VascuViz: a multimodality and multiscale imaging and visualization pipeline for vascular systems biology

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Despite advances in imaging, image-based vascular systems biology has remained challenging because blood vessel data are often available only from a single modality or at a given spatial scale, and cross-modality data are difficult to integrate. Therefore, there is an exigent need for a multimodality pipeline that enables ex vivo vascular imaging with magnetic resonance imaging, computed tomography and optical microscopy of the same sample, while permitting imaging with complementary contrast mechanisms from the whole-organ to endothelial cell spatial scales. To achieve this, we developed ‘VascuViz’—an easy-to-use method for simultaneous three-dimensional imaging and visualization of the vascular microenvironment using magnetic resonance imaging, computed tomography and optical microscopy in the same intact, unsectioned tissue. The VascuViz workflow permits multimodal imaging with a single labeling step using commercial reagents and is compatible with diverse tissue types and protocols. VascuViz’s interdisciplinary utility in conjunction with new data visualization approaches opens up new vistas in image-based vascular systems biology.

Recent advances in imaging methods have made image-based systems biology from the cellular to whole-organ spatial scale a reality1–3. Image-based vascular systems biology involves the acquisition and integration of high-fidelity vasculature related data across spatial scales, and its incorporation in computational models for a holistic understanding of the role of the vascular microenvironment (VME) in health and disease. However, this has remained challenging due to the lack of a versatile, multimodality, vascular imaging workflow. This is because labeling blood vessels in preclinical model systems to make them visible in one imaging modality, often precludes the use of other imaging modalities and tissue processing workflows. For example, traditional X-ray or computed tomography (CT)-visible vascular contrast agents4–6 tend to be hydrophobic and polarized, making them invisible on magnetic resonance imaging (MRI), incompatible with immunohistopathology or tissue clearing methods and therefore difficult to image with complementary imaging methods such as light-sheet (LSM) or multiphoton microscopy (MPM). In contrast, the water-soluble or fluorescent intravascular labels frequently used for optical imaging7–10 do not contain the contrast moieties necessary to make them visible in MRI or CT images. This limited blood vessel contrast also prevents integration of three-dimensional (3D) vascular imaging data acquired using one imaging modality with that acquired using complementary (endogenous or exogenous) contrast mechanisms from the same sample. This includes T1-weighted (T1W) and diffusion-weighted (DW) contrast from MRI, bone contrast from CT, immunohistochemistry (IHC) or cellular fluorescent protein expression from optical imaging, all of which provide invaluable structural and functional data for vascular systems biology applications. Although many laboratories have reported the development of new contrast agents11 and imaging techniques12–15 for preclinical vascular applications, these are not ideal for multimodality systems biology workflows. Most vascular contrast agents are optimized for a single imaging modality, tend to require laborious sample preparation16–18 or specialized synthesis protocols19, thereby limiting their widespread adoption20–23. While whole-brain24,25 and whole-organ26 vascular mapping techniques have been extensively reported, they often include tissue-destructive approaches27–29 that require specialized sectioning and imaging hardware in conjunction with sophisticated image reconstruction methods to generate 3D maps of the vasculature. Finally, integration of vascular contrasts across imaging modalities and spatial scales spanning several orders of magnitude is often challenging due to the need for externally visible fiducials for image coregistration, and complications that arise from the arboreal topology of the vasculature. Collectively, these hurdles prevent a ‘holistic’ mapping of the VME.

Therefore, we developed an easy-to-use method called VascuViz that circumvents the above-mentioned technical challenges and enables multimodality and multiscale 3D imaging of the vasculature in intact, unsectioned tissues using standard sample preparation protocols and commercially available reagents (please see Supplementary Table 1 for a summary of the advantages of VascuViz compared to other vascular imaging workflows). In VascuViz, we combined a water-soluble CT contrast agent (BriteVu, Scarlet Imaging) with a fluorescently labeled MRI contrast agent (Galbumin-Rhodamine B, BioPAL Inc.) to obtain a compound that makes the macro- and microvasculature simultaneously visible in high-resolution imaging with MRI, CT and optical techniques.
while also being compatible with conventional tissue processing protocols (for example, IHC and tissue clearing). We demonstrate the widespread use of VascuViz for vascular systems biology applications by: (1) conducting concurrent high-resolution 3D MRI, CT and optical imaging of the murine vasculature and integrating these multiscale data with complementary endogenous and exogenous image contrasts from the same sample; (2) showing its compatibility with tissue clearing and conventional histopathology workflows using paraffin-embedded or frozen tissue sections; (3) exploiting multimodality imaging to characterize the VME in a breast cancer model from the whole-tumor to cancer cell scale; (4) visualizing multimodality 3D maps of the neurovascular system in the murine brain; (5) simulating hemodynamic contrast from high-resolution 3D neurovascular data and integrating it with cellular scale maps of astrocytic coverage; (6) multimodality 3D mapping of the vasculature in different murine organ systems and (7) mapping in vivo blood flow changes to ex vivo 3D vasculature data acquired from a murine brain. Collectively, we believe these innovations and their broad applicability will make VascuViz invaluable to researchers exploring questions about the VME, and myriad other image-based vascular systems biology applications.

Results

VascuViz’s multimodality and multiscale vascular imaging pipeline. Figure 1 provides an overview of our multimodality, multiscale imaging and visualization (VascuViz) pipeline. First, a Galbumin-Rhodamine (GalRh–BVu) mixture was prepared by combining a water-soluble CT contrast agent, BriteVu (Scarlet Imaging) with the fluorescently labeled MRI contrast agent, Galbumin-Rhodamine-B (BioPAL Inc.) (Fig. 1a). Next, intravascular labeling was achieved via transcardial fixation followed by perfusion with the GalRh-BVu mixture (Fig. 1b), after which tissues were excised and immersion fixed overnight. After the GalRh–BVu polymerized, tissues were imaged using endogenous (that is, due to intrinsic tissue properties) and exogenous (that is, due to an externally administered label) contrast MRI, CT and optical imaging. MRI images were acquired at 40–100 µm (that is, macroscopic) spatial resolution (Fig. 1c). Then CT imaging of the same sample was performed at 7.5–9 µm isotropic (that is, microscopic) spatial resolution (Fig. 1d). Next, 10–25 µm tissue sections were prepared and labeled using standard histopathology workflows that included IHC as well as hematoxylin and eosin (H&E) staining (Fig. 1e). Additionally, thick tissue sections (>1 mm) were optically cleared (Fig. 1f) and 3D images acquired using MPM or LSM at <1 µm (that is, microscopic) spatial resolution (Fig. 1g). Image-based hemodynamic contrasts (for example, blood flow) were computationally generated (Fig. 1h) using the high-resolution 3D microvascular morphology data. Finally, these multicontrast data at multiple spatial scales were processed and integrated into multi-layered 3D data volumes for vascular systems biology applications (Fig. 1i) in the murine brain, kidney, hind limb, an orthotopic breast cancer model and in bespoke data visualizations.

Concurrent imaging of vasculature and complementary contrasts. To demonstrate the visibility and compatibility of the polymerized GalRh–BVu (that is, polymer) with other image contrast mechanisms, we performed multicontrast imaging of different tissues with MRI, CT and optical microscopy. Gadolinium-induced shortening of the tissue T1 caused positive enhancement of blood vessels on T1W-MRI (Fig. 2a). The intravascular polymer did not interfere with either endogenous soft tissue (Fig. 2a) or DW-MRI contrast as is evident from the fractional anisotropy (FA) map (Fig. 2b) in which white matter regions in a murine brain (for example, corpus callosum) exhibited elevated FA values (>0.6) as expected. Moreover, this approach permitted combining these two complementary MRI contrast mechanisms (Fig. 2c). For CT imaging, positive vascular contrast from radio-opaque BVu (Fig. 2d) did not interfere with the X-ray attenuation of bone (Fig. 2e), permitting these two complementary image contrasts to be acquired simultaneously (Fig. 2f). MPM imaging revealed that the polymer-bearing vasculature (Fig. 2g) did not hinder cryosectioning or subsequent immunofluorescent labeling of frozen murine brain sections for gliarial fibrillary acidic protein (GFAP) expression (Fig. 2h). This enabled the concurrent imaging and visualization of GFAP distribution relative to the neurovasculature as shown in Fig. 2i. Analogously, stably transduced green fluorescent protein (GFP) expressing MDA-MB-231 breast cancer cells (Fig. 2k,l) and red fluorescent polymer-bearing blood vessels (Fig. 2j,l) could be visualized in tissue sections from an orthotopic breast tumor xenograft. In the same tumor sample, one could also image the endogenous second harmonic generation (SHG) signal of collagen (Col) fibers (Fig. 2n,o) and fluorescent tumor blood vessels (Fig. 2m,o) without additional
tissue processing. Epifluorescence microscopy images of murine brain sections labeled with antibodies for smooth muscle actin (Fig. 2q,f) and laminin (Fig. 2f,u) in the green channel along with the polymer-bearing vasculature in the red channel (Fig. 2p,r,s,u) illustrate the compatibility of VascuViz with conventional immunofluorescence workflows. Finally, the polymer-bearing murine tissues could also be optically cleared using the PEGASOS protocol as illustrated in the insets of v–x for the brain (v), kidney (w), and hind limb (x) tissues, respectively. High-resolution 3D vasculature data could be acquired using LSM from optically cleared GalRh–BVu bearing brain (v), kidney (w) and hind limb (x) tissues, and combined with complementary endogenous contrasts such as that from the muscle fibers (x). Scale bars a–f, v–x, 1 mm; g–u, 50 μm. The transparency of the FA map in c was adjusted to enhance the visibility of the soft tissue contrast in the T1W image. d,f, Blood vessel diameters were scaled by x1.5 for data visualization. The CNR of the images in g–i,p,r,v–x was enhanced by normalizing image intensities to 0.1% of their dynamic range followed by 3D median filtering. Images in p–x were enhanced with background subtraction. The upper and lower bounds of the intensity range in merged images i,l,o,r,u were adjusted for visualization purposes without any changes to the original data.

