

The creation of human liver vascular models towards artificial tissue generation

Wesley Brooks Fabyan

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Reading Committee:

Kelly R. Stevens, Chair

Cole A. DeForest

Jonathan T.C. Liu

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Wesley Brooks Fabyan

University of Washington

Abstract

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Wesley Brooks Fabian

Chair of the Supervisory Committee:
Associate Professor Kelly Stevens
Bioengineering

The liver's intricate architecture is critical for its physiological function, and its disruption is pathologic. Liver cirrhosis, the 11th most common cause of death globally, is marked by significant architectural changes and decreased function in the liver, requiring liver transplant in severe cases. The high demand for liver transplantation and unmet need for liver tissue is inspiring research efforts towards creating artificial liver tissue *in vitro*. As the liver's function is closely tied to its structure, the development of functional artificial liver tissue may necessitate recapitulating native liver structure in artificial tissue. Towards this end, we first helped elucidate multilobular human liver architecture by creating the largest, cellularly resolved, 3D reconstructions of non-cirrhotic and cirrhotic human liver tissue known to date. We then compared the functional and structural features of non-cirrhotic tissue with cirrhotic tissue to help decipher which features may be essential for maintaining functional liver tissue. Our comparative multilobular analyses of non-fibrotic and cirrhotic tissues revealed that cirrhosis correlates with sinusoid zonation dysregulation, reduction in glutamine synthetase-expressing pericentral hepatocytes, regression of central vein networks, and fragmentation of biliary networks. Taken together, these observations suggest a pro-portalization/decentralization phenotype in correlation with human liver cirrhosis and emphasize the importance of the liver's central features for maintaining functional liver tissue. After gaining new appreciation for multilobular human liver architecture, we

next pursued computational modeling strategies to generate anatomically accurate human liver vascular designs for future incorporation into artificial liver tissue. Towards this end, we first acquired parameterized morphometrics and performed multiscale analyses on central vein, portal vein, and liver sinusoidal networks. We thereafter collaborated with Nervous System to computationally generate an interpenetrating central vein and portal vein networks design and a 3D sinusoidal mesh network design. Future computational work entails combining modeling methodologies to create interpenetrating central vein and portal vein networks connected by a 3D sinusoidal mesh, thereby creating a scalable multilobular human liver vascular model which we aim to incorporate into artificial liver tissue in future studies. Towards these future tissue engineering efforts, we lastly used DLP additive manufacturing and multi-LSL photodegradation to show feasibility for fabricating the initial interpenetrating networks design and 3D sinusoidal mesh network design respectively within biocompatible hydrogels. All in all, this dissertation work presented the largest cellularly-resolved 3D reconstructions of human liver tissue to date, revealed a pro-portalization/decentralization phenotype in correlation with human liver cirrhosis, generated anatomically accurate human liver vascular designs, and assessed the feasibility of two light-based bioprinting strategies for fabricating human liver vascular designs within biocompatible hydrogels. These collective efforts provide foundational knowledge on human liver architecture in its healthy and diseased states and offer insight into the computational modeling and subsequent fabrication of human liver vascular networks, which together may help advance tissue engineering initiatives towards creating functional artificial liver tissue in the future.

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Chapter 1: Introduction

1.1 Liver structure and function

The liver performs hundreds of functions, and its complex microarchitecture is integral to both its proper function and homeostasis [1]. The liver lobule – the main structural unit of the liver – is thought to be polygonal in shape and tessellate throughout the liver (Fig. 1.1a). At the vertices of the polygonal lobule are the portal tracts, which consist of portal veins that bring nutrient-rich deoxygenated blood from the intestine to the liver, hepatic arterioles that bring oxygenated blood into the liver, bile ducts that carry the hepatic byproduct bile out of the liver to the gallbladder and the small intestine, sympathetic nerves, and lymphatic vessels. At the center of the polygon is the central vein which transports processed blood out of the liver (Fig. 1.1b). Between the portal tracts and the central vein lies the parenchyma, which consists primarily of hepatocytes – the main functional cell type of the liver, bile canaliculi that collect and transport bile to the bile ducts, and specialized capillaries called sinusoids, which are highly-permeable to allow macromolecular exchange between the blood and hepatocytes in the perisinusoidal space [2]. Altogether, blood enters the liver through portal tracts at the vertices of the lobule, percolates inwards through the parenchymal space via the liver sinusoids, and exits through the central vein at the center of the lobule (Fig. 1.1c) [3, 4].

One critical aspect of the healthy liver lobule's cellular organization is its *functional zonation* where cells, specifically hepatocytes, are spatially divided into concentric functional zones that perform unique metabolic functions (Fig. 1.1c) [5]. Zone 1 consists of the portal regions of the lobule, wherein periportal hepatocytes are responsible for oxidative metabolisms including gluconeogenesis, cholesterol formation, and amino acid catabolism. Zone 2 pertains to the mid-lobular space where hepatocyte functions transition from periportal to pericentral roles with respect to their position on the portal-central axis of the lobule. Zone 3 consists of the central region of the lobule, wherein pericentral hepatocytes are responsible for detoxification, xenobiotic

metabolism, lipogenesis, glycogen synthesis, and glutamine formation [6]. The division of labor afforded by this functional zonation has likely evolved to 1) allow the tissue to efficiently perform hundreds of functions concurrently, and 2) enable the tissue to adapt quickly to nutritional requirements in various metabolic states [7]. Furthermore, these lobular zones have been shown to contain different types of vascular and biliary structures across the portal-central axis [8], which can be referred to as *structural zonation*. In the healthy, non-fibrotic liver, Zone 1 liver sinusoidal endothelial cells (LSECs) line the first entry points where blood from the portal tracts begins percolating towards the center of the lobule, and these Zone 1 LSECs are thought to scavenge low-density lipoproteins. Zone 2/3 LSECs are present in both the mid-lobular and pericentral zones and are believed to carry out functions related to blood-derived leukocyte adhesion and antigen clearance [8, 9]. The homeostasis of this intricate functional and structural zonation is critical for liver function and health.

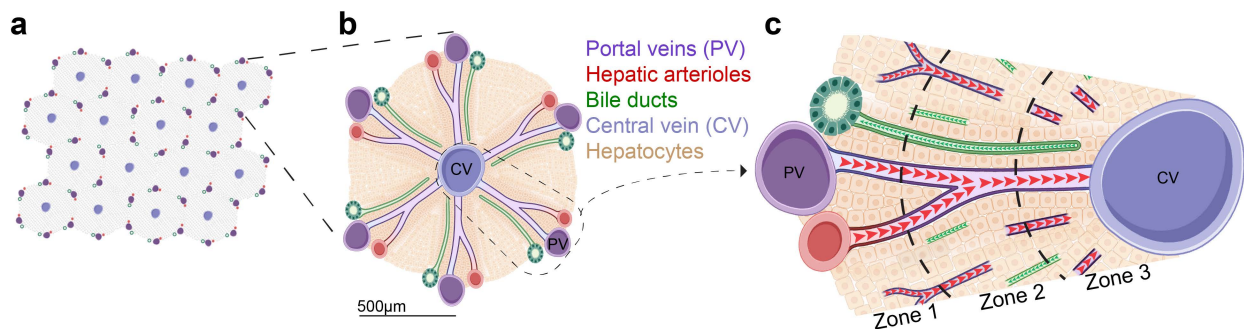


Figure 1.1: Liver lobule structure and functional zones. **a**, Depiction of polygonal lobules arranged within liver tissue. **b**, Traditional schematic of a single liver lobule with portal veins (PV), hepatic arterioles, and bile ducts at the vertices of the polygon and a central vein at its center. Hepatocytes and sinusoids (not shown) fill the space between vascular networks. **c**, The portal-central axis labeled with structural and functional zonation zones. Red arrowheads indicate that blood within the liver lobule flows from portal tracts to the central vein while green arrowheads denote that bile flows from central to portal areas. Schematics created in BioRender.

1.2 Liver cirrhosis and the need for artificial liver tissue

Liver disease is responsible for 2 million deaths per year worldwide, equating to 4% of all deaths globally each year [10]. Liver cirrhosis, an advanced stage of liver disease marked by increase of fibrotic scar tissue and impaired function in the liver, accounts for 1.16 million of the 2 million global liver disease deaths each year, making cirrhosis the 11th most common cause of death globally with a burden of 2.1% of all deaths [11]. Additionally, chronic liver disease has a high morbidity, ranking within the top 20 causes for highest disease-related disability-adjusted life years (DALYs) and years of life lost (YLL) [11]. Furthermore, the global burden of liver disease and cirrhosis is expected to increase [12, 13].

Liver cirrhosis may cause the liver's intricately organized microvasculature to become massively rearranged and result in significant functional deficiencies that lead to liver failure [14], and currently the only available treatment for liver failure is liver transplant [15]. Although liver transplantation is the second most common solid organ transplant surgery worldwide, less than 10% of the global liver transplant needs are currently met and the gap in liver organ supply and demand is widening each year (Fig. 1.2a) [11, 16]. In the United States, 10-20% of patients requiring a liver transplant die while waiting on the transplant list (Fig. 1.2b) [16]. Therefore, there is an urgent need to either decrease the number of patients requiring a transplant and/or increase the supply of livers for transplant.

To help close the gap between supply and demand for liver transplants, tissue engineers are working to build artificial liver tissue in the laboratory for disease modeling and eventually whole organ transplantation. Artificial liver tissue could help researchers model various liver diseases, such as cirrhosis, *in vitro* to improve the understanding of disease progression and provide a tool for testing drug treatments or interventions, which if successful could help reduce the number of patients requiring a liver transplant. Furthermore, artificial liver tissue may be able to recapitulate native liver function adequately enough to be transplanted directly into patients, thereby providing an alternative tissue source for liver transplantation and increasing the overall

supply of livers for transplant. Despite numerous advancements over the past several decades, tissue engineers have yet to create artificial liver tissue that replicates the functional complexity of native tissue. As the liver's function is closely tied to its structure, the development of functional artificial liver tissue may rely on the ability to recapitulate native liver structure in artificial liver tissue.

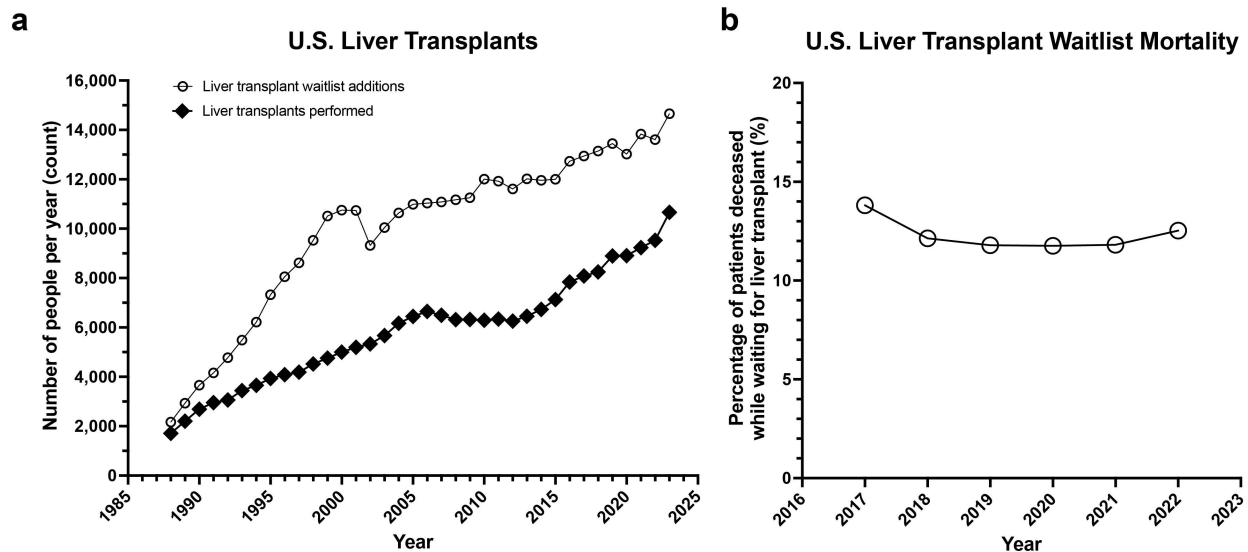


Figure 1.2: The unmet need for functional human liver tissue. **a**, Number of people added to the liver transplant waitlist each year and the number of liver transplants performed each year in the United States (1988-2023). **b**, Percentage of patients who die while waiting for a liver transplant each year in the United States (2017-2022). Data from: <https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/>.

1.3 Bioprinting

Tissue engineering research aims to create biomimetic tissues for applications ranging from *in vitro* disease modeling to *in vivo* regeneration, repair, and eventually whole organ replacement. However, challenges remain to incorporate cell spatial heterogeneity and construct intricate tissue architectures necessary for attaining physiologic function. Over the past several decades, tissue engineers have begun to employ 3D bioprinting to build 3D cellularized hydrogels

– the basis for artificial tissue – with unprecedented control and precision [17]. Common 3D bioprinting strategies typically rely on inkjet-, extrusion-, embedded-, or light-based technology [18, 19]. Inkjet-based bioprinting, employing similar technology as the common home/office inkjet 2D printer, deposits droplets of dilute biological solutions at determined positions to build a 3D object. Extrusion-based bioprinting, often referred to as direct ink writing, uses a spatially controlled nozzle to deposit biological material that thereafter thermally, chemically, or photochemically cures to form a 3D construct. Embedded-based bioprinting utilizes a support bath to hold extruded soft materials in 3D space while a curing or polymerization process occurs, and this technology can be leveraged to either bioprint large, unsupported cell-laden 3D constructs within a support bath (*i.e.*, FRESH printing) [20] or to create channel networks within a cell-laden bulk using sacrificial inks (*i.e.*, SWIFT biomanufacturing) [21]. Light-based bioprinting, such as digital light processing or laser-scanning lithography, uses photomasked light or a focused laser beam to selectively crosslink photopolymerizable bioinks to create 3D constructs. All of these bioprinting technologies are being utilized to print cells and biomaterials into 3D cellularized hydrogels resembling artificial tissues, yet the inability to establish adequate vasculature networks within bioprinted tissues has greatly limited the functionality and applications of these tissue engineering initiatives [19, 22].

1.4 Conclusion

The liver is an exceptionally complex organ responsible for numerous life-sustaining functions including detoxification, xenobiotic metabolism, gluconeogenesis, ammonia metabolism and production of bile, several blood proteins, and cholesterol [6]. The various functions of the liver are closely related to the liver's unique structure. In cirrhosis, the liver's vasculature networks often become significantly altered, and this structural rearrangement may have severe functional repercussions that lead to liver failure. Currently, liver transplantation is the only treatment for liver failure, and there is a large and growing gap in the number of livers available and the number of

patients on the transplant waiting list [11, 16]. To address this gap, tissue engineers aim to create artificial liver tissue for disease modeling and eventually whole organ transplantation. Towards this end, bioprinting offers unprecedented control and precision as a strategy for building artificial tissues [17]. However, despite numerous advancements over the past several decades, tissue engineers have yet to create artificial liver tissue that replicates the functional complexity of native tissue. As the liver's function is closely tied to its structure, the development of functional artificial liver tissue may rely on the ability to recapitulate native liver structure in artificial liver tissue. Interestingly, we discovered that there was a gap in knowledge of what human liver tissue truly looks like on the multilobular scale with cellular resolution. Therefore, our first step towards building native liver architecture in artificial tissues was to elucidate the organization of structural and functional features in human liver tissue across several lobules in 3D.

Chapter 2: Human liver imaging

2.1 Introduction

Much of what is currently known about human liver architecture with cellular resolution has been elucidated via traditional 2D histology – the interrogation of thin slices of tissue under a microscope. Although useful, 2D histology may leave gaps in our understanding of the liver's microstructure. For example, the 2D nature of histology can misrepresent actual biological structures: 1) 3D convoluted structures often appear as multiple distinct objects in a 2D cross-section, 2) cross-sections may not accurately capture the distribution of objects within a tissue volume, and 3) 2D slices may simply not contain rare or sparse objects [23]. Furthermore, sectioning tissue into thin slices may physically alter the examined structures before imaging.

To overcome some of these limitations, groups have begun performing 3D immunostaining and tissue clearing to enable deeper imaging into intact tissue samples. However, most prior 3D imaging of the liver has been performed in rodent liver tissue [24 – 32] or in humans on a scale substantively less than a single liver lobule [33, 34]. To date, no published study has visualized non-fibrotic (*i.e.*, normal) or cirrhotic human liver tissue across multiple lobules with cellular resolution, which is key for understanding how the 3D lobular architecture of human liver tissue remodels during cirrhosis and results in functional deficiencies.

Here, we developed accessible methods, which we call the LiverMap pipeline, to capture cellularly resolved human liver tissue on the mesoscale (4,000 x 4,000 x 500 μm), enabling visualization of several adjacent human lobules in 3D with cellular resolution over a depth of 500 μm . To achieve this, we acquired human liver specimens and applied 3D-optimized immunostaining to label LSECs, bile canaliculi, biliary epithelial cells, hepatic endothelial cells, smooth muscle cells, and pericentral hepatocytes. We then computationally reconstructed immunofluorescent signals to aid with visualization and quantification of features. Together, the human liver reconstructions created through the LiverMap pipeline offer a holistic, 3D view of

functional and structural features across several human liver lobules with cellular resolution, elucidating interlobular architectures in human liver tissue at resolution and scale that have not been previously observed.

2.2 Results

2.2.1 LiverMap pipeline for 3D visualization of human liver tissue

To visualize human liver tissue across several lobules with cellular resolution, we developed the LiverMap pipeline for 3D staining, clearing, reconstructing, and analyzing structures in human liver (Fig. 2.1). Towards this end, human non-fibrotic (*i.e.*, “normal liver”) control or cirrhotic liver samples were excised from either the livers of patients undergoing oncologic partial hepatectomy or from the removed livers of patients receiving liver transplantation, respectively (Fig. 2.1a). Non-fibrotic control samples were obtained from resected non-diseased human liver tissue located at least two centimeters away from any present tumor, a distance at which tissue is typically considered free from field effect [35, 36]. For all human liver specimens used here, an adjacent slice was also sectioned and stained via traditional 2D histology methods and assigned an ISHAK score to denote the degree of fibrosis by a blinded pathologist (Fig. 2.2). Next, each human liver sample was punched into 6 mm discs and sliced to a thickness of 2 mm, a size expected to contain several human liver lobules (Fig. 2.1a). Samples were then immunostained with various antibodies to identify hepatocytes, LSECs, portal and central veins, hepatic arterioles, bile ducts, and/or bile canaliculi (Table 1). Stained samples were optically cleared using the Ce3D protocol for lipid-rich, wholemount tissue (Fig. 2.1a) [37] and then tile-imaged with a confocal laser scanning microscope. Finally, commercially available software was utilized to computationally reconstruct immunofluorescent 3D human liver images via thresholding, segmentation, and binary interpretation for visualization and quantification (Fig. 2.1b,c; Fig. 2.3).

