Published in final edited form as:

Phys Med Biol. ; 66(8): . doi:10.1088/1361-6560/abf203.

Three-dimensional visualization and improved quantification with super-resolution ultrasound imaging - validation framework for analysis of microvascular morphology using a chicken embryo model

pek Özdemir¹, Kenneth Johnson¹, Shelby Mohr-Allen¹, Kara E Peak¹, Victor Varner¹, Kenneth Hoyt^{1,2}

¹Department of Bioengineering, University of Texas at Dallas, Richardson, TX, United States of America

²Department of Radiology, University of Texas Southwestern Medical Center, Dallas, TX, United States of America

Abstract

The purpose of this study was to improve the morphological analysis of microvascular networks depicted in three-dimensional (3D) super-resolution ultrasound (SR-US) images. This was supported by qualitative and quantitative validation by comparison to matched brightfield microscopy and traditional B-mode ultrasound (US) images. Contrast-enhanced US (CEUS) images were collected using a preclinical US scanner (Vevo 3100, FUJIFILM VisualSonics Inc.) equipped with an MX250 linear array transducer. CEUS imaging was performed after administration of a microbubble (MB) contrast agent into the vitelline network of a developing chicken embryo. Volume data was collected by mechanically scanning the US transducer throughout a tissue volume-of-interest in 90 μm step increments. CEUS images were collected at each increment and stored as in-phase/quadrature data (2000 frames at 152 frames per sec). SR-US images were created for each cross-sectional plane using established data processing methods. All SR-US images were then used to reconstruct a final 3D volume for vessel diameter (VD) quantification and for surface rendering. VD quantification from the 3D SR-US data exhibited an average error of $6.1\% \pm 6.0\%$ when compared with matched brightfield microscopy images, whereas measurements from B-mode US images had an average error of $77.1\% \pm 68.9\%$. Volume and surface renderings in 3D space enabled qualitative validation and improved visualization of small vessels below the axial resolution of the US system. Overall, 3D SR-US image reconstructions depicted the microvascular network of the developing chicken embryos. Improved visualization of isolated vessels and quantification of microvascular morphology from SR-US images achieved a considerably greater accuracy compared to B-mode US measurements.

Keywords

super-resolution ultrasound; microbubble contrast agents; microvascular morphology; chicken embryo

1. Introduction

Traditional ultrasound (US) imaging is not overly sensitive to slow blood flow in small vessels, and this motivated in part the development of microbubble (MB) contrast agents. In response to sufficiently high US pressures, MBs resonant and produce backscattered US signals with a nonlinear component. This nonlinearity helps make them differentiable from the surrounding tissue by an increased MB signal-to-tissue ratio, and allows more accurate visualization of small vessels due to the velocity independent MB properties (Averkiou *et al* 2020). MBs have a nontoxic gas core and after intravenous administration, they can be detected with high sensitivity by US imaging. With recent FDA approval of MBs for use in the United States, clinical use is increasing, particularly in patient populations where magnetic resonance imaging (MRI) and computed tomography (CT) contrast agents are contraindicated (Hull *et al* 2017). Contrast-enhanced US (CEUS) is a noninvasive and nonionizing imaging modality that enables an accurate assessment of blood flow and tissue perfusion (Saini and Hoyt 2014). CEUS has therefore been widely used to perform parametric analysis of blood dynamics to assist the diagnosis and treatment monitoring of diseases where tissue perfusion plays an important role (Hoyt *et al* 2015, Ghosh *et al* 2017a, Basavarajappa *et al* 2021).

While blood perfusion is defined by functional properties, CEUS-derived morphological features of microvessels are related to the structural organization of the microvascular networks. The complexity of the underlying microarchitecture can be defined by the number of vessels (NV) and bifurcations, geometric measures of mean vessel diameter (VD) and tortuosity, and microvessel density (MVD) as a measure of compactness. These morphological features are crucial because it is known that some more severe diseases, such as diabetes, begin developing impaired functionality at the capillary level (Tanigaki *et al* 2018, Ghosh *et al* 2019). Other severe diseases, such as cancer, can also trigger neovascularization as the disease progresses. The addition of structural information to the functional analysis of tissue microvascular networks may help improve disease management, particularly in the oncological setting (Hoyt *et al* 2015). Morphological features of microvascular networks can be clinically informative. For example, complex networks may assist in guiding repeat therapies while simpler networks may suggest a successful therapy response (Oezdemir *et al* 2020c). CEUS-derived quantification of microvascular morphology can expand our understanding of the chaotic development of pathologic angiogenesis by which tumors develop their own blood supply to receive nutrients and oxygen from the host vascular system (Folkman 2016).

Despite the advantages of CEUS, the spatial resolution of any US imaging system is fundamentally limited by diffraction to length scales of approximately half the wavelength of the transmitted beam. The recently introduced super-resolution US (SR-US) imaging technique is based on precise MB localization and has been shown to achieve a remarkably high spatial resolution beyond the diffraction limit (O'Reilly and Hynynen 2013, Christensen-Jeffries *et al* 2015, Errico *et al* 2015). With the use of SR-US imaging, it is now possible to provide functional and structural quantification of microvascular networks (Oezdemir *et al* 2020b). SR-US imaging offers the promise of more accurate identification

of microvascular morphology for the vessels below 50 μm in diameter (Brown *et al* 2020, Christensen-Jeffries *et al* 2020). For the most reliable morphological analysis derived from US studies, volumetric SR-US imaging is critical because microvascular networks inherently exist in three-dimensional (3D) space (Lin *et al* 2017, Heiles *et al* 2019, Zhu *et al* 2019, Harput *et al* 2020). While several important features of microvascular networks can be quantified from 2D and 3D SR-US images, detailed validation of these measurements is challenging.