Vascuviz is compatible with conventional histopathology workflows. To evaluate the compatibility of the GalRh–BVu polymer with standard histopathology workflows, we performed H&E staining on both paraffin-embedded and frozen tissue sections excited from mice perfused with it. Although paraffin embedding may quench the polymer’s fluorescence, bright-field microscopy of the same sample could be used to detect polymer-bearing blood vessels as shown in Extended Data Fig. 1a–d. The GalRh–BVu polymer appeared dark brown on H&E images as shown for the vascularized tumor rim (Extended Data Fig. 1a,b), and glomeruli (black arrows) and blood vessels of the kidney (Extended Data Fig. 1c,d). In contrast, the polymer’s fluorescence was preserved when H&E staining was performed on frozen tissue sections (Extended Data Fig. 1e–I). The cytoarchitecture of white matter fiber tracts and surrounding tissue were evident in H&E stained regions of the hippocampus (Extended Data Fig. 1e) and the cortex (CTX) (Extended Data Fig. 1f,g), respectively. H&E staining of tissue sections from a 4T1 breast tumor xenograft (Extended Data Fig. 1i,k) reveal the tumor cytoarchitecture. Extended Data Fig. 1h,j,l show that the red fluorescence from the same polymer-bearing vessels shown in the bright-field images in Extended Data Fig. 1g,i,k was detectable with fluorescence microscopy. These results clearly demonstrated that the vascular contrast agent combination did not interfere with

Fig. 2 | Concurrent imaging of GalRh–BVu bearing murine tissues with MRI, CT and optical contrast mechanisms. a, TIW-MRI of a GalRh–BVu bearing murine brain. b, DW-MRI-derived FA map of the same brain showing elevated (that is, >0.6) values in white matter regions. c, Composite of a and b, illustrating that complementary MRI contrast mechanisms can be simultaneously acquired in the presence of the GalRh–BVu polymer. d,e, Simultaneously acquired blood vessel (d) and bone contrast (e) from a GalRh–BVu bearing hind limb using CT. f, Composite of d and e, showing these contrasts did not interfere with each other. Here blood vessels are rendered in red and the bone in white. The presence of rhodamine in the polymer made blood vessels fluoresce (red channel) in MPM (g–l), epifluorescent microscopy (p–u) and SHG images (m–o). In murine brain tissue sections, the polymer (red channel) (g,p,s) did not interfere with fluorescence from other components such as GFAP labeled astrocytes (green channel) (h,l), smooth muscle actin (SMA) (q,r) or laminin (LAM) (t,u). In MDA-MB-231 breast tumor sections, the polymer (j,m) did not interfere with fluorescence from stably transduced GFP expression of MDA-MB-231 cancer cells (k,l) or the endogenous contrast (cyan channel) from fibrillar Col (n,o). v–x. The GalRh–BVu bearing tissues could be optically cleared using the PEGASOS protocol as illustrated in the insets of v–x for the brain (v), kidney (w), and hind limb (x) tissues, respectively. High-resolution 3D vasculature data could be acquired using LSM from optically cleared GalRh–BVu bearing brain (v), kidney (w) and hind limb (x) tissues, and combined with complementary endogenous contrasts such as that from the muscle fibers (x). Scale bars a–f, v–x, 1 mm; g–u, 50 μm. The transparency of the FA map in c was adjusted to enhance the visibility of the soft tissue contrast in the T1W image. d,f. Blood vessel diameters were scaled by x1.5 for data visualization. The CNR of the images in g–i,p,r,v–x was enhanced by normalizing image intensities to 0.1% of their dynamic range followed by 3D median filtering. Images in p–x were enhanced with background subtraction. The upper and lower bounds of the intensity range in merged images i,l,o,r,u were adjusted for visualization purposes without any changes to the original data.
standard histopathology assessments of tissue cytoarchitecture and provided a complementary vascular ‘tag’ for conventional H&E-based histopathological workflows.

**Multiscale characterization of the VME in a breast cancer model.** We applied our VascuViz pipeline to an orthotopic MDA-MB-231 breast cancer model to enable multicontact characterization of the VME from the whole-tumor to cancer cell spatial scale. Multicontrast imaging included the use of macroscopic MRI for blood vessel, soft tissue and cellularity (that is, necrosis versus healthy) contrast, mesoscopic CT imaging for vascular contrast, microscopic SHG imaging for contrast from Col fibers and MPM for contrast from fluorescent breast cancer cells. The macroscopic MRI data showed the whole-tumor (translucent blue boundary in Fig. 3a) surrounded by large (that is, 30–114 µm diameter) supporting blood vessels. Coregistration between contrast-enhanced TiT-W- and DW-MRI enabled simultaneous visualization of the tumor vasculature with FA (Fig. 3b) and apparent diffusion coefficient (ADC) (Fig. 3c) maps that reflected the cellularity in the VME. The average tumor FA (0.14±0.07) and ADC (0.57±0.18×10^{-3} mm^2 s^{-1}) were lower than that for the surrounding healthy tissue (0.2±0.07 and 1.3±0.34×10^{-3} mm^2 s^{-1}), respectively. Concurrently acquired 3D CT (Fig. 3d) data enabled computation of the intervessel distances in the VME as shown by the Euclidian distance map (EDM) in Fig. 3e. The mean and maximum intervessel distances in the tumor (80±56.5 and 320 µm) were greater than those for surrounding healthy tissue (53±48.8 and 150 µm), and complemented the cellularity data (that is, ADC maps) from DW-MRI (Fig. 3b–c). To directly correlate intervessel distance to cellularity, we divided the 3D EDM map into subregions corresponding to intervessel distances of 0–50, 51–150 and 151–350 µm, respectively (Extended Data Fig. 2a–c). Next, these subregion boundaries were mapped on to coregistered ADC maps (Extended Data Fig. 2d–f) to facilitate their segmentation into rim and central tumor subregions (Extended Data Fig. 2g–h). This approach revealed that ADC for the 151–350 µm EDM regions (5.4±2.2×10^{-3} mm^2 s^{-1}) was significantly elevated (P<0.001) relative to that for 51–150 µm (3.8±2.4×10^{-3} mm^2 s^{-1}) and 0–50 µm EDM regions (4.3±2.8×10^{-3} mm^2 s^{-1}), respectively (Extended Data Fig. 2i). Collectively, these integrated macroscopic and mesoscopic VME data demonstrated that avascular subregions (that is, regions with elevated intervessel distances) were more necrotic (that is, exhibited elevated ADC) relative to subregions that were well-vascularized (that is, regions with low intervessel distances) and nonnecrotic (that is, exhibited lower ADC).

Additionally, the macroscopic MRI and mesoscopic CT data could be integrated with microscopic SHG and MPM measurements for a 25-µm-thick tumor section (Fig. 3f) from the same sample. Figure 3g shows an example of a well-vascularized region of interest (ROI) (red square in Fig. 3f) from the tumor rim, while Fig. 3h shows a relatively avascular region from the tumor center (cyan square in Fig. 3f). To directly relate macro- and mesoscopic VME parameters to those assessed with microscopic SHG and MPM, we coregistered EDM data and the Col fractional area from SHG to the ADC (Fig. 3k) and FA maps (Fig. 3i) as shown in Fig. 3i,j, respectively. Next, we computed parameter distributions as a function of their distance from the tumor boundary (Extended Data Fig. 3a,b). These analyses revealed that the mean ADC and EDM were highest near the tumor center (x=2.5 mm) and lowest at the boundary (x=0.25 mm) (Fig. 3n). FA and Col fractional area (Fig. 3p) profiles showed the opposite trend with the highest values at the boundary and the lowest values near the tumor center. In contrast, both Col fractional area and EDM (Fig. 3m) and ADC and Col fractional area profiles (Fig. 3o) changed in the opposite directions from the tumor boundary to the center. Overall, EDM showed a positive correlation with ADC (R^2=0.43, P=0.0084) (Extended Data Fig. 3c) and was inversely correlated with Col fractional area (R^2=0.35, P=0.0183) (Extended Data Fig. 3d). A significant correlation was also observed between FA and ADC (R^2=0.68, P=0.0002) (Extended Data Fig. 3e), and between FA and Col fractional area (R^2=0.7, P=0.0001) (Extended Data Fig. 3f).

To visualize the potential impact of tumor microenvironmental factors on drug transport, we computed two-dimensional (2D) ‘hierarchical correlation’ plots using tumor boundary-to-center profiles of intervessel distance (that is, EDM) versus Col fractional area (Fig. 3q), EDM versus ADC (Fig. 3r), Col fractional area versus ADC (Fig. 3s) and Col fractional area versus FA (Fig. 3t). These plots helped identify regions of limited drug and nutrient delivery as defined in terms of elevated EDM (that is, intervessel distance), elevated ADC (that is, necrosis) and elevated Col fractional area (that is, extracellular matrix density)—all of which pose barriers to convective and diffusive transport in the tumor microenvironment. For example, near the tumor boundary (that is, 0–0.5 mm), intervessel distance and Col fractional area were positively correlated as a result of simultaneous decrease in both parameters (Fig. 3m,q), while ADC and intervessel distance (Fig. 3n,r) were negatively correlated. These factors are suggestive of efficacious (denoted by ‘+’) drug delivery in this region due to increased vascular density (that is, reduced EDM) and a concurrent reduction in Coll fiber density. In contrast, between 0.5 and 1.5 mm from the tumor boundary, one observes that elevated intervessel distance was negatively correlated with Col fractional area (Fig. 3m,q) and positively correlated with ADC (Fig. 3n,r). Simultaneously, a gradual decrease in Col fractional area was negatively correlated with ADC (Fig. 3o,s) and positively correlated with FA (Fig. 3p,t). These factors indicate intermediate (denoted by ‘–’) efficacy of drug transport in this region due to contrasting decreases in vascular density (that is, elevated EDM) and Col fiber density. Finally, for regions at distances >1.5 mm from the...
tumor boundary, the hierarchical correlation plots exhibited further increases in intervessel distance (Fig. 3m) accompanied by a sharp rise in ADC (Fig. 3n), and small increases in Col fractional area (Fig. 3o) and FA (Fig. 3p). Collectively, these observations suggest that this region will be characterized by poor (denoted by ‘−’) drug delivery attributable to necrosis (that is, elevated ADC) and enhanced barriers to convective (that is, stagnant Col fractional area) and diffusive transport (that is, elevated EDM).

Visualizing multimodality data for neurovascular systems biology. We demonstrated the feasibility of using the VascuViz pipeline to generate complementary multiresolution, multimodality 3D data from the murine brain for neurovascular systems biology investigations (Fig. 4). This included anatomical and DW contrast from macroscopic MRI, vascular contrast from mesoscopic CT and cellular contrast from LSM. Ten representative brain regions were segmented (Fig. 4a) and visualized in 3D: olfactory bulb, CT, caudate putamen, thalamus, amygdala, hypothalamus, hippocampus, striatum, cerebellum and brain stem (Fig. 4a). GalRh–BVu contrast-enhanced T1W-MRI data enabled visualization of large blood vessels (that is, diameters >40 µm) in different brain regions (Fig. 4a). Figure 4b shows the entire 3D microvascular network of the same brain derived from CT data acquired at 7.5 µm spatial resolution wherein blood vessels were scaled according to their mean diameters.
Visualizing multimodality 3D data from the murine brain for multiscale neurovascular systems biology.

