5,** fill to a 50 mL total volume with dH_2O, and dissolve. Adjust the pH to 7.3.

1.5.3. Aliquot 50 mL of the solution in 50 mL tubes. Add albumin V (0.65 g/50 mL) into solution E. Use one aliquot per animal. Place all aliquots in a pre-warmed water bath (37 °C).

2. Preparation of collagen sandwiches

2.1. On the day before primary hepatocyte isolation, prepare the first layer of the collagen I sandwich.

NOTE: Work on ice and use pre-chilled solutions, tips, plates, and tubes to minimize unwanted gelation of collagen I.

2.2. Neutralize the required amount of collagen I (from rat tail) according to the manufacturer's protocol. A volume of 100 µL of neutralized collagen (1.5 mg/mL) per experimental sample (3.5 cm dish) is required. To prepare 1 mL of neutralized collagen (1.5 mg/mL), add 100 µL of 10x DMEM, 11.5 µL of 1M NaOH, and 488.5 µL of dH_2O into 500 µL of collagen (stock concentration = 3 mg/mL). Check the pH of neutralized collagen with litmus paper (the pH should be ~7.5).

2.3. Disperse 100 µL of neutralized collagen solution evenly using a pre-chilled 200 µL tip over the surface of a 3.5 cm dish set on ice.

2.4. Incubate overnight under standard culture conditions (incubator with 5% CO_2 at 37 °C). On the day of primary hepatocyte isolation, add 1 mL of pre-warmed (37 °C) DMEM to the first collagen layer. Allow the collagen to rehydrate for at least 1 h at 37 °C.

3. Surgical procedure

3.1. Anesthetize the mouse by intramuscular injection of tiletamine (60 mg/kg of body weight), zolazepam (60 mg/kg), and xylazine (4.5 mg/kg).

3.2. Place the anesthetized mouse on a dissection mat and tape the lower and upper extremities to fix the mouse in a supine position. Swab the abdomen with 70% ethanol and open the abdomen with a V-shape incision from the pubic area to front legs. Fold the skin over the chest to uncover the abdominal cavity. Place the dissecting mat under a dissecting microscope.

3.3. Bend an insulin syringe needle (30 G) to a 45° angle. Expose the inferior vena cava (IVC) by moving the intestines and colon in the caudal direction.

3.4. Fill 2.5 mL of pre-warmed (37 °C) solution C into a 2 mL syringe with a cannula. Ensure that there are no air bubbles in the cannula or syringe.

3.5. Before cannulation of the IVC, reposition the liver lobes by pressing them up to the diaphragm with a wet (PBS) cotton swab. Place a silk suture around the IVC just below the liver (**Figure 1A,B**).

3.6. Inject 10 µL of heparin (5000 U/mL) into the portal vein using an insulin syringe with a 30 G needle bent at a 45° angle (**Figure 1C**).

3.7. To cannulate the liver, make a small incision with microsurgical scissors to the IVC directly next to the liver (below the suture; **Figure 1D**) that is large enough to insert the cannula. Secure the cannula in the position using sutures and two surgical knots (**Figure 1E**).

3.8. Cut the portal vein (**Figure 1F**) to allow the perfusion buffers to flow out from the liver to prevent expansion of the liver.

3.9. Connect the syringe prepared in step 3.4 to the cannula and manually perfuse the liver with 1.5 mL of pre-warmed solution C by slowly pressing the syringe (this should take ~15 s). The removal of blood from the liver by discoloration of the liver can now be observed.

3.10. Pre-fill a peristaltic pump with pre-warmed (37 °C) solution C. Check the perfusion apparatus and ensure that there are no air bubbles in the system.

3.11. Cautiously disconnect the cannula from the syringe and connect it to the tubing of the peristaltic pump. Work quickly but carefully and ensure that the cannula remains in position

and that no bubbles enter the tubing or cannula. Set the perfusion conditions at flow rate of 2.5 mL/min, and promptly initiate the perfusion.

3.12. Perfuse the liver with solution C for 2 min (5 mL of solution C). Change to solution D and continue the perfusion for an additional 10 min (25 mL of solution D).

3.13. Once the liver has been perfused, remove it from the abdominal cavity. The liver will now be very fragile and pale in color (**Figure 2**).

4. Isolation of primary hepatocytes

4.1. Remove the liver from the mouse. The cannula will still be tied to the liver, so use the forceps to lift the cannula with the liver, and carefully cut off all fascia connections. Transfer the liver to a 50 mL tube containing 20 mL of solution E.

4.2. Hold the cannula with the liver using forceps and disassociate the tissue by rubbing the liver around the wall of the tube to transfer the isolated hepatocytes into buffer. Keep the isolated cells on ice.

NOTE: Isolated primary hepatocytes are viable for several hours while kept on ice. Users can repeat steps 4.1 and 4.2 with the isolation of primary hepatocytes from another donor mouse, if necessary, or proceed to the next step.

4.3. Place a 70 µm nylon cell strainer on top of a 50 mL tube and filter the isolated cells.

4.4. Centrifuge the tube at 50 x *g* and 4 °C for 5 min. Aspirate the supernatant. To remove dead cells and increase the percentage of viable cells, resuspend the pellet in 20 mL of 40% percol in DMEM.

4.5. Centrifuge the tube at 50 x *g* and 4 °C for 5 min. Aspirate the supernatant containing dead cells and resuspend the pellet in 20 mL of solution E.

4.6. Centrifuge tubes at 50 x *g* and 4 °C for 5 min. Aspirate the supernatant and resuspend the pellet in 10 mL of solution E.

4.7. Check the primary hepatocyte yield and viability using trypan blue staining. Count the cell number using a Neubauer cell counting chamber and adjust the cell concentration to 5×10^5 viable cells/mL. Keep the cells on ice.

5. Cultivation of primary hepatocytes in 3D collagen sandwiches

5.1. Prepare hepatocyte culture medium (HCM). Add 15 µL of glucagon (1 mg/mL), 15 µL of hydrocortisone (50 mg/mL), and 40 µL of insulin (10 mg/mL) to 50 mL of complete medium (DMEM, high glucose, 10% FBS, 1% penicillin-streptomycin).

