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Multiscale and multi-modality visualization of angiogenesis in a human breast cancer model

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Abstract

Angiogenesis in breast cancer helps fulfill the metabolic demands of the progressing tumor and plays a critical role in tumor metastasis. Therefore, various imaging modalities have been used to characterize tumor angiogenesis. While micro-CT (μ CT) is a powerful tool for analyzing the tumor microvascular architecture at micron-scale resolution, magnetic resonance imaging (MRI) with its sub-millimeter resolution is useful for obtaining in vivo vascular data (e.g. tumor blood volume and vessel size index). However, integration of these microscopic and macroscopic angiogenesis data across spatial resolutions remains challenging. Here we demonstrate the feasibility of ‘multiscale’ angiogenesis imaging in a human breast cancer model, wherein we bridge the resolution gap between ex vivo μ CT and in vivo MRI using intermediate resolution ex vivo MR microscopy (μ MRI). To achieve this integration, we developed suitable vessel segmentation techniques for the ex vivo imaging data and co-registered the vascular data from all three imaging modalities. We showcase two applications of this multiscale, multi-modality

imaging approach: (1) creation of co-registered maps of vascular volume from three independent imaging modalities, and (2) visualization of differences in tumor vasculature between viable and necrotic tumor regions by integrating μ CT vascular data with tumor cellularity data obtained using diffusion-weighted MRI. Collectively, these results demonstrate the utility of ‘mesoscopic’ resolution μ MRI for integrating macroscopic in vivo MRI data and microscopic μ CT data. Although focused on the breast tumor xenograft vasculature, our imaging platform could be extended to include additional data types for a detailed characterization of the tumor microenvironment and computational systems biology applications.

Keywords

Breast cancer; Imaging; Multiscale; MRI; Micro-CT; MR microscopy

Introduction

Angiogenesis, the process of new blood vessel formation, plays a central role in breast cancer development, invasion and metastasis [1]. Therefore, new approaches for elucidating the angiogenesis pathway and the effects of anti-angiogenic therapies in breast cancer are required to confer survival benefits for patients [2]. Much of our understanding of angiogenesis has been the result of preclinical research [3] that includes a wide array of imaging studies [4]. Microscopic imaging with optical methods has revealed different aspects of tumor angiogenesis [5]. For example, intravital microscopy of window chamber tumor models has provided invaluable insights into vascular function, gene expression, the tumor microenvironment and drug delivery [6–8]. Compared to histology, advanced optical techniques do not require sectioning of the tumor and permit relatively larger spatial coverage. However, since most optical methods cannot image tissue deeper than ~ 1 mm, they are unsuitable for imaging the complete 3D vasculature of tumor xenografts [5]. Furthermore, since angiogenic gene expression has been shown to differ between orthotopically and ectopically (e.g. in window chambers) grown tumors [9], one requires a technique capable of imaging the whole tumor.

In contrast, one can characterize angiogenesis in vivo on the macroscopic scale using computed tomography (CT) [10], or magnetic resonance imaging (MRI) [11, 12]. MRI is a versatile imaging modality for assessing tumor angiogenesis because it permits the repeated measurement of numerous angiogenesis-related parameters such as blood volume, vessel size index and vessel permeability [13]. In vivo CT offers analogous parameters for quantifying in vivo angiogenesis [10, 14], but involves the use of X-ray radiation.

In spite of advances in imaging angiogenesis, integration of imaging data acquired at microscopic and macroscopic spatial scales is challenging for visualization [15] and computational applications [16]. Therefore, the goal of this study was to develop and validate an imaging platform for characterizing breast cancer angiogenesis at multiple spatial scales. We imaged orthotopic MDA-MB-231 human breast cancer xenografts using three imaging modalities: (1) in vivo susceptibility contrast-enhanced MRI at the macroscopic scale ($0.1 \times 0.1 \times 1$ mm³ resolution), (2) ex vivo magnetic resonance

microscopy (μ MRI) at the mesoscopic scale (40 μ m isotropic resolution) and (3) micro-CT (μ CT) at the microscopic scale (8 μ m isotropic resolution) (Fig. 1). Susceptibility contrast-enhanced MRI was used to measure in vivo tumor blood volume, while diffusion-weighted imaging was used to assess tumor cellularity by measuring the apparent diffusion coefficient (ADC). μ CT provided high-resolution 3D images of the whole tumor microvasculature without the need for tissue maceration. Co-registration of these complementary tumor angiogenesis data was facilitated by mesoscopic scale μ MRI, which exhibits both excellent soft tissue contrast (for co-registration with in vivo MRI) and visualization of blood vessel architecture (for co-registration with μ CT) as demonstrated by us earlier [17].

We demonstrate the utility of our multiscale imaging platform by: generating co-registered blood volume maps from macroscopic, mesoscopic and microscopic angiogenesis data acquired using different contrast mechanisms; and integrating tumor vascular data with complementary information on tumor viability to show how the distribution of tumor blood vessels varies between viable and necrotic tissue. The availability of a preclinical multiscale imaging platform can provide a wealth of clinically relevant information about tumor angiogenesis. This includes identification of novel drug targets, development of novel antiangiogenic therapies, tests of therapeutic efficacy, development of novel clinical biomarkers, correlation of genotype with phenotype and 'bioimage' based computational models for cancer systems biology.