We illustrate the integration of these data for the CTX (Fig. 4g–i), cerebellum (Fig. 4j–l), hippocampus (Fig. 4m–q) and thalamus (Fig. 4r–t). The soft tissue contrast from T1W-MRI (40 μm) was used as the anatomical reference (Fig. 4g–i,l,q,r–t) with red, blue and green colors representing diffusion along the medial-lateral, rostral-caudal and dorsal-ventral axes, respectively (Fig. 4i,l,q,t). The creation of these coregistered multisolution, multicontrast volumes permitted the simultaneous assessment of FA and local FV distributions in the cornu ammonis and the dentate gyrus fields of the hippocampus (Fig. 4n,u,v). Comparisons of the coefficient of variation (COV) between the cornu ammonis and the dentate gyrus fields revealed larger FV heterogeneity in cornu ammonis (COV = 0.43) compared to dentate gyrus (COV = 0.39). This correlated with the larger FA heterogeneity in cornu ammonis (COV = 0.53) compared...
to dentate gyrus (COV = 0.49). Finally, to facilitate the integration of macroscopic vascular contrasts with complementary LSM contrasts at the cellular scale, a 1.5-mm tissue section from the same brain was optically cleared and LSM data were acquired from the highlighted ROI (white square) from the CTX (Fig. 4g), cerebellum (Fig. 4j) and thalamus (Fig. 4r), and visualized as maximum intensity projection (MIP) images shown in Fig. 4m,w,x–z, respectively. Figure 4k,y,z illustrates how microscopic vascular contrast acquired with LSM can be complemented with GFAP coverage in the thalamus. Collectively, these results demonstrate the use of VascuViz for generating multicontrast 3D maps of different murine brain regions, and enabling the visualization of complementary information ranging from the spatial scale of the whole brain down to that of individual capillaries, without necessitating additional sectioning or processing of brain tissue.

**Integrating in vivo cerebral blood flow dynamics with 3D neurovascular data.** To further demonstrate the use of the VascuViz pipeline for neurovascular systems biology applications, we mapped in vivo cerebral blood flow (CBF) changes in response to carbogen gas inhalation assessed with dynamic laser speckle contrast (LSC) imaging (7.7 μm spatial resolution), to ex vivo microvasculature and anatomical data acquired using 3D CT imaging (7.4 μm) (Extended Data Fig. 5 and Supplementary Video 1). An anesthetized, head-fixed mouse (Extended Data Fig. 5a) was made to breathe room air for 3 min, followed by carbogen (that is, 95% O2 and 5% CO2) gas for 2 min and room air again for 5 min (Extended Data Fig. 5b), and the functional hyperemic response (that is, CBF increase due to the carbogen gas perturbation) was imaged continuously via a thinned-skull (Extended Data Fig. 5a) using in vivo LSC imaging (Extended Data Fig. 5f–k). Initial 2D intrinsic optical signal (IOS) imaging facilitated the in vivo visualization of small (that is, diameter <40 μm), medium (that is, diameter <60μm) and large (that is, diameter >60μm) cortical blood vessels (Extended Data Fig. 5c,d and Extended Data Video 1). Extended Data Fig. 5f–k depicts the in vivo functional hyperemic response in the same blood vessels at representative time points during baseline, carbogen inhalation and recovery phases of the perturbation model. These functional in vivo data revealed an increase in CBF between 3 and 5 min in response to carbogen inhalation followed by a gradual return to baseline during the next 5 min. A maximum CBF increase of 85% was observed for medium and large blood vessels in contrast to a 45% increase for small blood vessels relative to global mean baseline CBF (Extended Data Fig. 5e). We then euthanized the animal, processed it with the VascuViz workflow (Extended Data Fig. 5i) and imaged the skull and cerebrovasculature with micro-CT and confocal microscopy (<1 μm). Ex vivo CT imaging facilitated 3D visualization of the cranial anatomy and underlying vasculature (Extended Data Fig. 5m–o), as well as coregistration with in vivo CBF maps via cranial and vascular landmarks. As before, blood vessels were clearly visible at multiple spatial scales ranging from the cortical vascular network (Extended Data Fig. 5m–o) down to individual microvessels (Extended Data Fig. 5p,q) in the same sample. We also created a compelling four-dimensional (4D) visualization in which the in vivo functional hyperemic response was mapped on to the 3D anatomical and microvasculature data as shown in Extended Data Fig. 5r–w. In this visualization, one can clearly appreciate the time evolution of the functional hyperemic response to carbogen inhalation, which is even more apparent in the accompanying video (Supplementary Video 1). Collectively, these results demonstrate the use of VascuViz for conducting structural and functional assessments of the neurovasculature by exploiting complementary image contrast derived from the surrounding VME.

**Hemodynamic modeling using VascuViz-derived 3D vascular data.** We demonstrate the feasibility of generating simulated hemodynamic contrasts (for example, blood flow) in subcortical regions (for example, thalamus) of the murine brain (shown in Fig. 4) using an ‘image-based’ computational modeling approach. The microvascular network used for the blood flow simulations is shown in Fig. 5a. It consisted of 12,857 segments and corresponded to a field of view (FOV) of 300 × 300 × 100 μm3. Microscopic resolution (0.33 μm) LSM data revealed that blood vessel diameters ranged from 1.4 to 42 μm with a mean value of 3.2 ± 3.1 μm (Fig. 5b). Before applying our blood flow model to these data, we validated our approach using a widely used and publicly available dataset derived from a 546-segment rat mesentry microvascular network15 (Extended Data Fig. 6a). We observed excellent agreement between our simulated blood flow rates and discharge hematocrit and those reported in Pries et al.15 as indicated by R2 = 0.99 and 0.93, respectively (Extended Data Fig. 6b-c). Our results were also found to satisfy the Fahraeus and phase separation effects that are known to be substantial in the microcirculation15 (Extended Data Fig. 6e,f). Following validation, we applied our blood flow model to the thalamic microvasculature by imposing a pressure differential ranging from 75 mmHg in inlet vessels to 15 mmHg in outlet vessels, and obtained the intravascular pressure distribution shown in Fig. 5c. The resulting blood flow was then visualized on a log scale as shown in Fig. 5d. This image-based simulation resulted in a mean blood flow velocity of 0.7 ± 4.1 mm s−1, which was within the experimentally observed and simulated ranges reported by others for the murine brain (see Supplementary Table 2 for a summary). Our simulated blood flow rates (nl min−1), intravascular pressures (mmHg) and shear stresses (dyne cm−2) were also in excellent agreement with previously reported simulations for the murine brain (Supplementary Table 2).

In our hemodynamic simulations, we accounted for the vascular shrinkage that results from the use of dehydrating reagents during tissue clearing (Fig. 5a). A vessel shrinkage-based scaling factor (V) was defined as the ratio of maximum blood vessel diameter measured from the CT image to that measured from LSM data in the same FOV, and was equal to 1.25 (that is, case I). We also considered the scenario in which the entire tissue and blood vessels shrank proportionately due to optical clearing (that is, case II). To account for this, a second tissue shrinkage-based scaling factor (T) was defined as the ratio of the brain's dimensions in the rostral-caudal and dorsal-ventral directions before and after optical clearing. This yielded a mean T value of 1.67. Collectively, these two cases represented the lower (1.25) and upper bound (1.67) of blood vessel distortions attributable to tissue handling, processing and dehydration caused by optical clearing. Comparison between the blood flow and hematocrit distributions for case I (V, T) and case II (T) are presented in Fig. 5e,f, respectively. We found that the mean diameter of large vessels (that is, diameters >8 μm) in case II (13 ± 6.3 μm) was slightly greater than those for case I (12.5 ± 5.1 μm). Consequently, this resulted in an elevated mean blood flow for case II (7.0 ± 57.4 nl min−1) relative to case I (2.8 ± 18.9 nl min−1). Despite these differences, the mean values of all the simulated parameters were within previously reported ranges for the murine brain (see Supplementary Table 3 for a summary). Figure 5g–j demonstrates the feasibility of combining simulated hemodynamic contrast with complementary contrast (that is, GFAP expression) acquired with LSM at 0.33 μm. We observed that large blood vessels (that is, 25–40 μm diameter) in the neurovascular network exhibited elevated blood flow rates (86.7 ± 250.6 nl min−1) and colocalized with regions of high GFAP coverage (<4%) (Fig. 5i,k). Analogously, small blood vessels (that is, 1.4–8 μm diameters) exhibited lower blood flow rates (0.2 ± 0.9 nl min−1) and colocalized with regions of low GFAP coverage (<4%) (Fig. 5l,j). Collectively, these results demonstrate how the VascuViz pipeline enables image-based simulations of multiple hemodynamic contrasts as well as their integration with complementary LSM contrasts at the microscopic scale.
Fig. 5 | Computationally generated hemodynamic contrast from VascuViz-derived 3D neurovascular data. The polymer-bearing murine brain tissue section was optically cleared and imaged at a high spatial resolution (0.33 μm) using LSM. a, A 300 × 300 × 100 μm³ FOV of the thalamic microvasculature was extracted from the 3D LSM data. b, Diameter map of the microvascular network in a, wherein each blood vessel was scaled and color coded by its mean diameter (1.4–42 μm). c, Map of the simulated intravascular pressure (mmHg) wherein each blood vessel was scaled by the mean diameter and color coded by the average pressure across its end points. d, Map of the simulated blood flow (nl min⁻¹) wherein each blood vessel was scaled by the mean diameter and color coded by the log of the blood flow. e,f, Scatter plots of blood flow (nl min⁻¹) versus blood vessel diameter (μm) (e) and hematocrit versus blood vessel diameter (μm) (f). Data are shown for two cases: case I (yellow circles) used a vessel-based scaling factor (Vₜ = 1.25) and case II (green circles) used a tissue-based scaling factor (Tᵢ) and blood flow (nl min⁻¹). Overlay (−)-location. g, Map of the LSM-derived fractional GFAP volume (%) (0.33 μm) as shown in g–k. g, Map of the LSM-derived fractional GFAP volume (%) corresponding to a z-location 30 μm below the top plane in volume (a). h, Blood flow (nl min⁻¹) map corresponding to the same z-location. g–i. Overlay (i) of the fractional GFAP volume (%) (g) and blood flow (nl min⁻¹) (h). i–k, White boxes in i highlight ROI in which elevated blood flow (nl min⁻¹) colocalized with high fraction of GFAP labeling (>4%), and ROI in which low blood flow (nl min⁻¹) (j) colocalized with low fraction of GFAP labeling (<4%) (k).

