Journal of Visualized Experiments

Determining Bile Duct Density in the Mouse Liver

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59587R1
Full Title:	Determining Bile Duct Density in the Mouse Liver
Keywords:	liver development; bile duct; cytokeratin; Notch signaling; Alagille syndrome; Jag1
Corresponding Author:	Hamed Jafar-Nejad Baylor College of Medicine Houston, TX UNITED STATES
Corresponding Author's Institution:	Baylor College of Medicine
Corresponding Author E-Mail:	hamedj@bcm.edu
Order of Authors:	Joshua M Adams
	Hamed Jafar-Nejad
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Houston, TX, USA

TITLE:

Determining Bile Duct Density in the Mouse Liver

AUTHORS AND AFFILIATIONS:

Joshua M. Adams[1-3], Hamed Jafar-Nejad[1,3]

[1]Program in Developmental Biology, Baylor College of Medicine, Houston, TX, USA

[2]Medical Scientist Training Program (MSTP), Baylor College of Medicine, Houston, TX, USA

[3]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

Corresponding Author:

Hamed Jafar-Nejad

hamedj@bcm.edu

Email Addresses of Co-authors:

Joshua M. Adams (jmadams@bcm.edu)

KEYWORDS:

liver development, bile duct, cytokeratin, Notch signaling, Alagille syndrome, *Jag1*

SUMMARY:

We present a rather simple and sensitive method for accurate quantification of bile duct density in the mouse liver. This method can aid in determining the effects of genetic and environmental modifiers and the effectiveness of potential therapies in mouse models of biliary diseases.

ABSTRACT:

Mouse is broadly used as a model organism to study biliary diseases. To evaluate the development and function of the biliary system, various techniques are used, including serum chemistry, histological analysis, and immunostaining for specific markers. Although these techniques can provide important information about the biliary system, they often do not present a full picture of bile duct (BD) developmental defects across the whole liver. This is in part due to the robust ability of the mouse liver to drain the bile even in animals with significant impairment in biliary development. Here we present a simple method to calculate the average number of BDs associated with each portal vein (PV) in sections covering all lobes of mutant/transgenic mice. In this method, livers are mounted and sectioned in a stereotypic manner to facilitate comparison among various genotypes and experimental conditions. BDs are identified via light microscopy of cytokeratin-stained cholangiocytes, and then counted and divided by the total number of PVs present in liver section. As an example, we show how this method can clearly distinguish between wild-type mice and a mouse model of Alagille syndrome. The method presented here cannot substitute for techniques that visualize the three-dimensional structure of the biliary tree. However, it offers an easy and direct way to quantitatively assess BD development and the degree of ductular reaction formation in mice.

INTRODUCTION:

The biliary tree is a critical part of the mammalian liver, allowing the passage of bile from hepatocytes into the gut. Intrahepatic bile ducts (BDs) are formed by cholangiocytes, which differentiate from bipotential hepatoblasts through Notch and TGFβ signaling[1,2]. Proper specification and commitment of cholangiocytes and their assembly into mature BDs are critical for the development of the intrahepatic biliary tree. As the liver grows during development or upon organ regeneration, the biliary system needs to develop along the liver to ensure proper bile drainage. Moreover, a number of syndromic and non-syndromic diseases result in the paucity of intrahepatic BDs[3]. In addition, a number of acute and chronic liver diseases give rise to so-called ductular reactions in the liver, which are defined as the presence of a significant number of cells that express biliary markers but do not necessarily arise from biliary cells or form patent BDs[4]. In the multisystem disorder Alagille syndrome (ALGS), haploinsufficiency of the Notch ligand jagged1 (*JAG1*) results in poor BD formation and cholestasis[5,6]. Our lab recently demonstrated that a previously generated *Jag1* heterozygous mouse line[7] is an animal model of BD paucity in ALGS[8]. In this mouse model of ALGS, cholangiocytes are still present. However, they fail to commit to incorporation into mature, patent BDs[8]. Therefore, analysis of the liver in a model of BD paucity requires more than the apparent presence or absence of cholangiocytes. It is important to accurately assess the degree to which mature BDs are present in the liver.

In anatomic pathology, there are accepted quantitative methods for assessing whether BD paucity exists[9]. For example, studies on ALGS in human patients often quantify the BD to portal vein (PV) ratio by analyzing at least 10 portal vessels per liver biopsy[9,10]. Analysis of the shape and overall presence or absence of patent BDs, combined with serum chemistry, can provide valuable information about BD development in mice[11-13]. However, mice can lose a significant number of BDs with only a modest increase in serum bilirubin level[8]. Accordingly, a quantitative method that evaluates the number of BDs present per PV can provide a more direct measure of the degree of BD paucity in mice. In a recent report, we quantified the number of BDs per PV across all liver lobes and reported a significant decrease in the BD to PV ratio in *Jag1+/–* animals[8]. During the course of our analysis, we noticed that despite the significant variation in the degree of inflammatory response and ductular reactions, the BD to PV ratio does not show much variability[8]. Moreover, quantification of the BD to PV ratio allowed us to demonstrate that removing one copy of the glucosyltransferase gene *Poglut1* in *Jag1+/–* animals can significantly improve their BD paucity[8]. In a *Jag1+/+* background, conditional loss of *Poglut1* in vascular smooth muscle cells results in a progressive increase in BD numbers, which is modest (20-30%) at P7 but becomes prominent in adults[8]. Again, this technique allowed us to show that even at P7, the increase in BD density in these animals is statistically significant. Of note, the increased BD density in this genotype at four months of age was validated through resin cast analysis as well.[8] These observations and other reports which measured BD density in different ALGS mouse models[14,15] prompted us to incorporate this method into our overall strategy to analyze biliary defects in various mutant and transgenic mice.

Here, we detail a straightforward technique which can be used to examine the degree of BD paucity in mouse models of liver disease (**Figure 1**). In this method, co-staining with cholangiocyte markers cytokeratin (CK) 8 and CK19 (hereafter wide-spectrum CK, wsCK) is used

to visualize BDs and unincorporated cholangiocytes in the mouse liver. An antibody against alpha-smooth muscle actin (αSMA) is added to the staining to label vessels. Systematic analysis of the BD to PV ratio in a section covering all liver lobes ensures that a large number of PVs are analyzed for each genotype. Since our method relies on quantifying BDs and PVs in 2D images, it is not suitable for studying the effects of a given mutation on the 3D structure of the biliary tree or the integrity of the small biliary conduits. Nevertheless, it provides a simple and objective strategy for investigators to assess biliary development in the mouse.

PROTOCOL:

All animals were housed in a barrier animal facility at Baylor College of Medicine per Institutional Animal Care and Use Committee guidelines and under approved animal protocols.