Materials and methods

Breast cancer model

Human MDA-MB-231 breast cancer cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO), supplemented with 10 % fetal bovine serum and penicillin–streptomycin (Sigma-Aldrich). Three million cells were orthotopically inoculated in 50 μ l of Hanks balanced salt solution (Sigma-Aldrich) in the left thoracic mammary fat pad of ten female athymic NCr nu/nu mice. All animal studies were performed in accordance with the institutional Animal Care and Use Committee guidelines.

Imaging protocols

Tumor xenografts were imaged with in vivo MRI, ex vivo μ MRI and μ CT. Five tumors were imaged at post inoculation week three (PIW3) and another five at PIW5.

In vivo MRI protocol—For in vivo MRI, mice were anesthetized with a cocktail of ketamine and acepromazine, and the tail veins were cannulated for administration of the superparamagnetic iron oxide contrast agent Feridex[®] (Bayer Healthcare Pharmaceuticals Inc., Wayne, NJ). The mice were placed into a Bruker 9.4 Tesla horizontal bore MRI scanner with an 18 mm solenoid radio frequency (RF) coil and kept under isoflurane anesthesia while the body temperature was maintained at 37 °C using a heating blanket. The xenografts were imaged using the following pulse sequences: (1) Diffusion weighted (DW) images were acquired using a DW spin-echo sequence: echo time (TE) = 26.6 ms, repetition time (TR) = 1,000 ms, number of averages (NA) = 2. One non-DW image and three DW-images were acquired with a b-value of ~ 300 s/mm² and diffusion sensitizing gradient orientations

of [1,0,0], [0,1,0] and [0,0,1]. (2) T2*-weighted (T2*w) images were acquired using a two-dimensional (2D) multi-echo gradient-echo (MGE) sequence with the following parameters: TE = 4.2/7.2/10.2/13.2/16.2/19.2 ms, TR = 800 ms, flip angle (θ) = 90°, NA = 4, in-plane resolution = $100 \times 100 \mu\text{m}^2$, slice thickness = 1 mm. Next, Feridex® (3.73 mg/ml Fe in saline) was injected into the catheterized tail vein at a dose of ~25 mg/kg of Fe. After allowing 5 min for the contrast agent to equilibrate, T2*w scans were acquired to obtain post-contrast images.

Tumor sample preparation for ex vivo imaging—After in vivo MRI, each tumor was perfusion fixed followed by perfusion with the radio-opaque silicon-rubber compound Microfil® (FlowTech Inc., Carver, MA), according to a method described by us in [17]. After letting the Microfil® cure for ~60 min, the tumor and surrounding connective tissue were excised and fixed in zinc formalin. To preserve the anterior-posterior orientation of the tumor following excision, a cotton string soaked in Gd-DTPA (Magnevist®, Bayer Healthcare Pharmaceuticals) enriched water was attached with superglue along the anterior-posterior axis of the tumor. Two days later, excess connective tissue was trimmed from the tumor while leaving the cotton string attached.

MR microscopy (μ MRI) protocol—Twenty-four hours before μ MRI, each tumor was immersed in Phosphate Buffered Saline (PBS) doped with 1 mM Magnevist® for soft tissue contrast enhancement via shortening of the tissue T1. Right before the μ MRI scans, each tumor was placed in an NMR tube filled with Fomblin® (Solvay Solexis, Milano, Italy), which provides background free MRI images because it does not supply any proton signal. Fomblin also prevents sample dehydration. Any trapped air bubbles were removed using a vacuum pump.

The tumors were imaged on a vertical bore Bruker 9.4 Tesla spectrometer using a 10 mm volume RF coil (Bruker BioSpin Corp, Billerica, MA) and a T2*w MGE sequence with the following parameters: TE = 4.9/9.7/14.5/19.3/24.0/28.8 ms, TR = 150 ms, θ = 30°, NA = 14, resolution = $40 \times 40 \times 40 \mu\text{m}^3$.

μ CT protocol—Following μ MRI, samples were imaged by Numira Biosciences (Salt Lake City, UT) on a high-resolution, volumetric μ CT scanner (μ CT40, ScanCo Medical, Brüttisellen, CH). All images were acquired with the following parameters: 8 μm isotropic resolution at 55 kVp, 300 ms exposure time, 2,000 views and 5 frames per view.

Segmentation of tumor blood vessels

Since we were interested in characterizing the tumor vasculature using multiple imaging modalities, it was necessary to develop vessel segmentation pipelines unique to each contrast mechanism. A brief description ensues.

μ MRI vessel segmentation—3D MGE μ MRI images corresponding to the first echo were imported into ImageJ (Rasband, W.S., National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>). Blood vessels appear dark in the μ MRI images due to the lack of mobile water protons in the polymerized Microfil®. The tumor

vasculature was segmented from the background tissue in multiple steps as summarized in Fig. 2a.