5.2. Evenly disperse 2 mL (5 x 10^5 cells/mL) of viable primary hepatocytes in a pre-coated 3.5 cm dish. Incubate the cells with 5% CO_2 at 37 °C for 3 h.

5.3. Prepare neutralized collagen I (100 µL/3 cm dish; i.e., a sufficient volume for all dishes as described above [step 2.1]).

5.4. After 3 h, carefully remove the medium and unattached cells and add 100 µL of neutralized collagen I to each 3.5 cm dish to form the top layer of collagen sandwich on cells. Incubate the collagen sandwich under standard cell-culture conditions (5% CO_2 at 37 °C) for 40 min. After a 40 min incubation, carefully add 2 mL of HCM.

5.5. Culture for 3–8 days, depending on the formation of bile canaliculi. Check the culture every day under a microscope (**Figure 3**). Change the HCM every second day.

6. Immunolabeling of primary hepatocytes in 3D collagen sandwiches

6.1. Remove the media from the hepatocyte sandwiches, then wash carefully with pre-warmed PBS. Fix the sandwich cultures with 1 mL of 4% paraformaldehyde in PBS for 30 min at room temperature (RT).

6.2. After fixation, wash the sandwiches 3x for 10 min in 2 mL of PBS + 0.1% Tween 20 (PBS-T).

6.3. Permeabilize cells with 1 mL of 0.1 M glycine, 0.2% Triton X-100 in PBS at RT for 1 h. Wash 3x for 10 min in PBS-T.

6.4. Gently disturb the top layer of collagen using a 10 µL loading tip connected to a vacuum aspiration to ensure sufficient antibody penetration.

6.5. Block with 1 mL of 5% BSA in PBS-T (i.e., blocking solution) for 2 h. Incubate with primary antibodies diluted in blocking solution overnight at RT. Wash 3x for 15 min in PBS-T.

6.6. After washing, incubate with secondary antibody at 37 °C for 5 h. Wash 3x for 15 min in PBS-T.

6.7. Mount with anti-fade mounting media (see **Table of Materials**) for microscopy.

REPRESENTATIVE RESULTS:

Mouse primary hepatocytes were isolated and seeded in 3D collagen sandwiches. Bile canaliculi between two adjacent cells started to form within several hours after seeding. Cells formed clusters and self-organized in an approximately regular network of bile canaliculi within 1 day (**Figure 3**). Within 3–6 days, clusters of 5–10 cells were usually observed, with fully polarized hepatocytes forming a canalicular network (**Figure 3**).

Treatment of primary mouse hepatocytes in 3D collagen sandwiches with either toxin (ethanol) or cytoskeleton-altering drugs (e.g., blebbistatin, okadaic acid) resulted in changes in the hepatocyte cytoskeleton, canaliculi width, shape, and number of bile canaliculi illustrated by immunolabeling with an antibody to keratin 8 (the most abundant keratin in hepatocytes), phalloidin (visualizing F-actin), and antibody to tight junction protein zonula occludens-1 (ZO-1; **Figure 4**).

Ethanol treatment had only a mild effect on organization of keratin 8; however, it increased the tortuosity (as seen from F-actin staining) and distribution of bile canalicular widths (**Figure 4**). The signal intensity of ZO-1 staining was decreased in ethanol-treated bile canaliculi compared to untreated controls, suggesting a loss of tight junctions after ethanol treatment. The inhibition of actomyosin contractility with blebbistatin significantly affected the shape and number of bile canaliculi. The regular canalicular network was reorganized, compared to untreated hepatocytes, into disorderly shaped bile canaliculi with an increased incidence of thick rounded bile canaliculi instead of thin long ones (as seen in the histogram of canalicular widths).

Additionally, treatment with okadaic acid (OA) inhibiting phosphatases strongly affected the physical properties of keratins, as previously shown[8,17]. OA changes the solubility of keratin filaments; thus, the treatment resulted in profound reorganization of the keratin meshwork, which collapsed into large perinuclear aggregates. Both F-actin and tight junction protein ZO-1 were not localized into any particular structures, suggesting almost complete disappearance of organized bile canaliculi and a complete loss of hepatocyte polarity. The remaining bile canaliculi were significantly narrowed compared to untreated controls, as seen in the canalicular width histogram (**Figure 4**).

To correlate microscopic observations of changes in the hepatocyte cytoskeleton with the hepatocellular biochemical response to treatment, the protocol also measured levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) (two liver enzymes that are commonly used as hepatocellular injury markers) in supernatant from the 3D collagen sandwiches (**Figure 5**)[18]. Ethanol treatment significantly elevated the levels of both ALT and AST, suggesting severe hepatocellular injury. Blebbistatin treatment did not lead to any considerable changes in both ALT and AST levels compared to okadaic acid treatment, which triggered mild biochemical changes with increased levels of ALT, but no change in levels of AST. Thus, biochemical markers of hepatocellular injury measured in vitro from hepatocyte supernatant correlate with the cytoskeletal changes observed by immunostaining.

FIGURE AND TABLE LEGENDS:

Figure 1: Opening of the abdominal cavity and cannulation of the IVC. The abdomen is opened with a V-shape incision from the pubic area to front legs. The skin is folded over the chest to expose and enlarge the abdominal cavity. To expose the IVC, the intestines and colon are carefully moved caudally. (**A, B**) Prior to cannulation of the IVC, liver lobes should be

repositioned by pressing them upwards to the diaphragm with a PBS-wetted cotton swab. The IVC is then carefully separated from surrounding tissues, and a silk suture is placed around the IVC in close proximity of the liver. Panel B represents schematics of the abdominal cavity shown in panel A. The liver lobes, gut, inferior vena cava (IVC, red), and sutures are indicated. (**C**) Heparin is injected into the portal vein (PV, arrow) with an insulin syringe (30 G needle bent at 45° angle). (**D**) To cannulate the liver, the IVC is incised directly next to the liver (below the suture). (**E**) The cannula is inserted and secured with sutures by tying two surgical knots. (**F**) The portal vein is fully cut to allow free buffer outflow, preventing liver expansion.