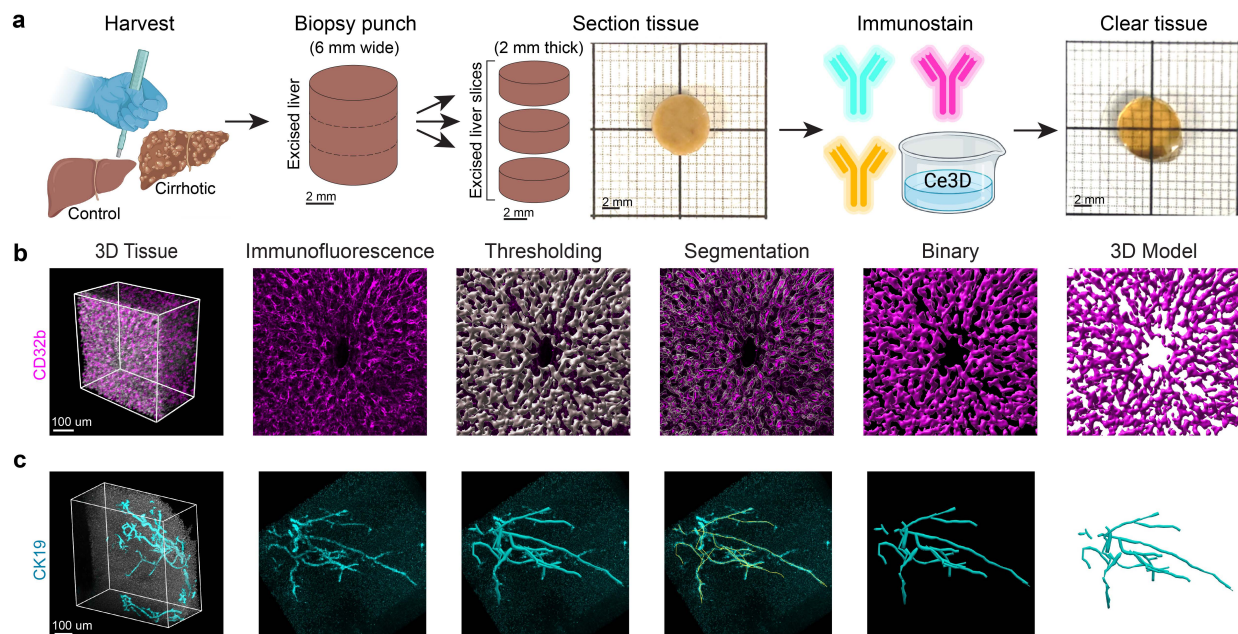


Figure 2.1: Schematic of the LiverMap pipeline. **a**, Human liver tissue is collected during surgical resection of non-fibrotic control liver (Harvest, left) and cirrhotic liver (Harvest, right). Next, biopsy punches are taken of the excised liver tissue and sliced into 2 mm thick sections before being stained with fluorescent molecules or antibodies and optically cleared via Ce3D for 3D imaging (schematic created with BioRender). **b**, **c**, After cleared tissues are imaged in 3D, immunofluorescent signals are used to computationally create object surfaces using Imaris 9.7 software (**b**) and vessel networks using Vesselucida software (**c**) for visualization and quantification purposes.

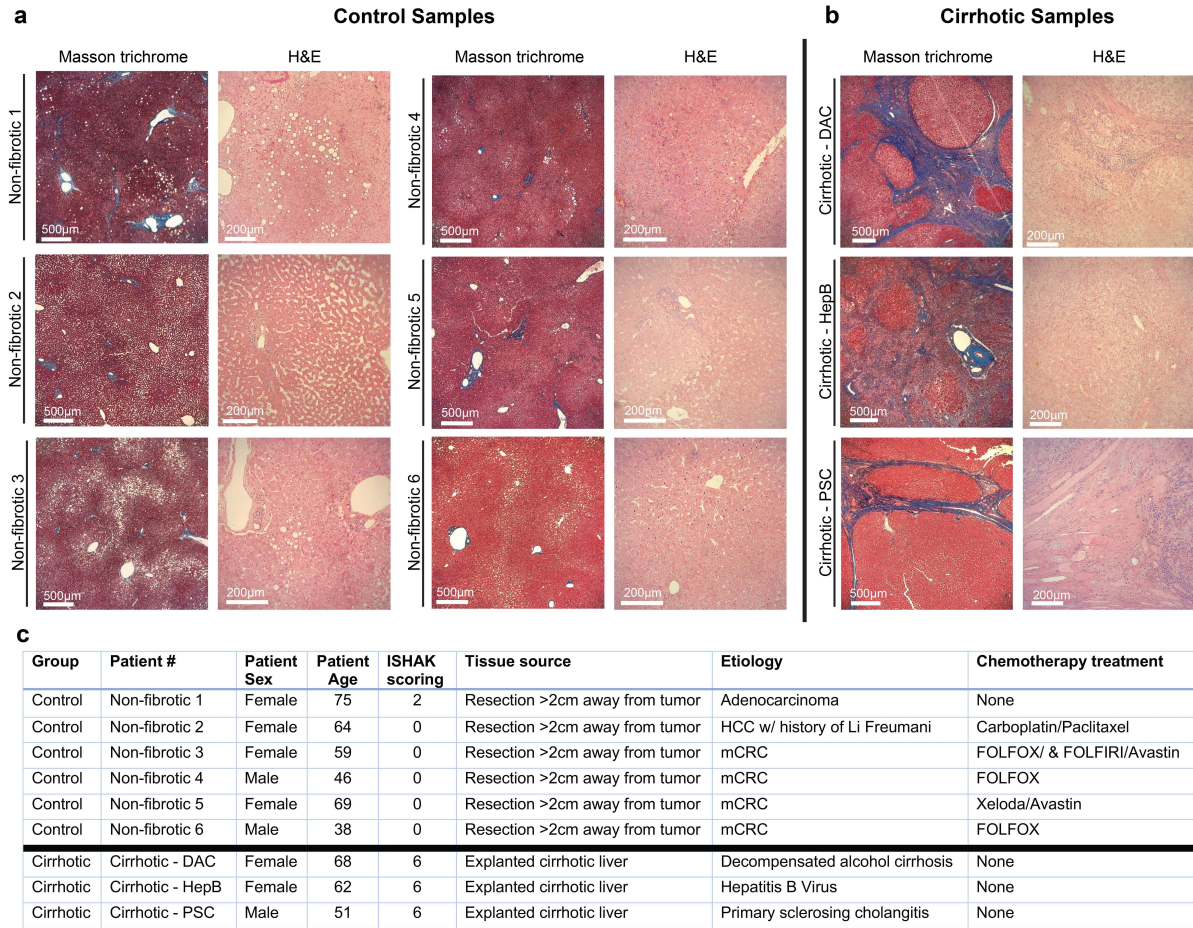


Figure 2.2: Histology and patient information of non-fibrotic and cirrhotic samples. a, b Masson trichrome and hematoxylin and eosin (H&E) staining for control samples (**a**, n=6) and cirrhotic samples (**b**, n= 3; DAC, decompensated alcohol cirrhosis; HepB, hepatitis B virus; PSC, primary sclerosing cholangitis). For Masson trichrome staining, collagen fibers stain blue, nuclei stain dark blue or black, and muscle fibers, cytoplasm, and keratin stain red. For the hematoxylin and eosin staining, nuclei stain blue via hematoxylin and extracellular matrix and cytoplasm stain red/pink via eosin. **c**, Patient information table showing group, patient biological sex, age, blinded pathologist ISHAK scoring, tissue source, etiology and chemotherapy treatment if applicable (HCC, hepatocellular carcinoma; mCRC, metastatic colorectal cancer; FOLFOX, folinic acid/fluorouracil/oxaliplatin; FOLFIRI, folinic acid/fluorouracil/irinotecan; Avastin, bevacizumab; Xeloda, capecitabine).

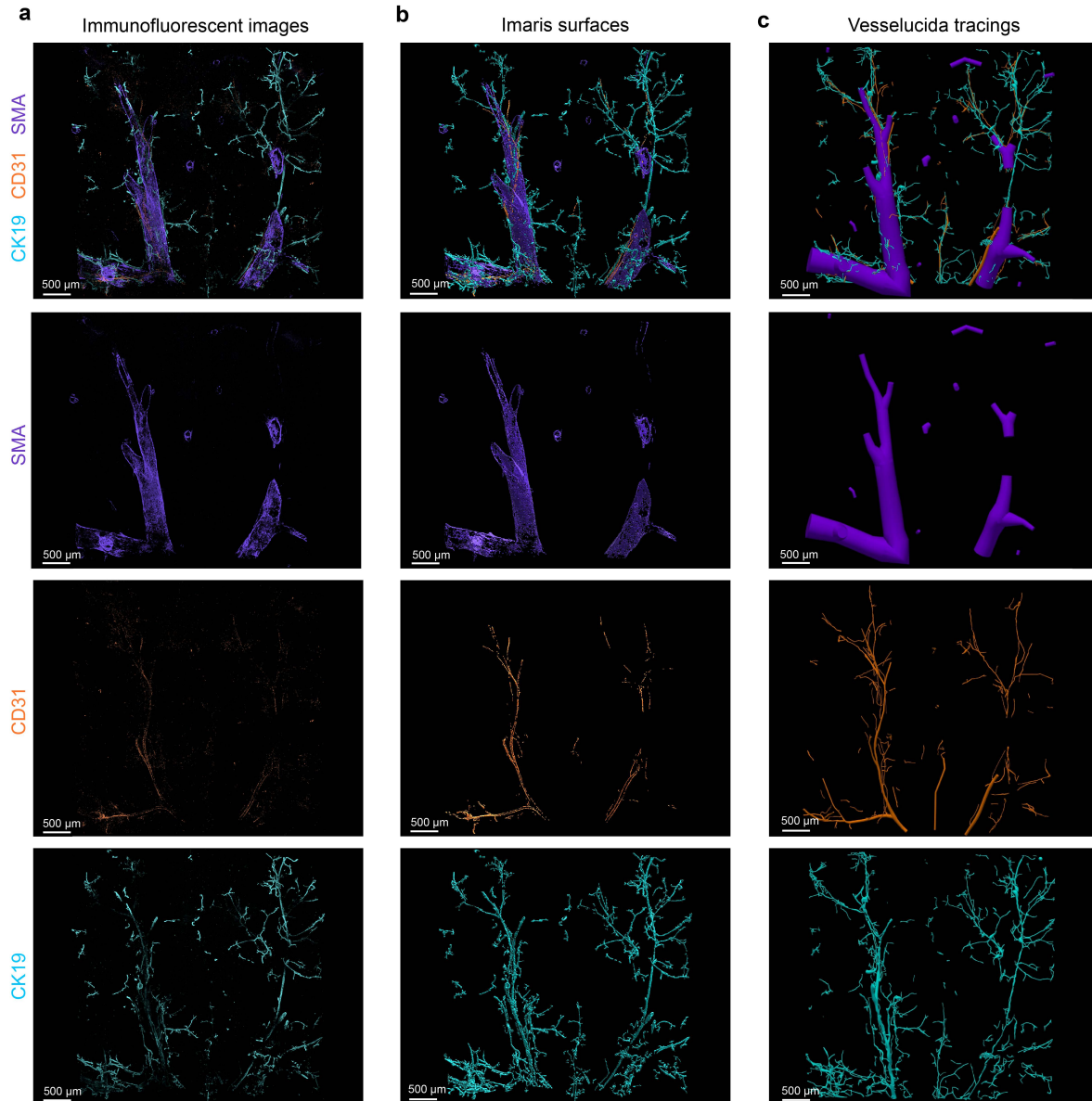


Figure 2.3: Computational reconstruction integrity of human liver images. **a**, Immunofluorescent images of control tissue with SMA (purple) staining smooth muscle cells of central and portal veins, CD31 (orange) labeling hepatic arterial endothelial cells, and CK19 (cyan) marking biliary epithelial cells. **b**, Imaris surface renderings of immunofluorescent signals in **a**. **c**, Vesselucida tracings of immunofluorescent signals in **a**.

2.2.2 Multiscale visualization of architectural features in human liver tissue

We leveraged the LiverMap pipeline to expand upon prior human liver tissue 3D imaging volumes to map how cellular and structural features were arranged across several length scales in 3D space. To do this, we first visualized how multiple lobules tessellate across a volume of human liver (Fig. 2.4a, left; Fig. 2.4b). We identified the center of each polygonal liver lobule, the central vein which transports processed blood out of the liver (Fig. 2.4a, center), by immunostaining the hepatocytes immediately surrounding the central vein using glutamine synthetase (GLUL; Fig. 2.4b) and manually tracing the central vein lumen through the 3D image stack. The polygonal vertices at the peripheral edge of each lobule were identified by immunostaining the bile ducts in portal tracts via cytokeratin 19 (CK19; Fig. 2.4b). We were able to visualize and reconstruct portions of 17 different liver lobules across a 4 mm x 4 mm x 500 μm volumetric space (Fig. 2.4b). The median average human liver lobule polygonal radius was 595 μm ($n=17$; range: 428 to 717 μm), which is consistent with the findings of prior studies of human liver [33, 38, 39, 40].

We next sought to map volumetric topology of mesoscale transport networks in the portal tracts and lobules. To do this, we stained, imaged, and reconstructed portal veins that bring partly deoxygenated but nutrient-rich blood to the liver, hepatic arterioles that bring oxygenated blood into the liver, and bile ducts that carry the hepatic byproduct bile out of the liver to the gallbladder and the small intestine (SMA, CD31, and CK19 respectively; Fig. 2.4c). SMA also stains central veins and thus demarcated the center of lobules (Fig. 2.4c, SMA marked by asterisks). The resultant 3D visualizations demonstrated how bile ducts (CK19, cyan) and hepatic arterioles (CD31, orange) extend, wrap, encircle, and branch alongside large interlobular portal veins (SMA, purple) (Fig. 2.4c). These reconstructions volumetrically displayed how several portal tracts together encase a liver lobule (Fig. 2.4c, left, lobule centers denoted by asterisk-labeled central veins).

As a next step, we sought to further visualize transport networks within a given lobule (Fig. 2.4a, center). We first used CD32b to visualize vascular sinusoids, which are specialized capillaries lined with highly-permeable endothelial cells (i.e., LSECs) that facilitate macromolecular exchange between the blood and hepatocytes. 2D histological studies have previously shown this marker is highly expressed in Zone 2/3 human LSECs in the mid-lobular and pericentral regions of the lobule and lowly expressed in Zone 1 human LSECs in the portal regions of the lobule (Fig. 2.4a, center) [9]. To visualize the sinusoids, we computationally created binary reconstructions of immunofluorescent CD32b signal to visualize all cells expressing CD32b regardless of expression level (Fig. 2.4b,d, magenta). We then isolated a single lobule via Imaris clipping planes using portal features as lobular boundaries. LSEC zonation was further visualized by pseudo-coloring immunofluorescent signal intensity (Fig. 2.4d, bottom), which showed roughly a radial gradient pattern with LSECs most strongly expressing CD32b at the central region of the lobule.

We further sought to visualize not only microscale vascular transport networks within the lobule but also the bile canalicular networks, which are 1-2 μm wide interconnected spaces between adjacent hepatocytes that collect and carry byproduct bile salts produced by hepatocytes to the bile ductules [2]. To do this, we immunostained for multidrug resistance-associated protein (MRP2), a transporter located on the canalicular (apical) surface of hepatocytes [41]. The resultant cellular and network-level volumetric images of CD32b⁺ LSECs and MRP2⁺ bile canaliculi depicted the complexity of how these two micro-transport networks interweave in 3D (Fig. 2.4e). These surfaces also demarcated hepatocyte location, as these cells have surfaces that face both the sinusoids (Fig. 2.4e, magenta) and bile canaliculi (Fig. 2.4e, green). Altogether, the LiverMap pipeline enabled capture and 3D visualization of cellularly resolved tissue volumes that are ~1,600-fold larger than prior studies for multiscale reconstruction of 3D human liver structure.

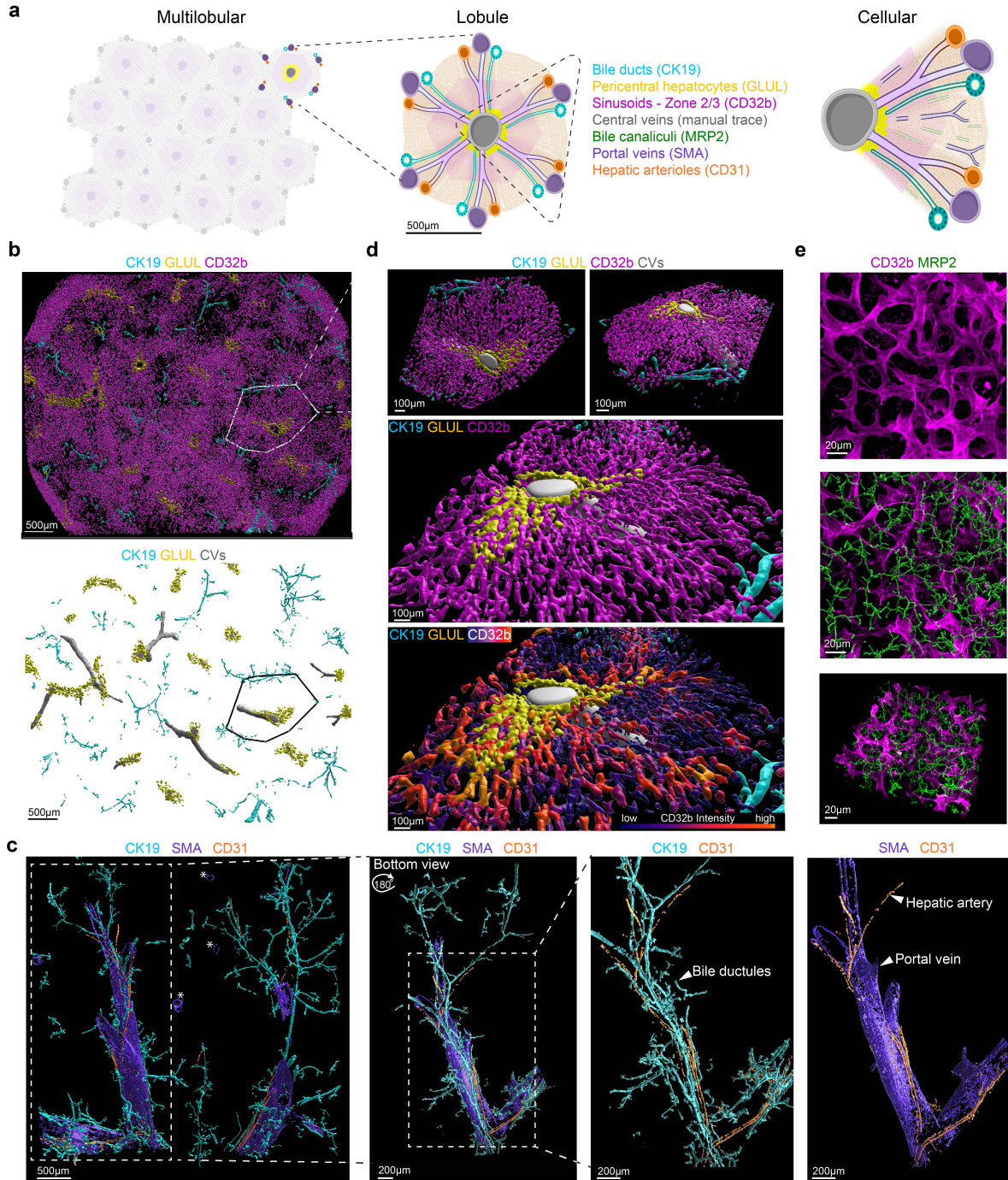


Figure 2.4: Multilobular reconstructions of zoned and architectural features. **a**, Native liver structure consists of repeating polygonal functional units called lobules, which have a central vein (SMA) at its center, Zone 2/3 LSECs (CD32) and pericentral hepatocytes (GLUL) radially organized around the central vein, and portal tracts (portal veins, SMA; bile ducts, CK19; hepatic

arterioles, CD31) at its vertices (schematic created with BioRender). **b**, Multilobular imaging and 3D modeling of zonation features: Zone 2/3 LSECs, bile ducts, and pericentral hepatocytes. CD32b (magenta) is expressed highly by Zone 2/3 LSECs. CK19 (cyan) marks biliary epithelial cells. GLUL (yellow) labels pericentral hepatocytes. Top, Imaris surface rendering of CK19, GLUL, and CD32b immunofluorescent signals with an approximate lobule border (white polygon). Bottom, Imaris surface rendering of CK19 and GLUL immunofluorescent signals with manually-traced central veins (grey) and an approximate lobule border (black polygon). **c**, Multilobular imaging and 3D reconstructions of architectural landmarks: portal veins, central veins, bile ducts and hepatic arterioles. SMA (purple) marks smooth muscle cells of portal veins and central veins; central veins are denoted by asterisks (left). CD31 (orange) labels vascular endothelial cells, especially bright on hepatic arterial endothelium. CK19 (cyan) marks biliary epithelial cells. Left, Whole-biopsy punch image of CK19, SMA and CD31 surface renderings. Center left, Bottom view of zoom section in left panel rotated 180 degrees about the y-axis. Center right, Zoom-in of bile ducts (CK19) and hepatic arterioles (CD31). Right, Zoom-in of large portal vein (SMA) and wrapping hepatic arterioles (CD31). **d**, Top, Zoom in of isolated lobule in **b** (left, top-view; right, angled-view). Middle, Close-up of lobule showing GLUL-expressing hepatocytes surrounding the central vein (grey). Bottom, Intensity-based pseudo-coloring of CD32b expression showing that the highest CD32b signal expression (orange) is located near the central vein and seemingly decreases with distance along the central-portal axis. **e**, Immunofluorescent image of hepatic sinusoids (top, magenta, CD32b) with bile canaliculi (green, MRP2) surface renderings (center) or MRP2 immunofluorescent signal (bottom).