First, a tubeness filter was used to determine how ‘tube-like’ each 3D structure in the image was. This filter is based on the computation of a 3D Hessian matrix which is described in detail in [18]. It first convolves the 3D image data with a spherical Gaussian kernel with a standard deviation ‘ σ ’ that sensitizes the filter to tubes or vessels of different radii. We determined optimal values of $\sigma = 0.8, 1.0, 1.2, 1.4$ by visual inspection and based on recommendations for morphological segmentation described in [18]. The outputs of the tubeness filter were combined via a voxel-wise ‘maximum’ operation (i.e. output intensity of voxel i $image3_i = \max(image1_i, image2_i)$) and a conservative threshold was applied to the result, which identified the most ‘tube-like’ structures and resulted in an initial binary vascular tree. Second, for regions that exhibited low contrast between the vasculature and tumor tissue, we enhanced the vessel extraction using a C-means clustering approach. The software MIPAV (Medical Image Processing, Analysis, and Visualization; National institute of Health, Bethesda, MD) was used to automatically classify three to five regions of different image intensities in the raw μ MRI data. A lower threshold was applied to the tubeness output, which identified vessels in low contrast regions as well as ‘non-vascular’ structures in the tumor tissue. The latter were removed by masking out the regions of intermediate image intensity determined by the C-means classes.

The tubeness filter was not sensitive to vessels with diameters greater than approximately four μ MRI voxels, which led to holes in these vessels, and using larger σ would have resulted in an unwanted dilation of smaller vessels. Therefore, in the third step, these vessels were segmented separately using C-means clustering. The class of voxels exhibiting the lowest signal intensity accounted for vessels greater than $\sim 80 \mu\text{m}$ in radius that were not completely segmented in the previous steps. Next, the outputs of all three vessel extraction steps were combined, and a 3D morphological closing operation was performed in ImageJ with a spherical structuring element of one voxel radius to fill remaining holes. Finally, to eliminate small, disconnected objects in the extracted vasculature, 3D regions smaller than six connected pixels determined using a 26-pixel-connected neighborhood were removed.

Due to the excellent soft tissue contrast of μ MRI, it was possible to manually mask non-tumor tissue using the segmentation editor in the Amira[®] visualization platform (VSG Inc., Burlington MA). This tumor mask was multiplied with the resulting binary vasculature to exclude non-tumor vasculature from the surrounding tissue.

μ CT vessel segmentation—Due to the radio-opacity of Microfil[®], all tumor blood vessels appeared bright in the μ CT images and the vasculature was extracted using a similar procedure as for the μ MRI data (Fig. 3a). First, the 3D μ CT data were imported into ImageJ and the tubeness filter applied with $\sigma = 1.0$ and 1.5 . The results were combined via a ‘maximum’ operation and then subjected to a 3D mean filter to fill holes and create a well-connected vascular structure, which was then thresholded to produce a binarized vasculature. This threshold was chosen by visual comparison with the raw data and a compromise was made between segmenting small or low intensity blood vessels, dilating vessels and including noise. The selected threshold resulted in some noise (which was removed in the

final processing step) while resultant vessel diameters were visually consistent with the raw data. To fill holes in large caliber blood vessels, a conservative intensity threshold was applied to the raw μ CT data to segment vessels larger than approximately four μ CT voxels in diameter, which were then combined with the initial binarized vessels. The final binarized vasculature underwent a 3D closing operation and in the final step, regions smaller than 27 connected voxels were removed.

Co-registration of in vivo MRI and μ MRI data

The unprocessed in vivo MRI and μ MRI data were co-registered using Amira[®]. The string fiducial attached to the tumor to indicate the anterior-posterior axis for ex vivo imaging helped to orient the μ MRI dataset relative to the in vivo MRI data. A thorough comparison of the tumor shapes between both datasets was then used to define the final rigid-body transformation for the alignment of the μ MRI tumor volume to the in vivo MRI volume. For the computational execution of the 3D co-registration, a Lanczos interpolation was applied to register the volumes while preserving voxel size. The same transformation was used to co-register the μ MRI vascular data and the tumor mask to the in vivo MRI data. The tumor mask was downsampled to the in vivo MRI spatial resolution.

Co-registration of μ MRI and μ CT data

The co-registration of the unprocessed 3D μ CT data to the transformed μ MRI data was performed in two steps using Amira[®]. (1) Since the string fiducial was visible in both μ MRI and μ CT datasets, it was used for an initial manual alignment of the two image volumes. The transformation was executed using Lanczos interpolation while preserving the voxel size. (2) To improve the manual alignment, two fiducial sets were created by manually placing landmarks on corresponding vessels in the raw μ MRI and μ CT datasets. Following slice-wise inspection of the 3D μ MRI and μ CT data, characteristic vessel bifurcations or conspicuous vessels were identified in both datasets. Between 5 and 10 landmarks were set per tumor and used for the rigid-body landmark registration in which the μ CT data was automatically aligned with the μ MRI data by minimizing the spatial differences between the manually set landmarks in both datasets. The extracted μ CT vasculature was transformed with the same parameters as those obtained from the registration of the raw μ CT data with the μ MRI data.

Assessment of μ MRI and μ CT blood vessel segmentation by optical microscopy

The quality of the vessel extraction was assessed by visual comparison with optical microscopy of whole mount tumor sections. Due to positive contrast of the Microfil in μ CT images, it was possible to segment the superficial tumor blood vessels. The same vessels were then imaged by bright-field microscopy and visually compared to the μ CT data. Superficial vessels were not visible in the μ MRI data since both the microfilled vessels and background appear dark, so intratumoral vessels were compared with optical microscopy images as follows. A 1 mm thick tumor section was optically cleared by sequential immersion in a 50 % (v/v) water-glycerin mixture followed by increasing concentrations every 24 h to 75, 85 and 100 %. A bright-field image of the tumor section was then acquired at 2 $\times$ magnification.