Most CEUS imaging studies to date have relied on use of vascular flow phantoms for *in vitro* validation (Viessmann *et al* 2013, Espíndola *et al* 2020) and histological biomarkers of MVD or prior knowledge about healthy versus tumor tissue for *in vivo* validation (Gessner *et al* 2012, Rao *et al* 2016, Panfilova *et al* 2019, Chen *et al* 2020). Recently, a CEUS study using a chicken embryo model demonstrated that image-derived morphology metrics (e.g. intervessel distance and microvascular tortuosity) were correlated to local tissue hypoxia (Lowerison *et al* 2020). Herein, we present an automated approach for 3D SR-US imaging and morphological analysis of microvascular networks. SR-US image-derived VD were extracted using an automated thinning algorithm. Accuracy was validated with matched brightfield microscopy images of vessel structures from the same chicken embryo model. The primary novelty of this research lays in improved visualization and validation of an approach to measure microvascular morphology features using 3D SR-US imaging.

2. Materials and methods

2.1. Phantom materials

A tubing material of regenerated cellulose (Dow Corning Corp, Midland, MI) with a 200 μm internal lumen diameter was immersed and secured inside a water filled container. Two different phantom setups were used for US system configuration testing and custom software calibration. In the first experiment, a single straight tube was used to circulate MBs, whereas in the second experiment, two crossing straight tubes were US imaged. With these flow phantoms, scale parameters for a vessel enhancement filter, were optimized using several different US image and focal depths.

2.2. Chicken embryo model

Fertilized white leghorn chicken eggs were obtained from a commercial vendor (Texas A&M University, College Station, TX) and stored in a forced-draft incubator (GQF Manufacturing Company Inc., Savannah, GA) maintained at 37 °C and 60% humidity until Hamburger–Hamilton stage 18 (HH18, 3 d). Eggs were then transferred to an open 3D printed polymer boat with a window created on one of the sides by gluing a thin polyester film (McMaster-Carr, Elmhurst, IL). A total of eight chicken embryos were used for this study.

2.3. Ultrasound imaging protocol

CEUS imaging was performed using a preclinical US scanner (Vevo 3100, FUJIFILM VisualSonics Inc., Toronto, Canada) equipped with an MX250 linear array transducer. This transducer has a center frequency of 21 MHz (axial resolution $\approx 75 \mu\text{m}$), bandwidth from

15 to 30 MHz, and a 403 μm slice thickness as specified by the vendor (FUJIFILM VisualSonics). A custom MB contrast agent was formulated using established methods (Sirsi and Borden 2009). For the phantom studies, 2 μl of MBs were diluted in 100 ml of degassed water and perfused through the cellulose tubes using a syringe pump at a rate of 2 ml min⁻¹ similar to the blood flow of a chicken embryo (Johnson *et al* 2020, Lowerison *et al* 2020). For the chicken embryo studies, 5 μl of the MB solution was loaded into a pulled glass pipette. MB size and concentration was measured using a microparticle sizing instrument with a 50 μm aperture (Multisizer 4e, Beckman Colter, Brea, CA) (Bellary *et al* 2020). Physical measurement of MB size and concentration found a mean diameter of $2.6 \pm 1.3 \mu\text{m}$ and 1.4×10^{10} MBs ml⁻¹, respectively. While visualizing the embryo with a stereomicroscope (Carl Zeiss Microscopy, White Plains, NY) connected to a digital camera, MBs were slowly introduced into the embryo's vitelline network by microinjection for a duration of about 5 s. Both phantoms and chicken embryos were US imaged as the transducer was mechanically scanned over a volume-of-interest (VOI) in 90 μm step increments using the Vevo Imaging Station (FUJIFILM VisualSonics Inc.). Note that MBs circulated throughout the microvascular network of the chicken embryo until the heart stopped, which could take up to 30 min according to our observations. The volume of the circulating blood in the chick embryo on day 4 was estimated to be $44 \pm 12 \text{ mm}^3$ (Kind 1975). As detailed in figure 1, a series of CEUS images were acquired at each increment and stored as in-phase/quadrature (IQ) data ($N=2000$). All scan parameters, such as time gain compensation and system power (1%) were saved as a preset and fixed for all experimental studies. Volume US datasets from eight different embryos were saved for offline processing and analysis.

2.4. Ultrasound image processing

All image processing, quantification, and visualization was performed using custom MATLAB software (MathWorks Inc., Natick, MA). For each CEUS dataset, a singular value filter (SVF) was first applied to the IQ frames (Mauldin *et al* 2010). SVF separates the stationary speckle signal (tissue) from the nonlinear MB signal of interest. After the removal of tissue signal, MBs were detected by an 8-connected component analysis. A single MB was represented as a connected component. The centroid of each connected component was then determined and mapped as described previously (Ghosh *et al* 2019). The centroid of each connected component was then determined and mapped. At any tissue cross-section, a sequence of 2000 CEUS images were collected and a SR-US image was created by counting the number of MBs found at each pixel location. Lastly, SR-US data from each spatial position were resampled to reconstruct an isotropic 3D SR-US volume.

A multiscale vessel enhancement filter was applied to the 3D SR-US volume to further enhance microvascular structures (Frangi *et al* 1998, Özdemir *et al* 2020a). Note this method has been widely used in CT and MRI angiography to improve diagnostic quality. Vessels were assumed to be tubular structures having a Gaussian profile and detected using the second derivative of Gaussian kernels of a specific dispersion. This parameter helps to detect a range of large and small vessels with wider and thinner Gaussian kernels, respectively. Next, we introduce a vesselness function $V_{\alpha}(\gamma)$ defined as:

$$V_o(\gamma) = \begin{cases} 0 & \text{if } \lambda_2 > 0 \text{ or } \lambda_3 > 0 \\ (1 - e^{-\left(\frac{R_A^2}{2a^2}\right)})e^{-\left(\frac{R_B^2}{2\beta^2}\right)}(1 - e^{-\left(\frac{S^2}{2c^2}\right)}) & \text{elsewise} \end{cases}, \quad (1)$$

where $\lambda_{1,2,3}$ are the eigen values from the Hessian matrix, and:

$$R_B = \frac{|\lambda_1|}{\sqrt{|\lambda_2|\lambda_3|}}, R_A = \frac{|\lambda_2|}{|\lambda_3|}, S = \sqrt{\sum_{j=1}^3 \lambda_j^2}. \quad (2)$$