Figure 2: Representative liver images before and after perfusion. (**A, B**) The cannulated liver was resected and perfused for 12 min at a flow rate of 2.5 mL/min. Note the significantly discolored liver after perfusion (B) compared to the freshly resected liver (A).

Figure 3: 3D collagen sandwich culture of primary mouse hepatocytes. Representative bright-field images of mouse primary hepatocytes cultured for 1, 2, and 3 days in 3D conditions. It should be noted that larger clusters of highly organized cells are formed after 3 days in culture. Boxed areas show ~3x magnified images. Arrowheads indicate the bile canaliculi. Scale bar = 100 µm.

Figure 4: Evaluation of the morphological response to toxic stress by immunofluorescent microscopy. Primary mouse hepatocytes cultured in 3D collagen sandwiches were treated with toxins (ethanol, blebbistatin, or okadaic acid) on day 3 of culture. Fixed cells were stained to visualize cytoskeletal components: keratin 8 (green), F-actin (red), and zonula occludens-1 (ZO-1, magenta) by immunofluorescence. The toxic treatment led to disorganization of visualized cytoskeletal components, and it reduced the number and increased the tortuosity of bile canaliculi. Canalicular widths were measured in both untreated and treated hepatocytes and are depicted as histograms of widths distribution. Scale bar = 100 µm.

Figure 5: Biochemical analysis of the response of 3D hepatocyte collagen sandwiches to toxic injury in vitro. ALT and AST, well-established markers of hepatocellular injury, were measured in supernatant from 3D hepatocyte collagen sandwiches treated with toxins (ethanol, blebbistatin, and okadaic acid). ALT and AST were elevated in treated cells compared to untreated ones. Data are reported as arithmetic means ± SEM.

Table 1: Stock solution A recipe.

Table 2: Stock solution B recipe.

Table 3: Stock solution C recipe.

Table 4: Stock solution D recipe.

Table 5: Stock solution E recipe.

DISCUSSION:

The use of mouse primary hepatocyte cultures is important for in vitro studies to better understand the signaling processes involved in the establishment of hepatocyte polarization, proper canalicular structure formation, and bile secretion. The challenges in isolation and long-term culture of mouse primary hepatocytes in 2D culture have driven the invention of several technical approaches with increased isolation effectivity and longevity of isolated cells, each with several advantages and disadvantages. It is now widely accepted that 2D cultures of primary hepatocytes mimic only limited number of attributes of liver biology for a short period of time. Thus, 3D cultivation in a collagen sandwich arrangement is widely replacing the 2D conditions, particularly when focused on function of the cytoskeleton in liver biology (e.g., toxic drug effects or spatial organization of bile transport).

Since the 1980s, several protocols for isolation of mouse hepatocytes with various modifications have been described. The two-step collagenase perfusion approach has become widely used in many laboratories. The addition of gradient centrifugation into the isolation protocol allows the removal of dead cells[19,20] and significantly increases the number of viable cells (here, routinely to ~93%). Even though this step extends the handling time of the cells and results in reduced cell numbers[21], this step is viewed as necessary in the 3D collagen sandwich culture for proper bile canalicular network formation. Additionally, it is more important to proceed quickly and accurately during perfusion steps, which shortens the time the cells are handled.

Other important factors that increase the viability of the cells and their ability to form canalicular networks in 3D is the usage of freshly prepared solutions and avoidance of bubbles during perfusion. Therefore, solutions should be prepared on the day of mouse hepatocyte isolation, and the peristaltic pump and tubing should be checked when changing solutions. If the protocol is closely followed, the isolation of primary hepatocytes should be successful with a high yield of viable cells.

Another critical factor in long-term 3D hepatocyte culture is the initial source of primary hepatocytes that are used. It is important to use animals that are 8–12 weeks old, which serve as optimal donors of hepatocytes. The use of hepatocytes from older animals was not as successful in long-term culture, as these hepatocytes changed their morphology more often, depolarized, and ceased to form canalicular networks. Also, plating hepatocytes on properly neutralized collagen gel formed from relatively highly concentrated solution is a vital step. In most protocols, concentrations of about 1 mg/mL are used. After many optimizations, a concentration of 1.5 mg/mL is optimal for long-term hepatocyte cultivation and provides highly organized hepatocytes with formed bile canaliculi.

This easy-to-follow protocol allows long-term cultivation of primary mouse hepatocytes. Representative results demonstrate a broad spectrum of use for 3D cultured primary mouse hepatocytes when studying the role of cytoskeletal components in bile canaliculi formation.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