Assessment of μ MRI and μ CT co-registration using blood volume maps

The co-registration of extracted μ MRI and μ CT vasculature was visually evaluated by overlaying the extracted tumor vasculature obtained from both modalities. Moreover, we compared the co-registered blood volume maps from the two modalities. Fractional blood volume (FBV) maps for μ MRI and μ CT data were computed by superimposing a spatial grid of 1 mm isotropic voxels on each co-registered dataset and computing the fractional occupancy of vessels within each grid voxel. Linear regression between the μ MRI and μ CT FBV was used to assess the quality of co-registration and vessel segmentation.

In vivo MRI blood volume maps

It has been shown that the change in the gradient-echo relaxation rate or $R2^*$ (i.e. $1/T2_{\text{post}}^* - 1/T2_{\text{pre}}^*$) is a measure of the total blood volume [19]. Thus, for each voxel of the in vivo MGE images, the signal intensity versus echo-time curve was fit with a mono-exponential function, which yielded pre- and post-contrast $R2^*$ maps. An F-test was used to select only those voxels for which the full exponential model fit the data significantly better ($p < 0.05$) than the reduced constant model. Next we performed a voxel-wise computation of $R2^*$ to create in vivo $R2^*$ maps. Voxels with negative $R2^*$ were excluded from all analyses.

The $R2^*$ maps were converted to FBV maps using an equation derived by Tropres et al. [20]:

$$\text{FBV} = \frac{3}{4\pi} \frac{\Delta R_2^*}{\gamma \Delta \chi B_0} \quad (1)$$

where γ is the gyromagnetic ratio of the ^1H nucleus, $B_0 = 9.4$ T the external magnetic field strength and $\chi = 0.112$ ppm (in cgs units) the difference in magnetic susceptibility of blood before and after contrast agent injection as measured by us earlier [21].

Comparison of multi-modality blood volume measurements

The measurement of the fractional blood volume of the tumors was compared for the three imaging modalities. The in vivo MRI, μ MRI and μ CT FBV maps were computed using the same spatial grid to make comparison of FBV maps possible. For the purposes of this study, the high spatial resolution (8 μm isotropic) μ CT data were treated as the ‘gold standard’ to which the in vivo MRI and μ MRI data were compared. For each imaging modality the FBV values were pooled for PIW3 and PIW5 groups, and quantile–quantile (Q–Q) plots of in vivo MRI and μ MRI versus μ CT FBV measurements were used to examine the similarity between their distributions. Nineteen quantiles (i.e. the 5th, 10th, 15th ... 95th) were computed for the Q–Q plots.

Vascularization in viable and necrotic tumor regions

For a representative tumor sample, we characterized differences in vasculature between viable and necrotic tumor regions of interest (ROI). Since necrotic ROI are known to exhibit a higher apparent diffusion coefficient (ADC) than viable tumor tissue [22], we used an in vivo ADC map to define the necrotic ROI. The ADC map was computed from the in vivo

DW MRI data using DTI-Studio (H. Jiang and S. Mori, Radiology Department, Johns Hopkins University, Baltimore, MD, USA). The MR signal intensity depends on the ADC and the b-value according to:

$$S=S_0 \times e^{-b \times \text{ADC}} \quad (2)$$

where S and S_0 are the MR signal intensities with and without diffusion weighting, respectively. The ADC value along each gradient direction ADC_x , ADC_y and ADC_z was obtained by solving Eq. 2 for ADC for the three DW images acquired with orthogonal diffusion-sensitizing gradients. The final ADC map is a map of the mean diffusivity:

$$\text{ADC}=1/3 (\text{ADC}_x+\text{ADC}_y+\text{ADC}_z) \quad (3)$$

This ADC map was smoothed, and for one 1 mm slice we used C-means clustering to create four classes of ADC values. The class of highest ADC values of $(0.43-0.93) \times 10^{-3} \text{ mm}^2/\text{s}$ was used to define the necrotic ROI. Voxels with high ADC values at the tumor edges were excluded.

A nearest-vessel distance map was computed for the 1 mm thick section of the μCT image corresponding to the ADC slice. This was done in Amira[®] by calculating the Euclidean distance between every μCT voxel within the 1 mm section and the nearest tumor blood vessel. The nearest-vessel distances were then averaged over the z-direction (i.e. the 1 mm thickness) to obtain a mean nearest-vessel distance map, which was overlaid with the necrotic ROI.

Results

Multi-modality vascular data were acquired from ten breast cancer xenografts at complementary spatial scales. However, motion artifact precluded computation of in vivo blood volume maps for one tumor, and two other tumors could not be imaged with μMRI because of sample dehydration and incomplete tumor exsanguination, respectively. Consequently, these tumors were excluded from further analyses.

Tumor blood vessel segmentation from μMRI data

Figure 2b–j illustrates the outputs of the steps necessary to reliably extract the breast tumor vasculature from the 3D μMRI data. Figure 2b shows the raw μMRI data in which the microfilled blood vessels appear dark. Figure 2c shows the result of the “tubeness” filter on the raw μMRI data wherein the most tube-like structures are brightest. The result of the C-means clustering for the raw data is presented in Fig. 2d. Figure 2e demonstrates that a “tubeness” filter alone was not sufficient for extracting all blood vessels because large caliber tumor blood vessels and those in regions of low contrast-to-noise ratio were not segmented by this method. Figure 2f illustrates how the C-means clustering successfully filled holes in tumor blood vessels larger than $\sim 80 \mu\text{m}$ in radius from the raw μMRI data. Figure 2g shows how the combination of C-means clustering and “tubeness” filtering

permitted extraction of blood vessels with low contrast relative to surrounding tumor tissue. In Fig. 2h, the results of these three approaches are overlaid, indicating that each step detects a different subset of vessels. In Fig. 2i one can see how the morphological 3D “closing” operation is able to fill small holes remaining in the extracted tumor vasculature. Figure 2j illustrates the final extracted 3D tumor vasculature.