1. Collection of mouse liver tissue

1.1. Preparation of mouse for liver harvest

1.1.1. Euthanize the mouse using isoflurane.

1.1.2. Perform cervical dislocation of the mouse to ensure death.

1.1.3. Make a transverse incision approximately one inch below the rib cage.

1.1.4. Expose the entire ventral surface of the liver.

1.2. Collection of the mouse liver

1.2.1. Carefully, with small scissors, cut through the ligaments connecting the liver to other organs in the abdomen.

1.2.2. Cut through the common BD to detach the liver from the intestine.

1.2.3. Carefully remove the liver by holding onto the gallbladder and immediately place in a 50 mL tube filled to three-quarters by 4% paraformaldehyde (PFA).

2. Fixation and embedding the liver in paraffin

2.1. Fixation

2.1.1. Fix the liver tissue for 48 h in 4% PFA at 4 °C.

2.1.2. Wash the tissue with 70% EtOH for 1 h at 4 °C.

2.1.3. Wash the tissue twice with 95% EtOH for 1 h each at 4 °C.

2.1.4. Wash the tissue twice with 100% EtOH for 1 h each at 4 °C.

2.2. Clearing

2.2.1. Wash the liver tissue with clearing agent (**Table of Materials**) three times for 30 min each at room temperature.

NOTE: The liver should feel rigid following the third wash.

2.3. Embedding in paraffin

2.3.1. Place the tissue cassette in a tissue mold in paraffin wax for 3 washes, 30 min each. Wax should be preheated to 60 °C.

2.3.2. Fill the tissue mold with paraffin wax to three-quarters height and keep on a heating block at 60 °C.

2.3.3. Place the liver in the mold with the ventral side facing up.

2.3.4. Carefully remove the mold from the heating block.

2.3.5. Place the top of the cassette on the mold and top off with hot liquid paraffin.

2.3.6. Allow the mold and block to cool to room temperature overnight.

NOTE: Tissue blocks can now be stored at room temperature.

3. Sectioning liver tissue

3.1. Preparation of the block for sectioning

3.1.1. Place the mold on ice for 5 min before removing the block from the mold.

3.1.2. Place the block on ice with a lab tissue paper present between block and ice.

3.1.3. Keep the block on ice when not sectioning for best tissue slicing results.

3.2. Sectioning the liver blocks

3.2.1. Using a microtome, begin by sectioning through the superficial, dorsal side of the liver. Sections should be 5 µm.

3.2.2. Check the superficial sections under a dissection microscope to ensure sections are not sheared or folded.

3.2.3. Take a section of the liver that includes the caudate lobe.

NOTE: For some blocks, you will have the left, medial, right and caudate lobes on the same tissue slice.

3.2.4. For those blocks where all four lobes are not present on the same slide, continue to slice until the left, medial and right lobes are present on the same slide.

4. Immunohistochemistry for wsCK and αSMA

4.1. Processing of slides for immunohistochemistry

4.1.1. Select one slide per genotype to be analyzed.

4.1.2. Wash the slide for 15 min in Xylene, 100% EtOH, 95% EtOH and finally 70% EtOH (3 x 5 min in each solution).

4.1.3. Wash the slide for 5 min in deionized H_2O.

4.1.4. Immerse the slide in the antigen retrieval solution (Tris-based, high pH).

4.1.5. Heat under pressure in a pressure cooker for 3 min at 10 psi.

4.1.6. Allow the slide to cool to room temperature (approximately 35 min).

4.2. Blocking the tissue sections

4.2.1. Using a Pap Pen, outline the sections on the slide.

4.2.2. Apply phosphate-buffered saline (PBS) + 0.1% Tween to cover the section twice, 5 min each.

4.2.3. Make blocking buffer by mixing Normal Goat Serum (NGS) at 1:50 in PBS + 0.3% Triton. To have enough buffer for both blocking and primary antibody application, 100 µL per section is sufficient.

4.2.4. Apply 100 µL of blocking solution per section.

4.2.5. Incubate the slides covered with the blocking solution at 4 °C for 1 h.

4.3. Staining for wsCK and αSMA

4.3.1. Dilute anti-CK8 and anti-CK19 antibodies[16] (Developmental Studies Hybridoma Bank,

TROMA-I and TROMA-III, respectively) 1:20 in blocking buffer to stain for wsCK. Dilute the anti-αSMA antibody[17] (**Table of Materials**) to 1:200 in the same buffer.

4.3.2. Apply 100 µL of the diluted antibody solution containing all three antibodies to each section.

4.3.3. Incubate the slides covered with the antibody solution at 4 °C overnight.

4.3.4. Wash the slides with PBS + 0.1% Triton three times, 5 min each.

4.3.5. Dilute secondary antibodies (anti-rat-Alexa488 and anti-mouse-Cy5) 1:200 in PBS + 0.3% Triton.

4.3.6. Apply 100 µL of the secondary antibody solution containing both secondary antibodies to the slides.

4.3.7. Incubate at room temperature for 1 h.

4.4. DAPI nuclear staining and mounting

4.4.1. Wash the slides three times, 5 min each.

4.4.2. Apply 100 µL of DAPI (1:3000) to each section for 10 m.

4.4.3. Apply Antifade Mounting Medium (**Table of Materials**) to the slides and place a glass coverslip on top of the tissue sections. Leave the slides at 4 °C overnight. Seal the slides the next day.

4.4.4. Store the slides at 4 °C and image within 1 week of mounting.

5. Imaging and quantification of BDs

5.1. Imaging liver sections

5.1.1. Prior to imaging, blind yourself to the genotype of the sample with help from a lab member. Ensure all imaging files are devoid of genotype or other specific identifying information besides an animal/sample number.

5.1.2. Using a fluorescent microscope, take 20X images at 1X zoom of each section and ensure that every portal vessel across the liver is imaged. Include the left, medial, right and caudate lobes.

NOTE: We usually find 60-90 portal tracts per animal depending on the size of the liver.

5.1.3. To identity the portal vessels, look for αSMA plus wsCK staining. Structures that are αSMA positive but lack wsCK staining are not portal structures.

5.2. Identification and counting of BDs

5.2.1. Create a spreadsheet with the following columns: Animal/Sample Number, Image Number, Number of Portal Vessels and Number of BDs.

5.2.2. Going through each image, identify and record the number of portal vessels per image.

5.2.3. Identify patent BDs in each image by the presence of cholangiocytes (wsCK+) surrounding a definable lumen. Structures should be distinct and separated by mesenchyme from other wsCK+ cells.