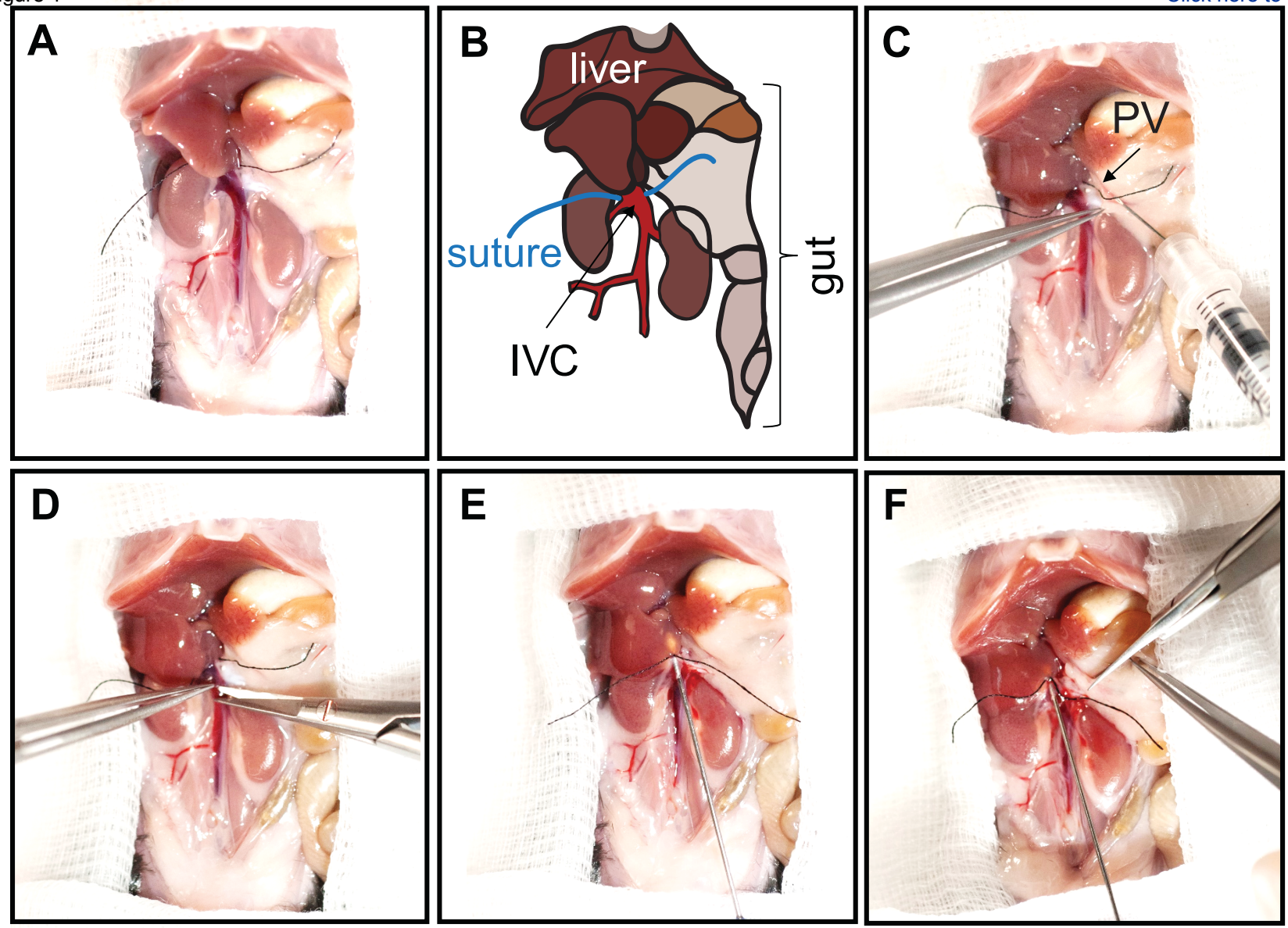

Figure 2

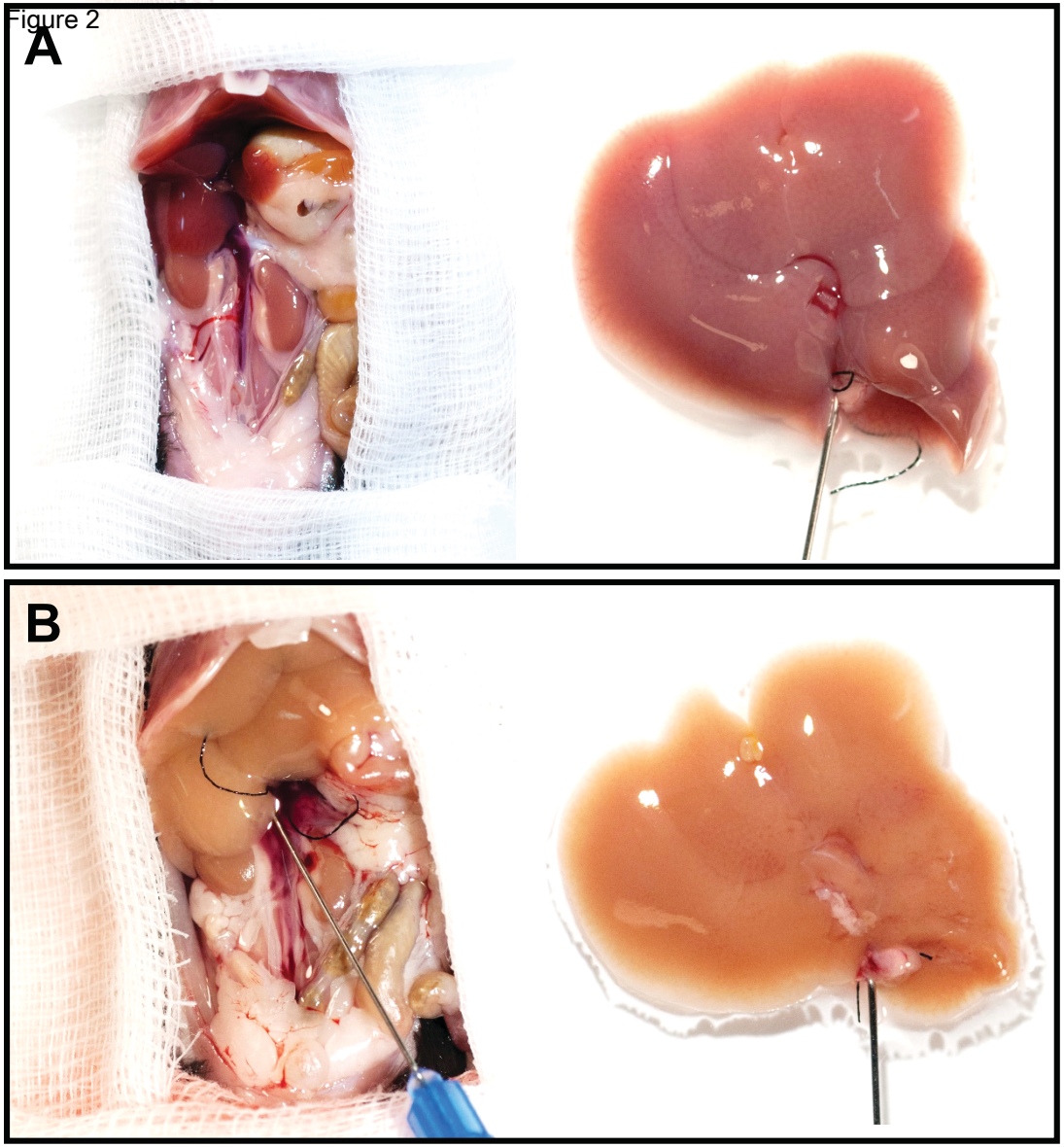

Figure 3

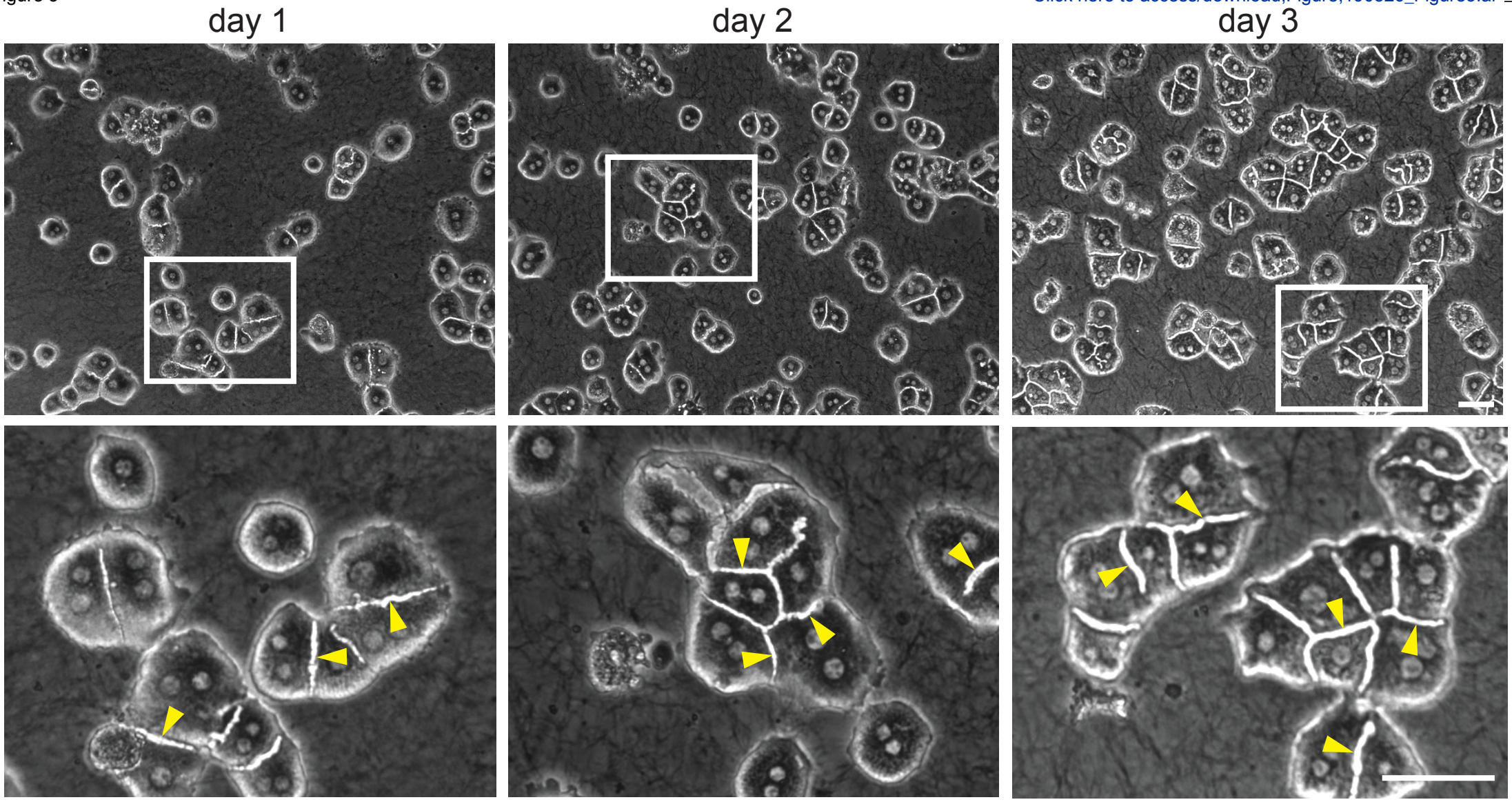

Figure 4

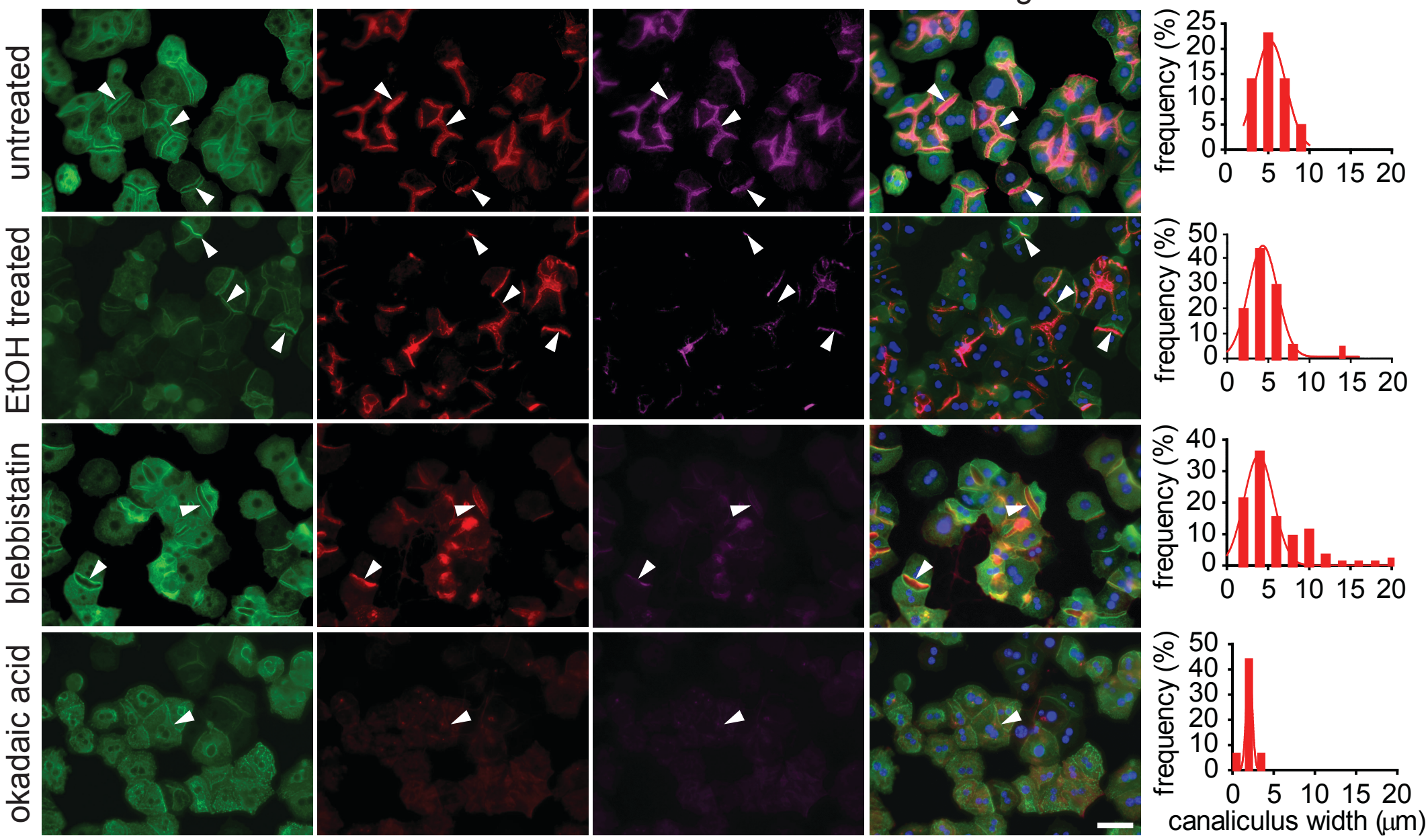

Figure 5

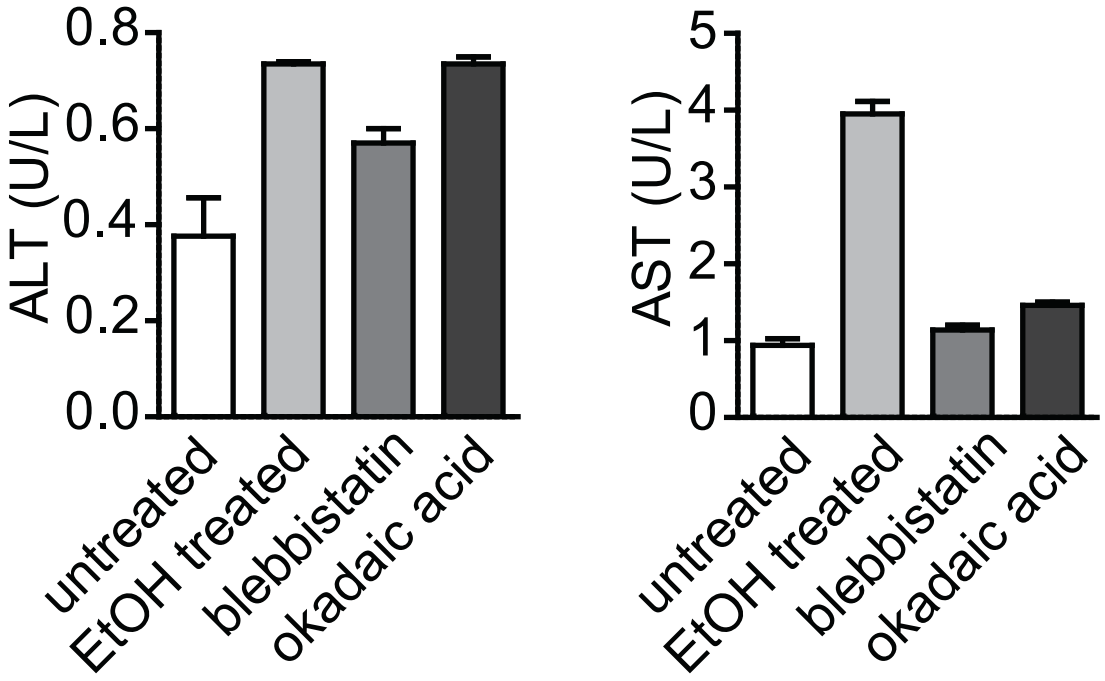

Table 1

Stock Solution A (10x)		
Reagent	Final concentration (g/liter)	
NaCl	80	
KCl	4	
$MgSO_4 \cdot 7H_2O$	1.97	
$Na_2HPO_4 \cdot 2H_2O$	0.598	
KH_2PO_4	0.6	

Stock Solution B (10x)		
	Final	concentration
Reagent	(g/liter)	
NaCl	69	
KCl	3.6	
KH_2PO_4	1.30	
$MgSO_4 \cdot 7H_2O$	2.94	
$CaCl_2$	2.772	

Solution C	
Reagent	
Stock Solution A (10x)	5 mL
$NaHCO_3$	0.1094 g
EGTA	0.0095 g
dH_2O	to 50 mL

Solution D	
Reagent	
Stock solution A (10x)	3 mL
$NaHCO_3$	0.065 g
$CaCl_2$	0.0125 g
dH_2O	to 30 mL