Tumor blood vessel segmentation from μ CT data

The results of the μ CT vessel extraction are demonstrated in Fig. 3b–f. The raw μ CT data is displayed in Fig. 3b in which the microfilled blood vessels appear bright. Figure 3c shows the result of the tubeness filter on the raw μ CT data, which was then subjected to a 3D mean filter and thresholded to obtain the vasculature shown in red in Fig. 3d. A few remaining holes in large caliber tumor blood vessels remained as illustrated in green. The final extracted tumor blood vessels after 3D morphological closing and small region removal are displayed in Fig. 3e. To better visualize the result of this 3D vessel segmentation pipeline, a volume rendering of the vasculature for a 0.5 mm tumor slice is shown in Fig. 3f.

Assessment of μ MRI and μ CT derived tumor vasculature

We visually evaluated the extraction of tumor blood vessels from μ CT data by directly comparing the resulting vasculature with an optical microscopy image. Figure 4a, b shows that a large number of blood vessels were visible in both the μ CT and bright-field image, while vessels smaller than $\sim 8 \mu\text{m}$ were only visible in the latter. A similar comparison was carried out between the μ MRI-derived tumor vasculature and bright-field microscopy. Figure 4d, e shows the concordance between the vasculature extracted from μ MRI and a bright-field image of a closely matching 1 mm tumor section.

Assessment of μ MRI and μ CT co-registration

The co-registered and extracted μ MRI and μ CT vessels were overlaid as shown in Fig. 4c to permit visual assessment of the co-registration. This overlay illustrates the large degree of overlap between the extracted tumor vasculature from the two datasets and confirms the inter-modality co-registration. In addition to the spatial overlap, we observed dilation of the μ MRI-derived blood vessels compared to both optical microscopy and μ CT-derived vessels (Fig. 4c–e). Small vessels ($<20 \mu\text{m}$ radius) were only visible in the μ CT data and not in the μ MRI data (Fig. 4c).

Additionally, we used linear regression for a voxel-wise comparison of the μ MRI and μ CT-derived FBV (Fig. 4f). The R^2 values for the linear regression ranged between 0.60 and 0.87 ($p < 0.001$) with a mean R^2 of 0.72 ($n = 8$ tumors). The μ MRI-derived FBV overestimated the μ CT-derived FBV for all tumors.

Comparison of multi-modality blood volume measurements

We used the multi-modality FBV maps to compare the blood volume distribution between the different image contrast mechanisms. Figure 5a–f illustrates the spatial distribution of FBV in a tumor slice from all three imaging modalities. There was good spatial agreement of the μ MRI and μ CT FBV maps, but absolute FBV values were higher for the μ MRI data. The mean μ MRI derived FBV was approximately twofold higher for the PIW3 tumors and

threefold higher for the PIW5 tumors, compared to the μ CT FBV (Fig. 5j). Although in vivo MRI-derived FBV maps showed less spatial agreement with those computed from μ MRI and μ CT, all three modalities were capable of visualizing the elevated FBV of the tumor rim compared to the center. The mean in vivo MRI FBV values overestimated those derived from μ CT by a factor of two (Fig. 5j). Q–Q plots of in vivo MRI and μ MRI FBV versus μ CT FBV were strongly linear for the PIW3 tumors. This suggests that the shapes of the in vivo MRI and μ MRI FBV distributions were similar to that of the μ CT FBV distribution (Fig. 5g). Interestingly, the in vivo MRI resembled the μ CT FBV distribution more closely than the μ MRI did. The slope of the in vivo MRI and μ MRI FBV versus μ CT FBV Q–Q plot was >1 , indicating that the former overestimated the latter by a scaling factor. An identical trend was observed for the Q–Q plots of the PIW5 tumors (Fig. 5h). Finally, Fig. 5i illustrates that the FBV assessed with all three modalities decreased with tumor volume.

Vascularization in viable and necrotic tumor areas

An application of our multi-modality imaging approach was the characterization of differences in vascularity between viable and necrotic tumor regions. Figure 6 demonstrates how the necrotic region of a tumor, as identified from in vivo ADC measurements, co-localizes with an area exhibiting large distance from the nearest blood vessels, calculated from the μ CT data (Fig. 6c). In addition, an earlier stage (PIW3) tumor with no identifiable necrotic regions exhibited higher ADC values in areas that were at larger distances from the nearest blood vessels (Fig. 6g).

Discussion

Quantifying angiogenesis-induced changes in the tumor vasculature from medical images is often challenging due to limited contrast-to-noise ratio, image artifacts and spatial resolution limitations. Additionally, each imaging modality has its own unique biophysical contrast mechanism, strengths and weaknesses when it comes to imaging the tumor vasculature as summarized in Table 1. In this work, we exploit the advantages of in vivo MRI and ex vivo μ MRI and μ CT to develop an integrated platform for characterizing angiogenesis at multiple spatial scales in a human breast cancer model.