Multimodality 3D vascular mapping in other murine organ systems. We showcase how the VascuViz pipeline can also be used to conduct 3D mapping of the vasculature in other organs besides the murine brain, such as the hind limb (Fig. 6a–g) and kidney (Fig. 6h–m) for which public 3D anatomical atlases are not currently available. The soft tissue contrast from T1W-MRI (40 μm) facilitated slice-by-slice anatomical segmentation and manual annotation of these organs (Fig. 6a,h). The hind limb tissue was segmented into ten main muscle groups: tibialis anterior, extensor digitorum longus, soleus, gluteus maximus, biceps femoris anterior, biceps femoris posterior, semitendinosus, rectus femoris, vastus medialis and vastus lateralis as illustrated in the MRI-derived volume rendering shown in Fig. 6b. High-resolution CT images (9 μm) revealed the 3D architecture of the whole hind limb vasculature (Fig. 6c–e) and bone tissue (Fig. 6d,e). A vascular landmark-based coregistration of the macroscopic MRI and mesoscopic CT data facilitated mapping of the murine hind limb vasculature in different muscle groups (Fig. 6c). Figure 6f,g demonstrates the feasibility of using 3D LSM (2.6 μm) to map the muscle fiber morphology using endogenous fluorescence contrast from the same sample. Similarly, kidney tissue was segmented into the CTX, medulla and renal pelvis using the soft tissue contrast from T1W-MRI (40 μm). Next, CT data (Fig. 6i) was coregistered with the T1W-MRI data using the same vascular landmark-based approach to coregister the 3D kidney vasculature (Fig. 6j) from CT with that acquired using T1W-MRI. This approach also enabled the use of tissue masks from T1W-MRI (for example, renal CTX) to visualize vasculature in a given ROI, as shown for the renal CTX (Fig. 6k). Finally, we demonstrated the
feasibility of using ultrahigh-resolution nano-CT (2 μm) and LSM to map the kidney glomerular morphology as illustrated in Fig. 61,m, respectively.

Discussion

Image-based vascular systems biology necessitates a versatile multimodality imaging approach that enables acquisition of vascular data in a single tissue from the macroscopic whole-organ scale to the mesoscopic vascular network scale, down to the microscopic scale of individual cells without requiring additional sample processing for each complementary imaging method used. To achieve this, we developed a method called VascuViz that enables concurrent multimodality, multiscale imaging and 3D visualization of the VME using MRI, CT and optical microscopy in intact, unsectioned tissues.

There have been recent reports of elegant tissue preparation and imaging approaches for microvascular applications in a range of preclinical models1,5,7,9,10,16–18. However, many of these techniques are not suitable for multimodality imaging workflows (as summarized in Supplementary Table 1) as they require specialized sample preparation protocols (for example, resin embedding, cell labeling, optical clearing, decalcification), involve tissue sectioning before imaging or both1,11. The use of unique or bespoke vascular tags and labels (discussed in detail below) often precludes the use of complementary imaging methods due to their deleterious effects on endogenous and exogenous tissue contrast, and tissue sectioning hampers subsequent 3D imaging and histopathological analyses. Collectively, this limits the use of such approaches in multimodality imaging workflows. In contrast, some nondestructive techniques17 do enable high-resolution 3D vascular imaging in tissue samples, but are often limited by their need for specialized hardware. For example, high-resolution X-ray synchrotron imaging16,17 requires a particle accelerator to generate high energy X-rays, which limits its accessibility and widespread use in multimodality imaging workflows.

As shown, VascuViz overcomes these limitations and enables multimodality, multiscale, 3D imaging and visualization of the vasculature with MRI, CT and LSM/MPM in intact, unsectioned tissues. The systemic perfusion of the entire animal during the VascuViz protocol ensured that the vasculature in any organ system could be imaged, and integrated with images of the same sample acquired with complementary (endogenous or exogenous) image contrast mechanisms without the need for additional tissue preparation or imaging hardware. VascuViz expedited multiscale imaging workflows because it did not involve relabeling of the blood vessels before multimodality imaging, IHC or optical clearing. It
also enabled the incorporation of high-fidelity 3D microvascular data in computational biology applications, such as hemodynamic simulations of blood flow in tissues. Finally, VascuViz is applicable for imaging healthy or pathologic vasculature in any preclinical disease model.

Conventional vascular contrast agents and intravital labeling methods have not been amenable to multimodality imaging workflows due to the following limitations. The synthesis of many vascular contrast agents is optimized for maximal contrast-to-noise ratio (CNR) with a single imaging modality or a given contrast mechanism, such as radiopaque compounds for CT, gadolinium chelates for MRI and fluorescent probes for optical imaging, which limits their use with complementary imaging modalities. Recently, a few dual modality (for example, MRI/optical or CT/optical) preclinical vascular contrast agents such as Galbumin-Rhodamine (Biopal, Inc.) and Pu4ii (vQtech) have become commercially available. However, these too have been optimized for initial imaging with MRI or CT followed by fluorescence microscopy and are invisible to other imaging modalities. Additionally, the physiochemical properties of vascular contrast agents such as their lipophilicity, water insolubility, polarization on polymerization, mismixed viscosity for vascular filling, toxicity and burdensome handling have limited their use for multimodality imaging pipelines that culminate in immunohistopathology workflows. Some vascular contrast agents have been shown to leak from perfused organs even in combination with other agents, preventing their use in tissue clearing protocols and subsequent imaging with LSM/MPM. Finally, other characteristics such as the low CNR of intravitral labeling methods (for example, intravitral lectins, anti-CD31 antibodies) in comparison with lumen-filling gels, and the similarity of X-ray attenuation coefficients of radiopaque compounds (for example, Microfil) to bone necessitate additional sample preparation steps such as combining vascular labeling with bone decalcification, combining two or more antibody labels and specialized image processing routines for vascular data extraction.

Here we demonstrated that our GaIRh–BVu combination notably simplified the multimodality vascular imaging workflow in preclinical models by obviating the need to relabel blood vessels differently for each modality. Also, one did not need to expend resources on quality control during synthesis of the polymer’s components or on reproducibility of the conjugated fluorophores since they are commercially available. In contrast to the lengthy sample preparation processes required for intravascular resins (typically 4 days) or knife-edge-scanning-microscopy protocols (typically 1–3 weeks), the tissues bearing polymerized GaIRh–BVu could be concurrently imaged with commercially available MRI, CT or 2D/3D optical microscopy systems within 24 h of fixation, and also be successfully integrated and detected in routine histopathology workflows based on paraffin-embedded or frozen tissue sections.

We also demonstrated the compatibility of GaIRh–BVu polymer with tissue clearing workflows that enabled submicron resolution imaging of the vasculature with LSM/MPM. Moreover, Extended Data Fig. 7 shows successful LSM imaging of a GaIRh–BVu polymer-bearing and optically cleared brain 11 months after sample preparation. This confirms the long-term stability of the polymer and its associated contrast moieties in the vasculature for up to a year. In the future, VascuViz could be adapted to be compatible with tissue clearing protocols geared toward specific organ systems such as the kidney or muscle. The GaIRh–BVu polymer enabled comprehensive mapping of the VME by enabling the integration of 3D vasculature with complementary contrast mechanisms acquired from the same tissue at spatial scales ranging from the macroscopic whole-organ level (for example, DW-MRI) to the microscopic or cellular level (for example, Col fibers, cancer cells). This study reports a multimodality, multiscale pipeline for image-based vascular systems biology that is compatible with such a plethora of ex vivo imaging methods (please see Supplementary Table 1 for a summary of these capabilities and a comparison with other techniques reported in the literature).

We demonstrated the use of VascuViz for multimodality characterization of the VME in an orthotopic MDA-MB-231 breast cancer xenograft. At the whole-tumor (that is, macroscopic) scale, our observations of intratumoral necrosis were consistent with similar DW-MRI based measurements in breast tumor xenografts that showed decreased FA and elevated ADC in these regions. Additionally, analysis at the vascular network (for example, mesoscopic) scale exhibited a strong positive correlation between elevated ADC and EDM, suggestive of necrosis at the tumor center. These observations were consistent with necrosis resulting from diffusion- or perfusion-limited hypoxia due to an abnormal, angiogenic and heterogeneous tumor vasculature. The lack of GFP-expressing tumor cells in the tumor center assessed at the microscopic scale using MPM further corroborated these observations.

Tumor boundary-to-center profiles facilitated comparisons between different image-derived VME parameters across spatial scales. For example, Col fractional area profiles revealed the greatest fiber density at the tumor boundary and lower fiber density toward the center. While heterogeneous distribution of Col fiber density between peripheral and central regions has been observed in different preclinical tumor models, the elevated peripheral Col fiber density in our preclinical breast cancer model is consistent with the well-known invasiveness of the MDA-MB-231 breast cancer cell line. Our whole-tumor analysis showed an inverse correlation between intervessel distance (that is, EDM) and Col fractional area. These observations are consistent with elevated measurements of microvascular and Col fiber density at the tumor boundary in contrast to the tumor center reported in other preclinical studies of breast cancer. Analogously, at the whole-tumor level we observed a positive correlation between FA and Col fractional area profiles, consistent with previous studies implicating the role of Col fibers in modulating the directionality of water diffusion in a similar breast cancer model. Finally, we also observed a negative correlation between ADC and Col fractional area profiles, which might be indicative of the presence of a hypoxic phenotype characterized by large intervessel distances, decreased Col fiber density and elevated ADC at the tumor center.

Efficacious drug and nutrient delivery in the VME requires both perfusive transport in the blood, and diffusive or convective transport into the surrounding extracellular matrix. In solid tumors, these mechanisms are often hindered by the abnormal architecture of the vasculature and/or density of extracellular matrix components (for example, Col fibers). VascuViz made it possible to visualize the mechanistic barriers to drug transport in the tumor microenvironment by spatially correlating empirical measurements of intervessel distance from CT, Col fractional area from MPM and ADC from DW-MRI using hierarchical correlation plots. At a local level (that is, at discrete distances from tumor boundary), this approach revealed that peripheral tumor regions tended to be zones of efficacious delivery, while regions of poor delivery were located closer to the tumor center. In the future, one could use this approach to visualize the response to cancer therapies that target the vasculature and reduce intervessel distance (for example, antiangiogenic therapy) or alleviate Col-mediated solid stress (for example, collagenase therapy) to enhance intratumoral drug delivery. DW-MRI-derived maps of ADC and FA could act as surrogate markers of Col fractional area to further aid in such characterizations and have already been used as therapeutic biomarkers in cancer. Furthermore, such VME data could be incorporated in image-based computational models of drug delivery to explain the role of other microenvironmental factors such as interstitial fluid pressure and fluid flow on the efficacy of different therapeutic strategies. Collectively, these data demonstrate how VascuViz can...
provide mechanistic insights into the barriers to intratumoral drug transport via the integration of multiscale, multimodality imaging data and image-based hemodynamic modeling.

