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Visualizing surface area and volume of lumens in three dimensions using images from histological sections

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Summary

Visualizing the interior (lumen) of a tubular structure within tissue can provide a unique perspective on anatomical organization of the tissue. Portal tracts of the liver contain several vessels and ducts in various patterns of intertwining branches and are an example of such spaces. An inexpensive method, using light microscopy and a sample of conventionally stained canine livers, was used to colorize and allow visualization of the lumens of vessels within the portal tract in three dimensions. When the colour of the background was digitally cleared and the lumen filled with a solid colour, it was possible to measure areas and volumes of the portal vein, arteries, bile ducts and lymphatics. Significant differences between vessels and ducts across lobes and gender in control samples are discussed. Differences were also found between control and mixed breed dogs and between controls and a dog that died of accidental traumatic haemorrhage. These differences are discussed in relation to visualizing lumens using images generated from a light microscope. Vessels in plants such as xylem and continuously formed spaces resulting from ice formation are other examples where this technique could be applied.

Keywords

3-D reconstruction; histology; liver; negative space; portal tract

Introduction

Visualizing internal structures of biological systems is crucial to a comprehensive understanding of the function of various components of that system. Since radiography was

first described in the late 1800s, a number of invasive as well as noninvasive techniques have been used to visualize internal structures of biological systems and in some cases produce three-dimensional (3-D) reconstructions of various components of those systems.

Techniques such as computer tomography (CT) microcomputer tomography, nuclear magnetic resonance, CAT, synchrotron radiation imaging and confocal laser microscopy have more versatility and resolution than radiography but require expensive equipment, considerable expertise and are limited in the size of structures than can be resolved. For that reason, although important information has been obtained with these systems (Bae, *et al.*, 1993; Fiebig *et al.*, 2012; Tabibian *et al.*, 2013), they may be unsuitable for particularly small structures and for many laboratories, budget limitations preclude their use.

The purpose of these methods is to visualize solid structures within tissues and, generally, adequate information is obtained to allow diagnoses of a specific problem. In some cases, however, visualizing spaces which might be filled with liquid or air, provides a different perspective that may be crucial to understanding the biology of the system. Conducting vessels that are filled with liquids such as those found in the portal tracts of liver tissue are an example of a biological system that is problematic to examine. Micro-computer tomography has been used to visualize the biliary tract of liver tissue (Fiebig *et al.*, 2012) and although excellent 3-D renditions of the liver were obtained, the resolution was not great enough to image smaller vessels such as arteries or arterioles nor to observe the lumen of those vessels.

A 3-D reconstruction technique has been described that uses pixel-based images (Livingston *et al.*, 2010). In this procedure, conventionally stained tissue was photographed with a consumer grade camera and internal structures were visualized in 3-D by digitally clearing the background of images and aligning them to producing a 3-D reconstruction. Although images at virtually any resolution can be used, it is not possible with that method to visualize the lumens within the portal tract in three dimensions.

In this study, we present a technique to visualize the lumen of tubular structures, compare volumes and reconstruct that surface area in three dimensions. We demonstrate the use of this technique by investigating differences in areas of vessels, bile ducts and lymphatics within portal tracts of canine liver tissue.

Materials and methods

Samples of canine livers were obtained from three sources: (1) 4 Beagle dogs (two male and two female) that were bred for research purposes, maintained in accordance with North Carolina State University IACUC regulations and obtained after euthanasia; these served as untreated control dogs. (2) Four mixed breed dogs that were obtained from a local pound after euthanasia. (3) A single dog that died from haemorrhagic shock (HS) following internal blood loss due to an automobile accident which served as an example of HS. This diagnosis was confirmed by post-mortem examination which revealed that the dog had died from massive blood loss due to abdominal trauma. All dogs were young adults, but specific ages not known. The body weight of the dogs ranged from 10 to 22 kg.

Samples were collected from the left lateral and the right medial lobe of each dog; they were fixed by immersion in 10% neutral buffered formalin and processed using a routine protocol for paraffin sectioning. Tissue was serially sectioned at 6μ and stained with a Safranin, Hematoxylin, and fast-green triple stain and photographed with a Sony DSC707 camera mounted on a Nikon Eclipse 50i microscope at $20\times$.