Due to its high spatial resolution, μ CT is able to resolve blood vessels down to the capillary level [23]. Although the 8 μ m isotropic resolution μ CT employed in this study cannot resolve the smallest capillaries ($\sim 2\text{--}3$ μ m diameter) and angiogenic sprouts, we were able to confirm with optical microscopy that μ CT could recapitulate the 3D tumor vasculature down to ~ 8 μ m diameter vessels. Moreover, our μ CT data is 3D, covers the whole tumor and tumor vessel radii over several orders of magnitude. Therefore, we regarded μ CT as the ‘gold standard’ for 3D blood vessel visualization in whole tissue specimens. Others have also demonstrated the utility of μ CT for quantifying tumor angiogenesis down to the capillary level [23], as well as for validating tumor vascular response to antiangiogenic therapies [24–26]. Moreover, we recently demonstrated μ CT’s usefulness as a tool for validating the accuracy of in vivo susceptibility-contrast based MRI biomarkers of breast cancer angiogenesis such as the FBV, vessel size index and vessel density [21].

In this study, we employed ‘mesoscopic’ scale μ MRI to facilitate co-registration between macroscopic in vivo MRI and microscopic μ CT angiogenesis data. The intermediate ($\sim 40 \mu\text{m}$) spatial resolution of μ MRI in conjunction with its excellent soft tissue contrast facilitates co-registration and distinguishing tumor tissue from normal tissue for ROI analyses. The spatial resolution of μ MRI is limited by the MRI scanner hardware such as the magnetic field and gradient strengths, and by the acquisition time that increases with spatial resolution. In the current study, the intermediate spatial resolution of μ MRI did not permit imaging of vessels smaller than $20 \mu\text{m}$ in radius while the median vessel radius assessed using μ CT was $\sim 10 \mu\text{m}$ [21]. Consequently, partial volume effects led to overestimation of absolute blood vessel size by μ MRI, and the μ MRI-derived FBV values were approximately twofold to threefold higher than those derived from μ CT. However, in spite of this resolution limitation, μ MRI-visible vessels greatly enhance the co-registration of in vivo MRI and μ CT data to enable multiscale imaging of the tumor vasculature. The strength of μ MRI therefore does not lie in resolving small capillaries, but in imaging larger blood vessels while simultaneously providing excellent soft tissue contrast. By exploiting the range of available contrast mechanisms, μ MRI can provide detailed information about the tumor tissue that can complement high-resolution vascular images from co-registered μ CT data.

In vivo MRI is the only imaging modality used in this study that is translatable to patients for repeated and noninvasive tracking of disease progression and anti-angiogenic treatment response [13, 21]. Subject motion and low spatial resolution make the computation of in vivo angiogenic biomarkers and direct spatial correlation with the μ CT data challenging. This was apparent from the co-registered FBV maps of all three modalities wherein the in vivo FBV maps did not exhibit voxel-wise agreement with the ex vivo FBV maps. However, the in vivo FBV maps did show spatial similarities with the ex vivo FBV maps, such as elevated blood volume in the tumor rim compared to the center. In addition, the Q–Q plots validated the usefulness of in vivo FBV as a clinical biomarker for angiogenesis since it resembled the μ CT FBV distribution more closely than the μ MRI FBV distribution, and was sensitive to the shift in FBV values exhibited by tumors at different stages. The shift towards lower FBV values in PIW5 tumors could be due to larger relative areas of necrosis or lower FBV values in viable tumor regions. Compared to μ CT, in vivo MRI overestimated the FBV. A detailed description of the possible biophysical factors (e.g. contrast agent dose, magnetic field strength etc.) contributing to this overestimation can be found in [21]. An extensive comparison of FBV, vessel size and vessel density for μ CT and in vivo MRI in that study showed a similar overestimation of blood vessel size. Finally, that study conclusively demonstrated that relative MRI derived parameters (i.e. those based only on $R2^*$ and $R2$ measurements) may be better biomarkers of angiogenesis than absolute MRI derived measures (e.g. FBV, which requires knowledge of the intravascular susceptibility difference and yields an absolute blood volume with the appropriate unit). In addition, others and we have previously shown that there is a significant correlation between several in vivo MRI angiogenesis biomarkers and their μ CT analogs [21, 27].

Our multi-modality visualization of differences in tumor vascularity between viable and necrotic tumor tissue takes advantage of the high spatial resolution provided by μ CT and the sensitivity of diffusion-weighted MRI to the underlying tissue cytoarchitecture. The integration of these complementary physiologic data helps us understand the relationship

between tumor vascularization and development of necrosis. In our breast cancer model, necrotic tumor areas co-localized with poorly vascularized areas. Moreover, the distance of voxels in the necrotic areas to the nearest blood vessel often exceeded $\sim 200\ \mu\text{m}$, which is the typical diffusion distance of oxygen in tissue [28]. In addition, smaller tumor regions with higher ADC values tended to exhibit larger distances to the nearest vessel, suggesting that necrotic areas were probably developing because of diffusion-limited hypoxia.