A number of studies using MRI, positron emission tomography and ultrasound imaging have demonstrated that FV can serve as a metric of healthy brain function\(^{27,38}\). Therefore, our ability to directly quantify FV from high-resolution 3D CT data is useful for the structural and functional characterizations of the vasculature in the brain\(^{49}\) as well as other organs\(^{50}\) (for example, kidney, heart). Our FV estimates for the murine brain obtained from CT were in agreement with other CT-based measurements reported in the literature\(^{48}\). Although one cannot quantify the contribution of capillaries at 7.5 µm isotropic spatial resolution, other high-resolution imaging studies have shown that they contribute to less than 1.5% of the FV in the murine brain\(^{49}\). Our multiscale assessment of the murine hippocampus showed that DW-MRI-derived FA, which is sensitive to underlying microstructure, was more heterogeneous than CT-derived FV that represents vascular morphology. This heterogeneity of FA in the hippocampus could be due to the presence of microscopic features such as axons and other cellular components that are known to influence DW-MRI contrast\(^{47}\). Nonetheless, one could use VascuViz to obtain LSM-derived microvasculature distributions for comparison with DW-MRI data to better understand and model the sensitivity of DW-MRI to the underlying microvasculature\(^{56}\). Moreover, high-resolution microvasculature data could also be incorporated in physiologically accurate biophysical models that explain the effect of changes in microvascular geometry on MRI contrast mechanisms\(^{57}\), which remains an underexplored area of research. This would be especially useful for characterizing microvascular changes in applications such as brain tumors\(^{58}\), stroke\(^{59}\) and Alzheimer’s disease\(^{60}\) in which the vascular phenotype is altered.

We demonstrated the feasibility of integrating in vivo functional imaging data with VascuViz-derived ex vivo imaging contrasts in the murine brain. The ability to coregister dynamic functional imaging data (for example, changes in CBF in response to a carbon monoxide gas challenge) with the underlying microvascular network and 3D bone contrast illustrates the applicability of VascuViz for functional and physiological assessments of the neuro-VME (Supplementary Video 1). One could envision using VascuViz to integrate structural and functional imaging data (for example, in vivo oxygen saturation\(^{61}\), vascular permeability\(^{62}\)) to assess vascular remodeling associated with bone regeneration or wound healing\(^{63}\) in preclinical models. In the future, the generation of such integrated 4D (that is, 3D + time) data could provide a more complete characterization of the structural and functional changes in the VME due to disease progression or treatment\(^{45,64,66}\).

We showcased the use of VascuViz in yielding high-fidelity 3D microvascular data from deep (that is, subcortical) regions of the murine brain for ‘image-based’ hemodynamic simulations. Since our image-based hemodynamic modeling approach is modality-agnostic, it would work with high-fidelity 3D vascular network data derived from either LSM of optically cleared samples, or high-resolution CT or MPM data from uncleared tissues. Previous simulations of murine brain blood flow have been performed for the CTX using microvascular network measurements from two-photon microscopy that are limited to a depth of roughly 600 µm. In this study, we demonstrated the feasibility of acquiring high-resolution (0.33 µm) high-fidelity 3D microvascular network data from deep murine brain regions such as the thalamus, and incorporating it in the generation of additional hemodynamic contrast. We presented two approaches for scaling blood vessel diameters and lengths to account for either vascular or gross tissue shrinkage resulting from the use of dehydrating agents during optical clearing of the samples. We found that in both cases, while the simulated hemodynamic data were within the ranges reported in the literature (Supplementary Table 2), the mean perfusion estimated from the vessel-based scaling approach (13 mL min\(^{-1}\) per 100 g) was lower than that for tissue-based scaling approach (40 mL min\(^{-1}\) per 100 g) as well as the ranges reported (40–165 mL min\(^{-1}\) per 100 g) for the murine CTX\(^{37,48}\). This may be due to our choice of zero flow boundary conditions at the capillaries\(^{49}\). When conducting hemodynamic simulations, obtaining a unique blood flow solution for a microvascular network requires the specification of complete boundary conditions, that is, experimental perfusion or pressure measurements at all inlets and outlet vessels as well as discharge hematocrit measurements at all inlet vessels. Large microvascular networks can have hundreds to thousands of blood vessels as inlets and outlets. Unfortunately, such measurements are rare for murine tissues because it is challenging to acquire in vivo data over a large 3D FOV (for example, 500 × 500 × 500 µm\(^3\)), for hundreds to thousands of vessel segments at capillary spatial resolution. While some measurements have been made in mesentery\(^{52,53}\) and cortical\(^{60,61}\) vascular networks, boundary condition measurements for a subcortical network are even more challenging since most in vivo optical imaging techniques are limited to a depth <1 mm from the surface of the brain\(^{12}\). Therefore, to more accurately model tissue perfusion one could optimize inlet/outlet pressures and shear rates with respect to target values assessed from in vivo blood flow measurements\(^{62}\). To this end, one could also use VascuViz to integrate in vivo functional imaging data (for example, blood flow measurements with laser speckle imaging or MPM) with high-resolution ex vivo vasculature data acquired from the same tissue. Since glial cells have been shown to play a major role in regulating CBF\(^{63}\), we also demonstrated the feasibility of combining simulated blood flow data with astrocytic coverage at the microscopic scale assessed with LSM (0.33 µm). Consistent with their role in modulating perfusion\(^{63}\), we observed that large blood vessels (that is, 25–40 µm diameter) exhibiting elevated blood flow rates colocated with regions of high astrocytic coverage in contrast to smaller blood vessels (that is, 4–8 µm diameter) exhibiting lower blood flow rates. Collectively, VascuViz enabled the integration of microvascular data into image-based hemodynamic models and facilitated comparisons between mesoscopic and microscopic aspects of the circulation. We expect this image-based hemodynamic modeling approach to be adaptable to investigations of the structural and function of the circulation in other tissues such as the retina, placenta, heart, liver and lung.

We developed VascuViz to be a multimodality imaging pipeline that exploits the strengths of a wide range of ex vivo imaging approaches and made it applicable to any organ system and preclinical disease model. Although the use of commercially available imaging reagents lowers the barriers to accessibility of the imaging equipment and reproducibility regarding data acquisition and integration, some imaging systems (for example, light-sheet microscopes) might not be as widely available as others (for example, MRI and CT). However, this would not limit the VascuViz’s use as it permits adaptation of the imaging workflow. For example, since blood vessels are simultaneously visible in each imaging modality, the absence of a modality would not hinder VascuViz’s ability to integrate vascular contrast with complementary contrasts at other spatial scales. We were able to demonstrate the strengths of CT imaging for mapping the 3D macro- and microvasculature in whole organs, and its use in multimodal image coregistration via the use of vascular fiducials instead of surface-based fiducials. Moreover, VascuViz provides a range of options for optical imaging of the GalRh–BVu perfused samples ranging from conventional 2D epifluorescence microscopy to 3D MPM, SHG and LSM. Finally, the VascuViz protocol does not limit the size or volume of the tissues that can be imaged. Sample volumes can range from a tissue subregion (for example, a muscle group as shown in Fig. 6f) to whole organs (for example, the hind limb in Fig. 6a–g or the brain in Fig. 4), or an entire organism (for example, whole-mouse). As long as the sample can be accommodated in the gantry of the
imaging system being used, one can acquire and integrate multimodal and multiscale imaging data using VascuViz. As demonstrated here, one could envision the widespread integration of the VascuViz workflow with in vivo functional imaging data (for example, fMRI, positron emission tomography, ultrasound) acquired from the same sample in the near future.

In this paper, we have presented a new multimodality, multiscale vascular imaging pipeline using MRI, CT and optical imaging with an intravascular contrast agent mixture, GalRh–BVu. This method dubbed VascuViz, has direct applications in image-based vascular systems biology of the brain, kidney and hind limb as well as preclinical disease models such as breast cancer. In addition to enabling high-resolution vascular mapping in intact unsectioned tissues, our method provides the ability to integrate vascular data with multiple image contrast mechanisms and spatial scales ranging from individual endothelial cells to the whole-organ. It is our hope that these advances in preclinical vascular imaging in conjunction with the new visualization approaches presented here will open up new vistas for image-based systems biology of the vasculature, and help answer important questions in the broader field of microcirculation and its role in health and disease.

Online content
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Methods
Preparation of the GalRh–BVu mixture. To prepare the GalRh–BVu mixture, one part of the BritéVu CT contrast agent (Scarlet Imaging) was mixed with 4.5 parts of distilled water and 2% w/v BritéVu Enhancer (Scarlet Imaging). The solution was warmed to 60–70°C before removing it from the heating plate and adding the Galbumin-Rhodamine B, a fluorophore conjugated MRI contrast agent (BioPAL Inc.) at a concentration of 0.96 mg ml⁻¹. The final GalRh–BVu mixture was maintained at roughly 40–50°C until it was ready to be perfused into the animal.

Vascular labeling with GalRh–BVu. All animal experiments were conducted in accordance with an approved Johns Hopkins University Animal Care and Use Committee (IHU ACUC) protocol. Mice were housed in barrier cages, under a standard dark/light cycle with ambient temperature and humidity conditions in the Johns Hopkins University animal facility, which is accredited by the American Association for the Accreditation of Laboratory Animal Care, and meets the National Institutes of Health standards as set forth in the ‘Guide for the Care and Use of Laboratory Animals.’ The GalRh–BVu mixture was injected transcardially into the vasculature of three anesthetized orthotopic MDA-MB-231 tumor bearing NCr nu/nu mice, one healthy NCr nu/nu mouse and one orthotopic 4T1-BALB/C tumor bearing mouse. Please see the accompanying protocols document for a detailed perfusion procedure.

MRI. Please see the accompanying protocols document for preimaging sample preparation steps. Samples were imaged on a 9.4T vertical bore MRI scanner (Bruker BioSpin Corp.) using either a 10- or 20-mm radio frequency volume coil and ParaVision (v.5.1) MRI software. T1W images were acquired using a 3D DW GRASE sequence with TE/TR = 4.2/400 ms, four averages and 40-µm isotropic spatial resolution. Diffusion tensor imaging data were acquired using a 3D DW GRASE sequence with TE/TR = 32/800 ms, 12 echoes per excitation, two averages, diffusion gradient duration/separation of 2.8/10 ms and 16 directions with a b value of 1,700 mm² s⁻¹ and 100-µm isotropic spatial resolution, zero-padded to 50 µm during reconstruction.

CT imaging. Please see the accompanying protocols document for preimaging sample preparation steps. CT imaging was performed on a SkyScan 1275 scanner (Bruker) using the following acquisition parameters: 0.5 mm aluminum filter, 55 kVp, 145 µA, 335 ms exposure time, 0.2 rotation step and three averages. The brain and kidney samples were imaged at 7.5-µm isotropic spatial resolution, respectively. 3D CT image reconstruction was performed using the NRecon software (v.1.7.0.4). Ultra-high spatial resolution (2.5 µm) data of the kidney glomeruli were acquired and reconstructed using the nano-CT Xadia Versa 520 scanner at the Biotechnology Resource Center Imaging Facility at Cornell University using the following parameters: 120 kVp, 145 µA, 0.2 rotation step and three averages.