Image processing

JPEG images were imported into Adobe After Effects (Version CS6) as a sequence and digitally cleared of background colour using the “colour key” subroutine. Cleared images were exported as TIFF images, brought back into After Effects as footage and manually aligned. Aligned and cleared images were again exported with a pure white background, then brought into After Effects as a sequence. The rotobrush tool in After Effects was used to automatically find the edges of lumens (interiors of veins, arteries, bile ducts and lymphatic ducts) in each image. The information used to delineate edges in the first image was automatically applied to the subsequent image. The software usually anticipated changes in the subsequent image making the process somewhat automatic. Slight manual manipulations were necessary in some cases to ensure correct identification of the lumen.

The lumens of each vessel within portal tracts from 12 consecutive sections were filled with colour to allow visualization and to determine area. Veins were coloured blue, arteries red, bile ducts green and lymphatic spaces yellow. Images with the four different colours were imported into Sigma Scan Pro (not presently supported by the designer) and the pixel number for each vessel was quantified. Although we did not use the software Image J (<http://rsbweb.nih.gov/ij/>) it could also be used to quantify number of pixels. Absolute data used in the principal component analysis (PCA) was obtained by converting number of pixels to mm^2 . This was done by calculating the number of pixels per mm in a standard image using a microscope reticule.

To select for portal tracts that were likely to be at the same level of the branching pattern we constrained the size of portal tracts by excluding those with a diameter smaller than 80μ and larger than 275μ . Portal tracts branch progressively from the hilus of the liver to the subcapsular region of the liver (MacSween's Pathology of the Liver, Burt *et al.*, 2012) and because of this, relationships between the bile duct, portal vein and hepatic artery can be altered depending on their position. For example, in human liver in the region beneath the capsule up to 30% of portal tracts can lack the portal vein leaving only a ‘dyad’ of the hepatic artery and bile ducts (Crawford *et al.*, 1998).

To ensure that portal tracts were cut in cross section and to avoid regions where oblique sections of the portal structures would be cut, three portal tracts were selected in each tissue sample that were in an axis perpendicular to the cut surface.

Statistical analysis

Several statistical analyses were conducted to illustrate how specific research questions could be explored using image data.

Principal component analysis—To investigate associations among vessel and duct sizes, PCA was applied to the correlation matrix of dog-specific means of nonnormalized vessel areas using SAS 9.3 (SAS Institute, 2010). PCA is a statistical method that can be used to identify common themes within the available variables, reducing a larger number of variables – in this case, four: areas occupied by the vein, artery, bile duct, lymphatic duct – to a smaller number of components – in this case, two – that may have meaningful interpretations. A goal of PCA is to account for a large portion of the variation present in the larger number of variables using few components. PCA derives eigenvalues and eigenvectors from the correlation matrix of the original set of variables. Components having eigenvalues greater than 1.0 were retained and plotted. Examination of the eigenvectors provided interpretations of each component.

Analysis of variance (ANOVA)—ANOVAs were conducted on percentages of portal tract area occupied by each of the vein, artery, bile duct and lymphatic duct within each section. To account for having multiple sections per portal tract and, for some dogs, evaluation of two portal tracts, mixed effects ANOVAs with a repeated measures structure were used for these analyses using SAS's PROC MIXED. Source of dog, gender, liver lobe were considered fixed factors, dog and tract nested within dog were random factors. In the analyses of three sources of dog, contrasts were used to compare each pair of sources. Means and standard errors were estimated by least squares from within the ANOVA.

Results and discussion

Liver tissue was selected to illustrate the technique described because it contains several tubular structures in which imaging and area measurements of the lumens would provide information that would be difficult and expensive to obtain by other means. Blood is supplied to the liver from two sources, the hepatic artery, derived from the aortic blood flow and the portal vein that arises from the veins draining the intestines and the spleen. Alterations in hepatic blood flow are determined by the hepatic artery buffer response in which hepatic arterial flow adjusts to alterations in portal vein flow. Consequently, reduction in venous flow leads to an increase in arterial flow. Portal vein flow, however, is not autoregulated, but varies depending on the amount of blood flowing from the intestines. The distributing branches of these vessels are bundled together in a sheath of connective tissue that is separated from the hepatocytes. The portal tract also contains the bile duct which drains bile to the intestine and the lymphatics that carry lymph. Lymph is formed when fluid leaks from blood vessels; it is driven by differences in intravascular blood pressure and protein content. This fluid is carried away by the lymphatics. Collectively, this structure is termed the portal tract. Because of the variation in size and the interrelationship of the structures within the portal tract, we determined that this would be an appropriate model system to demonstrate the usefulness of the technique described here.