Due to the range of available MRI contrast mechanisms, one can foresee several potential applications of this novel imaging platform. For instance, one could relate in vivo measures of vascular function to the tumor vessel architecture by overlaying maps of K_{trans} (i.e. the volume transfer constant between blood plasma and extracellular, extravascular space) obtained from dynamic contrast enhanced (DCE)-MRI with the well-resolved μCT vasculature. This would facilitate interpretation of K_{trans} maps that depend on both blood flow and vessel permeability [29]. We recently demonstrated that the high resolution μCT -derived vasculature can be used in more realistic computational models of tumor blood flow [30] and in ‘bioimage informatics’ approaches for simulating tumor hemodynamics [31]. In conjunction with histology, these developments help bridge the genotype to phenotype divide and are a major step towards creating tumor vasculature-based ‘digital atlases’ for cancer systems biology applications.

More recently, investigators have begun to employ realistic vascular geometries to elucidate the biophysics underlying the different MRI contrast mechanisms [32] as well as improve the accuracy of clinical biomarkers of tumor angiogenesis [33]. For example, Pannetier et al. developed a computational tool for simulating DCE-MRI experiments and validated it by testing it on a 2D mouse brain cortical vascular network acquired using two-photon laser scanning microscopy [34, 35]. We recently extended this approach by incorporating the ‘whole-tumor’ 3D vasculature in biophysical models of dynamic [36] and steady-state susceptibility contrast (DSC or SSC) MRI [32].

The multi-modality nature of the present study necessitated the development of suitable vessel segmentation techniques for the 3D μCT and μMRI data, since each imaging method has its own unique contrast mechanism [37]. For optimal results, our technique incorporated vessel enhancement [18], intensity thresholding and binary morphological operations. Although in some cases, simple intensity thresholding could be applied to isolate vascular structures from the background tumor tissue, we demonstrated that the quality of vessel segmentation (i.e. vessel completeness, elimination of ‘holes’ and isolated voxels) was greatly improved by additional pre- and post-processing steps. One limitation of our vessel segmentation procedure was that it involved the manual determination of segmentation thresholds. Although this made our procedure operator dependent, it resulted in higher-fidelity tumor vasculature than automatic thresholding alone. Both correlation analysis and visual inspection demonstrated that high-quality co-registration was achievable between the μMRI and μCT -derived tumor vasculature. While it would have been ideal to conduct a vessel-to-vessel co-registration for the whole tumor, even the slightest misalignment between the two vascular structures would affect the computed correlation coefficient. Since sample embedding and fixation for μMRI and μCT imaging can introduce tissue deformation as discussed in [17], tissue distortions on the order of the μMRI spatial resolution of $\sim 40\ \mu\text{m}$

would adversely affect the correlation at the original spatial resolution. Collectively, these data demonstrate that although not without limitations, our multiscale multi-modality imaging platform is a valuable tool for studying tumor biology at multiple spatial scales. In addition, although it necessitates the availability of different imaging modalities, the integration of multiscale, multidimensional data is a powerful platform for visualizing and integrating angiogenesis-related data in pre-clinical cancer models.

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Fig. 1.

Multiscale and multi-modality imaging of tumor angiogenesis. **a** *In vivo* MRI with its range of intrinsic and extrinsic contrast mechanisms enables the assessment of *in vivo* angiogenic parameters, but has a spatial resolution limited to the macroscopic scale ($\sim 100\ \mu\text{m}$). **c** Micro-CT (μ CT) allows us to visualize blood vessels directly and quantify angiogenesis down to the microscopic spatial scale ($\sim 8\ \mu\text{m}$) of a capillary. **b** Therefore, here we suggest that MR microscopy (μ MRI) with its intermediate ($\sim 40\ \mu\text{m}$) spatial resolution is useful for integrating angiogenesis data from *in vivo* MRI and μ CT.

Fig. 2.

Illustration of vessel extraction steps for μ MRI data. **a** Flow chart of segmentation procedure. Letters **b–j** indicate output images corresponding to each segmentation step. **b** Slice of raw T2*w μ MRI data (TE = 4.9 ms). Blood vessels appear dark due to presence of waterless Microfil. **c** Result of the tubeness filter after combining the filter outputs using $\sigma = 0.8, 1.0, 1.2, 1.4$ with a maximum intensity arithmetic operation. **d** Result of C-means clustering with five classes. The blue arrow indicates class 1 and the red arrows denote classes 3 and 4, respectively. **e** Result of applying a conservative threshold to the output of

the tubeness filter (*green*), overlaid on the raw μ MRI data. **f** Class 1 from (**d**) shown in *blue*. **g** Result (*red*) of applying a lower threshold on the output of the tubeness filter and masking out regions corresponding to classes 3 and 4 in (**d**). **h** Overlay of (**e**), (**f**) and (**g**). Co-localization of *green* and *red pixels* shown in *yellow*, *red* and *blue* overlap shown in *purple pixels* and all *three colors* add up to *white pixels*. **i** *Yellow* structures are the combination of (**e**), (**f**) and (**g**) while *red voxels* were added by the 3D closing operation. **j** Final extracted blood vessels after 3D region removal of <6 connected voxels. (Color figure online)

Fig. 3.