5.2.4. Count each patent BD and place in the same column as the image number.

5.2.5. Do this for each image taken of a portal vessel.

5.2.6. Calculate the sum of all portal vessels and all BDs in the liver sample.

5.2.7. Calculate the BD to PV ratio for the liver sample.

REPRESENTATIVE RESULTS:

We previously documented biliary defects in *Jag1+/–* animals, a mouse model of ALGS[8]. To determine the BD to PV ratio, we sectioned P30 mouse livers and co-stained them for CK8 and CK19 (wsCK) along with the vascular marker αSMA. We then imaged all the portal vessels in each of the liver lobes. As shown in **Figure 2A**, we defined PVs as αSMA-stained vessels that have adjacent wsCK staining (arrowheads). The αSMA-stained structures without wsCK were central veins and should not be included in the analysis (arrow).

Once portal vessels were identified, we identified patent BDs by their characteristic shape. As shown in **Figure 3**, patent ducts have a clearly definable lumen that is surrounded by wsCK+ cholangiocytes. The ducts are usually separated from nearby ducts or cholangiocytes by mesenchyme (arrowhead). wsCK+ cells that do not have a definable lumen, are attached to adjacent cells, or appear in isolation, are not counted toward the total number of BDs (arrows). **Figure 3A** shows a wild-type liver section with a PV which is associated with a fully patent duct along with several unincorporated cells. **Figure 3B** is a representative liver section from a P30 *Jag1+/–* animal. No patent BDs are present around the three PVs in this section. All wsCK+ cells are unincorporated and therefore should not be counted. This image highlights the importance of careful BD counting, as presence or absence of wsCK+ cells does not differentiate the *Jag1+/–* and wild-type livers.

As demonstrated in **Figure 1D**, analysis of the BD to PV ratio involves counting every PV in the liver section along with the total number of patent BDs present around each portal vessel.

While analyzing the *Jag1+/–* livers, we noticed that different lobes are not necessarily affected to the same extent in these animals (unpublished data). Therefore, we usually count portal vessels across the left, medial, right and caudate lobes to ensure complete liver coverage. Following tabulation of total portal vessels and BDs, the ratio is calculated for the whole section.

In **Figure 4**, we showed the analysis of the BD to PV ratios for 3 wild-type and 3 *Jag1+/–* animals. This graph shows how the two genotypes can be readily distinguished based on BD counts. Additionally, this method provides a quantitative measure for analysis of the degree of rescue of the *Jag1+/–* phenotype by genetic manipulations, as reported previously[8].

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the experimental process. (A). The liver is harvested whole from the mouse. **(B).** Liver samples are fixed for 48 h, dehydrated and cleared. **(C).** The liver tissue is embedded in paraffin. Sections are made and placed on charged slides and stained for wsCK and αSMA. **(D).** Slides are imaged, and the number of PVs and BDs are recorded. The BD to PV ratio (BD:PV) is calculated for the entire liver section. HA, hepatic artery; CV, central vein.

Figure 2: Distinguishing central veins from portal vessels. (A). A portal vessel is identified by the presence of αSMA staining and surrounding wsCK+ cholangiocytes (arrowheads). **(B).** Central veins are identified by the presence of αSMA staining with no wsCK+ cholangiocytes present around the structure (arrow). (**A' and B').** Grayscale images showing the αSMA channels from A and B, respectively. Scale bar in A is 100 µm and applies to all panels.

Figure 3: Identification of patent BDs. (**A-A').** In P30 wild-type livers, we count round to ellipsoid structures with a discernable lumen surrounded by wsCK+ cholangiocytes as a patent BD (arrowhead). Cholangiocytes which are not surrounding a lumen are considered unincorporated and are not counted (arrows). (**B-B').** In *Jag1+/–* livers, cholangiocytes are still present (arrows). However, most are not incorporated into patent BDs. Scale bar in A is 100 µm and applies to all panels.

Figure 4: The BD to PV Graph from P30 mouse livers. BDs and PVs are quantified and the BD to PV ratio is generated. As reported previously[8], *Jag1+/–* animals have a characteristic and significant decrease in BD to PV ratio compared to wild-type animals. For statistical analysis, two-way *t*-test was performed. Horizontal lines show means. *** $P<0.001$.

DISCUSSION:

Analysis of BD development and repair in mice is an important tool in studying the pathogenesis and mechanism of cholestatic disorders. In addition, development of new therapies is in part dependent upon establishing a reproducible and preferably quantifiable phenotype. Current phenotyping in mouse models usually involves serum chemistry, liver histology and immunostaining for cell-type specific markers. Although these techniques generate valuable information about the structure and function of the biliary system, they do not provide a direct measure of the effects of a given genetic manipulation on the number of

BDs. In anatomic pathology, BD paucity in human patients is determined through the analysis of BD to PV ratio in a biopsy section[9]. While clinicians employ serum chemistry analysis to determine the severity of cholestasis and liver disease in ALGS and other cholestatic diseases[18,19], histological assessment in mouse models is critical to both understanding the effects of disease modifiers on BD development and the effectiveness of therapies on restoring normal duct development. This is in part because mice can have a severe decrease in the number of patent BDs but still show only a modest increase in the serum bilirubin level[8], likely due to the highly efficient bile drainage in the mouse liver. Our previous work has shown that *Jag1* heterozygosity results in impaired BD maturation but not the absence of cholangcioytes[8]. Thus, to analyze BD development and assess disease severity in mouse models, it is not sufficient to merely examine the absence or presence of cholangiocytes. To address this issue, here we have presented a simple method for objective measurement of patent BD numbers in the mouse liver.

Our analysis is dependent on proper fixation and embedding of the mouse liver. Livers are embedded ventral side up to ensure similar sectioning from one sample to another. This is the most stable position. The sections used for staining must be deep enough in the liver lobes, as there are differences in the number of BDs and the size of PVs in the periphery versus the hilum of the liver. We occasionally see portal vessels that are cut longitudinally. In those cases, there is usually a neighboring BD that is also cut longitudinally and appears like a long open tube. To ensure reproducibility, we count these long tubes as a single BD. Identification of patent BDs is unambiguous for the most part. However, some biliary structures appear lumenized but do not show the round to ellipsoid morphology typically seen in normal BDs[14]. In our hands, this can sometimes result in one structure being called a BD by an investigator but not by another colleague. Therefore, to ensure consistency and reproducibility in data analysis and presentation, we recommend that all samples related to a specific project be analyzed by two investigators independently.