Correlation structure of vessel areas

Principal component analysis—The PCA (Fig. 1 and Table 1) helps to describe how individual dogs differ and how areas of the different vessels relate to each other in explaining variability among dogs. This analysis indicated that principal components 1 and 2

together explained 94.2% of the variability present among dogs in the mean areas of the four vessels within portal tracts (Table 1). The sizes of the vein, artery and bile duct were correlated (all three were either larger or smaller in a given dog), whereas the size of the lymphatic duct was independent of the size of the other three structures. The size of the beagles' vein/artery/bile ducts in combination was larger than similar structures when compared to pound dogs and the HS dog (Fig. 1). The HS dog had larger lymphatic ducts than the beagles and pound dogs (Fig. 1). The increase in hepatic lymphatic volume following shock would be expected because in shock dogs outflow veins are constricted causing an increase in hydrostatic pressure within blood vessels, driving fluid out of vessels to form edema fluid (Friedman, 1951). Because of these differences between groupings of canines using absolute data, as well as differences between the sizes of portal tracts in different sites in the livers, we elected to normalize the data by calculating percentages of total area for each vessel in the remaining analyses.

Correlation—For the Beagle group of dogs none of the percent areas of ducts were correlated with the size of the portal tract. For the pound dogs, percent artery was negatively correlated (i.e. the artery occupied a larger percentage of smaller portal tracts and a smaller percentage of larger portal tracts) and percent bile duct was positively correlated with portal tract area (Table 3). However, the biological significance of these relatively modest correlations is unclear. In the HS dog both the vein and bile duct were positively correlated (these structures occupied a larger percentage in larger portal tract areas and a smaller percentage in smaller portal tracts). The lymphatic duct size was negatively correlated with total tract area, occupying a larger percentage in smaller portal tracts and a smaller percentage in larger portal tracts. The correlations suggest that the relative area of individual ducts within the portal tract may be related, sometimes inversely, to the total area of the portal tract.

Beagle controls

Purebred Beagle dogs that had been raised under controlled conditions were selected as a likely uniform population to see if differences in portal tract structures and total volumes could be determined between genders or different liver lobes. As expected, there were only minor differences within this population of purebred dogs (Table 3). Statistically significant differences, where noted, were associated with minor absolute differences with the sample size used here.

On average, the portal vein occupied 90.5% of the area of the portal tracts sampled. Arteries made up 2.5%, bile ducts, 1.7% and the lymphatic space 4.5% of total portal tract area.

Gender comparisons—When lobes were combined, the total area of the portal tracts was significantly smaller in male dogs (23.1 mm²) than in females (27.1 mm²; Table 3). Despite the larger portal tract area in female dogs, the percent of the portal tract area taken up by the portal vein, hepatic arteries and bile ducts were significantly larger in the female dogs (Table 3). These data suggest that there may be a structural difference in the liver of male and female Beagles. However, although statistically significant, the differences are modest and

the clinical significance is not clear with the limited number of samples in this demonstration.

Interlobe comparisons—When male and female dogs were combined and separate lobes were analysed individually, the total portal tract area was larger in the left medial lobe (27.8 mm²) than that of the left lateral lobe (22.2 mm²; Table 4). However, the proportions of the portal tracts filled by the portal vein or hepatic arteries were similar between the two lobes. The relative areas of the bile ducts and the lymphatic ducts were both significantly larger in the medial than in the left lobe. The reason for this difference is not clear. Possibly, there is more compression of the left medial lobe due to contact with adjacent lobes and the gall bladder that stimulate lymph formation compared to the left lateral lobe found at the periphery of the liver.

Gender and lobe differences—The significant ANOVA interactions between gender and lobe (Table 5) suggest that differences between left lateral and left medial lobes are gender-specific. For example, for percent portal vein, female left and medial lobes averaged 93.4% and 89.6%, respectively, but male left lateral and left medial averaged 89.3% and 91.1%, respectively.