2.3 Discussion

Here, we introduce the LiverMap pipeline (Fig. 2.1) and present the first 3D visualization of human liver tissue across several lobules with cellular resolution (Fig. 2.4). Prior human liver 3D imaging studies with cellular resolution have imaged from a portal vein to a central vein, about

500 μm in length and 100 μm in width, to a depth of roughly 100 μm , using the portal-central axis as a surrogate for the entire lobule [33]. Our 3D images, measuring 4,000 μm by 4,000 μm in xy-dimension and 500 μm in the z-dimension, capture a volume roughly 1,600 times larger than the previously captured human liver tissue volume at cellular resolution. Through leveraging the LiverMap pipeline, we mapped mesoscale interlobular structures and examined how the portal tracts, pericentral hepatocytes, and sinusoids are arranged in 3D space across several human liver lobules. Together, our efforts provide methods for staining, clearing, reconstructing, and analyzing structures within the human liver and unveil never-before-seen-in-3D human liver structures across multiple size scales. Further implementation of the LiverMap pipeline may provide new insights into the human liver's 3D lobular architecture and enable more accurate modeling and future engineering of 3D human liver tissue.

2.4 Methods

Human liver samples

Patients undergoing surgical resection at the University of Washington Medical Center were consented under IRB approved protocol (#00001852, Liver and Abdominal Tumor Biorepository) for tissue donation and access to de-identified demographic and clinical data. Non-cirrhotic control samples were obtained from partial hepatectomy (n=6), where each sample was dissected at least two centimeters away from any present tumor to ensure the sample was free from mass effect [35, 36]. Cirrhotic liver samples were collected from livers in patients undergoing transplant (n=3). Tissue sections (~2 x 2 x 2 cm) not needed for pathologic evaluation were fixed in 10% formalin and sliced with a vibratome (Leica VT 1200S) to a thickness of 2 mm. Tissue was fixed for 48 to 72 hours at 4°C before being transferred to phosphate-buffered saline at 4°C until further use. Pieces of each tissue sample were sectioned and stained with hematoxylin and eosin (H&E) and Masson trichrome (MT) to evaluate tissue structure (H&E) and fibrosis content (MT) using conventional methods (Fig. 2.2). A pathologist then performed a

blinded ISHAK grading of the H&E and MT images for each patient sample to confirm the non-fibrotic and cirrhotic tissue groupings (Fig. 2.2c). The histological, demographic, pathologic, and clinical details of all samples in this work are summarized in Figure 2.2.

LiverMap immunostaining and tissue clearing

For immunostaining, tissue samples were stained with antibodies or small molecules to identify bile ducts (cytokeratin 19, CK19), pericentral hepatocytes (glutamine synthetase, GLUL; cytochrome P450 2E1, CYP2E1), Zone 2/3 LSECs (CD32b), portal and central veins (smooth muscle actin, SMA), hepatic arterioles (CD31), bile canaliculi (multidrug resistance associated protein 2, MRP2), F-actin filaments (phalloidin) and/or cell nuclei (Hoechst) (Table 1). For tissue clearing, the Ce3D protocol for lipid-rich, wholemount tissue was utilized [37].

Briefly, human liver tissue samples were punched into 6 mm discs using biopsy punches, sliced to a thickness of 2 mm, washed in fresh phosphate-buffered saline (PBS) for one hour, and then blocked in Ce3D alternative blocking buffer (1x BD Perm/Wash buffer + 1% bovine serum albumin + 1% normal donkey serum + 0.3% Triton X-100 in PBS) overnight at 37°C with gentle shaking. Next, samples were incubated in primary antibodies diluted in Ce3D alternative blocking buffer with 5% dimethyl sulfoxide (DMSO) for 24 hours at 37°C with gentle shaking (Table 1). If primary antibodies were conjugated to a fluorophore, samples were protected from light from this step on. Samples were then washed with Ce3D wash buffer (0.2% Triton X-100 + 0.5% 1-thioglycerol in PBS) for six to eight hours at room temperature with gentle shaking, changing the buffer every two hours. Washed samples were then incubated in fluorophore-conjugated secondary antibodies diluted 1:400 in Ce3D alternative blocking buffer with 5% DMSO overnight at 37°C with gentle shaking and were protected from light from this step on. Samples were next washed in Ce3D wash buffer for six to eight hours at room temperature with gentle shaking, changing the buffer every two hours. Finally, samples were transferred to Ce3D clearing solution with Hoechst 33342 (1:400 dilution) for overnight incubation at 37°C with gentle shaking, allowing

for water-clearing solution exchange within the tissue. After 12 hours, the samples were transferred to fresh Ce3D clearing solution and imaged. For long-term storage, samples were placed in multi-well plates, and plates were covered in parafilm and aluminum foil and stored at 4°C.

Table 1: Antibodies and small molecules

Target	Antibody/Small molecule	Vendor	Dilution
Bile ducts	Anti-Cytokeratin 19 (EP1580Y)	Abcam #AB52625; RRID: AB_2281020	1:100
Glutamine synthetase	Anti-Glutamine Synthetase (GS-6)	Sigma #MAB302 RRID: AB_2110656	1:100
CYP2E1	Anti- Cytochrome P450 2E1	Abcam #AB28146 RRID: AB_2089985	1:100
Zone 2/3 LSECs	Anti-Human Fc gamma RII/CD32b	R&D #AF1330 RRID: AB_354737	1:100
Smooth muscle cells	Anti-alpha Smooth Muscle Actin (1A4)	Sigma #A21202 RRID: AB_476701	1:500
Endothelial cells	Anti-CD31/PECAM-1 (JC/70A) – DyLight 550	Novus #NB600-562R	1:400
Bile canaliculi	Anti-MRP2 (EPR10997(2))	Abcam #AB187644	1:100
F-actin	Phalloidin – Alexa Fluor 488	ThermoFisher #A12379	1:100
Nuclei	Hoechst 33342	ThermoFisher #H3570	1:400

Confocal imaging and image processing

Human liver samples were submerged in Ce3D clearing solution within glass-bottom dishes and covered with coverslips to keep the samples flat before being imaged on a confocal laser scanning microscope (Leica TCS SP8) with a 10x 0.4 numerical aperture dry objective (Leica). Hoechst, Alexa Fluor 488, Alexa Fluor 555 or DyLight 550, and Alexa Fluor 647 were excited with 405, 488, 552, and 638 laser lines, respectively, and detected with hybrid detectors (HyD). z-stacks were acquired through Leica Application Suite X (LAS X) software with the bottom of the stack set ~5 μ m below the start of the visible signal and with the top of the stack set at the end of the visible signal, with a z-compensation set within LAS X to increase laser power and gain throughout the stack as necessary. To capture the 6 mm biopsy punches, tiles of 4 x 4 image stacks were stitched using the Mosaic Merge function within LAS X. Stitched confocal z-stack

images were then visualized and processed using Imaris 9.7 (Bitplane). First, z-stack images converted from .lif file format to .ims using Imaris File Converter 9.7 (Bitplane). Images were then processed with the following steps: 1) Flip Channels in the Z direction for all channels; 2) Normalize Layers of all channels; 3) Background Subtraction (Filter Width: 10um) of all channels; 4) Normalize Layers of all channels, 5) Rotate Channels counter clockwise for all channels about X-axis, Normalize Layers for all channels, Rotate Channels clockwise for all channels about X-axis; 6) Rotate Channels counter clockwise for all channels about the Y-axis, Normalize Layers for all channels, Rotate Channels clockwise for all channels about Y-axis.

Computational reconstructions for visualization and quantification

Imaris 9.7 (Bitplane) software was used to generate a surface rendering of the immunofluorescent signal from each antibody in all samples for enhanced visualization and feature volume quantification. The following parameters were used for the Imaris Surface Creation Wizard: Surface Grain Size = 4.01 μm ; Background Subtraction - Diameter of Largest Sphere = 15.0 μm ; Threshold - Background Subtraction manually adjusted for each sample to help account for signal variability; Voxel Filter or Volume Filter to exclude small surfaces created from background signal. Any significant background signal or tissue autofluorescence that was captured in the surface reconstructions was manually identified and removed. **Central vein visualization:** For visualization purposes, central veins were identified via positive glutamine synthetase staining or positive SMA staining outside of portal tracts, and central vein surfaces were created by manually tracing lumens throughout the tissue using autofluorescence (Fig. 2.4). **Lobule measurements:** Lobule boundaries were identified and isolated via recognition of portal tracts and the use of Imaris clipping planes. The Imaris measurement tool was then used to measure the distances between each central vein and all its nearest surrounding portal tracts for every full or partial lobule (n=17) identified in the representative sample (non-fibrotic #6). Each full or partial lobule's measurements were then averaged together to get an average lobule radius

for each full or partial lobule. Average lobule radii were then put in rank order, and the median lobule radius was identified and reported. **Immunofluorescent pseudo-coloring:** For intensity-based pseudo-coloring of the CD32b signal, the Split Touching Objects option was enabled and a custom color gradient with an Intensity Mean range of 3,000 units was utilized to visualize reconstructed CD32b-expressing sinusoid segments throughout the tissue sample. These Imaris surfaces aided in CD32b immunofluorescent signal visualization (Fig. 2.4d; Fig. 3.2d).

Chapter 3: Architectural remodeling during liver cirrhosis

3.1 Introduction

Liver cirrhosis, responsible for 2.1% of all deaths per year worldwide [11], typically originates from consistent exposure to damaging agents, such as alcohol (decompensated alcohol cirrhosis), fatty acids (metabolic dysfunction-associated steatotic liver disease), hepatitis B or C viruses, and/or drug abuse or misuse [42]. In some cases, the damaging agent or root cause that leads to cirrhosis may be unknown, such as in primary sclerosing cholangitis which is a rare disease that causes cirrhosis characterized by bile duct inflammation and scarring [43]. The cirrhotic etiology may dictate the presentation of abnormal vascular and/or biliary architecture in a patient's cirrhotic liver [14, 44, 45, 46]. Structural abnormalities due to cirrhosis include ductular reaction which appears as an increase in number of small bile ductules throughout the tissue [45], sinusoid capillarization where LSECs lose their fenestrations and portal and arterial blood supplies may directly shunt to the central vein [47, 48], perisinusoidal space scar formation which hinders blood – hepatocyte interaction [48], and central vein regression which may contribute to portal vein hypertension and other organism-wide circulatory problems [14, 44, 49]. Although there have been numerous studies on human liver cirrhosis, no published study has compared non-fibrotic liver tissue with cirrhotic human liver on the multilobular scale with cellular resolution, which is pivotal for elucidating alterations to the liver's interlobular functional and structural features during cirrhosis.

Towards this end, we utilized the LiverMap pipeline to compare functional and structural features of non-fibrotic tissue and cirrhotic tissue across several lobules in 3D (Fig. 3.1). Uncovering how cirrhosis-induced adaptive remodeling occurs across several lobules may help create more accurate liver disease models and lead to the development of treatments or interventions for liver cirrhosis. Furthermore, the identification of structural and functional changes

that lead to liver failure may enhance our understanding of how human liver structure relates to function, and thereby help inform efforts to engineer functional artificial liver tissue.

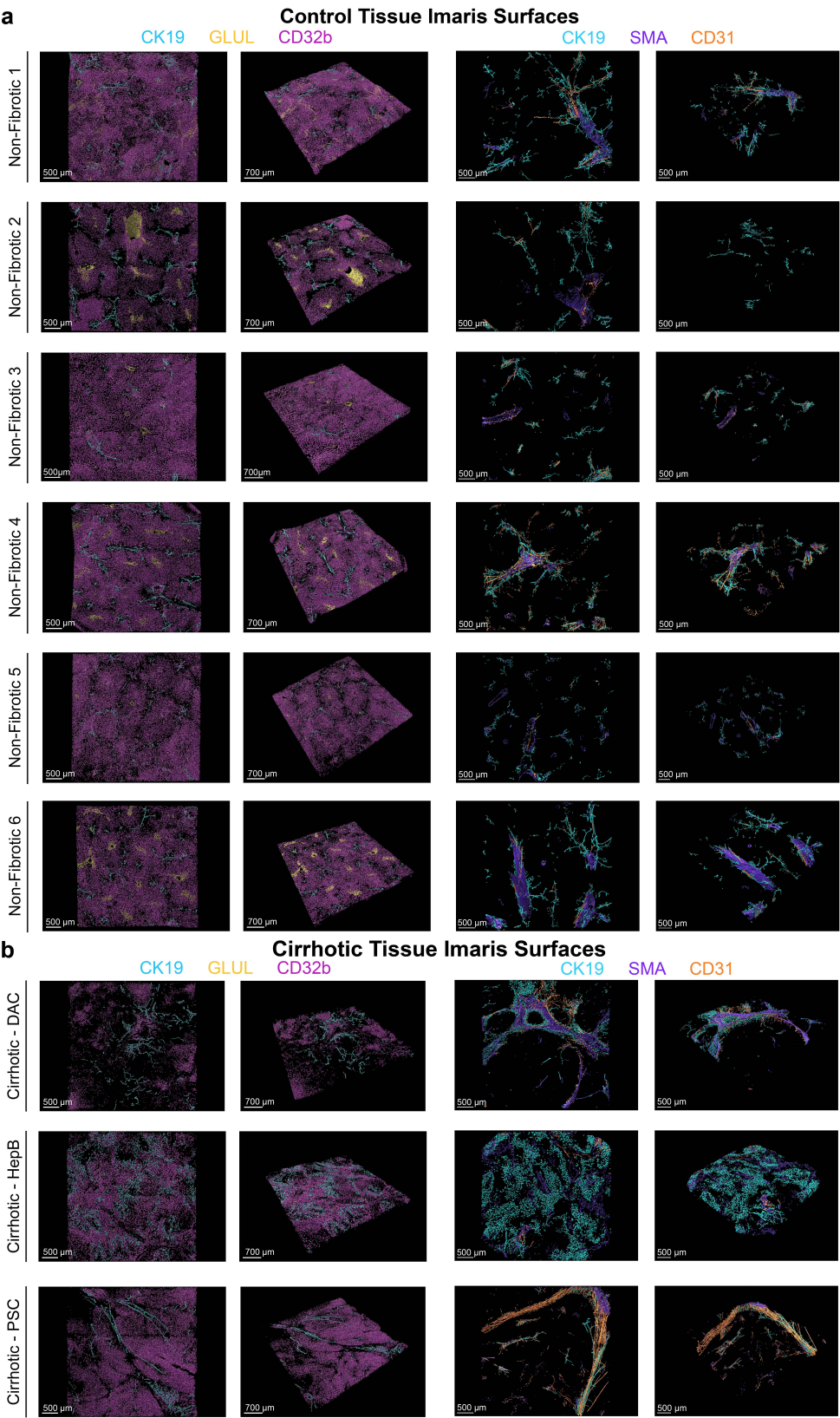


Figure 3.1: Reconstructions of non-fibrotic and cirrhotic human liver samples. Depictions of Imaris surfaces reconstructing zonation features (left) and architectural features (right) for all patients from the non-fibrotic control group (**a**, n=6) and the cirrhotic group (**b**, n= 3; DAC, decompensated alcohol cirrhosis; HepB, hepatitis B virus; PSC, primary sclerosing cholangitis).

3.2 Results

3.2.1 Dysregulation of human hepatocyte and sinusoid zonation in cirrhosis

Zonation of the liver lobule is essential for healthy liver function as it allows the numerous tasks of the liver to be delegated across cell subpopulations. Most prior investigations of liver zonation have focused primarily on hepatocytes, such as defining hepatocyte periportal functions and pericentral functions. Interestingly, recent research suggests that LSECs are also spatially zoned across the lobule [9]. However, these studies relied on 2D traditional methods and characterized only LSECs in normal liver tissue. To the best of our knowledge, the zonation of LSECs have not yet been directly compared spatially with classical hepatocyte functional zonation markers, such as cytochrome P450 2E1 (CYP2E1) and glutamine synthetase (GLUL) which denote pericentral hepatocytes [50]. Additionally, the extent to which LSECs are spatially volumetrically zoned in both normal and cirrhotic tissue remains unclear.

To address this knowledge gap, we first sought to benchmark how LSEC zonation correlates with hepatocyte zonation [7]. To do this, we employed the LiverMap pipeline to visualize the extent to which LSEC expression of CD32b correlates spatially with hepatocyte expression of zonation markers CYP2E1 and GLUL (Fig. 3.2). We found that expression levels of CD32b in LSECs and CYP2E1 in hepatocytes in normal non-fibrotic human liver tissue closely correlated spatially across both multilobular line profiles and 3D signal intensity plots, exhibiting gradient patterns that emanated from the center of the lobules (Fig. 3.2a-c). Conversely, GLUL was confined to the innermost layers of hepatocytes surrounding the central vein, as shown previously

by us and others [51], and thus overlapped spatially with only the most centrally located CD32b⁺ LSECs (Fig. 2.4; Fig. 3.2d,e).