Here, $V_o(\gamma)$ results in greater values for tubular structures in 3D space and consequently, background noise is suppressed, i.e. nontubular structures. Previous experience has revealed that enhancement of the vascular structures is helpful to improve individual vessel segmentation before performing any diameter measurements. The vesselness function is needed to enhance connectivity between pixels belonging to the same vessel segment. This function improves the process in two main ways. First, it suppresses background structures that are not necessarily tubular. And second, it enhances the tubular structures at different scales so we retain smaller vessels in the image as connected components during binarization. This is requisite for creating an accurate 3D centerline of the vessel segment, which relies on pixel connectivity of binary images. It was not possible to create accurate 3D centerlines without applying this multiscale vessel enhancement due to the presence of non-connected vessel segment components. Segmentation is performed using an adaptive thresholding algorithm (Bradley and Roth 2007). This method finds a local threshold to create a binary volume that represents vessels as foreground voxels. This binary volume was then used for morphological image processing

3D SR-US image-based morphological analysis was automated such that centerlines of the vasculature from the binary volume were extracted and a connected component analysis was performed. A 3D parallel medial-axis-thinning algorithm was then used for centerline extraction (Lee 1994). This method uses a morphological thinning operator that removes the voxels starting from the vessel border until it represents each vessel segment by a voxel-thick line (centerline) in volume space (Kollmannsberger *et al* 2017). Also called a skeleton, the centerlines have the same topology as the original vasculature. Vessel groups containing different vascular branches are defined by 26-voxel connectivity, where connected voxels are defined as two adjacent voxels sharing their faces, edges, or corners. Following the thinning process, a standard pruning algorithm is used to remove all branches shorter than 10 voxels in length (Gonzalez and Woods 2018). Branching points are also removed to create isolated vessel segments and the shortest vessel segments are identified as two-voxel connected centerlines. VD for each point on the centerline were estimated using another morphological operator called the distance transform. The Euclidean distance of each centerline point to the nearest point on the vessel border is computed and multiplied by the voxel size to obtain the VDs for the 3D SR-US data. VD were measured for each centerline voxel and the mean value was taken for the entire vessel segment. Each embryo needed up to 30 min of processing time to create the final skeleton using an Alien Aurora desktop computer (Dell Inc., Round Rock, Texas). Parallel processing was performed to reduce computation time for creation of the SR-US images. Skeletonized microvasculature

and diameter measurements at each centerline point were used for the surface reconstruction and for the extraction of morphological features, namely, number of bifurcations, NV, mean vessel length (VL), and mean vessel tortuosity (VT). VL is computed as the total vessel arc length. VT is the ratio of the VL to the distance between vessel end points. The VDs from microscopy and B-mode US images were measured using the reference method full-width-half-maximum (FWHM) for randomly selected individual vessels within the VOI. A mean FWHM from a single vessel segment is computed as the average of three randomly selected locations on the same vessel segment. Maximum intensity projection (MIP) images are created using the maximum values of the image stack. A control point registration method from the MATLAB Image Processing Toolbox (MathWorks Inc.) was used to apply affine realignment between the three imaging modalities. Control points were selected on the static image (microscopy) and moving image (B-mode US or SR-US MIP) and the spatial transformation was performed to locate the same vessels on all three different imaging modalities.

Finally, surfaces of the individual vessels were reconstructed based on the centerline and diameter values at each centerline point to improve visualization (Koci ski *et al* 2016). The final surfaces of isolated vessels were calculated as follows. First, the gradient vector was calculated to show the proper orientation of the microvasculature at each centerline point. A plane orthogonal to the gradient vector was established to determine surface reference points. Using sequential orthogonal planes, all surface reference points are connected to create right triangles that build the surface of the microvasculature. Visualization improvement was defined by 3D SR-US volume and surface renderings generated from microvascular centerlines to provide the shape information of a specific vessel in volume space, and by assessing the ability to detect vessels with diameters less than 75 μm . Both types of improvements were validated using co-registered brightfield microscopy images that were used to identify the matching vessels by qualitative assessment.

2.5. Performance metrics

The VD parameter was used to quantitatively compare FWHM microscopy, B-mode US, and thinning-based 3D SR-US-derived image diameter measurements. Unpaired *t*-tests between microscopy and 3D SR-US measurements, and between microscopy and B-mode US diameter measurements, were performed to test for differences in VD. Further, average diameter values for individual vessel segments were used to assess the accuracy in VD between the microscopy images and SR-US or B-mode US images using the following metrics:

$$\text{Absolute Error } (\mu\text{m}) \nabla = \left| \text{VD}_{\text{SR-US}} - \text{VD}_{\text{Microscopy}} \right|, \quad (3)$$

and

$$\text{Percent Error}(\%) = \frac{\Delta}{\text{VD}_{\text{Microscopy}}} \times 100. \quad (4)$$

From equation (3), the root-mean-square error (RMSE) for VD measures is computed between those derived from microscopy and each of 3D SR-US images as follows:

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^N (\text{VD}_{\text{SR-US}(i)} - \text{VD}_{\text{Microscopy}(i)})^2}{K}}, \quad (5)$$

where i is the individual diameter measurement index and K is the number of vessel segments that were analyzed. Note that $\text{VD}_{\text{SR-US}}$ was replaced with $\text{VD}_{\text{B-mode}}$ for the B-mode US measurements.