Anti-CK8 has been shown to mark immature and mature biliary cells, while anti-CK19 only marks mature biliary cells[12,20]. Therefore, even if a PV is not associated with a mature BD, it can be readily differentiated from central veins because of the presence of CK8+ cells. Using these two antibodies in combination with anti-αSMA ensures complete coverage of portal tracts in our quantification. Moreover, in our hands, the individual CK19 or CK8 staining of the biliary cells generates a relatively weak signal and is associated with some background staining. Mixing the two CK antibodies results in a consistently strong signal in biliary cells and therefore facilitates the quantification**.**

Maturation of the intrahepatic biliary tree occurs in a hilar-to-peripheral direction and continues postnatally[21]. Indeed, there are many more immature cholangiocytes in early postnatal livers, especially in peripheral areas, which are at the leading front of postnatal liver expansion. Moreover, we observed a change in BD numbers as the animals age[8], with more ducts in older animals. In addition, some portal structures contain multiple BDs while others have one or no ducts, particularly along the liver periphery. We use a microscopy slide covering all liver lobes for each animal and systematically quantify the BD to PV ratio across the whole

slide. While this is not essential, it ensures that ample portal tracts are analyzed for each animal regardless of age and liver size (60-90 PVs per liver depending on the genotype and age). Moreover, by analyzing all PVs on the slide, we ensure that both more mature hilar and less mature peripheral areas are counted at all stages of liver development. Covering all liver lobes for each animal can also decrease variability in measurements if a given mutation does not affect BD development uniformly across the liver.

The method is limited in distinguishing smaller bile conduits from groups of unincorporated biliary cells. The bile ductules[4] are usually too small to be recognized as a lumenized structure in the magnification that we use to analyze these stainings. Therefore, they are likely to be excluded from our quantifications, and thus the method is skewed towards identifying medium to large ducts. Despite this limitation, the method described here readily distinguishes between the *Jag1+/–* and *Jag1+/+* livers. Moreover, it is sensitive enough to detect the partial rescue of the *Jag1+/–* BD paucity upon simultaneous loss of one copy of *Polgut1+/–* and the modest increase in BD density in *Jag1+/+* animals with conditional loss of *Poglut1* in vascular smooth muscle cells[8]. These observations indicate this method's usefulness in determining alterations in BD density in various genetic backgrounds, even when the changes in BD number are modest.

In recent years, visualization of the 3D structure of the biliary tree has been used by several groups to analyze BD development[22-25]. These elegant methods rely on filling the biliary tree from the common BD by ink or resin, and therefore examine the patency of the biliary system. Moreover, they provide information about the 3D structure of the biliary tree and its formation in the liver periphery as the liver grows, which cannot be assessed by 2D assessment used in protocols like the one presented here. However, successful performance of these 3D visualization techniques requires considerable expertise[26]. In contrast, the technique presented here is rather straightforward and can be executed by any group with access to routine equipment for histological and imaging analysis. Moreover, analysis of the sections double-stained for wsCK and αSMA will also show whether ductular reactions or vascular smooth muscle cell abnormalities exist in the liver[8,27,28]. We suggest that quantifying the BD to PV ratio in the whole liver sections can provide a sensitive and reproducible measure of biliary development in mice and can serve as a relatively easy technique to help the investigators decided whether they should consider more sophisticated techniques like 3D visualization of the biliary tree.

ACKNOWLEDGMENTS:
The authors acknowledge support from the National Institutes of Health (NIH) (R01 GM084135 and R01 DK109982), a Pilot/Feasibility Award from the Texas Medical Center Digestive Disease Center under NIH P30 DK56338, and an Alagille Syndrome Accelerator Award from The Medical Foundation.

DISCLOSURES:
The authors have no conflict of interest.

REFERENCES:

1 Zong, Y. et al. Notch signaling controls liver development by regulating biliary differentiation. *Development.* **136** (10), 1727-1739 (2009).

2 Clotman, F. et al. Control of liver cell fate decision by a gradient of TGFβ signaling modulated by Onecut transcription factors. *Genes & Development.* **19** (16), 1849-1854 (2005).

3 Karpen, S. J. Update on the etiologies and management of neonatal cholestasis. *Clin Perinatol.* **29** (1), 159-180 (2002).

4 Roskams, T. A. et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology.* **39** (6), 1739-1745 (2004).

5 Oda, T. et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nature Genetics.* **16** (3), 235 (1997).

6 Li, L. et al. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nature Genetics.* **16** (3), 243 (1997).

7 Xue, Y. et al. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Human Molecular Genetics.* **8** (5), 723-730 (1999).

8 Thakurdas, S. M. et al. Jagged1 heterozygosity in mice results in a congenital cholangiopathy which is reversed by concomitant deletion of one copy of Poglut1 (Rumi). *Hepatology.* **63** (2), 550-565 (2016).

9 Hadchouel, M. Paucity of interlobular bile ducts. *Seminars in Diagnostic Pathology.* **9** (1), 24-30 (1992).

10 Emerick, K. M. et al. Features of Alagille syndrome in 92 patients: frequency and relation to prognosis. *Hepatology.* **29** (3), 822-829 (1999).

11 Poncy, A. et al. Transcription factors SOX4 and SOX9 cooperatively control development of bile ducts. *Dev Biol.* **404** (2), 136-148 (2015).

12 Hofmann, J. J. et al. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. *Development.* **137** (23), 4061-4072 (2010).

13 McCright, B., Lozier, J. & Gridley, T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development.* **129** (4), 1075-1082 (2002).

14 Andersson, E. R. et al. Mouse Model of Alagille Syndrome and Mechanisms of Jagged1 Missense Mutations. *Gastroenterology.* **154** (4), 1080-1095 (2018).