Illustration of vessel extraction steps for μ CT data. **a** Flow chart of μ CT vessel segmentation procedure. Letters **b–e** indicate output images corresponding to each segmentation step. **b** Slice of raw μ CT data. Blood vessels appear bright due to presence of radio-opaque Microfil. **c** Result of the tubeness filter after combination of the filter outputs using $\sigma = 1.0, 1.5$ with a maximum intensity arithmetic operation. **d** Results (*red*) of the tubeness filter after undergoing a 3D mean filter and thresholding, and thresholded raw data shown in *green*. *Yellow pixels* indicate overlap of the *red* and *green pixels*. Holes in large vessels were filled by thresholding the raw data, as indicated by *green pixels*. **e** Final extracted blood vessels after 3D closing operation and isolated 3D region removal of <27 voxels in *red*. **f**

Volume rendering of the segmented vasculature from a 0.5 mm tumor slice. (Color figure online)

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Fig. 4.

Assessment of vessel extraction and co-registration of μ MRI and μ CT. **a** Visualization of an isosurface view of the tumor vasculature extracted from the volumetric μ CT data in Amira. **b** Fluorescence image of the breast tumor xenograft overlaid with a bright-field image of the microfilled vasculature ($\times 4$). The images were overlaid and processed in ImageJ. Arrows indicate vessels visible in both (a) and (b). **c** Overlay of the μ MRI (green) and μ CT (orange) derived blood vessels of a breast tumor xenograft after co-registration. On the right is a zoomed version wherein white arrows indicate vessels that can easily be seen in the μ MRI and μ CT data. Small vessels were not always visible in the μ MRI image while they were resolved with μ CT as indicated by red arrows, which is most apparent in the tumor rim. **d** Cropped view of a slice through the extracted μ MRI-derived tumor blood vessels. Red arrows indicate structures that are also visible in the bright-field image in (e). **e** Bright-field image of a 1 mm tumor slice. Structures visible in (d) and (e) are indicated by red arrows. **f** Scatter plot of the μ MRI FBV versus the μ CT FBV for a representative breast tumor xenograft with the regression line shown in red. (Color figure online)

Fig. 5. Multi-modality blood volume measurements for tumors at different stages. Co-registered fractional blood volume (FBV) maps for a 1 mm thick tumor slice overlaid on the corresponding anatomical spin-echo images of a PIW3 tumor **a–c** and PIW5 tumor **d–f** computed using in vivo MRI, μ MRI and μ CT, respectively. Zero FBV values are not mapped. **g, h** Q–Q plots of pooled FBV values for five PIW3 (**g**) and three PIW5 (**h**) tumors illustrate the similarity of in vivo MRI (*plus symbol*), μ MRI (*open circle*) FBV distributions

to those of μ CT. **i** Plots of tumor-wise median FBV versus tumor volume. **j** Table of mean FBV values and standard deviation for data pooled for PIW3 and PIW5 tumors

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Fig. 6.

Differences in vascularization between viable and necrotic tumor regions. **a** Smoothed in vivo ADC map of a 1 mm thick tumor slice of a PIW5 tumor wherein the tumor region is delineated in *red*; **b** Classification of tumor tissue by C-means clustering of the smoothed ADC map in (**a**). The class corresponding to the highest ADC values was defined as the necrotic area (visualized by yellow border); **c** *color map* of the Euclidean distance of every voxel from the nearest blood vessel, averaged over the 1 mm tumor slice. A volume rendering of the blood vessels is shown in *gray/white* with the necrotic ROI (outlined in *black*). The necrotic ROI co-localizes with a poorly vascularized tumor region with large distances to the nearest blood vessels; **d**, **e** enlarged regions from the necrotic ROI and rim. **f** In vivo ADC map and **g–i** nearest vessel distance map for an early stage PIW3 tumor. (Color figure online)

Table 1

Overview of the resolution, advantages and disadvantages of the three imaging modalities employed for multiscale angiogenesis imaging

Imaging modality	Spatial resolution	Advantages	Disadvantages
Ex vivo μ CT	30–1 μ m isotropic Current study: 8 μ m	High resolution, 3D volumetric imaging of blood vessels Synchrotron radiation based CT scanners can resolve submicron structures and capillaries, albeit only for small (1–2 mm) samples Whole-tumor coverage without sample sectioning	Successful perfusion of CT contrast agent is necessary Vessel segmentation from images can be challenging Large sized datasets (GB) Requires specialized high-resolution CT scanners
Ex vivo μ MRI	100–20 μ m isotropic Current study: 40 μ m	Excellent soft tissue contrast permits distinguishing tumor tissue and normal tissue Wide array of complementary contrast mechanisms such as diffusion tensor imaging Whole-tumor coverage without sample sectioning	Long data acquisition times Partial volume artifacts for vascular sizes below the available spatial resolution Resolution ultimately limited by scanner hardware and water diffusion
In vivo MRI	~0.1 mm isotropic for pre-clinical imaging Current study: $0.1 \times 0.1 \times 1 \text{ mm}^3$	Several acquisition methods (i.e. pulse sequences) are translatable to clinic Reliable estimates of several 'angiogenesis biomarkers' such as blood volume, vessel density, average vessel size, perfusion, permeability etc. Sensitive to blood vessels that are perfused Repeated measurements possible Excellent soft tissue contrast Versatile: allows acquisition of complementary data, e.g. blood oxygenation level dependent (BOLD) contrast	Vascular parameters are estimated based on a wide variety of tracer-kinetic models Measurement of absolute values challenging due to wide array of noise sources, (e.g. respiration, motion etc.), difficulty in defining an arterial input function and accurately measuring plasma contrast agent concentrations