In female Beagles, the proportion of the portal tract occupied by the artery was essentially the same in the left lateral lobe and the left medial lobe. However, for males, the artery area was proportionately larger in the left lateral lobe than the medial lobe. In males and females the relative area of the bile duct in the left lateral lobe was considerably smaller than in the left medial lobe; this difference was more pronounced in females than it was in males (Table 5). In females, the proportion of portal duct area occupied by the lymphatic duct in the left lobe was smaller than that of the left medial, whereas in males the lymphatic duct area of left lateral lobe was proportionately greater than that of the left medial lobe. Fluid movement from blood vessels is known to be regulated by the hydrostatic (blood pressure) and oncotic forces (Levick, 2010), therefore, a greater arteriolar flow to this lobe and the associated greater blood pressure perhaps led to a higher volume of lymph production, and was reflected in the larger area of lymphatic ducts in this lobe. Although blood pressure can change in short periods, blood protein levels are unlikely to have changed during the period of our study. The statistical significance of the analyses suggests that the method is capable of determining the kinds of differences measured here but a larger sample size may provide greater clinical relevancy.

Comparison of beagles to dogs from other sources

HS dog—The portal tracts from the individual HS dog that died from accidental trauma-induced lethal loss of blood differed from the mixed-breed pound dogs and the Beagle dogs in two major respects (Table 6). The portal vein area was greatly reduced, as would be expected following blood loss. Typically blood loss leads to shock which generates a redirection of blood from the gastrointestinal tract to the heart, brain and lungs. Thus a smaller diameter portal vein would be expected. Also, there was an increase in the lymphatic duct area. This is likely a result of the species-specific response to shock in dogs which involves constriction of sphincters surrounding the veins draining the liver, causing

congestion and edema formation, which would induce a distending of the lymphatic ducts (Friedman *et al.*, 1951).

Mixed-breed dog comparisons—The area of the portal tracts in the mixed-breed dogs was smaller than that of the Beagles (Table 6). Furthermore, compared to Beagles, the percentage of the portal tract occupied by the portal veins and hepatic arteries was smaller in the mixed-breed dogs, whereas the lymphatic duct percentage was larger in the mixed-breed dogs. Possibly plasma protein levels are lower in pound dogs than in laboratory maintained dogs which could stimulate lymph formation. Bile duct area, as a percentage of the portal tract, did not differ between the Beagles and mixed-breed dogs (Table 6).

3-D reconstruction

Images from 295 sections of a portion of a portal tract from a mixed-breed dog were used to build a 3-D reconstruction (S1, supplemental video). Freeze frames from the video animation of that reconstruction are shown in Figure 2. The parallel branching patterns of the portal vein, hepatic artery and the bile duct can be evaluated in panels A–J (Fig. 2). The close relationship between the branching patterns of the hepatic artery and the bile ducts are especially evident in panel J. For a better visualization of this reconstruction the reader should access the supplemental video S1. This video enables a three dimensional appreciation of the spatial relationships between the main structures within the portal tract. The videos linked below show similar patterns in other portal tracts.

http://www.youtube.com/watch?v=4GHgS_ORxcw

<http://www.youtube.com/watch?v=1biAhHBZAtM>

Conclusion

Using the technique of filling lumens with false colour to evaluate anatomic structures *in situ* at the light microscopic level we have demonstrated that the total and relative area of structures such as blood and lymphatic vessels and bile ducts can be determined. This relatively simple and inexpensive method allows imaging of microscopic structures that are currently beyond the resolution of many more sophisticated techniques. Even greater resolution of smaller calibre branches would be possible by using images of higher magnification. In addition, three dimensional reconstructions of *in situ* microscopic structures can be facilitated which enables a more robust understanding of the spatial relationships of different structures. Using this approach, morphologic analysis of quite small anatomic structures can be accomplished. As these reconstructions are from normal dogs, typical relationships can be established. Similar reconstructions from a larger sample of livers with abnormalities such as HS, congenital vascular disorders or congenital biliary disorders would be useful to better understand the spatial relationships that occur with abnormal development and function. In addition, the technique would be applicable to other biological tissue such as plants. Imaging the lumen of water/nutrient conducting vessels in plants would provide a better functional understanding of how disease might alter those structures and affect water and nutrient flow.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Principal component analysis of canine liver portal tracts. Note: B, Beagle.