Table 5

Solution E	
Reagent	
Stock solution B (10x)	5 mL
$NaHCO_3$	0.1 g
glucose	0.045 g
dH_2O	to 50 mL

Name of Material/ Equipment
35 mm TC-treated culture dish
50 mL Centrifuge tubes, SuperClear, Ultra High Performance
70 µm nylon cell strainer
Albumin Fraction V
Arteriotomy Cannula (1mm)
Calcium Chloride
Collagen I. from Rat tail (3mg/ml)
Collagenase (from Clostridium Hystolyticum)
D(+) glucose monohydrate
Dissecting microscope
DMEM, high glucose
EGTA
FBS
Glucagon
Glycine
Heparin (5000 U/mL)
Hydrocortisone
Insulin
Insulin syringe (30G)
Magnesium Sulphate Heptahydrate
microsurgical forceps
microsurgical forceps
microsurgical scissor
Paraformaldehyde
Penicillin-Streptomycin
Percoll
Peristaltic Pump Minipuls Evolution
Potassium Chloride
Potassium Phosphate monobasic
ProLong Gold Antifade Mountant
Refrigerated centrifuge
Round cover glasses, 30 mm, thickness 1.5
Silk braided black
Sodium Chloride
Sodium Hydrogen Carbonate
Sodium hydroxide
Sodium Hydroxide
Sodium Phosphate Dibasic Dihydrate
Syringe 2 ml
Triton X-100
Tween 20

Water bath
Whatman membrane filters nylon, pore size 0.2 µm, diam. 47 mm
Whatman pH indicator papers, pH 6.0-8.1
Zoletil

Company

Corning
VWR
Biologix
ROTH
Medtronic
Sigma-Aldrich
Corning
Sigma-Aldrich
ROTH
Zeiss
Sigma-Aldrich
ROTH
Gibco
Sigma-Aldrich
Pufferan
Zentiva
Sigma-Aldrich
Sigma-Aldrich
BD Medical
ROTH
FST
FST
FST
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2629-990
VET00083

Comments/Description

Dear Dr. Steindel,

Thank you for the review of our manuscript “Isolation and 3D Collagen Sandwich Culture of Primary Mouse Hepatocytes to Study The Role of Cytoskeleton In Bile Canalicular Formation In Vitro” (JoVE60507) and for granting us a two-week resubmission period.

We are submitting a new version of the manuscript, which has been revised according to the editorial and reviewers’ suggestions. Overall, we found the comments very helpful and constructive. We believe that alterations of the text will significantly raise the impact of our protocol.

A detailed description of all changes made and our responses to the reviewers’ comments point-by-point are listed below, and all changes are indicated (in red) throughout the manuscript. Further, the manuscript have been proofread by native speaker and major changes were made in the text. We acknowledge the reviewers’ contributions in helping to improve our work and we hope the manuscript is now suitable for publication in *Journal of Visualized Experiments*.

Yours sincerely,

Martin Gregor

Editorial comments:

> *1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Following the editorial recommendation, the text of the manuscript was proofread by native speaker and major changes are indicated (in red) throughout the manuscript.

> *2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5” x 11”) page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.*

Following the editorial recommendation, the text of the manuscript was formatted.

> *3. Please include email addresses for all authors in the manuscript.*

Following the editorial recommendation, E-mail addresses of all authors were included.

> *4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*

Following the editorial recommendation, all trademark symbols were removed.

5. Keywords: 1. Please provide at least 6 key words or phrases.

Following the editorial recommendation, we included the sixth key word ("hepatocellular damage").

6. Summary: 1. Please include a separate Summary section (before the abstract) that clearly describes the protocol and its applications in complete sentences between 10¬ and 50 words, e.g., "Here, we present a protocol to …

Following the editorial recommendation, we included short Summary (50 words, lines 22-25).

7. Protocol: 1. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps .

Following the editorial recommendation, the whole protocol was thoroughly revised. Corresponding changes are indicated by tracking mode throughout the manuscript.

8. Specific Protocol steps: 1. The 'REAGANTS' and 'EQUIPMENT' are unnecessary (and not generally in line with JoVE format), provided everything is listed in the Table of Materials.

Following the editorial recommendation, the 'REAGENTS' and 'EQUIPMENT' sections were removed from the text. Items listed originally in REAGENTS' and 'EQUIPMENT' sections are provided in the Table 1.

9. Specific Protocol steps: 2. 2.1.1: Litmus paper seems to be too imprecise to validate a pH of 7.3-7.4 .

We agree with editors that Litmus paper is imprecise to validate a pH of 7.3-7.4. The corresponding sentence was changed as follows: "Always check pH of neutralized Collagen with litmus paper (pH should be ~ 7.5)."

10. Figures and Tables: 1. Please remove 'FIGURE 2' etc. from the Figures themselves.

Following the editorial recommendation, the Figure numbers were removed from Figures.

11. Figures and Tables: 2. Figure 5: Please use 'L' instead of 'l'.

Following the editorial recommendation, the 'L' instead of 'l' was used in Figure 5.

12. Figures and Tables: 3. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Following the editorial recommendation, the embedded tables were eliminated from the manuscript and are provided as a separate .xls files. Title and a description for each table is provided after the Representative Results of the manuscript text.

13. References:1. Please do not abbreviate journal titles.

Following the editorial recommendation, the full journal titles are provided.

14. Table of Materials: 1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Following the editorial recommendation, we thoroughly revised Table of Materials for materials and equipment mentioned in the Protocol.

15. Table of Materials: 2. Please remove trademark (™) and registered (®) symbols from the Table of Material.

Following the editorial recommendation, trademark and registered symbols were removed.

Reviewer #1:

1. Hepatocytes forming "functional" canalicular network should be tested (e.g. transcellular transport of fluorescein diacetate).