15 Loomes, K. M. et al. Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. *Hepatology.* **45** (2), 323-330 (2007).

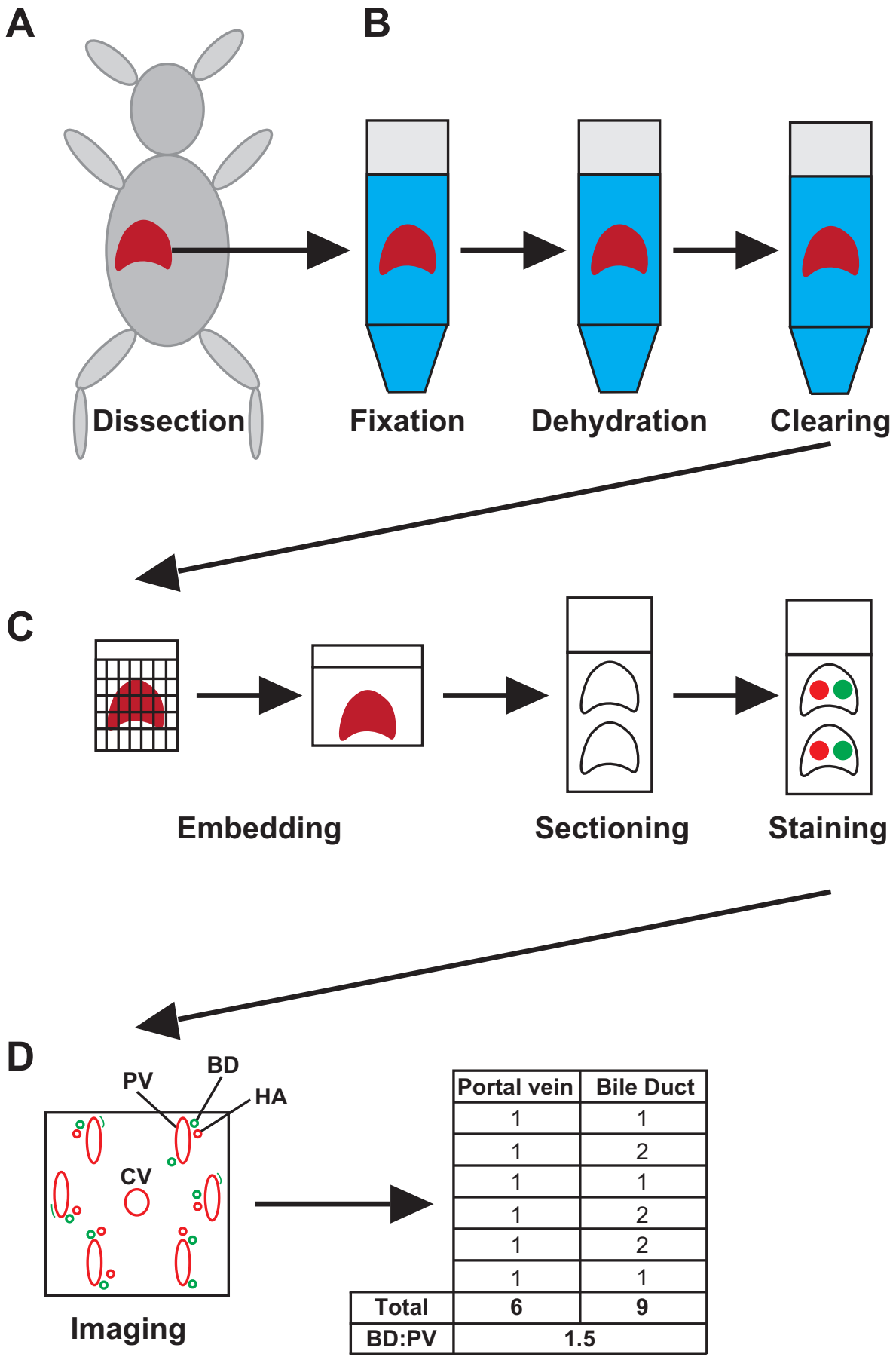
16 Brulet, P., Babinet, C., Kemler, R. & Jacob, F. Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation. *Proc Natl Acad Sci U S A.* **77** (7), 4113-4117 (1980).

17 Skalli, O. et al. A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol.* **103** (6 Pt 2), 2787-2796 (1986).

18 Kamath, B. M. et al. A longitudinal study to identify laboratory predictors of liver disease outcome in Alagille syndrome. *Journal of Pediatric Gastroenterology and Nutrition.* **50** (5), 526 (2010).

19 Mouzaki, M. et al. Early life predictive markers of liver disease outcome in an International, Multicentre Cohort of children with Alagille syndrome. *Liver International.*

36 (5), 755-760 (2016).
20 Shiojiri, N. Development and differentiation of bile ducts in the mammalian liver. *Microsc Res Tech.* **39** (4), 328-335 (1997).
21 Crawford, J. M. Development of the intrahepatic biliary tree. *Semin Liver Dis.* **22** (3), 213-226 (2002).
22 Kaneko, K., Kamimoto, K., Miyajima, A. & Itoh, T. Adaptive remodeling of the biliary architecture underlies liver homeostasis. *Hepatology.* **61** (6), 2056-2066 (2015).
23 Schaub, J. R. et al. De novo formation of the biliary system by TGFbeta-mediated hepatocyte transdifferentiation. *Nature.* **557** (7704), 247-251 (2018).
24 Sparks, E. E., Huppert, K. A., Brown, M. A., Washington, M. K. & Huppert, S. S. Notch signaling regulates formation of the three-dimensional architecture of intrahepatic bile ducts in mice. *Hepatology.* **51** (4), 1391-1400 (2010).
25 Tanimizu, N. et al. Intrahepatic bile ducts are developed through formation of homogeneous continuous luminal network and its dynamic rearrangement in mice. *Hepatology.* **64** (1), 175-188 (2016).
26 Walter, T. J., Sparks, E. E. & Huppert, S. S. 3-dimensional resin casting and imaging of mouse portal vein or intrahepatic bile duct system. *J Vis Exp.* (68), e4272 (2012).
27 Popper, H., Kent, G. & Stein, R. Ductular cell reaction in the liver in hepatic injury. *J Mt Sinai Hosp N Y.* **24** (5), 551-556 (1957).
28 Yimlamai, D. et al. Hippo pathway activity influences liver cell fate. *Cell.* **157** (6), 1324-1338 (2014).