Fig. 2. Still frames from a 3-D reconstruction of a canine liver portal tract. See supplemental video for a full 3-D animation. (A) 295 Individual sections of triple-stained tissue that were photographed and aligned in Adobe After Effects. (B) An image of the complete set of vessels and ducts in a canine portal tract compiled from the sections in A with the background digitally cleared and negative space coloured as Blue, portal vein; Red, arteries; Green, bile ducts; Yellow, lymphatics. (C) Portal vein in isolation demonstrating the typical branching pattern of veins. (D) Portal vein and hepatic arteries in isolation demonstrating the close relationship in branching patterns. (E) Portal vein and bile ducts in isolation. (F) Portal vein and lymphatics in isolation. (G) Hepatic arteries alone demonstrating the presence of two arteries in this portal tract. (H) Bile ducts alone demonstrating the typical branching

pattern. (I) Lymphatics alone. (J) Hepatic arteries and bile ducts demonstrating a very closely related branching pattern.

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Table 1

Eigenvectors and eigenvalues for PCA analysis

	<u>Principal component</u>	
	1	2
Eigenvectors		
Vein	0.583	0.087
Artery	0.578	0.115
Bile duct	0.559	−0.003
Lymphatic duct	−0.117	0.990
Eigenvalues	2.8	1.0
Proportion of variance explained	69.7%	24.5%

Table 2

Pearson correlations of total portal tract area to percent of the tract occupied by individual vessels

	Total area		
	Pound dogs	HS dog	Beagles
Vein (%)	0.01	0.46	0.00
Artery (%)	−0.52	0.14	0.07
Bile duct (%)	0.35	0.64	0.07
Lymphatic duct (%)	0.00	−0.64	−0.05

Table 3

Gender comparison of Beagle control dogs with lobes combined

Sex #Dogs #Tracts #Measurements	Males Two Beagles 144		Females Two Beagles 144		p Value
	Three per lobe, two lobes per dog	Mean	Three per lobe, two lobes per dog	Mean	
Vein (%)	90.23	0.13	91.26	0.15	0.034
Artery (%)	2.06	0.03	2.68	0.04	0.006
Bile duct (%)	1.58	0.03	1.96	0.04	0.015
Lymphatic duct (%)	5.18	0.12	3.73	0.10	0.012
Area (mm ²)	23.2	0.4	27.1	0.4	0.010

Table 4

Lobe comparison of Beagle controls with genders combined

Liver lobe #Dogs #Tracts #Measurements	Left lateral Four Beagles		Right medial Four Beagles		<i>p</i> Value
	12	144	12	144	
Mean	Mean	SE	Mean	SE	
Vein (%)	90.58	0.14	90.50	0.15	0.701
Artery (%)	2.86	0.05	2.73	0.04	0.131
Bile duct (%)	1.57	0.03	1.97	0.05	0.006
Lymphatic duct (%)	3.92	0.09	4.99	0.12	0.005
Area (mm ²)	22.2	0.6	27.8	0.2	0.003

Table 5

Interaction of lobe with gender of Beagle controls

Liver lobe #Tracts #Measurements	Females (two dogs)				Males (two dogs)			
	Left lateral		Right medial		Left lateral		Right medial	
	6 72	Mean SE	6 72	Mean SE	6 72	Mean SE	6 72	Mean SE
Vein (%)	93.37	0.12	89.63	0.24	89.31	0.19	91.09	0.20
Artery (%)	2.77	0.06	2.78	0.04	3.42	0.03	2.23	0.04
Bile duct (%)	1.62	0.03	3.28	0.06	1.32	0.04	1.74	0.06
Lymphatic duct (%)	2.66	0.10	5.13	0.18	5.49	0.18	4.87	0.15
Area (mm ²)	25.2	0.8	29.7	0.5	22.6	0.3	26.1	0.5

ANOVA and contrast <i>p</i> values				
Gender × lobe interaction		Female l. lateral vs. male l. lateral		Female rt. medial vs. male rt. medial
Vein (%)	0.005	0.003	0.042	
Artery (%)	0.005	0.011	0.011	
Bile duct (%)	0.006	0.027	0.003	
Lymphatic duct (%)	0.010	0.005	0.386	
Area	0.451		0.032	0.019

Comparison of Beagles to dogs from other sources

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