Immunofluorescent labeling and histology. Please see the accompanying protocols document for prelabeling tissue preparation steps. The polymer-bearer brain tissue sections were cut onto silanized slides for immunofluorescent labeling. Astrocytes were labeled with Alexa Fluor 488 conjugated monocular GFAP (GAS) mouse antibody (dilution 1:50, Cell Signaling), vasculature associated smooth muscle with FITC-conjugated α-smooth muscle actin antibody (dilution 1:50, Sigma-Aldrich) and blood vessel endothelium with anti-rabbit laminin antibody (dilution 1:100, Sigma-Aldrich). The secondary antibody used was goat anti-rabbit IgG Alexa Fluor 488 (10 µg ml⁻¹), ThermoFisher to complement rhodamine emission from the GalRh–BVu polymer. H&E staining on parafin-embedded tissues was conducted at the Johns Hopkins Pathology Core facility using their standard pathology workflow.

Tissue clearing. Please see the accompanying protocols document for details of the tissue clearing. The polymer-bearing murine brain, kidney and hind limb samples (1–2 mm) were optically cleared using the PEGASOS method.¹

Ex vivo optical imaging. Tissue sections (≤20µm) were imaged on a Nikon ECLIPSE-TS100 microscope (Nikon Instruments Inc.) at x2, x10 and x40 magnification with the appropriate filters for detecting immunofluorescence.

MPM. Tumor and brain tissue sections (25–50 µm) were imaged on an Olympus laser scanning FV1000 MPE multiphoton microscope. Incident laser light at 860 nm was used for acquiring the SHG images from tumor Col fibers as well as for the two-photon excitation of GFP from cancer cells, GFAP from brain astrocytes and rhodamine fluorescence in blood vessels. The SHG signal from Coll fibers was detected at 420–460 nm, GFP/GFAP fluorescence at 495–540 nm and rhodamine fluorescence at 575–630 nm, respectively. The 5 × 5 µm² tumor section was imaged at 1-µm intervals resulting in 3-µm z-step using a x10 objective while a x25 lens was used to acquire multiple 500 × 500 µm² FOV from the tumor rim and core at a 0.1-µm spatial resolution with a z-step of 2 µm. Similarly, a 200 × 200 µm² FOV was acquired for the brain sample at 0.1-µm spatial resolution with a z-step of 3 µm.

LSM. Optically cleared brain, hind limb and kidney tissue sections (>1 mm) were imaged on a light-sheet microscope (Ultramicroscope II, LaVision BioTec) using benzyl benzoate-PEG₄ as the mounting medium. Excitation wavelengths were 488 nm for Alexa 488 fluorophore and 561 nm for the GalRh–BVu polymer. The corresponding emission wavelengths detected were between 525–550 and 620–660 nm, respectively. 3D images of the brain vasculature were acquired at the following spatial resolutions using 1 µm z-step size: CTX, 5 µm; cerebellum, 0.65 µm and thalamus vessels and their astrocytic coverage, 0.33–0.56 µm. Similarly, 3D images of the glomeruli in the kidney CTX and hind limb vasculature were acquired at 0.6-µm lateral resolution.

In vivo optical functional imaging. To demonstrate the compatibility of the VascuViz technique with in vivo functional imaging workflows, a 6-week-old, homozygous Nu/nu male mouse (Jackson Laboratories) was maintained under 1% isoflurane (ISO-Flo, catalog no. 06–8550–2/B1) anesthesia mixed with air using a Vapomatic Model 2 vaporizer (AM Bickford, Inc.) and administered at 1.51 min⁻¹ via a nose cone. Next, a thinned-skull cranial window was surgically prepared according to ref. 16 after which the animal’s head was secured in a custom-designed stereotactic frame. Following this, in vivo optical imaging was conducted using a benchtop imaging system at high spatial (7.7 µm) and temporal (5 s) resolution. This included imaging of total hemoglobin (HbT) absorption or cerebral blood volume via IOS contrast imaging under 570 ± 5 nm illumination and CBF via LSC imaging under 632.8 nm illumination, respectively. First, baseline IOS and LSC data were acquired for 5 min under room air inhalation. Then, the animal was subjected to 20% oxygen (95% oxygen:5% carbon dioxide) gas inhalation for 5 min followed by room air inhalation for another 5 min (that is, recovery phase) with dynamic LSC data being continuously acquired. Following in vivo imaging, the animal was perfused with the vascular contrast agent combination of GalFITC-BVu (BioPAL Inc.) at a concentration of 0.45 mg ml⁻¹ and imaged ex vivo using 3D CT and confocal microscopy as described above.

Segmentation of vasculature from 3D CT and LSM/MPM data. Blood vessels were segmented from 3D images using the Interactive Learning and Segmentation toolkit Itlastik (v.1.3.9) as described in the accompanying protocols document.

CT-derived EDM map. The EDM was calculated from CT imaging data using the 26-voxel neighborhood chamfer metric in Amira (v.5.4).

Computation of FV and fractional area maps. To calculate the FV for each brain region, a 3D binary vessel mask was generated by implementing a logical AND operation between the binary tissue mask derived from T1W-MRI and the coregistered CT data. Next, using the ‘material statistics’ feature in Amira (v.5.4), we calculated the FV for each brain region as the ratio of the total number of blood vessel voxels from CT to the total number of tissue voxels from T1W-MRI. The mean FV of the whole brain was the average FV of the ten brain regions. Additionally, we used custom MATLAB code (v.R2017A) to generate local FV maps from binary 3D CT vascular data. To do this, we replaced each voxel with the vascular fraction computed in a sliding volume of 20 × 20 × 20 voxels. The FV varied from 0 to 1, where 0 indicated no blood vessel voxels detected in the window and 1 indicated that the window consisted only of blood vessel voxels. A similar approach was used to generate the GFAP fractional volume map from the 3D LSM vascular data. This included a preprocessing step to eliminate stripe artifacts in LSM images by applying a fast Fourier transform-based structural filter in FIJI (v.2.1). For the 2D data, MIP images from SHG and MPM data (coregistered with MRI and CT data) were used to generate maps of the Col fractional area and vascular fractional area.

DW-MRI-derived ADC, FA and 3D direction-encoded color maps. FA, ADC and 3D direction-encoded color maps were computed at 50-µm isotropic spatial resolution from DW-MRI data using DTI-Studio software as described previously.²

Boundary-to-center profiles of VME parameters. First, a 2D binary mask of the tumor was generated using FIJI (v.1.2). Then, a distance map was generated from the binary mask using custom MATLAB code (v.R2017A) having a set of contours that were parallel to the shape of the boundary as shown by white points in Extended Data Fig. 3a,b. Next, a set of radial lines that were oriented normal to each contour were generated as shown by the dashed red lines in Extended Data Fig. 3a,b. Using a grid of 150 (radial) × 210 (azimuthal) points mapped on to each parameter map (Extended Data Fig. 3a,b), a mean boundary-to-center profile was calculated and plotted using the Grapher 12 software.

Hierarchical correlation plots for identifying VME niches. To visualize the relationship between boundary-to-center profiles of EDM, Coll fractional area, ADC and FA, we generated hierarchical correlation plots using custom MATLAB code (v.R2017A). First, for each parameter’s profile, combinations of all possible subprofiles were computed by varying the number of data points in each. For example, the Pearson correlation coefficient computed and displayed at level 1 (that is, at the apex of the hierarchical correlation plot), corresponded to that
for the entire profile (that is, N data points) for each variable, while correlation coefficients displayed at level 2 (that is, just below the apex) corresponded to that between two subprofiles comprising (N - 1) data points for each variable and so on. In this plot, Pearson correlation coefficients < -0.5 were color coded black, those between ~0.5 and 0.5 color coded gray and those >0.5 color coded white. Thus, the hierarchical correlation plot provided a visualization of the spatial correlation (that is, at discrete distances from the tumor boundary) between variable pairs that characterized the VME. Moreover, in each plot we identified zones of efficacious (+t), intermediate (t) or poor (−t) drug transport in the tumor based on the spatial correlations between these image-derived parameters.

**Image-based hemodynamic modeling.** Reconstruction of the microvascular network. A GaRh–BVu polymer-bearing murine brain section (1.5 mm) was optically cleared and imaged at microscopic spatial resolution (0.33 μm) over a FOV of 900 × 900 μm2 using LSM. First, vascular skeletonization was performed as described above. Then, the 'film editor' tool in Amira (v.5.4) was used to visualize and remove isolated vessel segments at the domain boundary to yield a fully connected microvascular network that comprised 12,900 nodes and 12,899 vessel segments. We observed that a total of 64 nodes (that is, 0.4%) were of the fourth degree. As there was insufficient information available on the location of vascular trifurcations in the murine brain, we converted these fourth-degree nodes into bifurcating nodes according to the following geometric rules: fourth-degree nodes that were connected to at least one first-degree node were converted to third-degree nodes by removing the boundary node and creating two new nodes to represent this segment of the network. This was equivalent to removing isolated segments at the boundary as described earlier. Fourth-degree nodes that only had third-degree connections were converted into bifurcations by inserting a new node between the fourth-degree node and one of its neighboring nodes, and then connecting this new node with its two neighbors. These steps enabled us to generate a fully connected microvascular graph suitable for computational hemodynamic simulations.

**Scaling of blood vessel diameters and lengths.** To account for tissue and vascular distortions due to optical clearing, we computed two geometric scaling factors for blood vessel diameters and lengths. To do this, we first needed to define the inlets and outlets of the microvascular network. All Boundary conditions. measurements helped define the lower (1.25) and upper (1.67) bounds for the Blood flow model.

(15.1 distortions due to optical clearing, we computed two geometric scaling factors for Scaling of blood vessel diameters and lengths.

To account for tissue and vascular hemodynamic simulations. and then connecting this new node with its two neighbors. These steps enabled us to generate a new node between the fourth-degree node and one of its neighboring nodes, and then connecting this new node with its two neighbors. These steps enabled us to generate a fully connected microvascular graph suitable for computational hemodynamic simulations.

**Boundary conditions.** To assign boundary conditions for blood flow simulations, we first needed to define the inlets and outlets of the microvascular network. All nodes that were <8 μm and only had one connection (that is, boundary nodes) were assigned zero flow according to\(^{65}\). Next, from the remaining vessel segments (that is, 8–50 μm in diameter), inlets or outlets were identified as those segments with diameters larger than the mean diameter of 12 μm. Then, to distinguish inlets from outlets, additional criteria were imposed based on the known microvascular topology of the murine brain and other rodent models\(^{64}\). For example, inlets (that is, arterioles) were defined as having more branches and smaller mean diameter (15.1 μm) than outlets (that is, venules) (15.7 μm)\(^ {65}\). These criteria resulted in the identification of 14 inlet and 10 outlet nodes. The ratio of the number of inlets to outlets (1.4/1) in the microvascular network was in agreement with observations from other rodent models\(^ {65}\). Following this classification, constant pressure boundary conditions were prescribed at all inlets (75 mmHg) and outlets (15 mmHg)\(^ {65}\).