As the microvasculature can become disrupted during cirrhosis, we next sought to visualize spatial zonation patterns of LSECs across several lobules in both non-fibrotic and cirrhotic tissue. We stained six non-fibrotic and three cirrhotic tissue samples to identify sinusoidal endothelial cells, bile ducts, and pericentral hepatocytes (CD32b, CK19, GLUL respectively) [50]. We then created intensity-based pseudo-colored plots of CD32b immunofluorescent signal intensity across the multilobular tissue volume (Fig. 3.2d, orange denotes highest signal & dark blue represents lowest signal) as well as 3D surface plots of CD32b, CK19, and GLUL signal intensity from MIP images (Fig. 3.2e, z-values represent signal intensity). In non-fibrotic control human liver tissue, CD32b was most highly expressed at the center of each spaced liver lobule nearest to GLUL-expressing hepatocytes and displayed roughly radial expression gradients emanating from the pericentral region of each lobule and abating at the lobule edges demarcated by CK19⁺ bile ducts (Fig. 3.2d,e, left). Each roughly radial gradient of CD32b expression associated with each given liver lobule appeared to be interspersed regularly across non-fibrotic control human liver tissue. Conversely, in the cirrhotic tissue, this regular pattern of radial CD32b expression gradients appeared to be disrupted, with some but not all cells retaining CD32b expression (Fig. 3.2d,e, right). Furthermore, CD32b expression appeared to be interlaced with CK19 expression, suggesting that spatial dysregulation of zoned CD32b expression may accompany lobular vasculobiliary architectural changes in cirrhotic liver tissue. Lastly, similarly to that shown by others previously in 2D sections [52], we found a near absence of volumetric GLUL expression in the cirrhotic samples (Fig. 3.1; Fig. 3.2f), which may imply that CD32b dysregulation could be linked to disruption of hepatic zonation patterns.

In summary, the LiverMap pipeline enables visualization of volumetric functional and structural sinusoidal and hepatocyte zonation across several human liver lobules in 3D, and

through leveraging this pipeline, we found that cirrhosis correlates with the dysregulation of sinusoid zonation and a reduction in glutamine synthetase-expressing pericentral hepatocytes.

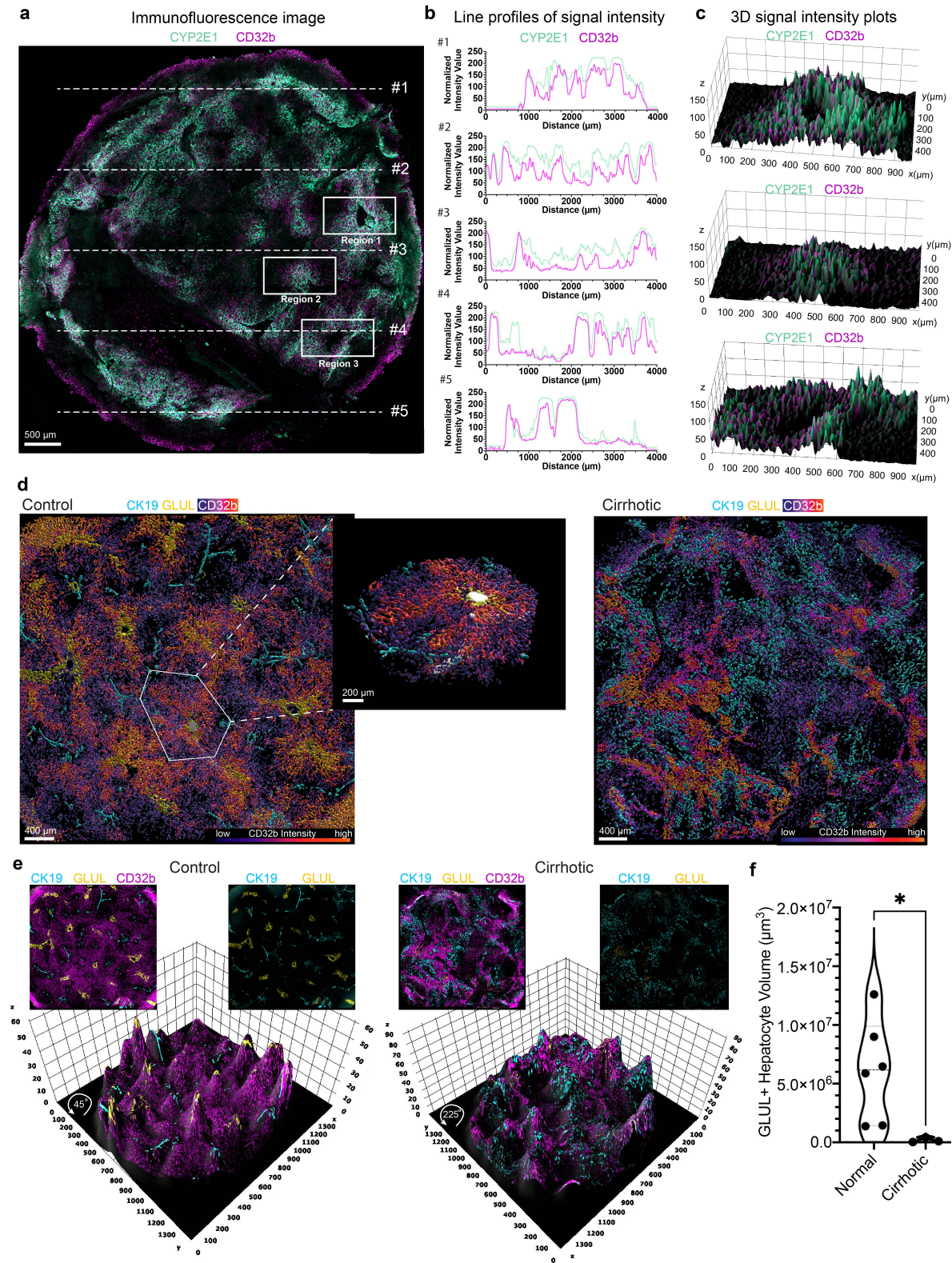


Figure 3.2: Dysregulation of human sinusoid zonation in cirrhosis. **a**, En face 3D MIP of control tissue with CD32b (magenta) most strongly staining Zone 2/3 LSECs and CYP2E1 (teal) staining metabolically-active pericentral hepatocytes. **b**, Spatial line profiles of CD32b signal intensity (magenta) and CYP2E1 signal intensity (teal) across horizontal lines denoted in **a**. From these line profiles, we can see that CD32b and CYP2E1 signals follow similar spatial trends across the sample. **c**, Angled 3D signal intensity plots showing pixel intensity on the z-axis corresponding to each pixel on the xy-plane of the three regions denoted in **a**. **d**, Left, control sample with CD32b surface pseudo-colored based on signal intensity. High CD32b regions (orange) are typically in the central and mid-lobular zones, and low CD32b regions (blue) are portal. Approximate lobule border drawn in white with inset showing an angled view of the isolated lobule from control tissue with a manually-traced central vein. Right, Cirrhotic sample with CD32b surface pseudo-colored based on signal intensity. High CD32b regions are less patterned than seen in the non-fibrotic control sample. **e**, Top, En face MIP of entire non-fibrotic control (left) and cirrhotic (right) biopsy punches labeled for CK19⁺ bile ducts (cyan), GLUL⁺ pericentral hepatocytes (yellow), and CD32b⁺ Zone 2/3 LSECs (magenta). Bottom, Angled 3D signal intensity plots showing pixel intensity (z-axis) corresponding to each pixel of the 2D image (xy-plane) for CK19, GLUL and CD32b. **f**, Statistical comparison of GLUL⁺ hepatocyte Imaris surface volumes for non-fibrotic control (n=6) and cirrhotic (n=3) tissues using a two-tailed Mann-Whitney test (*, $p < 0.05$; p-value = 0.0238).

3.2.2 Alterations to liver vascular networks in cirrhotic tissue

Functional zonation is largely associated with the vasculobiliary structural hallmarks of the lobules, such as the central veins from which zonation patterns typically emanate. We and others have reasoned that disruption in zonation patterns, such as the reduction we observed in GLUL-expressing hepatocytes, may stem from disrupted central vein network architecture [14, 53]. We

thus next set out to further map mesoscale 3D vasculobiliary network architectures in both non-fibrotic and cirrhotic human liver tissue.

To do this, we first reconstructed the central vein, hepatic arterial, and biliary networks within six non-fibrotic control tissues and three cirrhotic tissues (Fig. 3.3a,b). Parameterization and quantification of central vein networks demonstrated that normal non-fibrotic liver tissues had a higher number of central veins, a greater total network volume, and more network endings than cirrhotic tissues (Fig. 3.3c). While all cirrhotic tissue samples exhibited a reduction in central veins compared to normal non-fibrotic tissue, each cirrhotic etiology had distinct architectural arrangements of mesoscale hepatic arterial networks (Fig. 3.3b). Specifically, we observed an expansion of the CD31⁺ vessels demarcating both hepatic arterioles and also likely dysregulated sinusoids in the samples from patients with decompensated alcoholic cirrhosis and primary sclerosing cholangitis (Fig. 3.3b, top and bottom, respectively) but not in the sample from the patient with hepatitis B viral infection (Fig. 3.3b, center). These volumetric findings indicated and allowed the visualization of central vein network regression and hepatic arterial network disruption in cirrhotic human liver tissues with distinct etiologies.

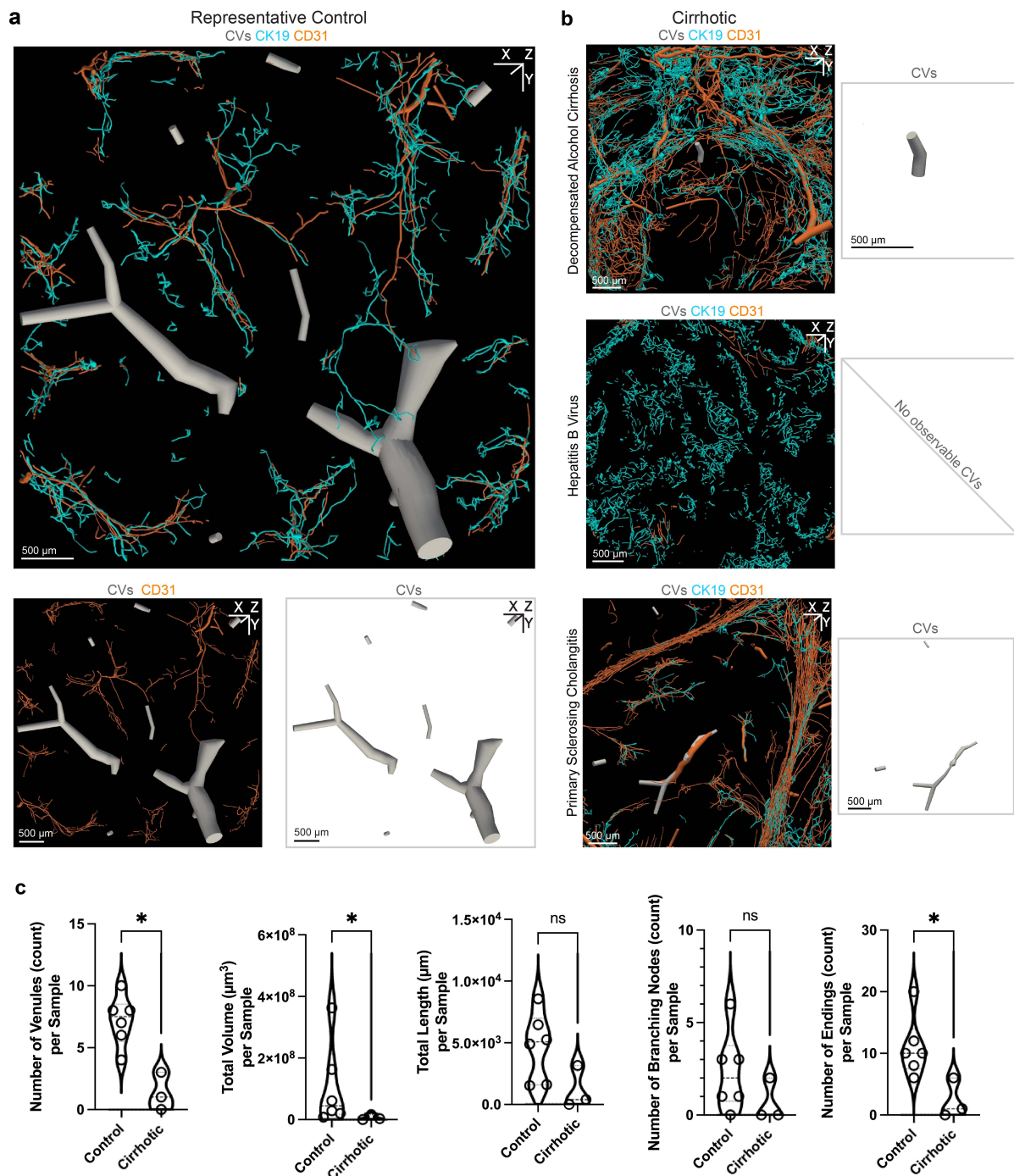


Figure 3.3: Decentralization of vasculature in cirrhosis. **a**, Top, En face 3D reconstructions of identifiable central veins (grey), CK19⁺ bile ducts (cyan), and CD31⁺ microvessels (orange) across representative non-fibrotic control human liver tissue. Bottom, depiction of vasculature (grey and orange) isolated from non-fibrotic control 3D model above. **b**, Left, En face 3D models of

identifiable central veins (grey), CK19⁺ bile ducts (cyan), and CD31⁺ microvessels (orange) across three cirrhotic human liver tissue sections with different etiologies. Right, 3D models of identifiable central veins (grey) visually isolated from adjacent cirrhotic samples. **c**, Morphometric measurements describing central vein networks in the control (n=6) and cirrhotic (n=3) groups. Data are represented by violin plots and were statistically analyzed via a two-tailed Mann-Whitney test (*, $p < 0.05$; ns, non-significant).

3.2.3 Architectural changes of the biliary network in cirrhosis

Bile ducts carry hepatic bile out of the liver to the gallbladder and the small intestine, which helps facilitate the excretion of hepatic metabolites and intestinal absorption of lipids and fat-soluble vitamins [54]. In liver cirrhosis, biliary networks can undergo significant remodeling and result in failure of bile to be transported out of the liver, thereby causing a buildup of bile in the liver and prompting bile leakage into the bloodstream – a condition called cholestasis [55]. Our initial vasculobiliary network reconstructions suggested architectural dysregulation in the CK19 biliary networks from patients with cirrhosis of various etiology (Fig. 3.1; Fig 3.3b). We thus sought to further investigate bile duct architecture in both normal and cirrhotic human liver tissue.

For the cirrhotic samples, we were particularly interested in 3D morphological analysis of a pathological bile duct phenotype called ductular reaction (DR), which resembles an expansion of small bile ductules and may be caused by the proliferation of cholangiocytes or hepatic progenitor cells, or by transdifferentiation of hepatocytes [56]. DR is not fully understood, but it is believed that the cell type of origin for DR may depend on the underlying pathology that caused the cirrhosis [46, 56]. Typically, 2D histology is used for the evaluation of ductular reaction; however, 2D analysis often cannot elucidate the extent to which such 3D structures are interconnected within the tissue, especially when structures are lumenized. As examples, we point to the morphological similarity of CK19⁺ features in several 5 μm -thick slices across two different human liver specimens (Fig. 3.4). Upon 2D inspection, the two samples appeared to have similar

presentation of CK19⁺ features (Fig. 3.4, left). However, using 3D volumetric imaging, we observed that one sample had CK19⁺ bile ducts forming interconnected networks (Fig. 3.4, top right), and the other sample had CK19⁺ features representing distinct, non-connected smaller structures (Fig. 3.4, bottom right). To our knowledge, ductular reaction architecture has not yet been visualized and quantifiably measured across several human liver lobules in 3D.

Towards this end, we created Imaris surface renderings of CK19 immunofluorescent stains within six non-fibrotic samples and three cirrhotic samples (Fig. 3.1). In the non-fibrotic samples, we observed biliary structures that extended and branched through tissue volumes, resembling network architectures across several lobules (Fig. 3.1; Fig. 3.5a). However, in the cirrhotic samples, each cirrhotic etiology here presented a unique CK19 biliary network architecture (Fig. 3.5b). We observed ductular expansion primarily within the thick fibrotic bands in the decompensated alcohol cirrhosis sample, discrete clusters of CK19⁺ cellular structures throughout the hepatitis B cirrhosis sample, and a combination of long bile ducts within thin fibrotic bands and bile duct segments within the bulk of the tissue in the primary sclerosing cholangitis sample (Fig. 3.5b).

To quantify the degree of ductular reaction in our samples, we calculated the volume of each discrete bile ductular structure in every non-fibrotic control and cirrhotic sample (Fig. 3.1) and generated exponential histograms of CK19⁺ ductular volumes for each sample (Fig. 3.5c). This method provides a new quantitative means to describe differences in ductular fragmentation between different diseased samples, a metric that is not possible to accurately derive from 2D images (Fig. 3.4; Fig. 3.5c). Here, the three samples with different cirrhotic etiologies had histograms of markedly different shapes (Fig. 3.5c), which reflected their distinctive ductular architectures visualized in 3D (Fig. 3.5b). The frequency counts for non-fibrotic or cirrhotic samples were then grouped and plotted as average frequency distributions (Fig. 3.5d, top). The resultant analyses showed that overall, cirrhotic samples on average had both a higher quantity of the smallest ($<10^3 \text{ } \mu\text{m}^3$ and $<10^4 \text{ } \mu\text{m}^3$) CK19⁺ structures (Fig. 3.5d, top) and a higher total

number of CK19⁺ structures (Fig. 3.5d, bottom), compared to non-fibrotic samples. Altogether, our method thus provided a means to quantitatively describe ductular remodeling and fragmentation in human liver cirrhosis, enabling us to quantitatively visualize the differing degrees of ductular fragmentation with regard to each cirrhotic etiology as well as conclude that on average cirrhotic samples had more fragmented ductular networks than those in the non-fibrotic human liver control samples.