Portal vein	Bile Duct
1	1
1	2
1	1
1	2
1	2
1	1
Total	**6**	**9**
BD:PV	**1.5**

Figure 1

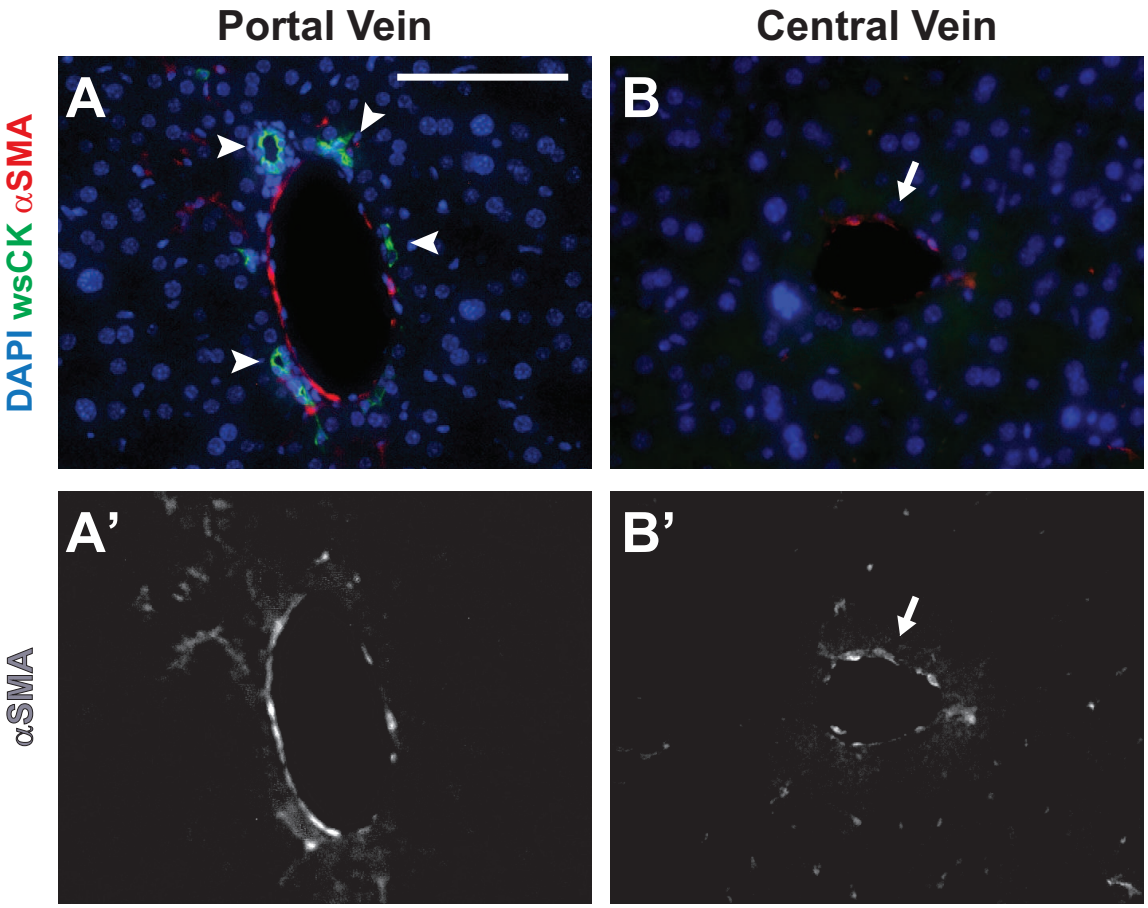


Figure 2

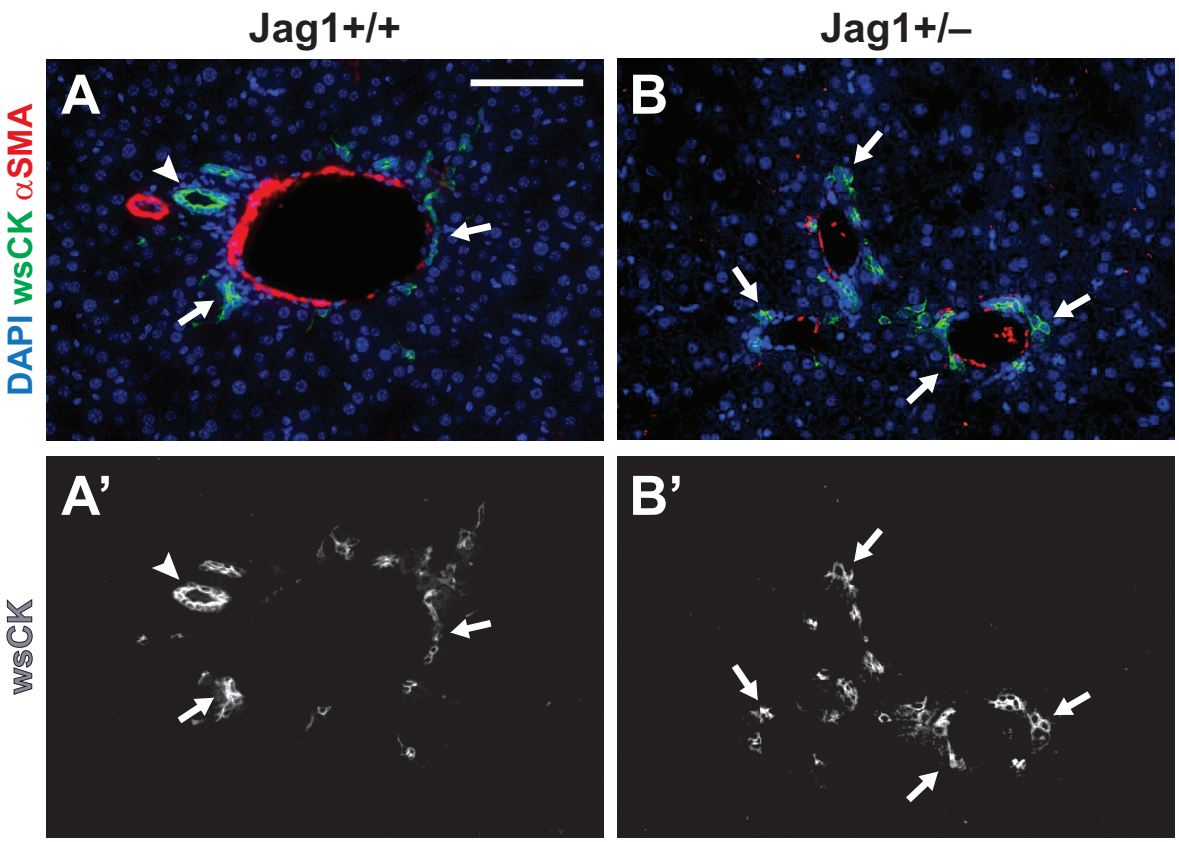


Figure 3

Figure 4

Click here to access/download;Figure;Adams-JOVE_Fig 4.eps

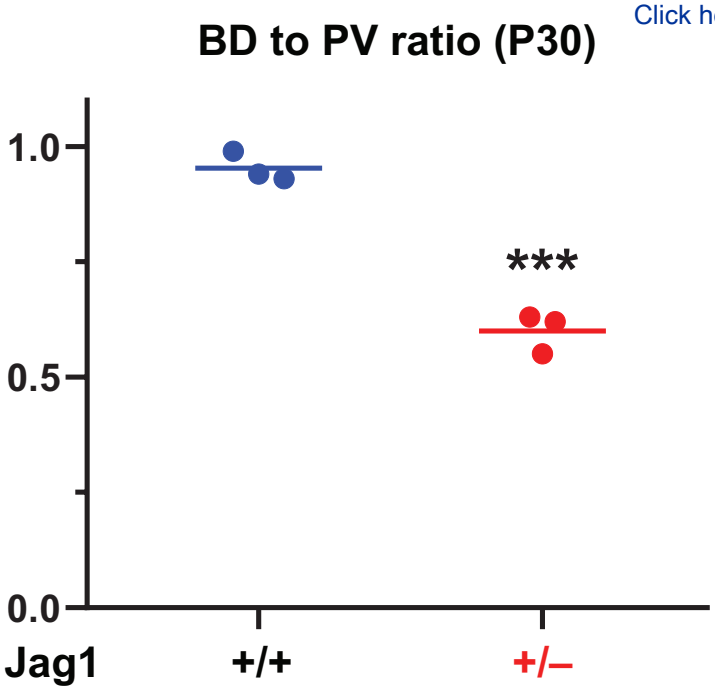


Figure 4

Table of Materials

Click here to access/download;Table of Materials;Adams-JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
Isothesia (Isoflurane)	Henry Schein	11695-6776-2
Desiccator	Bel-Art	16-800-552
10% PFA	Electron Microscopy Sciences	15712
50mL tube	ThermoScientific	339653
70% Ethanol	Decon Laboratories	2401
95% Ethanol	Decon Laboratories	2801
100% Ethanol	Decon Laboratories	2701
HistoChoice	VWR Life Sciences	H103-4L
Omnisette Tissue Cassette	Fisher HealthCare	15-197-710E
Macrosette	Simport	M512
Paraplast X-TRA	McCormick Scientific	39503002
Tissue Mold	Fisher Scientific	62528-32
Microtome	Microm	HM 325
Superfrost Plus Microscope Slides	Fisher Scientific	12-550-15
Xylene	Fisher Scientific	C8H10
Tris-Based Antigen Retrieval	Vector Laboratories	H-3301
Pressure Cooker	Instant Pot	Lux Mini
Mini Pap Pen	Life Technologies	8877
Polyoxyethylene 20 Sorbitan Monolaurate (Tween-20)	J.T. Baker	X251-07
Octyl Phenol Ethoxylate (Triton-X-100)	J.T. Baker	X198-07
Normal Goat Serum	Jackson Immunoresearch	005-000-121
anti-CK8	Developmental Studies Hybridoma Bank	TROMA-I
anti-CK19	Developmental Studies Hybridoma Bank	TROMA-III
anti-αSMA	Sigma Aldrich	A2547, Clone 1A4
anti-rat-Alexa488	ThermoFisher	A21208
anti-mouse-Cy5	Jackson Immunoresearch	715-175-151
DAPI	Vector Laboratories	H-1000
22x50 micro cover glass	VWR Life Sciences	48393 059
Fluorescence Microscope	Leica	DMI6000 B

Kimwipes	Kimtech Science	05511
VECTASHIELD	Vector Laboratories	H-1000

Comments/Description

clearing agent

Parrafin

Antibody Registry ID AB531826
Antibody Registry ID AB2133570

Antifade Mounting Medium

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	A Simple Method for Determining Bile Duct Density in the Mouse Liver
Author(s):	Josh Adams and Hamed Jafar-Nejad

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

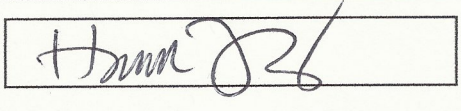
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Hamed Jafar-Nejad
Department:	Molecular and Human Genetics
Institution:	Baylor College of Medicine
Title:	A Simple Method for Determining Bile Duct Density in the Mouse Liver
Signature:	
Date:	12/18/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Hamed Jafar-Nejad, M.D.
Associate Professor
Dept. of Molecular & Human Genetics

Bing Wu, Ph.D.
Review Editor
JoVE

February 4, 2019

Dear Dr. Wu,

Thanks for overseeing the review of our manuscript. We are pleased to see the positive evaluation by the reviewers and appreciate all the constructive Editorial and reviewer comments. As discussed in the following point-by-point response, we have addressed all of the comments in the revised manuscript. We feel that these changes have improved our work and hope that you and the reviewers will find the revised version suitable to publication in JoVE.

Best regards,

Hamed Jafar-Nejad
Department of Molecular and Human Genetics
Program in Developmental Biology
Baylor College of Medicine

One Baylor Plaza, Room 919E, MS: BCM225, Houston, TX 77030
Phone: 713-798-6159 Fax: 713-798-8142
Email: hamedj@bcm.edu

EDITORIAL COMMENTS:

The manuscript has been modified and the updated manuscript, 59587_R0.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

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