3. Results

A set of 3D SR-US images were acquired from a slowly perfused flow phantom (internal VD of 200 μm) and volume reconstructions are presented in figure 2. Representing a simple vessel structure, the phantom containing a single straight tube was successfully visualized using a MIP from the SR-US data and in 3D space. The skeletonized image illustrates a simplified topology of this single vessel. Using data acquired from the flow phantom with crossing tubes, scaling parameter optimization of the vessel enhancement filter resulted in successful visualization of the 3D SR-US data and the derived topology of the vascular network. As further summarized in figure 2, mean internal VD measurements were found to be $204 \pm 19 \mu\text{m}$ and $206 \pm 12 \mu\text{m}$ for the straight and crossing tube phantoms, respectively. The corresponding VD measurements from the co-registered B-mode US images were $242 \pm 92 \mu\text{m}$ and $188 \pm 59 \mu\text{m}$, respectively.

Phantom studies demonstrated that 3D SR-US morphological analysis with multiscale vessel enhancement and surface reconstruction qualitatively improved the visualization of microvascular structures. A validation study using developing chicken embryos then showed promising results when compared to co-registered brightfield microscopy images. Representative B-mode US and SR-US images from this model system are presented in figure 3, which allows a qualitative comparison between the three different imaging methods for a VOI containing vessels with several varying morphological characteristics. A 3D reconstruction of the SR-US volume data provides a more comprehensive view of the microvascular network in the developing chicken embryo although some structures are disconnected due to the limited number of US frames (i.e. sparse dataset). The spatial gaps between the detected MBs were partially filled after the use of a multiscale vessel enhancement algorithm.

As detailed in figure 4, the SR-US image processing approach removes the unwanted stationary tissue signal while leaving the MB signal of interest for further analysis. Further highlighted, the microvascular network in the B-mode US image is obscured and vessels within are not clearly distinguishable due in part to insufficient spatial resolution and reverberation artifacts. However, the SR-US MIP image in figure 4 clearly depicts the microvasculature that was successfully perfused with MBs. When considering a specific VOI, the smallest vessel resolved was approximately 30 μm in diameter as estimated from the FWHM profiles shown. Here, VD was accurately estimated by the 3D SR-US-derived MIP image when compared to the reference standard brightfield microscopy image.

This specific vessel was isolated from the microvascular network and its surface was reconstructed for a qualitative comparison to the B-mode US image embedded in the width-depth plane. This example illustrates that the spatial resolution of the B-mode US image does not allow visualization of vessels that have a diameter below the US system resolution (about $75\text{ }\mu\text{m}$) while 3D SR-US MIP images can accurately delineate vessels with diameters as small as $30\text{ }\mu\text{m}$. The ability to reconstruct the surface from the 3D SR-US data using the simplified network topology, together with the VD, enables a more complete morphological analysis that can provide information about the complexity, geometric diversity, and the compactness of the underlying microvascular network.

A collection of vessels segments from a brightfield microscopy image and 3D SR-US data are shown in figure 5. The mean diameter of the sample vessels vary and range from 30 to $350\text{ }\mu\text{m}$. As a result of the thinning algorithm, representative samples of isolated vessel topologies containing the morphological information is depicted in volume space. According to the automatically extracted tortuosity measurements, for example, vessel 3 is the most tortuous vessel (14.0) and vessels 1, 5, and 4, have decreasing tortuosity contours of 2.7, 1.9, and 1.5, respectively. Vessel 2 is the simplest vessel with the least tortuosity (0.5), which implies it is a nearly straight segment. The centerlines of different microvascular segments from a developing chicken embryo are presented in figure 6, and allow for an analysis using morphological features from the network topology. Qualitatively, B-mode US images are first improved by the SR-US MIP, which enables visualization of the microvasculature. Further qualitative improvement is achieved after using the multiscale vessel enhancement filter, which provides a more connected microvascular structure in volume space after closer inspection. Improved connectivity resulted in more accurate segmentation and thinning for mapping the microvascular topology in a clear and simplified form of vessel centerlines. These qualitative results are also supported by the quantitative results summarized in figure 7. Diameter measurements from 15 individual vessel segments extracted from different chicken embryo datasets show good agreement between the brightfield microscopy and 3D SR-US images. On the other hand, B-mode US image measurements exhibited large deviations from the true values (i.e. microscopy quantification). Physical limitations of the US system prevent accurate B-mode US measurement of vessels with diameters below the resolution threshold, which is about $75\text{ }\mu\text{m}$ for the transducer used. On average, 3D SR-US had an average VD measurement error of 6.1%, including small vessels, while B-mode US-derived results exhibited an average error of 77.1% for vessels greater than this $75\text{ }\mu\text{m}$ diameter threshold, table 1. The absolute error between the 3D SR-US and microscopy image measurements was $4.8\text{ }\mu\text{m}$ for all 15 vessels studied and only $4.2\text{ }\mu\text{m}$ for the smaller vessels (those less than $75\text{ }\mu\text{m}$ in diameter). The RMSE between the 3D SR-US and microscopy measures was $7.4\text{ }\mu\text{m}$, while the RMSE between the B-mode US and microscopy was found to be considerably higher and $171.6\text{ }\mu\text{m}$. B-mode US image-derived estimations were found to be significantly different than those from the microscopy measurements ($p < 0.01$), which was not true for the 3D SR-US estimates ($p = 0.93$) and supports the previously observed qualitative improvements.

4. Discussion

It was shown that 3D SR-US image reconstructions could be obtained from CEUS data acquired at multiple tissue cross-sections on a preclinical US system without any hardware modifications. VD were quantified using co-registered B-mode US, SR-US, and brightfield microscopy images. An automated 3D SR-US morphological analysis was performed and VD measurements demonstrated greater accuracy when compared to B-mode US image-derived values and use of microscopy image measures as the reference standard. This work helps validate the accuracy of 3D SR-US imaging and quantification of microvascular morphology features.