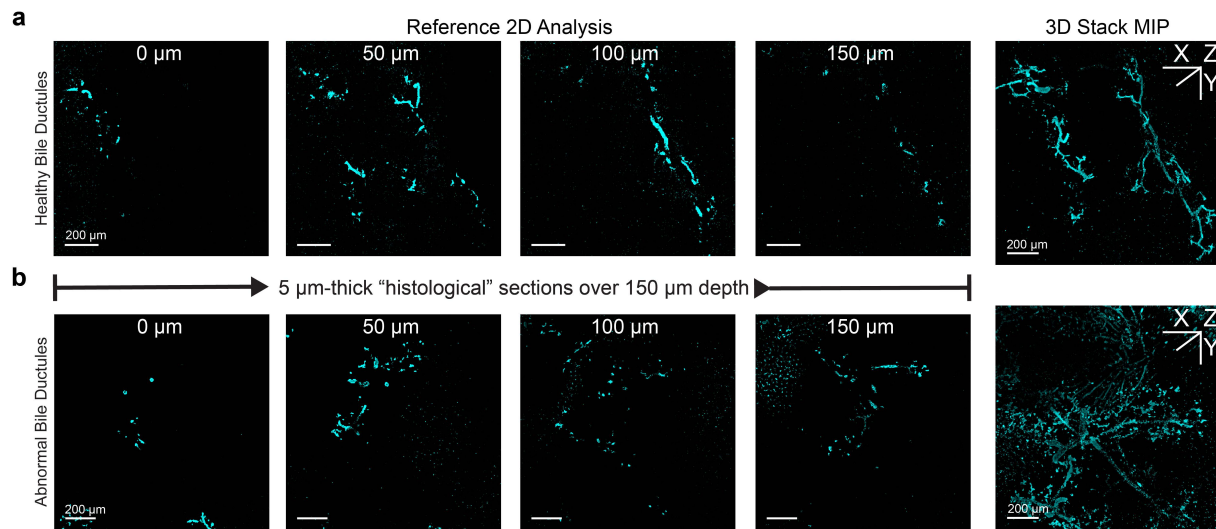


Figure 3.4: Benefits of 3D analyses for morphological evaluation. a, b, Left, Reference 2D histological analyses of 5 μm -thick sections at various z-depths ranging 0 μm to 150 μm of healthy bile ducts (a) and abnormal bile ducts (b). Right, 3D Stack MIP of the same CK19⁺ networks.

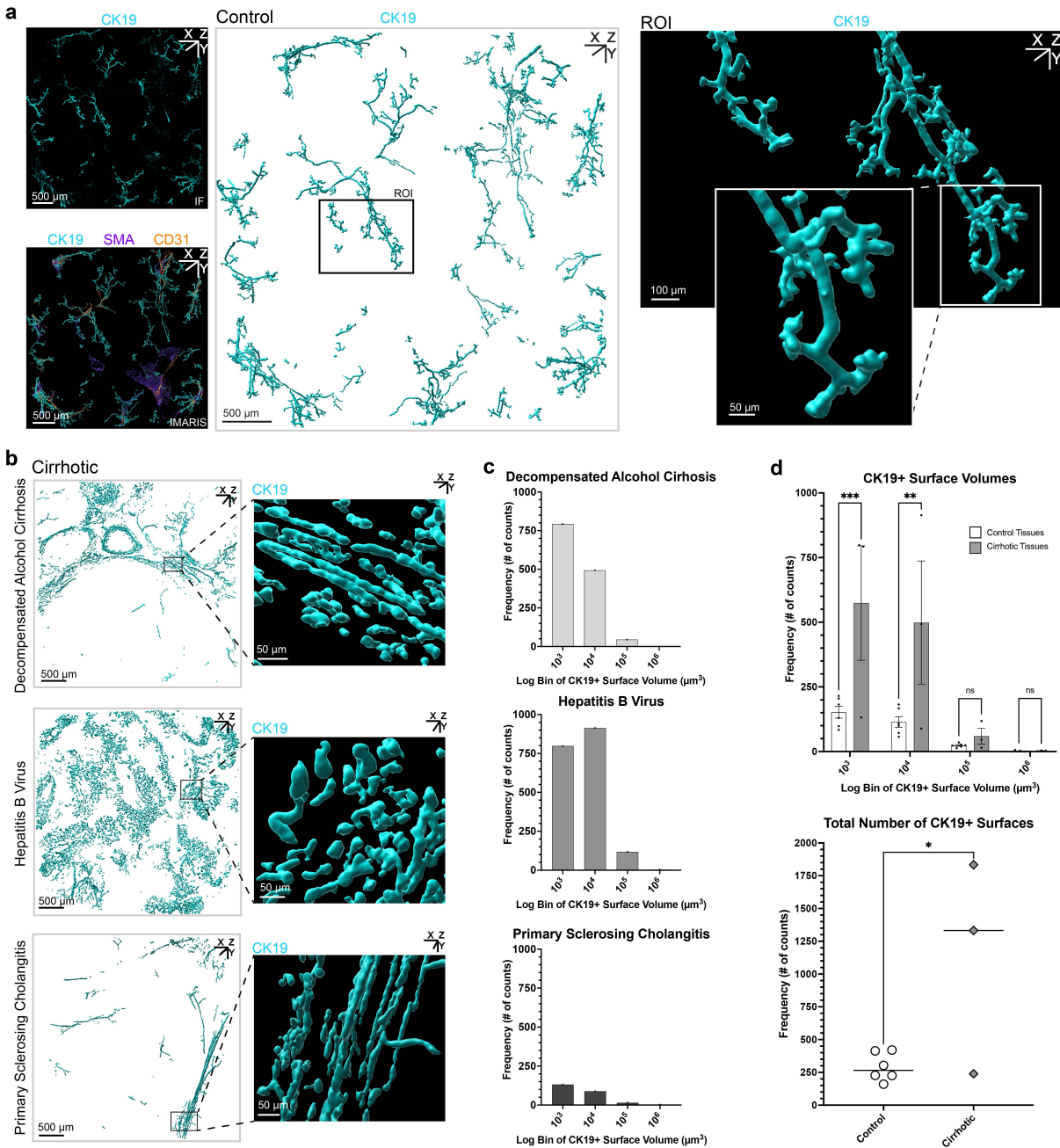


Figure 3.5: Cirrhotic bile duct networks exhibit more architectural fragmentation. **a**, Top left, en face immunofluorescence MIP (10x,16-tile merge) of CK19⁺ bile ducts in representative non-fibrotic control sample. Bottom left, Imaris surfaces of CK19⁺ bile ducts, SMA⁺ portal veins, and CD31⁺ hepatic arterioles from immunofluorescence signal in representative non-fibrotic control sample. Center, Imaris surfaces of CK19⁺ bile ducts in representative control sample with ROI box. Right, Zoom in of ROI identified in center panel with high zoom in panel showing a

selected bile duct. **b**, Imaris surfaces of CK19⁺ features in cirrhotic group with different etiologies. **c**, CK19 Imaris surface volumes from cirrhotic samples binned with regard to their log base 10 to create exponential histograms of CK19 surface volumes for each sample. **d**, Top, Comparison of non-fibrotic control (n=6) and cirrhotic (n=3) CK19 surface volume exponential histograms (error bars, +/- SEM). Exponential histogram bins were statistically compared via two-way ANOVA ($F = 14.90$; $DFn = 1$; $DFd = 28$) and Bonferroni multiple comparison test (***, $p < 0.001$; **, $p < 0.01$; ns, non-significant). Bottom, Comparison of total number of CK19 surfaces within non-fibrotic control (n=6) and cirrhotic (n=3) groups. Average total number of CK19 surfaces between groups were statistically compared via a two-tailed, unpaired t-test ($t = 2.679$; $df = 7$; *, $p < 0.05$).

3.3 Discussion

Liver cirrhosis is associated with several structural changes that may result in significant functional deficiencies and lead to liver failure. To date, no prior work has compared non-fibrotic liver tissue with cirrhotic human liver tissue on the multilobular scale with cellular resolution, which is an analysis that could improve our understanding of how the liver's interlobular functional and structural features rearrange during cirrhosis. Therefore, we leveraged the LiverMap pipeline to compare functional and structural features of non-fibrotic tissue and cirrhotic tissue across several lobules in 3D. Our analyses suggested spatial dysregulation of CD32b⁺ LSECs in cirrhotic tissue in comparison to non-fibrotic control tissue (Fig. 3.2), which could imply a disruption in critical Zone 2/3 LSECs roles such as functions related to blood-derived leukocyte adhesion and antigen clearance in cirrhotic tissue [9]. Additionally, we found a significant reduction in glutamine synthetase-expressing pericentral hepatocytes in cirrhotic tissue when compared to non-fibrotic tissue (Fig. 3.2f). As glutamine synthetase plays an important enzymatic role in nitrogen metabolism by catalyzing glutamate and ammonia to form glutamine, this observation may imply decreased ammonia metabolism for cirrhotic patients, thereby indicating a higher risk of hyperammonemia or a buildup of toxic ammonia in the blood as is commonly observed in patients

with cirrhosis and liver failure [57, 58]. Furthermore, our 3D analyses revealed a significant decrease in the presence of central veins within cirrhotic tissue in comparison to non-fibrotic tissue (Fig. 3.3). Since the central vein serves as the primary transporter of blood out of the lobule, fewer central veins may result in dysfunctional blood drainage from the liver and potentially contribute to portal vein hypertension, a clinically observed condition within cirrhotic patients [14, 44, 49]. Lastly, we provided a new quantitative means to describe CK19 biliary network fragmentation in non-fibrotic and cirrhotic human tissues. We found that the presentation of CK19 biliary networks varied substantially between cirrhotic etiologies (Fig. 3.5), which was reflected by each cirrhotic sample having CK19 volumetric histograms with markedly different shapes (Fig. 3.5c). We also found that on average cirrhotic samples had both a higher quantity of the smallest CK19 structures (Fig. 3.5d, top) and a higher total number of CK19 structures (Fig. 3.5d, bottom) than non-fibrotic samples. Together, these statistics indicated that the average cirrhotic bile ductular network was more fragmented than the average non-fibrotic control bile ductular network, which may imply abnormal biliary pressure and flow in cirrhotic tissue.

In summary, our 3D comparative analyses of non-fibrotic control and cirrhotic tissues indicated that cirrhotic tissue manifests a dysregulation of sinusoid zonation, reduction in glutamine synthetase-expressing pericentral hepatocytes, regression of central vein networks, and fragmentation of biliary networks, which altogether suggest a pro-portalization/decentralization phenotype in correlation with human liver cirrhosis. The finding that cirrhotic tissues adopt a phenotype with overexpressed portal features and under-expressed central features may help inform the creation of artificial liver tissue for liver disease modeling. Furthermore, as liver cirrhosis is associated with significant functional deficiencies, a lack of central features within cirrhotic tissue may suggest that the liver's central features are critical for maintaining liver function, which is a key notion that could assist future efforts towards creating functional artificial liver tissue.

3.4 Methods

Line profile analysis and 3D surface plots

2D maximum intensity projection (MIP) images of 500 μm -thick z-stack images were exported from Imaris 9.7 software as .tiff files. The 2D MIP images were then opened in FIJI software [59]. Line profile analysis (Fig. 3.2a,b) was performed in Fiji with the following pipeline: 1) Convert the stack to 8-bit; 2) Apply a 15 μm Gaussian Blur to help smooth the image and reduce noise; 3) Apply Equalize Histogram function to help normalize pixel intensities across images; 4) Employ Fiji's Plot Profile analysis tool for equally spaced horizontal lines throughout the sample. 3D signal intensity plots (Fig. 3.2c,e) were also generated in FIJI by selecting a rectangular region of interest and employing Fiji's 3D Surface Plot analysis tool with the following parameters: Grid Size: 512; Smoothing: 6.0; Perspective: 0.22; Lighting: 0.2; Scale: 1.73; z-Scale: 0.52.

CD32b pseudo-coloring

Imaris 9.7 (Bitplane) software was used generate a surface rendering of CD32b immunofluorescent signal as described previously (2.4 Computational reconstructions). To create a CD32b surface with intensity-based pseudo-coloring, the Split Touching Objects option was enabled and a custom color gradient with an Intensity Mean range of 3,000 units was utilized to visualize reconstructed CD32b-expressing sinusoid segments throughout the tissue sample.

Glutamine synthetase volumetric measurements

Using Imaris 9.7 (Bitplane) software, each sample was processed to normalize the total image volume to 4,000 x 4,000 x 300 μm . For each sample's normalized tissue volume, glutamine synthetase immunofluorescent signal was rendered into Imaris surfaces, and the total surface volume for each sample was calculated via the Imaris statistics module (Fig. 3.2f).

Central vein morphometrics

Vesselucida (MBF Bioscience) was used for the reconstruction and morphometric analysis of central veins (Fig. 3.3). Before performing morphometric analysis within Vesselucida software, images were resized to 25% using the Resize tool within LAS X software. This provided sufficient image resolution for analysis while reducing the file size to meet program and computing limitations. After resizing, a semi-manual process was used to trace branching structures in Vesselucida. First, the automatic tracing module was used to identify the microstructures with diameters of approximately 15 μm , such as CD31⁺ microvessels and small bile ductules. Then, larger vessels or ducts were manually traced, and these were manually connected to smaller automatically traced vessels. Central veins were manually traced in their entirety as these features were all too large for the automatic tracing in Vesselucida to identify reliably. From these tracings, Vesselucida generated morphometric data describing the architecture of traced networks.

Ductular fragmentation quantification

The volume and quantity of each discrete CK19⁺ structure in every non-fibrotic control and cirrhotic sample was calculated and recorded via the Imaris statistics module (Fig. 3.1). Exponential histograms of CK19⁺ surface volumes (bin width: $\log_{10}$ of surface volume in μm^3) for each non-fibrotic control sample and cirrhotic sample were then created (Fig. 3.5c). The frequency counts of the resulting exponential histograms from each sample were then grouped with their respective non-fibrotic or cirrhotic classification and plotted as average frequency distributions (Fig. 3.5d, top), and frequency counts of CK19⁺ surface volumes within each bin from the non-fibrotic group and cirrhotic group were statistically compared via a two-way ANOVA (Fig. 3.5d, top). Additionally, the average total number of CK19⁺ surfaces for the non-fibrotic group and cirrhotic group were plotted and compared via an unpaired t-test (Fig. 3.5d, bottom).

Statistics

Statistical comparisons were performed using a two-tailed Mann-Whitney test, a two-way ANOVA with a Bonferroni multiple comparisons test, or a two-tailed, unpaired t-test. Sample sizes for each dataset and relevant statistics are described in figure legends. All p -values <0.05 were considered statistically significant.

Chapter 4: Generating computational models of human liver vasculature

4.1 Introduction

The critical role of the liver's spatial architecture in sustaining liver function suggests that artificial liver tissue may require anatomically accurate structure to recapitulate physiologic function. As such, our ultimate goal is to build perfusable channel networks that mimic the architecture of human liver vasculature within hepatic constructs. Our imaging work elucidated densely packed sinusoids within lobules and hierarchical branching vascular networks across several liver lobules in 3D for the first time in human liver tissue. Despite providing a ~1,600-fold increase in imaging volume of human liver tissue over prior work, we have yet to capture entire cellularly-resolved liver lobules in all three dimensions due to limitations in antibody depth penetration and the light scattering properties of tissue. As a result, the verbatim fabrication of our imaged networks would result in incomplete pieces of a multilobular network containing numerous terminal ends, making the perfusion of fabricated networks challenging. Additionally, for use within artificial tissue, the vascular network would need to abide by fabrication restrictions, such as a maximum bounding volume, and contain two main terminal endings to serve as a primary inlet and outlet for eventual perfusion of the artificial tissue. These requirements are unlikely to be met through the direct reconstruction of images taken of native human liver tissue. Therefore, we pursued computational solutions informed by morphometrics and observations from our human liver images to generate anatomically accurate human liver vascular designs.

4.2 Results

4.2.1 Parameterized morphometric data of liver vascular networks

To generate human liver vascular network designs via computational modeling, we first acquired parameterized network and feature morphometrics from our human liver images to inform computational modeling algorithms and ensure anatomical accuracy. Towards this end,

we analyzed non-fibrotic human liver images in Imaris software to calculate morphometrics pertaining to central vein networks, portal vein networks, and liver sinusoids across the portal-central axis.

We first evaluated the spacing between the central vein and portal vein networks by measuring the shortest observed distances between adjacent central vein and portal vein branches, which represented portal-central axes and thereby was used to approximate lobule radii (Fig. 4.1a,b). Through this, we found the median lobule radius to be 603 μm (mean: 584 μm ; range: 452 μm to 676 μm), which is consistent with the findings of prior studies [33, 38, 39, 40]. Upon inspection of isolated central veins and portal veins, we found that central vein diameters ranged from 53 μm to 212 μm , and portal vein diameters ranged from 41 μm to 358 μm (Fig. 4.1c,d). We then used these diameter measurements to calculate the Murray's law deviation of each observed bifurcation [60]. Murray's law deviation is defined as the parent vessel radius cubed divided by the sum of the cubes of daughter vessel radii, and this ratio can be used to describe flow efficiency within a branching network [61, 62]. From this, we found that central veins had a median Murray's law deviation of 1.42 (mean: 1.69; range: 1.14 to 3.19), and portal veins had a median Murray's law deviation of 1.35 (mean: 1.77; range: 1.09 to 4.24). Furthermore, we found that central vein branch segment lengths ranged from 393 μm to 1554 μm , and portal vein branch segment lengths ranged from 189 μm to 1214 μm . Additionally, we observed that the average branch angles of central veins and portal veins were $93^\circ \pm 19.6^\circ$ (range: 71° to 118°) and $78^\circ \pm 36^\circ$ (range: 26° to 153°) respectively (Fig. 4.1c,d). Lastly, using high-magnification images of non-fibrotic human liver tissue, we found that human liver sinusoids had an average diameter of 9.92 $\mu\text{m} \pm 2.75 \mu\text{m}$ and sinusoid diameters were on average smaller in the periportal region (8.89 $\mu\text{m} \pm 2.81 \mu\text{m}$) than in the pericentral region (11.30 $\mu\text{m} \pm 3.51 \mu\text{m}$) (Fig.4.2), which is an observation also noted in other works [63, 64].

Overall, our multiscale network analyses on human liver tissue revealed that lobule functional units are anatomically defined by interpenetrating networks of portal tracts and central

veins (Fig. 4.1). Furthermore, our human liver sinusoid reconstructions revealed that human sinusoids have a mesh-like topology (Fig 4.2), as opposed to the “wheel spoke” topology that is often described in existing lobule models throughout other works [5, 65, 66]. Together, these morphometric parameters and multiscale observations of human liver vasculature were used to inform algorithms to computationally generate models of interpenetrating central vein and portal vein networks as well as a 3D sinusoidal mesh network.