Done.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Not applicable.

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Done.

4. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done.

5. Please use h, min, s for time units.

Done.

REVIEWER #1:

Manuscript Summary:
Well written clear presentation of a useful methodology. Last paragraph of Discussion (lines 417-431) is excellent.

Major Concerns:

The manuscript states that there can be significant inter-lobe differences in bile duct density within a liver, and the authors strongly emphasize that, therefore, there is a consequential need for using a method that evaluates across the entire liver. Whereas there is no doubt that a full-liver analysis is good, the authors do not establish in their presentation that this concern is, indeed, valid, nor that a real

One Baylor Plaza, Room 919E, MS: BCM225, Houston, TX 77030
Phone: 713-798-6159 Fax: 713-798-8142
Email: hamedj@bcm.edu

(significant) need for this exists. The data presented do not support this mandate. To support this concern, I would need to see a an analysis of inter-lobe bile duct density variance in a substantial set of livers. If the authors cannot clearly establish that this is a true statistically significant need, and that erroneous conclusions are likely to arise from more restricted (e.g., single-lobe) analyses, then the language needs to be adjusted. Otherwise, there is a risk that this publication will mandate unnecessary overkill, and future research submissions in the field reporting completely robust conclusions might be rejected for not following an unnecessarily exhaustive prescription.

I would like to re-emphasize that this is a very good submission, and regardless of the outcomes of a study on inter-lobe variance, should be published in JoVE. However, the language used needs to be supported by data presented. In the absence of demonstrating a significant need, the authors could simply re-word their presentation to indicate that the method presented is an improvement over more restricted analyses, rather than presenting it as a need.

We fully agree with the reviewer. In the revised manuscript, we have adjusted the language to address this point. We removed the "variability" argument from the Introduction. Also, in the Discussion, we now write "We use a microscopy slide covering all liver lobes for each animal and systematically quantify the bile duct to portal vein ratio across the whole slide. While this is not essential, it ensures that ample portal tracts are analyzed for each animal regardless of age and liver size (60-90 PVs per liver depending on the genotype and age). Covering all liver lobes for each animal can also decrease variability in measurements if a given mutations does not affect bile duct development uniformly across the liver."

Minor Concerns:
Does use of the phrase: "Last but not least" (line 53) serve a purpose?

Rephrased as "In addition".

REVIEWER #2:

Manuscript Summary:
The authors describe a method to quantify biliary structures on tissue sections. By counting the average number of bile ducts associated with the clear lumen around each portal vein, they distinguished between wild-type and Jag1+/- mice.

Major Concerns:
Because the mutant obviously has abnormalities of the biliary structure, it is quite easy to show the difference against the wild type by any quantitative experiments. Therefore, the reviewer could not judge whether this method is useful to estimate abnormalities of the biliary structure.