Structural properties of vascular networks depicted in preclinical and clinical CEUS images have previously been investigated using a single cross-sectional plane (Hoyt *et al* 2015, Özdemir *et al* 2019a). While these studies demonstrated the potential of structural analysis from CEUS images, use of a single plane is a limitation and it was suggested that a 3D CEUS imaging approach could better capture the heterogeneous nature of tumor vascularization in volume space (Hoyt *et al* 2012a, 2012b) and allow isotropic measurements (Mahoney *et al* 2014). This current study provided a 3D morphological analysis of CEUS images and the use of super-resolution techniques provided a more precise and accurate depiction of morphometries extracted from the microvascular network of developing chicken embryos. Further, results were compared with morphological measurements from traditional B-mode US images to highlight the improvements.

Other studies have performed CEUS-derived morphological analysis using US systems with modified hardware components (Shelton *et al* 2015, Rao *et al* 2016, Lin *et al* 2017, Panfilova *et al* 2019). Although these studies validated MVD by histological analysis and found correlations between the CEUS-derived morphological features and tumor growth, a detailed validation of the underlying geometrical measurements was missing. Our study demonstrated a reliable multimodal imaging validation of VD using an *in vivo* embryo model and brightfield microscopy. This current work also showed that 3D SR-US image measurements are highly accurate compared to those derived using traditional B-mode US. With the 3D surface reconstruction of isolated smaller vessels, it is now possible to identify the same vessels for longitudinal studies and to track quantifiable morphological changes to assess disease progression, which typically manifests at the capillary level.

Chicken embryo models have been investigated in a non CEUS imaging study (Huang *et al* 2019). Compared to CEUS, contrast-free US is known to have limited sensitivity to slow blood flow. A recent CEUS study using chicken embryo models have shown that image-derived morphology metrics (e.g. intervessel distance and vascular tortuosity) were correlated to tissue hypoxia (Lowerison *et al* 2020). This particular study compared manually selected vessel profiles from brightfield microscopy to SR-US images acquired with a research US system, where morphological metrics were validated with histological analysis. Our approach in the current study is different from this work in several aspects. First, we used a preclinical US system with a smaller step size to collect the 3D CEUS data. Second, our automated 3D SR-US software measures the vessel centerlines based on a thinning algorithm. Third, and most importantly, we compare the validated

VD measurements with B-mode US images and demonstrate improved accuracy and visualization using 3D SR-US imaging.

Extracting vascular morphometric maps directly from B-mode US images can lead to systematic biases that result in erroneous measurements, particularly from smaller vessels. The effect of such a bias is reduced with use of 3D SR-US data for two principal reasons. First, the higher spatial resolution allows more accurate delineation of microvascular details. Second, 3D reconstructions of microvascular shape improves the geometric accuracy because the microvasculature inherently exists in volume space. The coherent geometry of the microvascular structures from a VOI can be better visualized and measured more accurately (10-fold improvement) based on the percent error in VD using the proposed method. It is important to be able to visualize the vessels with a volume and surface reconstruction because this will provide further details to the quantitative results. While a morphological analysis would use the mean diameter measurement as a metric of monitoring the shape alterations for individual vessels in longitudinal studies, volume and surface reconstructions provide further insight if the measured mean diameter is the same on all of the centerline voxels of the vessel or if there is some variations caused by vessel tapering. As an example of abnormal vessel shape, this information would further help understand chaotic structures of tumor angiogenic networks. Visualization of smaller blood vessels (i.e. those with diameters below the diffraction limit) is only possible with SR-US data as these structures are not readily visible in B-mode US images.

For some longitudinal studies, it is assumed that various types of artifacts are included in baseline and follow-up measurements, and these would contribute equally to erroneous results. It would therefore be beneficial to observe relative changes to capture a disease treatment response or to observe progressive tumor growth. Nevertheless, it is important to characterize the tumor microvasculature as accurately as possible using a noninvasive imaging method so as to appropriately plan a drug delivery and treatment study (Oezdemir *et al* 2020c). Being able to observe any sub-optimal vascularization and reduced tumor blood flow could guide the selection of alternative treatment strategies. For example, if the microvasculature has collapsed due to high intratumoral pressures, the angiogenic network could be normalized first (Hoyt *et al* 2010, Jain 2013). In this case, as a pre-therapeutic necessity, the vessels needed to be dilated to a certain diameter range to deliver any systemic anticancer drug efficiently. The software approach presented here is also appropriate for these types of drug delivery studies.

Compared to traditional B-mode US image analysis, 3D SR-US morphological analysis offers the prospect of measurement repeatability. Instead of single imaging plane, a volumetric representation can assist in repeating measurements for the same subject, which is important for monitoring drug response or status of disease progression. A reproducible quantitative morphometric analysis increases the reliability of the metrics, and this may improve drawing clinically-relevant correlations to the measured quantities. 3D isolation of individual vessels allows for more accurate monitoring of vessel shape alterations. In 2D, the binarization process creates many overlapping vessels, and this may cause the centerline detection to fail. Due to the heterogeneous nature of the tumor burden, a noninvasive 3D morphological analysis is the preferred method.

A potential limitation of the method presented herein is related to motion artifacts. When applying the proposed approach to complex microvascular structures and in the presence of tissue displacement, this method would presumably necessitate a fine motion correction strategy (Hingot *et al* 2017), which was not necessary in the present study due to negligible cardiac motion of the chicken embryo. All imaging was performed in live chicken embryos and it was noted that the heartbeat did tend to slow after introduction of the MB contrast agent. Subsequently, microvascular displacement was negligible relative to MB motion. An optimizer to correct any CEUS frame motion might have revealed any otherwise negligible microvascular pulsations. A multi-stage motion correction strategy for SR-US (Harput *et al* 2018, Özdemir *et al* 2019b) can be performed and will be needed with use of more complex *in vivo* models. Any of these respiratory, cardiac, or transducer motions would have a more profound impact on the skeletonization and thinning processing when devoid of a motion correction strategy. In future studies, we will explore use of motion correction before thinning and any subsequent microvascular measurements. The limitation of elevational plane thickness is minimized by having a smaller step size (90 μm) than the slice thickness (403 μm). This allowed overlap between sequential slices and a 90 μm window for updated and differentiable information between two slices.