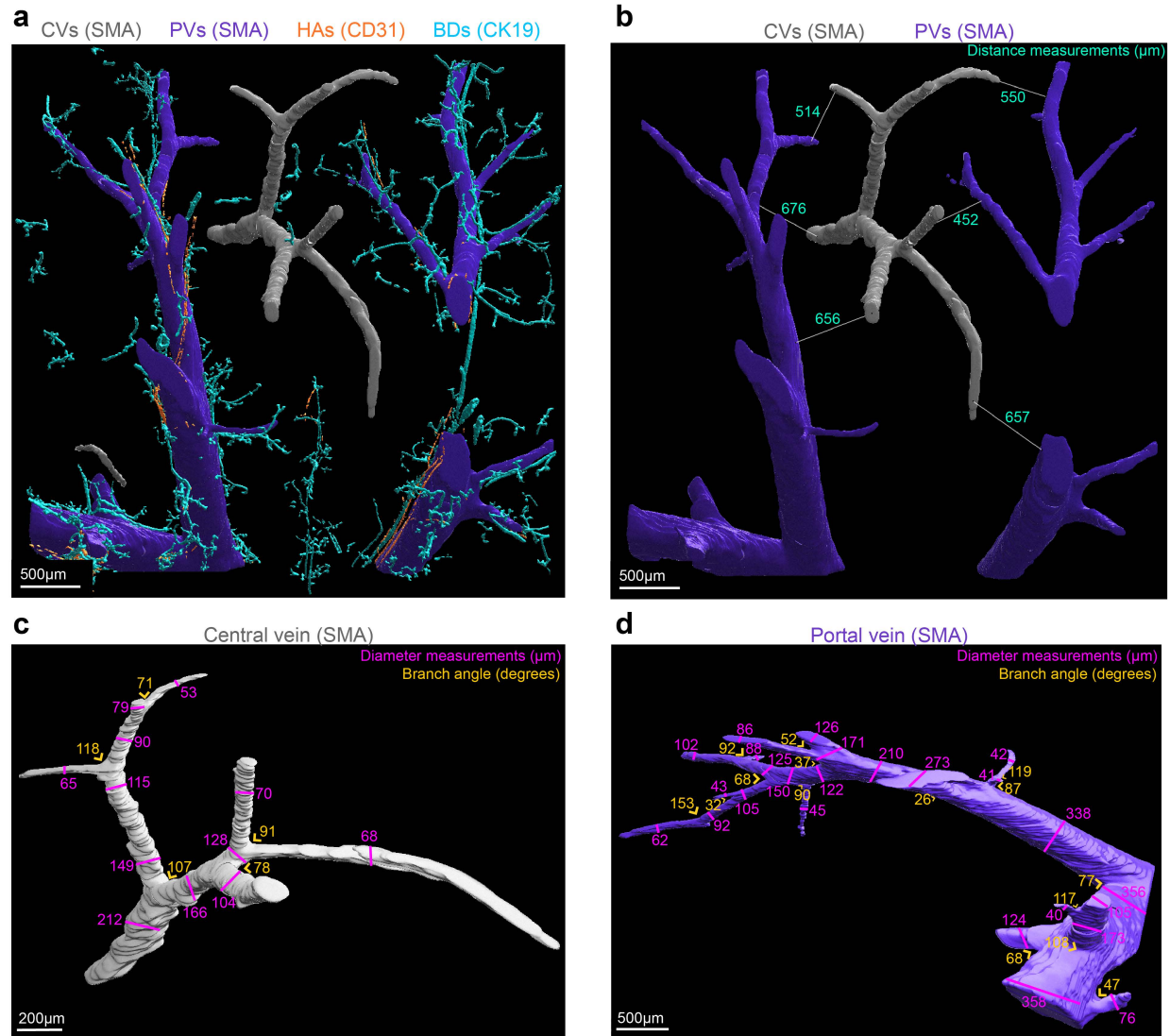


Figure 4.1: Central vein and portal vein morphometrics. **a**, Interlobular branching central veins (CVs, SMA⁺ and manual tracing, grey), portal veins (PVs, SMA⁺ and manual tracing, purple), hepatic arterioles (HAs, CD31⁺, orange), and bile ducts (BDs, CK19⁺, cyan). **b**, Shortest distance

measured (μm) between central vein and portal vein networks, approximating lobule radii. **c, d**, Vessel diameter measurements (μm , pink) and branch angles (degrees, yellow) of isolated central vein (**c**) and isolated portal vein (**d**).

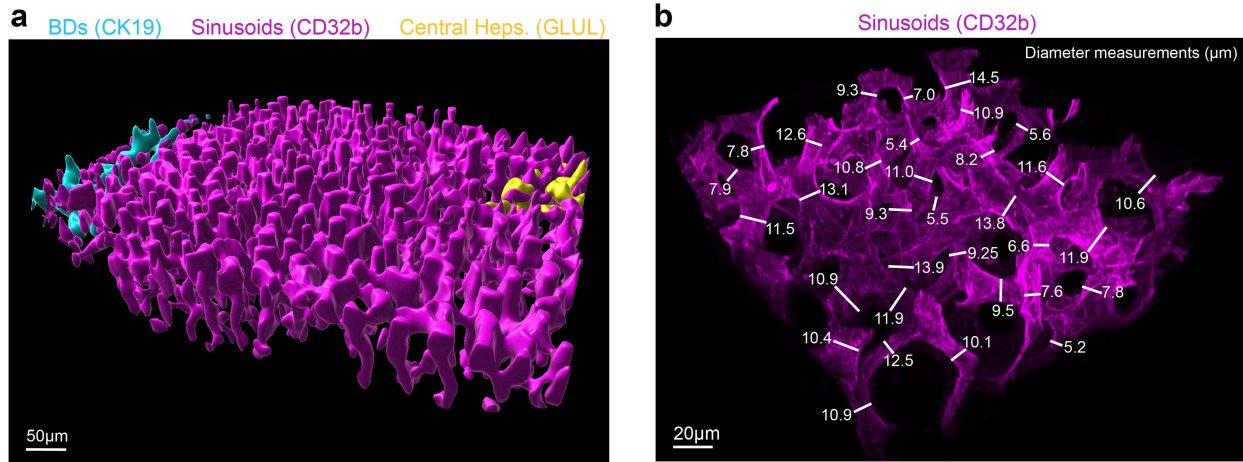


Figure 4.2: Sinusoid topology and diameter measurements. **a**, Reconstruction of a representative 20X image of human liver tissue showing 3D mesh-like topology of the sinusoids (CD32b, magenta) spanning from the portal region (bile ducts, BDs, CK19, cyan) to the central region (pericentral hepatocytes, Central Heps., GLUL, yellow). **b**, Representative 63X image of human liver sinusoids (CD32b, magenta) with overlaid sinusoid diameter measurements (μm , white).

4.2.2 Modeling central and portal veins as interpenetrating networks

We next set out to computationally model the interpenetrating central vein and portal vein networks revealed by our multiscale network analyses while implementing user-defined parameters. To accomplish this goal, we collaborated with Nervous System (Palenville, NY), a generative design studio that we have worked with on past projects [67, 68]. Through this collaboration, Nervous System expanded upon their prior space colonization algorithm [67] to develop an alternating probabilistic isogonic space colonization algorithm that supports the generation of multiple non-intersecting networks with regular spacing between them.

Briefly, the probabilistic isogonic space algorithm was initialized by growing the large-scale primary vein structure for the central and portal network (Fig. 4.3a,b). The central vein network was prioritized first as central feature architecture may be critical for liver function (Chapter 3) and our morphometric analyses revealed that this network generally has more consistent topology than the portal vein network (Chapter 4.1). Central vein and portal vein network morphometrics, such as vessel diameters, branch angles, segment lengths, Murray's law deviations, and network spacing measurements, were used to inform the growth and thereafter validate the anatomical accuracy of generated human liver vascular networks. The probabilistic extension of the space colonization algorithm modified the vein's growth rate to be proportional to the number of sources flowing to it, which promoted more hierarchical branching in the network. The algorithm proceeded by alternating growth phases between central and portal vein structures, while doubling the density of veins and implementing a regularization step at each phase until the design reached a desired network density (Fig. 4.3c-g). The regularization step -- consisting of trimming, smoothing, and repelling -- helped ensure that the networks conformed to user-defined design parameters and were evenly spaced. Specifically, the trimming component removed branches below a threshold length, thereby removing branches too small to contribute to meaningful network architecture and benefit network flow characteristics. The smoothing component used weighted Laplacian smoothing to reduce any vein segments with high curvatures as highly tortuous segments were not observed in our human liver images and their inclusion may disrupt fluid flow within the vascular model. Lastly, the repelling component simulated the veins as a stiff network using the method of Position and Orientation Based Cosserat Rods [69], while adding an inverse square repelling force from the opposing network, which together allowed the growing network to find an equilibrium position that was evenly spaced from the other network's segments.

Altogether, the alternating probabilistic isogonic space colonization algorithm produced a model of interpenetrating human central vein and portal vein networks with user-defined parameters (Fig. 4.3g). The current interpenetrating network design utilizes a bounding box of 16

x 8 x 6.5 mm and includes network branch diameters that range from 70 to 700 μm . Additional alterations to user-defined parameters will be employed as necessary to accommodate fabrication restrictions imposed by future manufacturing work.

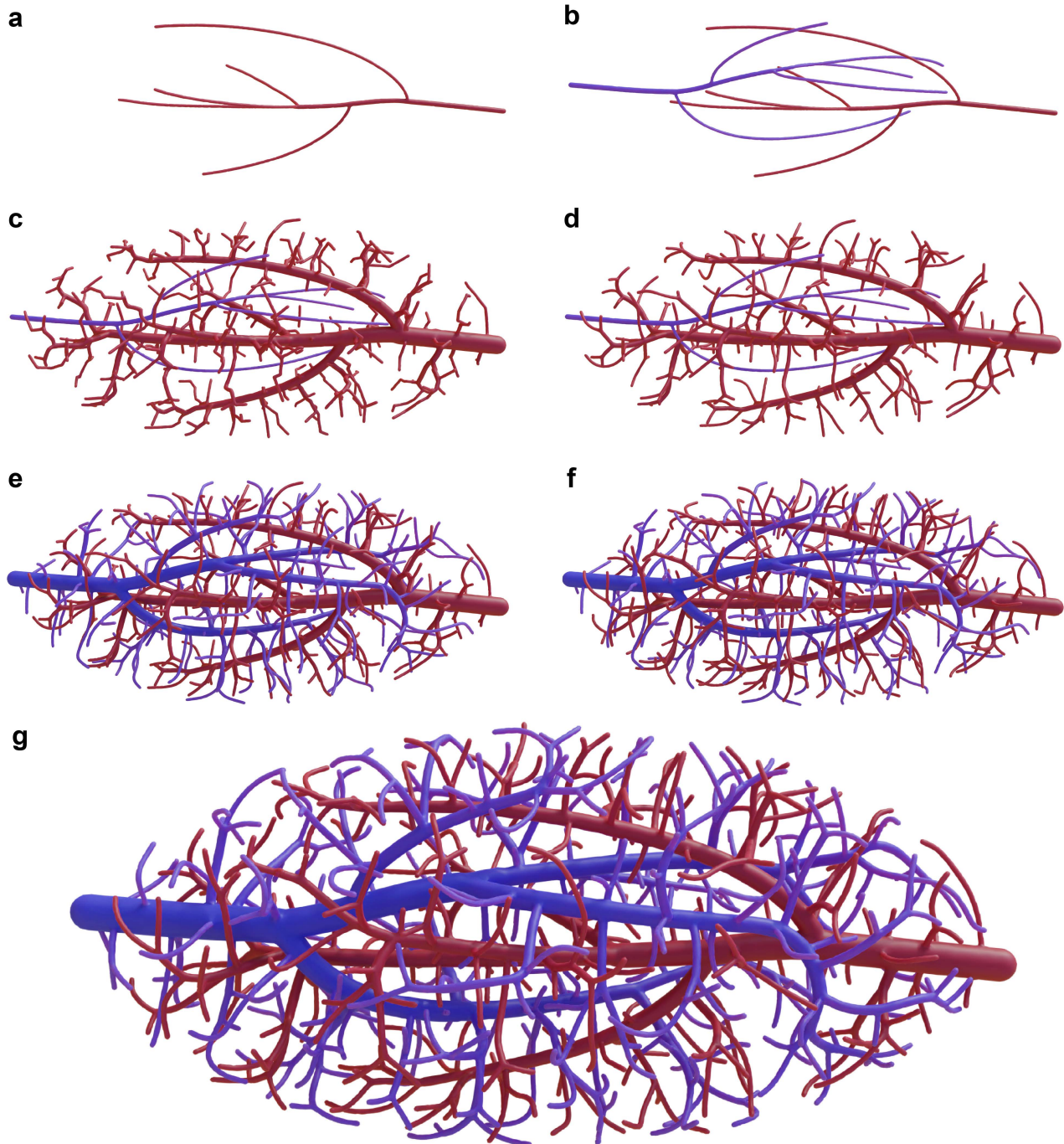


Figure 4.3: Interpenetrating network growth methodology. **a**, First, initialize large-scale growth of central vein network (red). **b**, Next, initialize large-scale growth of portal vein network (purple) and repel the networks to avoid intersections. **c,d**, Perform first isogonic growth step of the central vein network (**c**) and implement regularization steps to trim, smooth, and repel the central vein network from the large-scale portal vein network (**d**). **e**, Execute first isogonic growth step of the portal vein network and implement regularization steps. **f**, Perform second isogonic growth step of the central vein network and apply regularization steps. **g**, Lastly, implement second isogonic growth step of the portal vein network and apply regularization steps to create a model of interpenetrating central vein and portal vein networks within human liver tissue. Network renderings provided by Nervous System.

4.2.3 Modeling liver sinusoids as an elongated 3D mesh network

After building large-scale interpenetrating human liver vascular networks, we shifted our focus towards creating biomimetic architectures of the liver lobule's smallest vascular feature – the sinusoids. Liver sinusoids are highly permeable specialized capillaries that facilitate macromolecular exchange between blood and hepatocytes while transporting blood from the portal tracts to the central veins [2]. As mentioned priorly, our multiscale analyses of reconstructed 3D human liver tissue revealed that human sinusoids exhibit a mesh-like topology (Fig. 4.2), instead of the classic “spokes of a wheel” analogy that is used to describe hepatocyte and sinusoids organization in many textbook lobule models [5, 65, 66]. Therefore, we continued our collaboration with Nervous System to computationally model the sinusoids as an elongated 3D vascular mesh connecting portal and central vein networks.

As the character of the sinusoidal network differed substantially from the hierarchical portal and central vein networks, the modeling of the liver sinusoidal mesh network required a different computational approach than the generation of interpenetrating networks. Towards this end, Nervous System implemented an anisotropic centroidal Voronoi diagram to model the sinusoidal

mesh, using the edges of the diagram to form the network architecture. Briefly, a tetrahedral mesh was extracted encompassing the space between the portal vein and central vein networks by setting a minimum threshold for the angle formed by a point in space and the closest portal and central vein as well as a maximum threshold for the diameter of the closest veins. These thresholds helped ensure that the mesh would not include high curvature segments and that the sinusoids would only connect to the smallest vein segments as seen in our human liver images as well as described elsewhere [70]. Next, a direction field was defined and utilized to control an anisotropy metric for elongating Voronoi cells in the desired direction of flow (Fig. 4.4a). The anisotropy metric was encoded using a high dimensional embedding approach [71], which allowed the optimization of an isotropic, uniform centroidal Voronoi diagram in high dimensional space before its projection into 3D space to yield an elongated mesh network. Lastly, the channel diameter of the sinusoidal network was set to average $\sim 10\ \mu\text{m}$ and expand from $7\ \mu\text{m}$ to $12\ \mu\text{m}$ as the network spatially transitioned from periportal to pericentral to align with our and others' observations of human liver sinusoids [63, 64, 72]. Initial modeling of liver sinusoids as an elongated 3D mesh network was first performed with a minimal lobule structure, consisting of one central vein and three portal veins (Fig. 4.4b,c), before scaling up to use a hexagonal lobule structure with one central vein and six portal veins (Fig. 4.4d-f) as this is the common textbook depiction of a human liver lobule.

Through utilizing an anisotropic centroidal Voronoi diagram, we were able to build a 3D sinusoidal network model that simulated the mesh-like topology we observed in our multiscale 3D human liver analyses (Fig. 4.4). The 3D sinusoidal mesh network design with a hexagonal lobule structure occupies a bounding box of $3.5 \times 1 \times 1\ \text{mm}$ and entails a sinusoidal mesh network with diameters that expand from $7\ \mu\text{m}$ in the periportal regions to $12\ \mu\text{m}$ in the pericentral regions (Fig. 4.4d-f).

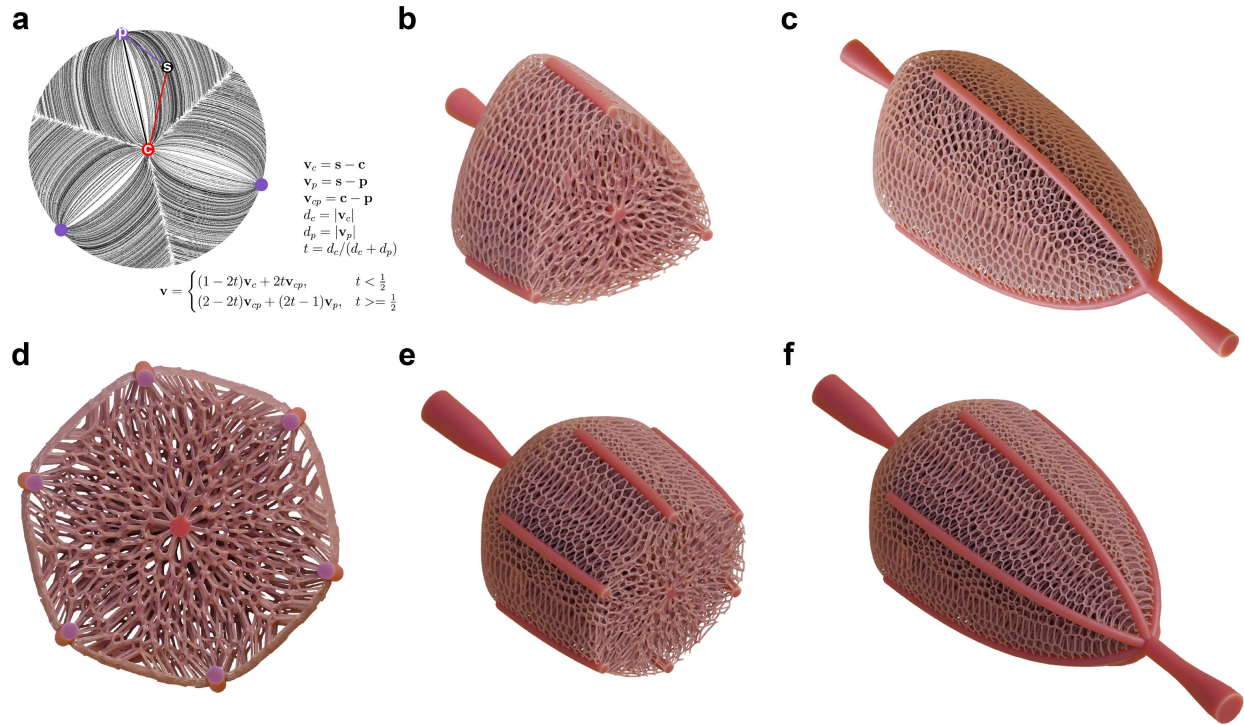


Figure 4.4: Modeling sinusoids as a 3D mesh connecting central and portal networks. a, Direction field diagram showing methodology used to form anisotropy of sinusoids (sinusoids, s, black; central vein, c, red; portal veins, p, purple). Equations represent vector calculations utilized to establish direction field with regard to spatial position. **b,c** Sectioned view (**b**) and full structure view (**c**) of 3D sinusoidal mesh with initial minimal 3D lobule design consisting of one central vein and three portal veins created via implementation of an anisotropic centroidal Voronoi diagram. **d,e,f**, Cross-sectional view (**d**), sectioned view (**e**), and full structure view (**f**) of 3D sinusoidal mesh network design with a hexagonal lobule structure consisting of one central vein and six portal veins. Schematics and renderings provided by Nervous System.