In our previous paper (Thakurdas et al, Hepatology, 2016), we have shown that this method not only shows the difference between BD numbers of wild-type and Jag1 heterozygous mutant, but can also detect a statistically significant but modest difference (20% to 30%) in BD numbers caused by several other genetic manipulations. Therefore, the presented method does not need a major defect in the number of BDs in order to detect a difference. In the revised version, we refer to the above-

One Baylor Plaza, Room 919E, MS: BCM225, Houston, TX 77030
Phone: 713-798-6159 Fax: 713-798-8142
Email: hamedj@bcm.edu

mentioned observations.

Minor Concerns:
The authors should show how many PV areas they need to count to determine the number of bile ducts for each animal.

We count all the PVs on a section from the whole liver. Depending on the liver size, this allows us to analyze between 60 and 90 portal veins per animal. This is added to the revised version.

Why the authors need to use anti-CK8 antibody in addition to anti-CK19 to recognize biliary structures? Which antibodies did the authors use?

We thank the reviewer for raising this point. We should have clarified the reason for this choice in the manuscript. CK19 marks mature biliary cells. In contrast, CK8 marks both mature and immature cholangiocytes. We use a 50:50 mixture of anti-CK18 and anti-CK19 antibodies for two reasons. First, even in wild-type animals, there are a number of PVs (especially in the peripheral areas of the liver) that do not have a large enough portal vein to be identified by immunofluorescent or immunohistochemical staining. In mutants affecting BD development, this can be even a bigger issue. If we only use CK19 staining, it will be difficult to differentiate those PVs from central veins, and this will distort the BD to PV ratio. To confidently distinguish PVs from central veins, a second antibody needs to be used together with CK19. We have chosen CK8, because it enhances the overall staining of BDs (please see the second reason) and because it will also provide an assessment of the degree of ductular reaction. An alternative strategy would be to co-stain the liver sections with CK19 and glutamine synthetase (GS), which exclusively marks the hepatocytes surrounding the central veins (Kuo et al, Molecular and Cellular Biology, 1988). Second, in our hands, individual CK19 or CK8 staining of the biliary cells generates a relatively weak signal and is associated with background staining. Mixing the two antibodies results in a consistently strong signal in biliary cells and therefore facilitates the quantification. In the revised Discussion, we have added a new paragraph to address this issue.

REVIEWER #3:

Manuscript Summary:
This is a manuscript describing a method for determining bile duct density in the mouse liver. The experimental protocol was simple and clear. The methods described in this paper can quantify the density of bile duct formation. However, several points in this manuscript should be changed.

Major Concerns:

#1. As described by the authors in Page 8, Line 378-380, there are differences in the sizes of portal vein between the hilum and peripheral portion of liver. Authors described that "every portal vessel across the liver is imaged": however, one portal vein is cut in cross-sectional, and another is cut in longitudinal axis. To improve reproducibility of this method, authors should define the inclusion and/or exclusion criteria of portal vein diameter to count the number of bile ducts.

One Baylor Plaza, Room 919E, MS: BCM225, Houston, TX 77030
Phone: 713-798-6159 Fax: 713-798-8142
Email: hamedj@bcm.edu

The reviewer makes an important point. We occasionally see portal vessels that are cut longitudinally. In those cases, there is usually a neighboring bile duct that is also cut longitudinally and appears like a long open tube. To ensure reproducibility, we count these long tubes as a single bile duct. We have described this in the second paragraph of Discussion in the revised manuscript.

#2. Page 8, Line 383-386. Authors described that "this can sometimes result in one structure being called a bile duct by an investigator but not by another colleague, … we recommended that all samples related to a specific project be analyzed by the same person". It seems to be scientifically strange.
To improve reproducibility of this method, another colleague should assess the result of bile duct density using same specimens, but not by a single researcher. Authors should change the description about this point.

Thanks for raising this point. What we wrote was meant to address an important issue about consistency of measurements across genotypes in a given project. However, having read the reviewer's comment, we realized that the original sentence did not take into account another equally important issue, namely reproducibility. We fully agree that having more than one investigator perform the counts is important. In fact, we have done this in our lab. When multiple individuals in our lab assessed bile duct counts of the same samples, we observed a variation of 5-10% which is consistent across all genotypes for each person. In other words, some investigators counted 5-10% fewer bile ducts for all genotypes compared to other investigators. Therefore, in the first submission we recommended that one person perform all of the quantifications for a project. In the revised manuscript, we have rephrased to suggest that at least two individuals perform all the counting of all samples to ensure both consistency and reproducibility.

Minor Concerns:
#3. Page 9, Line 413. "it in sensitive" is a spelling error.

Spelling error is corrected.

REVIEWER #4:

The authors describe a method to calculate the average number of bile ducts associated with each portal vein in sections covering all lobes of a mouse liver. In this method, the bile ducts are identified via light microscopy of cholangiocytes stained with antibodies to two different cytokeratins. These bile ducts are then counted and divided by the total number of portal veins present in the liver section, providing a quantitative relative assessment of bile duct to portal vein ratio across the liver. This method was able to clearly distinguish between liver sections of wild type mice and a mouse model of Alagille syndrome. This study makes a valuable contribution to the methodology available to study bile duct differentiation and formation in mouse models.

Specific comments:

1. The manuscript does not state clearly whether EVERY section needs to be stained and counted. They clearly make the point that the observed phenotype can vary across different liver lobes, but is it really necessary to stain every section? How many sections would that amount to in a typical situation (e.g., an

One Baylor Plaza, Room 919E, MS: BCM225, Houston, TX 77030
Phone: 713-798-6159 Fax: 713-798-8142
Email: hamedj@bcm.edu

8 week old adult liver, or a postnatal day 6 liver). Having done my own sectioning, I know very well that you sometimes lose some. How would this be accounted for? I feel that some additional details along these lines are essential for the manuscript.

We apologize for not clarifying this issue. Not every section needs to be stained and counted. We continue the sectioning until all lobes are present on a given slide. We then stain that single slide and quantify all PVs and bile ducts on it. In other words, for each animal we use one slide covering all lobes for quantification. We have updated the phrasing to make this clear.

2. The abbreviation wsCK is used multiple times in the manuscript prior to it being defined at the bottom of page 6 (first paragraph of the Representative Results section).

We have defined wsCK in the Introduction upon first use.

3. Line 371 (suggested change in capital letters): Thus, to ANALYZE bile duct development

Changed analysis to analyze as suggested.

4. Line 413: previously reported that it IS sensitive.

Corrected.

One Baylor Plaza, Room 919E, MS: BCM225, Houston, TX 77030
Phone: 713-798-6159 Fax: 713-798-8142
Email: hamedj@bcm.edu