As MB size is known to impact SR-US image quality, use of a larger MB contrast agent (Ghosh *et al* 2017b) and more sensitive US imaging strategy (Khairalseed *et al* 2019) may further help improve quantification of the finer microvascular detail in the developing chicken embryo and other tissue environments. Recently, a simultaneous comparison between different transmit pulsing strategies revealed that a combined CEUS approach can improve SR-US imaging by increasing the number of MBs detected during a given acquisition time period (Brown and Hoyt 2019). It was also shown that MB tracking can help improve delineation of smaller vessels (Song *et al* 2018). To use our quantification and visualization approach for SR-US imaging of human subjects, the image processing will need to address additional considerations, including the impact of US attenuation and the ability to detect MBs at tissue depth, acceptable motion correction accuracy, etc. Once adequate SR-US images are produced, our quantification and visualization approach could be applied without any known limitations. Although some promising results have shown that SR-US image can be formed using clinical data (Dencks *et al* 2019), 3D CEUS image acquisition needs to be assessed in a controlled clinical setup due to above noted (and other) potential concerns.

5. Conclusion

The work presented details a novel framework for image processing and validation of 3D SR-US. Morphological analysis of microvascular networks was validated using co-registered brightfield microscopy images. Microvessel segments with a diameter (size) below the B-mode US image resolution were visible in volume and surface reconstructions of the 3D SR-US image data.

Acknowledgments

This research was supported in part by NIH grants R01DK126833, R01EB025841, R01HL145147, and Texas CPRIT award RP180670. The authors thank Dr Shashank Sirsi for fruitful discussion and providing the US contrast

agent used in this study. The authors also acknowledge the Texas Advanced Computing Center at the University of Texas at Austin for providing HPC resources that have contributed to the research results reported within this paper. URL: <http://tacc.utexas.edu>.

References

- Averkiou MA, Bruce MF, Powers JE, Sheeran PS and Burns PN 2020 Imaging methods for ultrasound contrast agents *Ultrasound Med. Biol.* 46 498–517 [PubMed: 31813583]
- Basavarajappa L, Baek J, Reddy S, Song J, Tai H, Rijal G, Parker KJ and Hoyt K 2021 Multiparametric ultrasound imaging for the assessment of normal versus steatotic livers *Sci. Rep.* 11 2655 [PubMed: 33514796]
- Bellary A et al. 2020 Perfusion-guided sonopermeation of neuroblastoma: a novel strategy for monitoring and predicting liposomal doxorubicin uptake *in vivo* *Theranostics* 10 8143–61 [PubMed: 32724463]
- Bradley D and Roth G 2007 Adaptive thresholding using the integral image *J. Graph Tools* 12 13–21
- Brown KG, Ghosh D and Hoyt K 2020 Deep learning of spatiotemporal filtering for fast super-resolution ultrasound imaging *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* 67 1820–9 [PubMed: 32305911]
- Brown K and Hoyt K 2020 Simultaneous evaluation of contrast pulse sequences for ultrafast contrast-enhanced ultrasound imaging 42nd Annu. Inter. Conf. of the IEEE Eng. in Med. & Bio. Soc. (EMBC) 2020 2121–4
- Chen Q, Yu J, Rush BM, Stocker SD, Tan RJ and Kim K 2020 Ultrasound super-resolution imaging provides a noninvasive assessment of renal microvasculature changes during mouse acute kidney injury *Kidney Int.* 98 355–65 [PubMed: 32600826]
- Christensen-Jeffries K, Browning RJ, Tang M-X, Dunsby C and Eckersley RJ 2015 *In vivo* acoustic super-resolution and super-resolved velocity mapping using microbubbles *IEEE Trans. Med. Imaging* 34 433–40 [PubMed: 25265604]
- Christensen-Jeffries K et al. 2020 Super-resolution ultrasound imaging *Ultrasound Med. Biol.* 46 865–91 [PubMed: 31973952]
- Dencks S, Piepenbrock M, Opacic T, Krauspe B, Stickeler E, Kiessling F and Schmitz G 2019 Clinical pilot application of super-resolution US imaging in breast cancer *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* 66 517–26 [PubMed: 30273150]
- Errico C, Pierre J, Pezet S, Desailly Y, Lenkei Z, Couture O and Tanter M 2015 Ultrafast ultrasound localization microscopy for deep super-resolution vascular imaging *Nature* 527 499–502 [PubMed: 26607546]
- Espíndola D, DeRuiter RM, Santibanez F, Dayton PA and Pinton G 2020 Quantitative sub-resolution blood velocity estimation using ultrasound localization microscopy *ex-vivo* and *in-vivo* *Biomed. Phys. Eng. Express* 6 035019
- Folkman J 2016 How is blood vessel growth regulated in normal and neoplastic tissue? *Cancer Res.* 76 2854–6 [PubMed: 27197228]
- Frangi AF, Niessen WJ, Vincken KL and Viergever MA 1998 Multiscale vessel enhancement filtering *MICCAI* pp 130–7
- Gessner RC, Aylward SR and Dayton PA 2012 Mapping microvasculature with acoustic angiography yields quantifiable differences between healthy and tumor-bearing tissue volumes in a rodent model *Radiology* 264 733–40 [PubMed: 22771882]
- Ghosh D, Peng J, Brown K, Sirsi SR, Mineo C, Shaul PW and Hoyt K 2019 Super-resolution ultrasound imaging of skeletal muscle microvascular dysfunction in an animal model of type 2 diabetes *J. Ultrasound Med.* 38 2589–99 [PubMed: 30706511]
- Ghosh D, Xiong F, Sirsi SR, Mattrey R, Brekken R, Kim JW and Hoyt K 2017a Monitoring early tumor response to vascular targeted therapy using super-resolution ultrasound imaging *Proc. IEEE Ultrason Symp* 2017 1–4
- Ghosh D, Xiong F, Sirsi SR, Shaul PW, Mattrey RF and Hoyt K 2017b Toward optimization of *in vivo* super-resolution ultrasound imaging using size-selected microbubble contrast agents *Med. Phys.* 44 6304–13 [PubMed: 28975635]