4.3 Discussion

As the liver's unique architecture is integral to its proper function, we believe that establishing anatomically accurate vascular networks within artificial tissue may be key for recapitulating function. Unfortunately, the verbatim fabrication of our priorly imaged vascular networks (Fig. 3.1) would result in networks with terminal endings and no defined primary inlet

and outlet, making perfusion of the fabricated networks challenging. Therefore, we pursued computational modeling strategies to generate anatomically accurate human liver vascular designs. Towards this end, we first acquired parameterized central vein and portal vein network morphometrics from our human liver images, such as network spacing measurements, vessel diameters, Murray's law deviations, segment lengths, and branch angles (Fig. 4.1). We next used high-magnification human liver images to measure the diameter of liver sinusoids across the portal-central axis and found that sinusoids in the periportal region on average had smaller diameters than sinusoids in the pericentral region (Fig. 4.2). Overall, our multiscale network analyses of human liver vasculature revealed two key morphological aspects: 1) the central vein and portal vein networks resemble two interpenetrating networks, and 2) the sinusoids form a 3D mesh that connects central and portal vein networks. These findings inspired us to collaborate with Nervous System, a generative design studio, to create anatomically accurate computational models of human liver vasculature that replicated these key observations. Through this collaboration, we made a model of interpenetrating central vein and portal vein networks via implementation of a probabilistic isogonic space colonization algorithm informed by our network morphometrics (Fig. 4.3). Subsequently, we created a model of a 3D sinusoidal mesh network connecting the central vein and portal veins through utilization of an anisotropic centroidal Voronoi diagram and our sinusoidal morphometrics (Fig. 4.4). Future work entails combining the probabilistic isogonic space colonization algorithm with the anisotropic centroidal Voronoi diagram to create interpenetrating central vein and portal vein networks connected by a 3D sinusoidal mesh, thereby creating a scalable multilobular human liver vascular model. Ultimately, we hope these human liver vascular designs will be incorporated into artificial liver tissue to help support tissue viability and function in future studies. Towards these future efforts, we next began exploring our ability to be able to manufacture these highly complex designs with micrometric features using bioprinting technologies.

4.4 Methods

Computational reconstructions of human liver images

Imaris 9.7 software was used to create surface renderings of immunofluorescent signals in non-fibrotic human liver images. SMA stained both central veins and portal veins robustly at the tissue surface, but SMA signal intensity quickly diminished with z-depth, resulting in poor reconstructions via the Imaris Surface Creation Wizard. Therefore, portal vein and central vein surfaces were created via manual surface creation in Imaris by tracing each vessel's lumen on every odd-numbered z-stack image throughout the whole z-depth of each image. Central vein and portal vein lumens were identified by positive SMA staining at the tissue surface and thereafter differentiated due to association with portal tracts within non-fibrotic tissues. Reconstructions of CK19, CD31 and CD32b were all created using the Imaris Surface Creation Wizard as described priorly in Chapter 2.

Morphometric measurements of central veins, portal veins, and sinusoids

Network spacing measurements: The distances between each adjacent branch of the central vein and portal vein networks were measured at numerous points using the Imaris measurement tool, and the shortest observed distance between each central vein and portal vein branch segment pairing was recorded and used to approximate lobule radius. **Diameter and branch segment length measurements:** The Imaris measurement tool was used to manually measure the diameters and segment lengths of each branch of reconstructed central vein and portal vein networks. **Murray's law deviation:** Murray's law is a basic physical principle for transfer networks to achieve maximum efficiency of blood circulation and states that the cube of the radius of the parent vessel should be equal to the sum of the cubes of the radii of the daughter vessels [60]. Murray's law deviation is defined as the parent vessel radius cubed divided by the sum of the cubes of the daughter vessels radii [61, 62]. Using Microsoft Excel, the Murray's law deviation for each bifurcation within the portal vein and central vein networks was calculated.

Branch angle measurements: The Imaris polygonal measurement tool was utilized to trace each reconstructed branch of central vein and portal vein networks, and angles between traced segments were then calculated in Imaris software. Branch angle averages and standard deviations were calculated in Microsoft Excel. **Sinusoids diameter measurements:** Imaris 9.7 software was used to reduce immunofluorescent background signal in 63X and 20X images of non-fibrotic human liver tissue before the Imaris measurement tool was used to manually measure the diameter of sinusoids at numerous locations (n=253) across the portal-central axis in five samples. Microsoft Excel was then used calculate the averages and standard deviations of sinusoid diameters.

Chapter 5: Initial work towards the 3D fabrication of human liver vascular networks

5.1 Introduction

Vascularization is essential for providing dense, volumetric tissues with a supply of oxygen and nutrients and the removal of waste. Without proper vasculature, tissues thicker than $\sim 150\text{ }\mu\text{m}$, which is roughly the *in vivo* oxygen diffusion limit through cell-dense tissue [73], will become hypoxic and undergo necrosis. Similarly, artificial liver tissue created in the laboratory will also require vasculature for survival. Furthermore, as discussed, creating anatomically accurate vascular structure within artificial liver tissue may be necessary for recapitulating tissue function.

Over the past several decades, various bioprinting technologies have been utilized to build user-defined channel networks within artificial tissue to resemble vasculature. However, to date, no single comprehensive bioprinting strategy has been able to fully recreate biomimetic vasculature with native 3D geometric complexity, hierarchical branching, and cellular heterogeneity [18, 74]. Inkjet-based bioprinting struggles with the fabrication of overhanging, unsupported structures as each layer of printed droplets is supported only by the previously printed layer, which makes the biofabrication of complex, interweaving 3D geometries challenging [18]. Extrusion- and embedded-based bioprinting both rely on the use of nozzles for material deposition, which makes it difficult to construct hierarchical branching networks as this would require swift transitions to different diameter nozzles or the seamless utilization of multi-nozzle extrusion printing on the micrometric scale. Furthermore, extrusion- and embedded-based bioprinting strategies are unable to print complex, entangled 3D networks as this would require the nozzle apparatus to have free-range around the construct being printed, which is a capability these technologies have not yet attained. Lastly, light-based bioprinting cannot easily establish spatial cellular heterogeneity within fabricated cellular constructs as most printers that utilize light have a single resin vat containing a homogenous photocurable bioink. However, light-based technologies are capable of fabricating unsupported, micrometric features and printing

hierarchical branching networks with complex 3D geometries, such as entangled networks [68]. As such, we chose to pursue light-based bioprinting technologies, specifically digital light projection and multiphoton laser-scanning lithography, to assess our ability to fabricate our human vascular designs within biocompatible hydrogels and show the future feasibility of incorporating these networks in artificial liver tissues.

5.2 Results

5.2.1 Digital light projection fabrication of interpenetrating networks

We chose to test digital light projection (DLP) technology for fabricating the interpenetrating networks design (Fig. 4.3g) due to the design's high complexity, network entanglement, and large bounding box (16 x 8 x 6.5 mm), as well as our prior expertise with DLP additive manufacturing [68, 75]. DLP additive manufacturing is a form of mask-based photolithography that is capable of making cell-laden hydrogels with embedded user-defined networks [68, 75]. Briefly, the DLP system projects photomasked light ($\lambda = 405$ nm) onto a mirror positioned at 45° which orients digital photomasks onto a build platform. The z-axis motor moves by a fixed amount with each sequential photomask, and a 3D model is generated by curing a photosensitive prepolymer layer by layer with regard to the sequential photomasks that together reconstitute the 3D model. DLP additive manufacturing is relatively fast as it projects the entire xy-layer at each z-step to allow whole layer-by-layer printing, with print time only scaling in the z-dimension. As a result, DLP technology can be beneficial for printing constructs with xy-dimensions on the centimeter length scale. In DLP additive manufacturing, xy-resolution is dictated by the precision of the projected photomask while z-resolution is determined by the light attenuation properties of the bioink, thereby permitting DLP additive manufacturing to exhibit high resolution in the xy-dimensions but limited resolution in the z-dimension.

Our original DLP printer used in prior work, termed the SLATE printer [68], utilized an optical engine with a 1280 x 800 square pixel array to achieve 50 μ m voxel resolution and could

print open channel networks with a minimum diameter of 500 μm . Unfortunately, this channel resolution would have been insufficient for printing most of the branches in the interpenetrating networks design. To print smaller channels using DLP technology, we first collaborated with the Miller Laboratory at Rice University to build a high-resolution DLP printer that used an optical engine with a 1920 x 1080 square pixel array and a 10 μm voxel resolution to produce a 19.2 x 10.8 mm projection (Fig. 5.1a), following similar methodology as published in our prior work [68]. After significant printer and bioink optimization by both members of the Stevens Laboratory and Miller Laboratory, we found that the high-resolution DLP printer could reliably fabricate open channels networks with a minimum diameter of 100 μm , and in some cases achieve a minimum channel diameter of 60 μm (Fig. 5.1b).

After improving the minimum channel resolution of our DLP technology, we next used the high-resolution DLP printer to fabricate the full 16 x 8 x 6.5 mm interpenetrating network design (Fig. 5.2a,b). To print this design, we utilized a polymer ink consisting of 20 wt% poly(ethylene glycol) diacrylate (PEGDA, 3.4 kDa), 34 mM lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), 8.66 mM tartrazine, 10% glycerol, and 0.2 mg/mL FITC in phosphate buffered saline, which corresponded to a photosensitive polymer, photoinitiator, photo-absorber, viscosity agent, and imaging agent respectively. After printing, we were able to visualize large channel architectures via standard digital imaging (Fig. 5.2c,d). To determine the minimum open channel architecture that could be fabricated, we pipetted a solution containing 2 μm fluorescent polystyrene beads and India ink into the open ending of each network, which thereafter could be visualized by confocal microscopy and digital imaging respectively (Fig. 5.2e-g). However, as the two networks within the interpenetrating network design are independent and closed (*i.e.*, containing terminal branches and not innately perfusable), it is likely that many of the smallest branches did not fill with fluorescent beads or India ink, thereby limiting our imaging capabilities of the smallest branches (Fig. 5.2f,g). Confocal imaging of FITC stain within the bulk of the gel revealed many of the smallest diameter branches within the design, but without the presence of fluorescent beads

or India ink, we could not confirm if these branches were truly open. Upon isolating open branches that filled with fluorescent beads, we found that some of the design's smallest branches, designed as 70 μm in diameter, were fabricated with a diameter of 50 to 60 μm . By comparing the designed diameters to the measured fabricated diameters, we found that fabricated branch diameters were on average 18.6 μm smaller than designed, which is likely due to the overcuring of the polymer ink during printing (Fig. 5.2e). In future work, scaling the design to be larger by the percentage of overcuring may be able to yield channels of the designed diameters, and polymer ink modifications, such as increasing the concentration of tartrazine, may be able to minimize reductions in channel diameter due to overcuring. Lastly, as mentioned previously, future computational work will add sinusoids to the interpenetrating networks design, thereby eliminating terminal branches within the design and allowing us to evaluate the smallest open branches via network perfusion. However, once the sinusoids are added to the design, modifications to the DLP ink will likely be necessary to further improve the minimum channel resolution of our DLP technology and successfully print sinusoids with a channel diameter averaging 10 μm . Alternatively, DLP technology could potentially be used in tandem with other bioprinting technologies to fabricate the interpenetrating networks and sinusoids respectively.

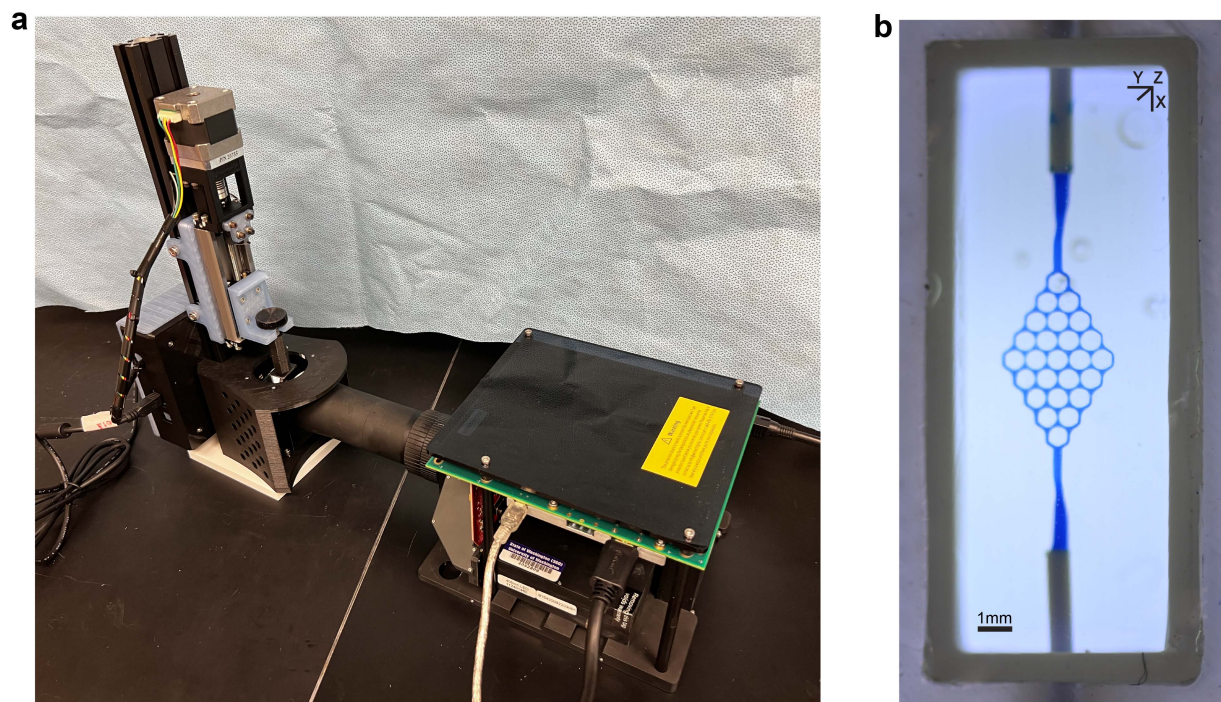


Figure 5.1: High-resolution DLP bioprinter. **a**, Photograph of high-resolution DLP bioprinter built by Wesley Fabyan (Stevens Lab), Sherina Malkani (Stevens Lab; Miller Lab) and Kevin Janson (Miller Lab). **b**, Image of a perfused network that was printed by Sherina Malkani using the high resolution DLP bioprinter. Minimum channel diameters in this network are 60 μm .

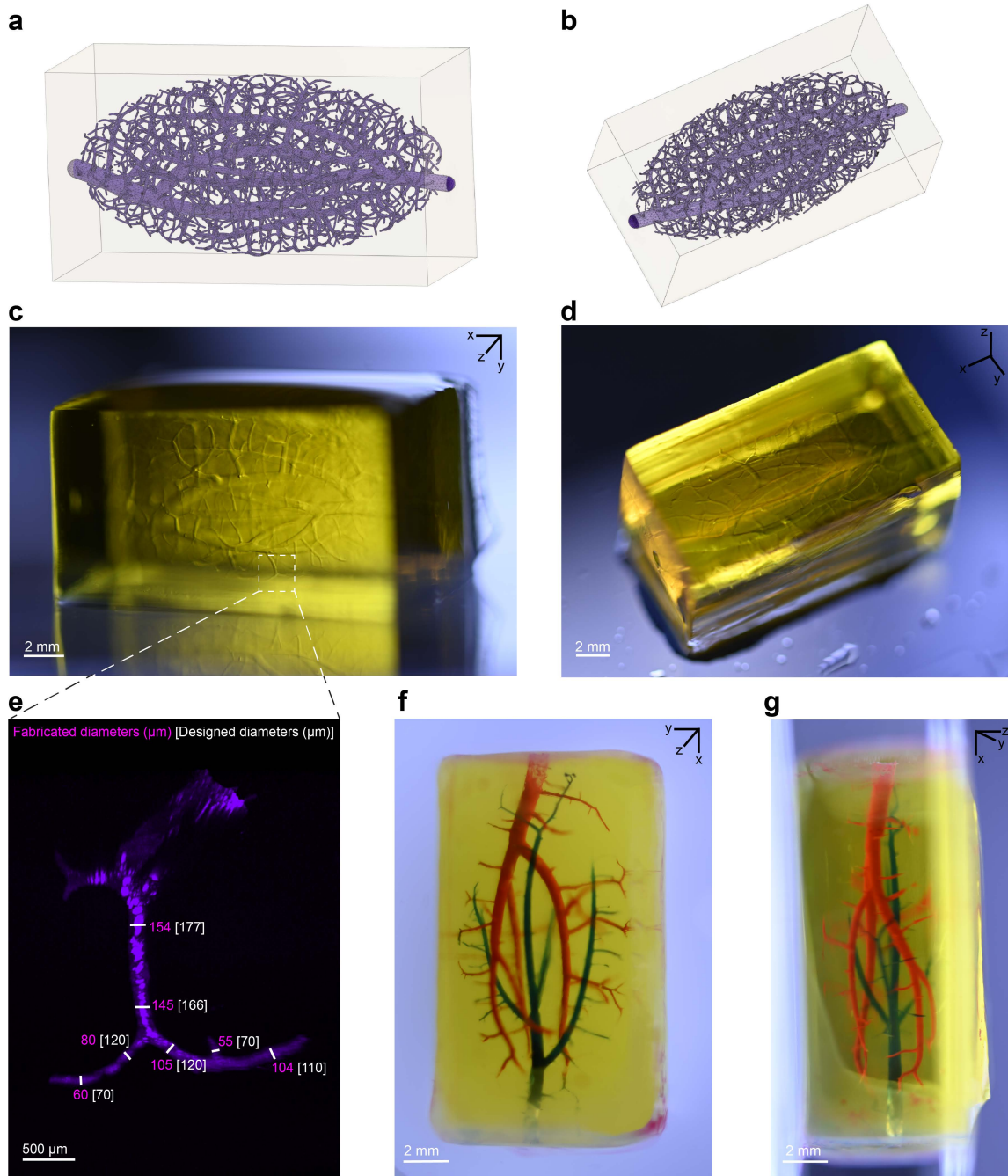


Figure 5.2: Interpenetrating networks design printed via DLP additive manufacturing. a, b, 3D computer aided design (CAD) model of interpenetrating networks design with bounding box measuring 15.9 x 8.6 x 7.1 mm. The z- and y-dimensions of the bounding box were extended by 300 μm both in the positive and negative directions to provide a sufficient wall thickness between the network design and the edge of the gel. c, d, Digital images of DLP-printed interpenetrating

network design matching the perspective of the CAD design in **a** and **b**, respectively. **e**, Immunofluorescent image of fluorescent polystyrene beads within selected network branch in **c** labeled with measured fabricated diameters (pink, μm) and CAD design diameters ([white], μm). **f, g**, Digital images of the top (**f**) and side (**g**) of DLP-printed construct with red and blue dye containing India ink and fluorescent polystyrene beads introduced to channel openings to provide contrast for visualization with digital and confocal imaging. DLP fabrication work and subsequent imaging was performed with assistance from Sherina Malkani (Stevens Lab; Miller Lab).