We agree with the reviewer that functionality of formed canaliculi can and shold be tested for physiologically relevant experiments. However, our aim was to provide protocol for "Isolation and 3D Collagen Sandwich Culture of Primary Mouse Hepatocytes to Study The Role of Cytoskeleton In Bile Canalicular Formation In Vitro". For this purpose is sufficient to observe formation of relevant cytoskeletal structures and of canalicular network. Further, we were unable to perform functional assays within the period provided by editor (14 days). To avoid possible misunderstanding, we removed the word "functional" from Representative Results sections (lines 272-273) and the sentence was changed as follows: "Within 3-6 days, clusters of 5-10 cells were usually seen, with fully polarized hepatocytes forming functional well-defined canalicular network (Figure 3).".

2. The morphological changes in the formation of the canaliculi as an indicator of polarization must be quantified more precisely (e.g. canaliculi lengths and/or total area canaliculi/cells per frame).

We agree with the reviewer that other morphological parameters then canalicular width can be retrieved from our 3D cultures. We based our analysis on our previously published analyses (Jirouskova et al., 2018, J. Hepatol), which steered interest of JoVE Editors. However, if the reviewer or the editor considers it beneficial, we are prepared to expand our current data set.

3. Specify the source, concentration and treatment conditions for ethanol, okadaic acid and blebblistatin. Cite the references for the antibodies/dyes used for cell staining.

Following the reviever's recommendation, the source, concentration and treatment conditions for ethanol, okadaic acid and blebblistatin as well as references for the antibodies/dyes were included.

4. - Lines 98-100; Percoll, Albumin and ProLong Gold are not enzimes

Following the editorial recommendation, the relevant part of the text was eliminated and relevan Table was restructured.

5. - Line 174 or 242; Specify the source and reference # of the 35 mm cell culture dishes or glass coverslips used for the immunolabeling/imaging

Following the reviewer's recommendation, the source and reference # of the 35 mm cell culture dishes or glass coverslips used for the immunolabeling/imaging are provided in Table 1.

> *6. - Line 177; dH_2O should be 388.5 µl. To clarify the calculation, the concentration of the collagen in the stock solution must be specified .*

Following the reviewer's recommendation, the whole sentence was altered as follows: "To prepare 1 mL of neutralized collagen (1.5 mg/mL) add 100 µL 10× DMEM, 11.5 µL of 1M NaOH and 488.5 µL of dH2O into 500 µL of collagen (stock concetration 3 mg/mL).".

> *7. - Line 259; Making holes into the top layer of collagen sandwich could disrupt the morphology of cells locally. Alternatively, a brief treatment with collagenase type I could be done before fixation.*

We agree with reviewer that making holes into the top layer of collagen sandwich could disrupt the morphology of cells locally. Therefore the whole sentence was rephrased as follows: "Gently disturb the top layer of collagen using a 10 µL loading tip connected to a vacuum aspiration pump to ensure better antibody penetration.". We are aware of collagenase alternative, nonetheless it generated frequently aberrant cytoskeletal structures when optimizing or protocol. The mechanical approach worked well in our hands and therefore we feel safe to keep it in the protocol.

Reviewer #2:

> *1. I would be happy to see the electron microscopy of the bile canaliculus. This step may add important aspects in the acceptance of the protocol by scientists.*

We agree with reviewer that the electron microscopy of the bile canaliculi would be interesting for wider scientific community. However, SEM approach is so time consuming technique that providing SEM micrographs would disallow to meet revision period (14 days) granted by Editor. Also, we feel that ultrastructural analysis goes beyond the scope of our present protocol.

> *2. Some typos and grammar style should be rectified.*

Following the reviewer's and editorial recommendation, the text of the manuscript was proofread by native speaker and major changes are indicated (in red) throughout the manuscript.

Reviewer #3:

> *1. - Preparation of the Collagen Sandwich, Line 173: You wrote you use 100 µl of neutralized collagen per 3 cm dish. A 3 cm dish is app. an area of 7.9 cm^2. That´s 14.1 µl collagen per cm^2 which is in my experience very less collagen for a proper 3D sandwich culture. We use in our lab app. 46 µl collagen per cm^2. Please check the numbers and confirm that 14.1 µl per cm^2 are sufficient to cover the whole plate with a proper gel amount .*

We agree with reviewer that usage of 100 µl of neutralized collagen per 3 cm dish appears as to be insufficient. In fact, we spent significant portion of time to optimize this particular step and it in its

current form it works well for both highly trained technicians and untrained students. Therefore we feel safe to keep the protocol as it is.

2. - The manuscript needs linguistic revision. For example…

Following the reviewer's and editorial recommendation, the text of the manuscript was proofread by native speaker and major changes are indicated (in red) throughout the manuscript.

3. - Line 115: Please add the protein concentration of the Rat Tail Collagen I

Following the reviewer's recommendation, the protein concentration of the Rat Tail Collagen I was included.

4. - Please add a sentence how you dilute your Rat Tail Collagen I to the final concentration of 1.5 mg/mL.

Following the reviewer's recommendation, the corresponding part of the text was revised as follows: "To prepare 1 mL of neutralized collagen (1.5 mg/mL) add 100 µL 10× DMEM, 11.5 µL of 1M NaOH and 488.5 µL of dH2O into 500 µL of collagen (stock concetration 3 mg/mL).".

5. - Line 143: Please add a space between numbers and their units. Better use a "x" than a dot to display the number of bound water molecules: e.g. MgSO4 x 7 H2O

Following the editorial recommendation, the corresponding part of the text was moved to Tables, where it was changed according to reviewer's recommendation.

6. - Line 185: Please add BW (body weight)

Following the reviewer's recommendation, BW (body weight) was added.

7. - Line 271: incomprehensible sentence, Please reconsider

Following the reviewer's and editorial recommendation, the text of the manuscript was proofread by native speaker.

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Author(s): K. KORELOVA, M. JIROUSKOVA, L. SARNOVA, M. GREGOD

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Title:	ISOLATION AND 3D COLLAGEN SANDWICH CULTURE OF PRIMARY MOUSE HEPATOCYTES TO STUDY THE ROLE OF CYTOSKELETON IN BILE CANALICULAR FORMATION IN VITRO
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