- Gonzalez RC and Woods RE 2018 Digital Image Processing (Delhi: Pearson Education)
- Harput Set al. 2018 Two-stage motion correction for super-resolution ultrasound imaging in human lower limb IEEE Trans. Ultrason. Ferroelectr. Freq. Control 65 803–14 [PubMed: 29733283]
- Harput Set al. 2020 3D super-resolution ultrasound imaging with a 2D sparse array IEEE Trans. Ultrason. Ferroelectr. Freq. Control 67 269–77 [PubMed: 31562080]
- Heiles B, Correia M, Hingot V, Pernot M, Provost J, Tanter M and Couture O 2019 Ultrafast 3D ultrasound localization microscopy using a 32×32 matrix array IEEE Trans Med. Imag 38 2005–15
- Hingot V, Errico C, Tanter M and Couture O 2017 Subwavelength motion-correction for ultrafast ultrasound localization microscopy Ultrasonics 77 17–21 [PubMed: 28167316]
- Hoyt K, Sorace A and Saini R 2012a Quantitative mapping of tumor vascularity using volumetric contrast-enhanced ultrasound Invest. Radiol 47 167–74 [PubMed: 22104962]
- Hoyt K, Sorace A and Saini R 2012b Volumetric contrast-enhanced ultrasound imaging to assess early response to apoptosis-inducing anti-death receptor 5 antibody therapy in a breast cancer animal model J. Ultrasound Med 31 1759–66 [PubMed: 23091246]
- Hoyt K, Umphrey H, Lockhart M, Robbin M and Forero-Torres A 2015 Ultrasound imaging of breast tumor perfusion and neovascular morphology Ultrasound Med. Biol 41 2292–302 [PubMed: 26116159]
- Hoyt K, Warram JM, Umphrey H, Belt L, Lockhart ME, Robbin ML and Zinn KR 2010 Determination of breast cancer response to bevacizumab therapy using contrast-enhanced ultrasound and artificial neural networks J. Ultrasound Med 29 577–85 [PubMed: 20375376]
- Huang C, Lowerison MR, Lucien F, Gong P, Wang D, Song P and Chen S 2019 Noninvasive contrast-free 3d evaluation of tumor angiogenesis with ultrasensitive ultrasound microvessel imaging Sci. Rep 9 4907 [PubMed: 30894634]
- Hull TD, Agarwal A and Hoyt K 2017 New ultrasound techniques promise further advances in AKI and CKD J. Am. Soc. Nephrol 28 3452–60 [PubMed: 28923914]
- Jain RK 2013 Normalizing tumor microenvironment to treat cancer: Bench to bedside to biomarkers J. Clin. Oncol 31 2205–18 [PubMed: 23669226]
- Johnson K, Özdemir I and Hoyt K 2020 Three-dimensional evaluation of microvascular networks using contrast-enhanced ultrasound and microbubble tracking Proc. IEEE Ultrason Symp 2020 1–3
- Khairalseed M, Özdemir I and Hoyt K 2019 Contrast-enhanced ultrasound imaging using pulse inversion spectral deconvolution J. Acoust. Soc. Am 146 2466 [PubMed: 31671995]
- Kind C 1975 The development of the circulating blood volume of the chick embryo Anat. Embryol 147 127–32
- Koci ski M, Materka A, Deistung A and Reichenbach JR 2016 Centerline-based surface modeling of blood-vessel trees in cerebral 3D MRA Sig. P Algo Arch Arrang. & Applic 2016 85–90
- Kollmannsberger P, Kerschnitzki M, Repp F, Wagermaier W, Weinkamer R and Fratzl P 2017 The small world of osteocytes: connectomics of the lacuno-canalicular network in bone New J. Phys 19 073019
- Lee T-C, Kashyap RL and Chu CN 1994 Building skeleton models via 3D medial surface/axis thinning algorithms CVGIP 56 462–78
- Lin F, Shelton SE, Espíndola D, Rojas JD, Pinton G and Dayton PA 2017 3D ultrasound localization microscopy for identifying microvascular morphology features of tumor angiogenesis at a resolution beyond the diffraction limit of conventional ultrasound Theranostics 7 196–204 [PubMed: 28042327]
- Lowerison MR, Huang C, Lucien F, Chen S and Song P 2020 Ultrasound localization microscopy of renal tumor xenografts in chicken embryo is correlated to hypoxia Sci. Rep 10 2478 [PubMed: 32051485]
- Mahoney M, Sorace A, Warram J, Samuel S and Hoyt K 2014 Volumetric contrast-enhanced ultrasound imaging of renal perfusion J. Ultrasound Med 33 1427–37 [PubMed: 25063408]
- Mauldin FW, Lin D and Hossack JA 2010 A singular value filter for rejection of stationary artifact in medical ultrasound Proc. IEEE Ultrason Symp 2010 359–62