**Blood flow model.** We adapted our recently reported blood flow model\(^ {65}\) for these data as follows: our model was based on the 1D formula of Poiseuille’s law and accounted for nonlinear rheological effects of blood flow\(^ {66}\). To solve for pressure at all the blood vessel junctions and subsequently blood flow rates in each vessel segment under the above-mentioned boundary conditions, a system of linear equations was derived to satisfy mass conservation at all the interior vessel nodes. To solve for hematocrit in each vessel segment, we used the in vivo viscosity law\(^ {67}\). To validate our hemodynamic model, we applied our blood transport model to a publicly available rat mesentry microvascular network\(^ {63}\) according to ref. \(^ {65}\). We then computed the correlation between our simulated blood flow rates, discharge hematocrit and discharge to tube hematocrit ratio and those reported in Pries et al.\(^ {65}\). Additionally, we determined whether the simulated fractional erythrocyte flow versus fractional blood flow distribution using our approach exhibited the phase separation effect reported by Pries et al.\(^ {65}\).

Integrating ex vivo 3D data from MRI, CT and optical imaging. Tumor data. We first segmented the tumor based on its soft tissue contrast on T1W-MRI images. ADC and FA maps were coregistered to the T1W-MRI data using a rigid body transformation in DiffeoMap (www.mristudio.org).\(^ {66}\) Tumor vascular CT data and CT-derived EDM maps were coregistered to the T1-WI-MRI data via a vascular landmark-based coregistration approach\(^ {68}\) using Amira (v.5.4). To coregister MPM data with MRI data, the tumor sample was embedded in an agarose block (Extended Data Fig. 4a) before CT imaging. This enabled us to match the sample’s orientation before CT imaging to that after MPM. Before sectioning of the tumor sample, the location of the cutting plane relative to the tumor center was visually aligned with the T1-WI-MRI data as illustrated in Extended Data Fig. 4a. This orientation remained throughout the entire cutting (Extended Data Fig. 4b) and cryosectioning (Extended Data Fig. 4c) process with the help of directional annotations on the sample.

MPM images were preprocessed using Fiji (v.1.2\(^ {2}\)). MIP images were calculated from vascular, Col and GPP image z-stacks (50 μm in thickness), respectively. Each intensity histogram was normalized to 0.1% of its dynamic range following a median Gaussian filter with a six-pixel kernel size. For each MIP, vessel label assignments were validated by visual inspection and regional boundaries edited for accuracy using the segmentation toolbox in Amira (v.5.4). To map the cornu ammonis and the dentate gyrus in the hippocampus, hippocampal data from the Australian Mouse Brain Mapping Consortium (AMMBC, www. imaging.org.au/AMMBC) were coregistered to the T1-WI-MRI data in Amira (v.4) after median filtering based on a normalized mutual information based affine transformation. Next, DW-MRI-derived FA, 3D direction-encoded color map data and vascular CT data were coregistered to T1-WI-MRI data as described above. The 3D spatial graph representing the neurovasculature was manually aligned with 3D T1-WI-MRI data by using the large blood vessels as internal landmarks.

**Brain data.** We first computed a nonlinear registration based on diffeomorphic mapping\(^ {69}\) between the DW-MRI data and a reference anatomical atlas\(^ {67}\) in 3D using FSL (FMRIB’s Diffusion Toolbox)\(^ {67}\) to transform FA into a common space. Then, a normalized mutual information based affine transformation was applied to register the MPM data to the MRI data. This alignment was possible because VascuViz made the vasculature concurrently visible in the MRI, CT and MIP images. Finally, the MPM image was coregistered to the target MRI slice using an affine transformation based on a normalized mutual information approach in Amira (v.5.4). To correlate MPM contrasts with those from MRI and CT, we used the average boundary-to-center profiles for each parameter as described previously.

**Hind limb and kidney data.** We first manually segmented ten muscle groups in the hind limb based on soft tissue contrast from T1-WI-MRI using Amira (v.5.4), and annotated each muscle by matching our data with previously reported data on muscle anatomy\(^ {67}\). Next, vascular CT data were coregistered to T1-WI-MRI data via a landmark-based registration approach that used fiducials placed on large free-floating blood vessels (>40 μm)\(^ {63}\) and bone. This enabled the 3D mapping and visualization of the hind limb vasculature in ten different muscle groups as well as around the bone. To combine 3D contrasts with the 2D vasculature, the tibialis anterior muscle was isolated, optically cleared and imaged to visualize muscle fibers and capillaries at 2.6 μm. Similarly, to perform 3D mapping of the vasculature in kidney, each of the CTX, medulla and renal pelvis were segmented based on soft tissue contrast from T1-WI-MRI data using Amira (v.5.4) that was followed by coregistration between vascular CT data and TWI-MRI data as described above. Then, blood vessels were segmented from the vascular CT images and combined with the cortical ROI using a logical AND operation to generate a CTX-specific 3D vascular tree.

**Integration of dynamic in vivo CBF data with ex vivo vascular CT data. Data preprocessing.** First, 2D CBF maps were computed from LSC data as described previously\(^ {70}\). Next, a background mask was generated using Ilastik (v.1.3)\(^ {71}\) and subtracted from the CBF maps to enhance the vascular CNR. Next, IOS data were used to quantify in vivo vascular morphology as described in the previous section. Ex vivo vascular and bone masks were also derived from 3D CT images using Ilastik (v.1.3). Finally, %ACBF was computed for each vessel with respect to a global mean baseline CBF (that is, %ACBF = (CBF – CBF\(_{\text{baseline}}\))/CBF\(_{\text{baseline}}\) × 100) to enable visualization of the functional hyperemic response.

**Coregistration of in vivo to ex vivo data.** The 2D maps of dynamic CBF data were coregistered to CT-derived 3D bone and neurovascular data via a landmark-based registration approach that used fiducials placed on blood vessels\(^ {69}\) and cranial landmarks (for example, the bregma). These combined data resulted in a 4D image volume that was then converted to a video of the dynamic functional hemodynamic response to carbon dioxide inhalation by using the ‘3D Explorer’ plugin in Amira (v.2.0), Visage Imaging).

**Statistical analyses.** Pearson correlations between boundary-to-center EDM profiles, ADC, FA and Col fractional area were calculated using NCSS statistical software (NCSS, v.11). A two-tailed, nonparametric Mann–Whitney U-test was used (α = 0.05) to determine whether there were any significant differences between the AUC distributions corresponding to the different EDM ranges. To demonstrate that the polymer did not interfere with other fluorescent moieties, optical microscopy was used to acquire multiple fields of view (FOV) from GaRh–BVu perfused tissue samples. The MDA-MB-231 breast tumor sample...
(FOV = 2) brain tissue (FOV = 1) were imaged with MPM. The brain sample was also imaged with epifluorescence microscopy (FOV = 2). Finally, to demonstrate the compatibility of the VascuViz workflow with tissue clearing, a GaRh–BVu perfused and PEGASOS cleared brain was imaged using LSM (FOV = 3).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that all the data supporting the findings of this study are included in the paper and its Extended Data and supplementary information files. This includes the availability of imaging data for: (1) the murine brain in Figs. 4 and 5, Extended Data Figs. 5 and 7, Supplementary Tables 2 and 3 and Supplementary Video 1; (2) the breast tumor xenograft in Fig. 3 and Extended Data Figs. 2 and 3 and (3) the kidney and hind limb in Fig. 6. Finally, IHC and H&E data are available in Fig. 2 and Extended Data Fig. 1. The mouse hippocampal data that were used for the labeling of the cornu ammonis and dentate gyrus layers can be accessed freely from the Australian Mouse Brain Mapping Consortium (AMBMC) weblink: www.imaging.org.au/AMBMC.

Code availability

MATLAB code used in the paper will be made available upon reasonable request from the corresponding author.

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Author contributions

Conception and optimization of the GaRh–BVu polymer and study design were carried out by A.B. and A.P. Sample preparation and ex vivo MRI were conducted by A.B., M.A. and A.P. Sample preparation and ex vivo CT were carried out by A.B., P.K., R.C.R. and A.P. Sample preparation and ex vivo optical imaging were performed by A.B., B.M. and A.P. Sample preparation and IHC were carried out by A.B. and A.P. Sample preparation and in vivo imaging were performed by J.S., Y.R., A.B. and A.P. In vivo data analysis and data integration were carried out by A.B., J.S., Y.R. and A.P. Ex vivo data analysis, data integration and visualization were carried out by A.B., B.M. and A.P. The manuscript was prepared by A.B., M.A. and A.P. Results, discussion and data interpretation were carried out by all authors, along with input and revisions.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | The GalRh-BVu polymer did not interfere with histopathological workflows. The GalRh-BVu polymer was compatible with H&E staining of paraffin-embedded (PE) tissues as shown for a 4T1 tumor xenograft sample (a-b), and a kidney sample (c-d). The intravascular polymer appeared dark brown on H&E images as seen in (b) the tumor rim vasculature from (a), and (d) renal cortex vasculature from (c). Black arrows point to a perfused glomerulus in (c-d). The polymer also did not interfere with H&E staining of frozen tissues as shown for the murine hippocampus (e) and cortex (f-g). In H&E labeled images, the cytoarchitecture of the hippocampus and cortex could also be complemented with the vascular visibility of the GalRh-BVu polymer (e-g). The GalRh-BVu polymer bearing blood vessels in (g) could also be imaged using fluorescence microscopy as shown in (h). Similarly, tissue cytoarchitectural alterations seen in a H&E stained 4T1 tumor xenograft sample (i, k) could be complemented with the vascular visibility of the GalRh-BVu polymer in bright field (j, l) and fluorescence microscopy images (j, l). N.B. The brightness and contrast of H&E images were adjusted for visualization purposes without any changes to the original data.
**Extended Data Fig. 2 |** Combining Euclidean distance maps derived from CT with ADC maps derived from DW-MRI in a human breast cancer model. 

**a-c,** A 2D section from the 3D Euclidian distance map (EDM) is shown with an overlay of white contour lines to highlight regions within intervessel distance ranges 0-50 µm (**a**), 51-150 µm (**b**) and 151-350 µm (**c**), respectively. Soft tissue contrast from T1W-MRI data of the same region was employed as the underlay image in (**a-c**). The contour lines corresponding to the inter-vessel distance ranges shown in (**a-c**) were mapped on to co-registered apparent diffusion coefficient (ADC) maps derived from DW-MRI (**d-f**), respectively. Rim and central tumor sub-regions from (**d**) and (**f**) were selected and visualized with volume rendered tumor blood vessels (red) derived from CT (**g-h**). Low ADC regions (blue-green) co-localized with regions with a high density of tumor vasculature (**g**), while high ADC regions (yellow-red) co-localized with regions exhibiting low vessel density (**h**). 