5.2.2 Multiphoton laser-scanning lithographic fabrication of 3D sinusoidal mesh

As our DLP technology is currently limited to a minimum channel resolution of $\sim 60\ \mu\text{m}$, we next investigated the use of laser-scanning lithography (LSL) for fabricating the 3D sinusoidal mesh network design (Fig. 4.4f) due to the technology's ability to achieve channel diameters on the order of single microns. LSL fabrication leverages single-photon (SP-LSL) or multiphoton (multi-LSL) technology and photo-additive and/or photodegradative chemistries to build 3D objects or networks within a hydrogel with high precision. SP-LSL directs a focused laser beam to a focal point within a bioink resin to initiate photoreactions, achieving submicron xy-resolution but lower z-resolution ($>25\ \mu\text{m}$) due to unwanted reactions above and below the focal plane. Multi-LSL, on the other hand, utilizes a pulsed near-infrared laser to prompt the absorption of two low-energy photons and confine photoreactions to a focal plane, which allows submicron xy-resolution and $2\text{-}3\ \mu\text{m}$ z-resolution [76]. Although providing high-resolution printing capabilities, LSL fabrication has drawbacks such as requiring expensive, specialized equipment and being time intensive, which often limits the total build volume.

The DeForest Laboratory at the University of Washington recently publicized the utility of their multi-photon technology, termed GIZMO, for light-mediated subtractive manufacturing [77]. Therefore, we collaborated with the DeForest Laboratory to fabricate the 3D sinusoidal mesh network design via GIZMO. Briefly, the GIZMO technology leveraged a multiphoton microscope

and an acousto-optic modulator directed by a custom MATLAB code to govern laser power and local light dosage in real time on a voxel-by-voxel basis while raster scanning through a photosensitive hydrogel. For photodegradative patterning, hydrogels were first formed via SPAAC step polymerization of PEG-tetraBCN (4.05 mM) and a bis-azide modified photodegradable ruthenium polypyridyl complex-based crosslinker (Rubpy, 8 mM) [78]. Next, the GIZMO apparatus applied a femtosecond-pulsed laser ($\lambda = 770$ nm) to induce two-photon absorption and trigger photo-scission of the Rubpy crosslinker within the hydrogel, resulting in localized material dissolution [77]. Using GIZMO's subtractive manufacturing capabilities, the DeForest Lab has been able to establish open channels down to a minimum diameter of 5 μm within hydrogels.

Leveraging GIZMO technology, we fabricated the 3D sinusoidal mesh network design to assess if multi-LSL could fabricate the 3D mesh with its high complexity and micrometric length scale (Fig. 5.3). After photopatterning, gels were stained with a fluorescent azide dye (Cy5-N₃, 1.25 μM), which bound to the stoichiometric excess of BCN groups included during gel formation, thereby allowing us to stain the bulk gel post-patterning and expose void channels via lack of fluorescence after imaging with a confocal microscope. By inverting the Cy5 fluorescent stain, we were able to see that the channel networks created via multi-LSL photodegradation resembled the 3D sinusoidal mesh network design (Fig. 5.3a,b). However, confocal imaging was only able to capture about half of the photopatterned structure due to the thickness of the gel and the high fluorescent signal from staining the bulk of the gel (Fig. 5.3b). We confirmed that photopatterning went through the entire depth of the gel by flipping the gel 180 degrees and imaging again, which revealed the other half of the photopatterned structure. Future improvements to imaging techniques will be necessary to capture the entire photopatterned structure via confocal imaging. Despite these imaging limitations, we next created Imaris surfaces of the inverted Cy5 fluorescent stain to aid with visualization of the fabricated 3D sinusoidal mesh (Fig. 5.3c). Lastly, we found the average diameter of the multi-LSL fabricated 3D sinusoidal mesh to be 10.05 μm $\pm$ 2.03 μm , which closely resembled the designed sinusoidal average diameter of ~ 10 μm (Fig. 5.3d). This

initial work indicated that multi-LSL is capable of creating the 3D mesh within the sinusoidal network design. Future work will improve imaging techniques for assessing channel architecture throughout the photodegraded construct and evaluate the capability of multi-LSL photodegradation to fabricate the entire 3D sinusoidal mesh network design.

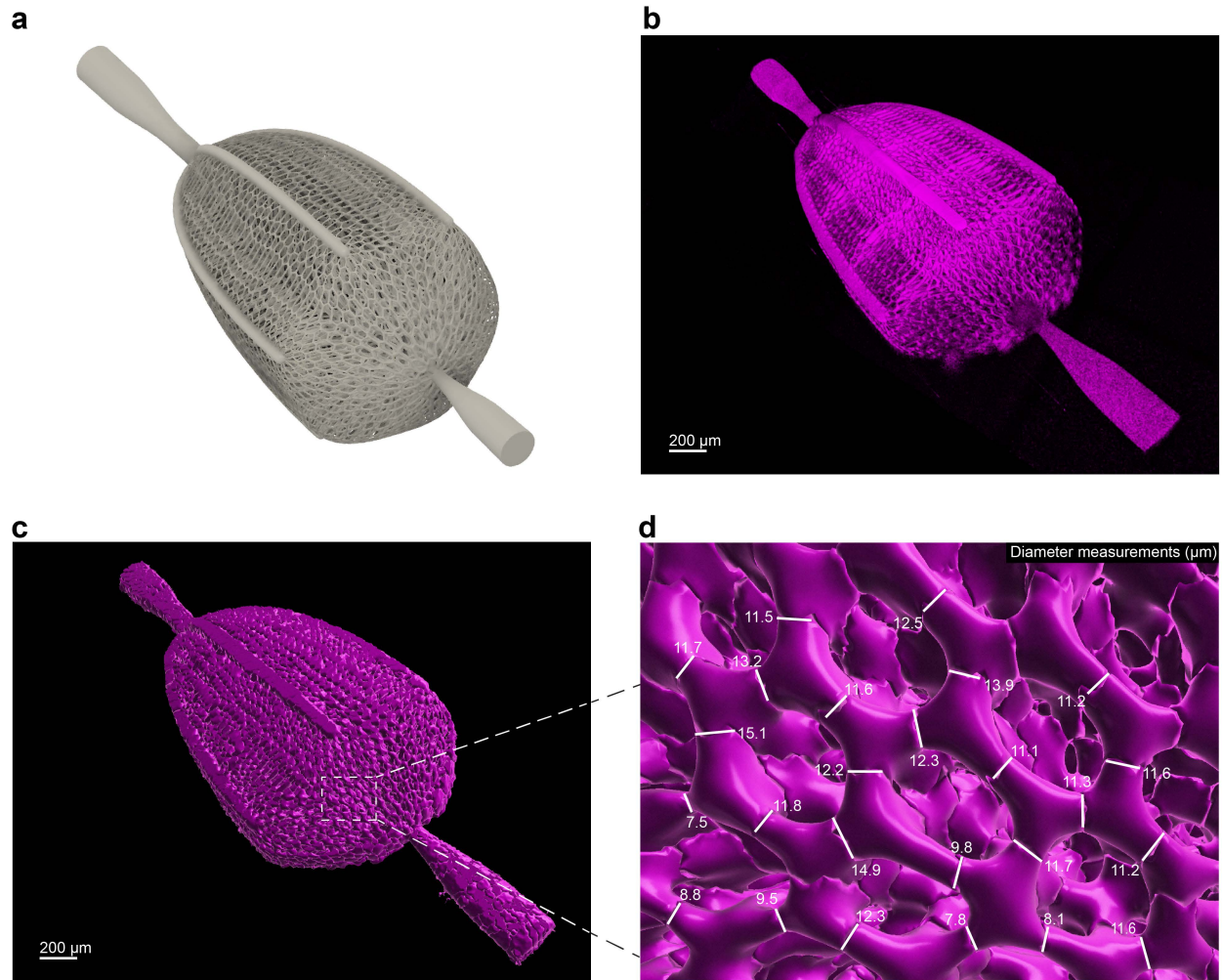


Figure 5.3: 3D sinusoidal mesh printed via multi-LSL photodegradation. **a**, CAD model of the 3D sinusoidal mesh network design with hexagonal lobule structure. **b**, Inverted Cy5 immunofluorescent signal showing multi-LSL photopatterned areas within the photodegradable hydrogel. **c**, **d**, Imaris surface rendering of inverted Cy5 immunofluorescent signal with zoom-in (**d**) showing representative measurements (μm) of fabricated sinusoidal mesh. Multi-LSL fabrication work and subsequent imaging was performed with assistance from Shiyu Yang (DeForest Lab).

5.3 Discussion

Similarly to native tissue, artificial tissue made in the laboratory will require vascular channels for the supply of oxygen and nutrients and the removal of waste. Additionally, vascular architecture, such as that seen in the human liver, may play a key part in maintaining tissue function, which suggests that building anatomically accurate vascular models within artificial tissue could be beneficial towards recapitulating function *in vitro*. Therefore, towards the future goal of building our human liver vascular designs within artificial tissues, we began exploring light-based bioprinting technologies due to their ability to fabricate complex 3D geometries containing hierarchical branching networks and micrometric features, which are attributes exhibited within our interpenetrating central vein and portal vein networks design and our 3D sinusoidal mesh network design.

We chose to explore DLP technology for fabricating the interpenetrating networks design due to the design's high complexity and size. However, we first had to build a high-resolution DLP printer in collaboration with the Miller Laboratory (Rice University) as our prior DLP printer could not fabricate channels with diameters less than 500 μm (Fig. 5.1). Next, we used our high-resolution DLP printer to fabricate the full 16 x 8 x 6.5 mm interpenetrating network design within a biocompatible hydrogel (Fig. 5.2). Our initial high-resolution DLP printing work showed feasibility for fabricating the current interpenetrating networks design with a minimum channel diameter of 70 μm , albeit with some observed overcuring yielding smaller fabricated channel diameters than designed. Future work aims to minimize the effects of overcuring on channel diameters and to computationally modify the interpenetrating networks design to include sinusoids. Once the sinusoids are added to the interpenetrating networks design, improvements to the DLP bioink and/or implementation of additional bioprinting technologies may be necessary to build the sinusoidal networks.

We next explored the use of multi-LSL for fabricating the 3D sinusoidal mesh network design as multi-LSL photodegradation can achieve channel diameters on the order of single

microns. Towards this end, we collaborated with the DeForest Laboratory (University of Washington) to implement their multi-LSL subtractive manufacturing technology. Our initial work with the DeForest Laboratory showed that multi-LSL photodegradation may be a feasible method for fabricating the 3D sinusoidal mesh within biocompatible hydrogel as fabricated sinusoidal diameters closely matched the design's sinusoidal diameters (Fig. 5.3). Future work entails improving imaging techniques for visualizing the full 3D sinusoidal mesh network design after being fabricated via multi-LSL photodegradation.

All in all, these initial fabrication results suggest that light-based bioprinting technologies may be able to build our human vascular designs within biocompatible hydrogels. Here, we explored DLP additive manufacturing and multi-LSL photodegradation for creating our interpenetrating networks design and our 3D sinusoidal mesh network design respectively. Although both methods of fabrication have shown feasibility for printing their respective design, future innovations and improvements in both DLP and multi-LSL will be necessary to fabricate multilobular vasculature with the complexity and scale of native human liver tissue. Nevertheless, these initial fabrication efforts show feasibility for incorporating these networks within biocompatible hydrogels, bringing us one step closer to incorporating these anatomically accurate networks within artificial tissues in the future.

5.4 Methods

Digital light projection additive manufacturing

DLP printing ink: Prepolymer ink was prepared containing 20 wt% poly(ethylene glycol) diacrylate (PEGDA, 3.4 kDa), 34 mM lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), 8.66 mM tartrazine, 10% glycerol, and 0.2 mg/mL FITC in phosphate buffered saline. **CAD processing:** The interpenetrating networks design was imported into Autodesk Fusion 360 and the design's STL was subtractive cut from a bounding box that encased the entire design to create positive volume for the bulk of the gel and negative volume for the channel networks. The 3D

CAD model was then scaled to 130% in z, sliced in CreationWorkshop with an anti-aliasing of 3, and processed in a custom MATLAB script to grayscale the image and even the inherent fluctuation in projection intensity emitted by the optical engine across the xy-plane. **DLP printing:** DLP printing was then performed with 10 μm z-steps, a laser power of 17.8 mW/cm², and 17 second exposures per layer after two bonding layers of 75 seconds each. Total print time was 7 hours and 35 minutes. Immediately after printing, the construct was submerged in 250 mL of phosphate-buffered saline and allowed to hydrate overnight. **Gel imaging and channel visualization:** After hydrating the gel, a Nikon digital camera was used to capture digital images of the gel. Next, a solution containing 50% 2 μm fluorescent polystyrene beads (Magsphere, Inc.; PSF-002UM - Red) and 50% India Ink (Dr. Ph. Martin's Bombay) was pipetted into the channel opening at each end of the gel, and once the solution had time to diffuse into major channels, the open ending of the gel was sealed by crosslinking a drop of 6% alginate with 1M CaCl₂. After sealing both ends, the gel was then imaged on a Nikon AXR confocal microscope using 488nm and 594nm lasers to visualize FITC within the bulk of the gel and 2 μm fluorescent polystyrene beads within channels. **Diameter measurements:** The diameters of isolated branches from the interpenetrating networks CAD design were measured using the Inspect / Measurement tool in Autodesk Fusion 360. Next, the confocal image of the gel was opened in Imaris 9.7 software, and the Imaris measurement tool was used to measure the diameters of immunofluorescent signal for the 2 μm fluorescent polystyrene beads within the same isolated branches to find actual manufactured diameters. As the polystyrene beads were added to channel networks after printing, we can say with confidence that these manufactured diameters pertain to open channel networks.

Multiphoton laser-scanning lithography for subtractive manufacturing

Pre-pattern gel formation: Photodegradable hydrogels were first formed via SPAAC step polymerization of PEG-tetraBCN (4.05 mM) and a bis-azide modified photodegradable ruthenium polypyridyl complex-based crosslinker (Rubpy; (Ru(2,2'-bipyridine)₂-(4-azidobutanenitrile)₂); 8

mM) as described priorly [78]. Additionally, AF488-N₃ was included in gel formation to help with visualizing photodegradation after printing. **Multi-LSL photodegradation:** For photopatterning, a Thorlabs Bergamo II multiphoton microscope driven by ScanImage software was used to emit a 770 nm femtosecond-pulsed laser with 15 repeats and 100% laser intensity, which initiated two-photon mediated cleavage of the Rubpy crosslinker. The microscope used a voxel resolution of 0.7 x 0.7 x 1 μm and a 360 x 360 μm field of view, and a platform motor was used to tile field of views together. **Gel imaging and channel visualization:** After photopatterning, gels were stained with a fluorescent Cy5 azide dye (Cy5-N₃, 1.25 μM), which bound to the stoichiometric excess of BCN groups included during gel formation. After staining, gels were soaked in 1X PBS for 2 hours to remove excess fluorescent Cy5 dye. Photopatterned gels were then imaged on a Leica Stellaris 5 confocal microscope using 496nm and 633nm lasers to visualize the AF488 and Cy5 respectively. Together, AF488 and Cy5 immunofluorescence were used to evaluate integrity of photopatterning. AF488 signal showed bulk gel architecture, however, as it was included during gel formation, there was a possibility that the lack of AF488 could be due to photobleaching during the photodegradation process. Therefore, the Cy5 azide dye was used to stain the bulk gel after printing and confirm photopatterning. Using these stains, the lack of fluorescence with the bulk of the gel indicated void channel networks. As such, the Cy5 fluorescence was inverted to highlight channel networks and allow visualization and surface creation in Imaris 9.7 software. **Diameter measurements:** The Imaris measurement tool was used to manually measure the diameter of multi-LSL fabricated sinusoids, denoted by inverted Cy5 fluorescence, at numerous locations (n=80) throughout the image stack. Microsoft Excel was then used calculate the average and standard deviation of fabricated sinusoid diameters.

Chapter 6: Conclusion

The liver's unique and complex microstructure plays an important role in maintaining its proper function. In liver cirrhosis, the liver's architecture often becomes significantly altered, which can lead to severe functional deficiencies and liver failure. In fact, liver cirrhosis is the 11th most common cause of death globally, amounting to 2.1% of all deaths worldwide each year [11]. The only current treatment for liver failure is liver transplant, and the gap in liver organ supply and demand is widening each year [11, 16]. To help close this gap, tissue engineers are working to build functional artificial liver tissue for disease modeling and eventually whole organ transplantation.

As the liver's function is closely tied to its structure, the development of functional artificial liver tissue may necessitate recapitulating native liver structure in artificial tissue. While working towards this initiative, we discovered that the architecture and cellular arrangement of human liver tissue on the multilobular scale with cellular resolution has not yet been uncovered, making the accurate recapitulation of native liver structure in artificial tissues not feasible. Therefore, our first step was to elucidate the organization of structural and functional features in human liver tissue across several lobules in 3D. Towards this end, we utilized immunostaining, tissue clearing, confocal microscopy, and digital reconstruction to create the largest, cellularly resolved, 3D reconstructions of human liver tissue known to date. Next, we compared the functional and structural features of normal non-cirrhotic tissue with cirrhotic tissue to help elucidate which features may be essential for maintaining functional liver tissue. Our collective comparative analyses of non-fibrotic and cirrhotic tissue suggested a pro-portalization/decentralization phenotype in correlation with human liver cirrhosis and emphasized the importance of the liver's central features for maintaining functional liver tissue.

With a new appreciation of multilobular human liver architecture, we next pursued computational modeling strategies to generate anatomically accurate human liver vascular

designs for future incorporation into artificial liver tissue. Towards this end, we first acquired parameterized morphometrics and performed multiscale analyses on central vein, portal vein, and liver sinusoidal networks, which revealed 1) the central vein and portal vein networks resemble two interpenetrating networks, and 2) the sinusoids form a 3D mesh that connects central vein and portal vein networks. We thereafter collaborated with Nervous System to generate anatomically accurate computational models of these two key observations. Future computational work entails combining modeling methodologies to create interpenetrating central vein and portal vein networks connected by a 3D sinusoidal mesh, thereby creating a scalable multilobular human liver vascular model. Ultimately, we hope this multilobular human liver vascular design will be incorporated into artificial liver tissue to help support tissue viability and function in future studies. Towards these future tissue engineering efforts, we lastly used DLP additive manufacturing and multi-LSL photodegradation to show feasibility for fabricating the interpenetrating networks design and the 3D sinusoidal mesh network design respectively within biocompatible hydrogels. Although both fabrication methods showed initial feasibility for printing their respective design, further improvements and innovations to both technologies will likely be needed in order to create the ultimate multilobular human liver vascular model.

All in all, this dissertation work presented the largest cellularly-resolved 3D reconstructions of human liver tissue to date, revealed a pro-portalization/decentralization phenotype in correlation with human liver cirrhosis, generated anatomically accurate human liver vascular designs, and assessed the feasibility of two light-based bioprinting strategies for fabricating human liver vascular designs within biocompatible hydrogels. These collective efforts provide foundational knowledge on human liver architecture in its healthy and diseased states and offer insight into the computational modeling and subsequent fabrication of human liver vascular networks, which together may help advance tissue engineering initiatives towards creating functional artificial liver tissue in the future.

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