- Oezdemir I, Javed K, Rijal G and Hoyt K 2019a Contrast-enhanced ultrasound imaging of acute changes in pancreatic cancer following targeted hyaluronan treatment Proc. IEEE Ultrason Symp 2019 2303–6
- Oezdemir I, Mohr-Allen S, Peak KE, Varner V and Hoyt K 2020a Three-dimensional super-resolution ultrasound imaging of chicken embryos—a validation framework for analysis of microvascular morphology Proc. IEEE Ultrason Symp 2020 1–4
- Oezdemir I, Peng J, Ghosh D, Sirsi S, Mineo C, Shaul PW and Hoyt K 2020b Multiscale and morphological analysis of microvascular patterns depicted in contrast-enhanced ultrasound images J. Med. Imaging 7 034001
- Oezdemir I, Shaw C, Eisenbrey JR and Hoyt K 2019b Improved quantitative contrast-enhanced ultrasound imaging of hepatocellular carcinoma response to transarterial chemoembolization Proc. IEEE 16th Inter. Symp. on Biom. Imag. (ISBI 2019) 2019 pp 1737–40
- Oezdemir I, Wessner CE, Shaw C, Eisenbrey JR and Hoyt K 2020c Tumor vascular networks depicted in contrast-enhanced ultrasound images as a predictor for transarterial chemoembolization treatment response Ultrasound Med. Biol 46 2276–86 [PubMed: 32561069]
- O'Reilly MA and Hynynen K 2013 A super-resolution ultrasound method for brain vascular mapping Med. Phys 40 110701 [PubMed: 24320408]
- Panfilova A, Shelton SE, Caresio C, van Sloun RJG, Molinari F, Wijkstra H, Dayton PA and Mischi M 2019 On the relationship between dynamic contrast-enhanced ultrasound parameters and the underlying vascular architecture extracted from acoustic angiography Ultrasound Med. Biol 45 539–48 [PubMed: 30509785]
- Rao SR, Shelton SE and Dayton PA 2016 The 'fingerprint' of cancer extends beyond solid tumor boundaries: assessment with a novel ultrasound imaging approach IEEE Trans. Biomed. Eng 63 1082–6 [PubMed: 26394410]
- Saini R and Hoyt K 2014 Recent developments in dynamic contrast-enhanced ultrasound imaging of tumor angiogenesis Imaging Med. 6 41–52 [PubMed: 25221623]
- Shelton SE, Lee YZ, Lee M, Cherin E, Foster FS, Aylward SR and Dayton PA 2015 Quantification of microvascular tortuosity during tumor evolution using acoustic angiography Ultrasound Med. Biol 41 1896–904 [PubMed: 25858001]
- Sirsi S and Borden M 2009 Microbubble compositions, properties and biomedical applications Bubble Sci. Eng. Technol 1 3–17 [PubMed: 20574549]
- Song P, Trzasko JD, Manduca A, Huang R, Kadirvel R, Kallmes DF and Chen S 2018 Improved super-resolution ultrasound microvessel imaging with spatiotemporal nonlocal means filtering and bipartite graph-based microbubble tracking Proc. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 65 149–67
- Tanigaki Ket al. 2018 Hyposialylated IgG activates endothelial IgG receptor FcγRIIB to promote obesity-induced insulin resistance J. Clin. Invest 128 309–22 [PubMed: 29202472]
- Viessmann OM, Eckersley RJ, Christensen-Jeffries K, Tang MX and Dunsby C 2013 Acoustic super-resolution with ultrasound and microbubbles Phys. Med. Biol 58 6447–58 [PubMed: 23999099]
- Zhu Jet al. 2019 3D super-resolution ultrasound imaging of rabbit lymph node vasculature in vivo by using microbubbles Radiology 291 642–50 [PubMed: 30990382]

Figure 1.

Diagram of the experimental design and the data processing strategy used for acquiring a sequence of contrast-enhanced ultrasound (CEUS) images and subsequent generation of the three-dimensional (3D) super-resolution ultrasound (SR-US) maps of microvascular structures. Two thousand frames of ultrasound (US) data was collected for each cross-section as the transducer was mechanically scanned over a defined volume-of-interest (VOI).

Figure 2.

Representative CEUS images from two phantoms containing either a (A) single straight tube or (B) two crossing tubes, all with an internal lumen diameter of $200\ \mu\text{m}$. After acquisition of the CEUS data, (D), (E) SR-US maps were created and displayed as 2-dimensional (2D) projections. Matched (F), (G) 3D SR-US image reconstructions are also shown in volume space. After binary image skeletonization as needed for morphological analysis of microvascular features, (H), (I) centerline segments were colorized to reflect local vessel diameter measurements. (C) Tube diameter measurements (mean $\pm$ standard deviation) were found to be $204 \pm 19\ \mu\text{m}$ and $206 \pm 12\ \mu\text{m}$ for the straight and crossing tubes, respectively.

Figure 3. Co-registered (A) brightfield microscopy, (B) SR-US, and (C) B-mode US images for a defined area (white box), which has been enlarged in (D)–(F), respectively. Panel (G) contains the 3D SR-US volume reconstruction of the microvascular network from a developing chicken embryo.

Figure 4.

Comparison of co-registered (A) B-mode US (A), (B) SR-US, and (C) brightfield microscopy images of a developing chicken embryo. Vessel cross-sectional line plots in (E) and (F) were taken from within the white box and estimated to be approximately $30\ \mu\text{m}$ and below the axial resolution of the US system used for data acquisition. (D) Zoomed-in area from the B-mode US image is shown into the width-depth plane (grayscale map) with the matched 3D SR-US surface reconstruction provided for qualitative comparison.

Figure 5. Individual vessels from a (A) brightfield microscopy image and (B)–(F) 3D SR-US images that were used to make select microvascular morphology measurements. Color bar represents vessel diameter (mm). Units for the 3D renderings are mm.

Figure 6.

CEUS images of five different developing chicken embryos whereby each row shows the intermediate steps of the image processing pipeline for each target. Visual inspection of the B-mode US images (column one) compared to the SR-US MIP (column two), spatially filtered SR-US MIP (column three), and extracted 3D network topologies (column four), reveals a clear representation of tissue microvascular networks using SR-US imaging.

Figure 7.

Comparison of vessel diameter validations using brightfield microscopy, B-mode US, and 3D SR-US images. Quantification from the 3D SR-US image reconstructions are in good agreement with the microscopic measurements. Vessels are ordered by diameter values and it was not possible to measure vessels from the B-mode US images that were smaller than the spatial resolution limits US system used for image acquisition.

Table 1.

Quantification error of vessel diameters when comparing B-mode ultrasound (US) and super-resolution US (SR-US) after comparison to co-registered microscopy-based (reference) measurements.

Imaging technique	Absolute error (μm)	Percent error (%)	<i>p</i> -value
B-mode US	113.9	77.1	0.007
3D SR-US	4.8	6.1	0.9