**i,** Box and whisker plots of whole-tumor ADC distributions showed that 151-350 µm EDM regions were significantly different ($p \ll 0.001$) from those for 51-150 µm EDM regions and 0-50 µm EDM regions using a two-tailed Mann-Whitney U-test at alpha = 0.05. The box and whisker plots corresponding to 0-50 µm, 50-150 µm and 150-350 µm EDM regions show the median, interquartile range (IQR) and the data within the Q1-1.5IQR and Q3+1.5IQR range. The upper and lower bounds of the displayed intensity range for the merged images shown in (**a-f**) were adjusted for visualization purposes without any changes to the original data.

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Extended Data Fig. 3 | Correlation between tumor boundary-to-center profiles for MRI-, CT- and optical imaging-derived vascular microenvironmental (VME) parameters in a human breast cancer model. a–b, For each VME parameter map (e.g. ADC), we overlaid a 2D grid of points along azimuthal (white points) and radial directions (dashed red lines). An enlarged view of the grid is shown in (b) wherein black arrows point to white contours that are located at the tumor boundary and at 1 mm and 2 mm normal to it. Next, boundary-to-center profiles were calculated along each dashed red line and an average radial profile generated for each VME parameter map. c, ADC correlated with EDM ($R^2 = 0.43$, $p = 0.0084$). d, Collagen (Col) fractional area correlated inversely with EDM ($R^2 = 0.35$, $p = 0.0183$). e, FA correlated inversely with ADC ($R^2 = 0.68$, $p < 0.0001$). f, FA correlated with Col fractional area ($R^2 = 0.7$, $p = 0.0001$). The Pearson correlation coefficients between each variable pair shown in (a–d) was significant.
Extended Data Fig. 4 | Steps for matching sample orientation between CT and MPM imaging. a, The sample was embedded in an agarose block prior to CT imaging and directional annotations made. Before sectioning the sample for MPM, the location of the cutting plane was determined relative to the tumor center by matching the embedded sample with its T1W-MRI image, as shown in (b). Red hatched line indicate where the sample was cut. This orientation was then preserved with the help of the directional annotations shown in (c) during cutting, and (d) during cryosectioning.
Extended Data Fig. 5 | Creating a 4D (that is 3D + time) visualization by mapping the temporal dynamics of the in vivo functional hyperemic response to 3D ex vivo neurovascular and anatomical data. a, A thinned-skull preparation for in vivo LSC and IOS imaging. b, The animal was made to breathe room air (AIR), carbogen (95% oxygen/5% carbon dioxide) gas (CARB), and room air (AIR) for 3, 2 and 5 minutes, respectively during which dynamic CBF data was acquired. Black arrows in (b) correspond to the time points (that is 0.4 and 2.5 min, AIR; 3.3 and 5 min CARB; and 7.5 and 10 min, AIR) for which the corresponding CBF maps are shown in (f-k). Black, dark gray and light gray squares (d-e) indicate large (i.e. 115 μm < diameter < 120 μm), medium (i.e. 40 μm < diameter < 60 μm) and small (i.e. 20 μm < diameter < 40 μm) blood vessels that were identified using the IOS image (c) and from which the mean in vivo CBF traces shown in (e) computed. e, Medium (dark gray trace) and large vessels (black trace) showed a larger peak increase in %ΔCBF w.r.t the global baseline than that exhibited by small vessels (light gray trace). f-k, Spatio-temporal evolution of CBF corresponding to the experimental paradigm in (b) illustrating the significant response to carbogen inhalation (h, i, l). The same sample prepared for ex vivo imaging in which the GalFITC-BVu perfused neurovasculature was visible (white contrast) in the intact skull. m-o, Concurrent visualization of the 3D skull anatomy (gray) and underlying neurovasculature (red) using ex vivo CT imaging (7.5 μm) (m-o). p, Fluorescent MIP images of perfused skull vessels from a tiled scan acquired at 10x with confocal microscopy (1.3 μm), and a 25x scan of the same sample acquired with MPM (0.6 μm) (q). The positive vascular contrast in CT data (m-o, r-w) was due to BVu while the GalFITC component provided fluorescence contrast in microscopy data (p-q). r-w, Integrated 4D volume created by co-registering the 2D in vivo CBF maps (f-k) to 3D ex vivo neurovascular and skull anatomy data (m-o) illustrating the time evolution of the functional hyperemic response. The displayed intensity range for (f-k) was adjusted for visualization purposes without altering the original data. A 0.25 minute moving average filter was applied to (e) to reduce noise. Scale bars: 1 mm unless stated otherwise.
Extended Data Fig. 6 | Validation of the image-based hemodynamic modeling approach. To validate our blood flow modeling approach, we employed a 546-segment vascular network of the rat mesentery (a) that was derived from high resolution intravital microscopy imaging data by Pries et al. To simulate pressure and blood flow values in all segments of the network, experimentally obtained blood flow rates were prescribed at 35 boundary segments while one boundary node was subjected to a constant pressure boundary condition. Blood flow and discharge hematocrit distributions in all 546 segments simulated using our approach (black dots) are compared against the solution of the fully determined system as obtained by Pries et al. We achieved an $R^2 = 0.99$ for blood flow rates (nl/min) and $R^2 = 0.93$ for discharge hematocrit distributions observing excellent agreement between these data. Moreover, the distribution of the ratio of discharge to tube hematocrit vs. vessel diameter satisfied the well-known Fahraeous effect and showed an $R^2 = 0.94$ against the simulated values reported by Pries et al. (d). Finally, fractional erythrocyte flow ($FQE_α$) vs. fractional blood flow ($FQB$) distributions obtained using our approach satisfied the phase separation effect (e) in agreement with the distributions reported by Pries et al. (f). Open and filled circles correspond to data points for daughter vessels $α$ and $β$ at diverging bifurcations. Collectively, these plots demonstrate the validity of our image-based hemodynamic modeling approach and its utility for predicting functional properties of micro-vascular networks. Panel (f) reproduced with permission from.
Extended Data Fig. 7 | The GalRh-BVu polymer remained stable for at least 11 months after sample preparation. a, LSM image of the thalamic vasculature in a murine brain acquired at 0.33 μm spatial resolution at 6 months after sample preparation. b, LSM of the same field of view acquired at 0.56 μm spatial resolution at 11 months after sample preparation. Here, vascular contrast was enhanced by normalizing the image intensity to 0.1% of the dynamic range followed by 3D median filtering (radius = 2 voxels).
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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

In vivo imaging data were acquired on a bespoke bench-top optical imaging system controlled via MATLAB code {R2017A}. All ex vivo imaging data were acquired using commercial software associated with the imaging hardware employed. This included the image acquisition software for the Bruker BioSpin 9.4T vertical bore MRI scanner (i.e. ParaVision version 5.1), Bruker Skyscan 1275 scanner (i.e. Skyscan 1275 control software version 1.0.12 and NRecon 1.7.0.4), Olympus laser scanning FV1000 MPE multiphoton microscope (i.e. FV10-ASW viewer software), LaVision BioTec Ultramicroscope II light sheet microscope (i.e. ImSpector version 5.1.328) and Nikon ECLIPSE-TS100 epifluorescence microscope (i.e. SPOT imaging software version 5.6).

Data analysis

Data analysis was performed using: FUI version 2.1 (image and preinstalled plug-ins), MATLAB R2017A, ilastik 1.3, AMIRA 5.4, NCSS 11, and Grapher 12. Diffgeomap, DTI-Studio.

For manuscripts utilizing custom algorithms or software that are not central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitLab). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that all the data supporting the findings of this study are included within the paper and its supplementary information files. This includes the availability of imaging data for: (i) the murine brain in Figures 4 and 5, Supplementary Figures 5 and 7 and Supplementary Tables 2 and 3; (ii) the breast tumor...
Field-specific reporting

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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

We conducted ex vivo imaging of murine organs harvested from five different animals (n=5) perfused with the vascular contrast mixture. The brain (n=1) of the first mouse was imaged in vivo and ex vivo. Sample size for the remaining mice were as follows: The brain (n=1) and hind limb (n=1) were harvested from the second mouse; kidney (n=1) from the third mouse; MDA-MB-231 breast cancer xenograft (n=1) from the fourth mouse; and the 4T1 breast cancer xenograft (n=1) from the fifth mouse. We did this to ensure that organ systems could be independently labeled and imaged in different animals, and to demonstrate the utility of our multimodality imaging method for different vascular systems biology applications in the preclinical setting.

Data exclusions

N/A  No data were excluded from the analyses.

Replication

We demonstrated that the vasculature of different animals (n=5) could be repeatedly labeled and imaged ex vivo. Our intravascular label was concurrently visible across multiple ex vivo imaging modalities (i.e. MRI, CT, and multiphoton microscopy) in multiple organ-systems and fields-of-view derived from different animals. Moreover, co-localization of the intravascular contrast polymer with immunohistochemical markers of the vasculature (e.g. laminin, α-SMA etc.) as well as H&E staining provided additional validation of the approach. Our ability to successfully image the fluorescence of an optically cleared brain sample using light sheet microscopy (LSM) at time six and eleven months after sample preparation also demonstrated the long-term stability of the intravascular label. Finally, successful application of the VascoViz workflow in healthy tissues (e.g. brain, kidney and hind limb) as well as in diseased tissue (e.g. breast cancer xenograft), demonstrated the replicability of the approach.

Randomization

N/A  Randomization was not applicable to the study because we did not seek to assess any difference effects between groups in this study. Our main goal was to demonstrate the utility of our multimodality imaging method for different vascular systems biology applications in the preclinical setting.

Blinding

N/A  Blinding was not applicable in this study because it was not designed to assess any group effects. Our main goal was to demonstrate the utility of our multimodality imaging method for different vascular systems biology applications in the preclinical setting.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐  | Antibodies            |
| ☒  | Eukaryotic cell lines |
| ☒  | Palaeontology and archaeology |
| ☒  | Animals and other organisms |
| ☒  | Human research participants |
| ☒  | Clinical data         |
| ☒  | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☐  | ChIP-seq              |
| ☒  | Flow cytometry        |
| ☒  | MRI-based neuroimaging |

Antibodies

The following antibodies were used:
1. Alexa Fluor® 488 conjugated monoclonal GFAP (GAS) mouse antibody (Cell Signaling, 36555).
2. FITC-conjugated monoclonal α-Actin, α-smooth muscle antibody (Sigma-Aldrich, F3777).
3. Polyclonal anti-rabbit laminin antibody (Sigma-Aldrich, L9393).
4. Alexa Fluor® 488 conjugated polyclonal goat anti-rabbit IgG secondary antibody (ThermoFisher, A32731).

Validation

Since immunostaining was performed on healthy tissues, we followed vendor-provided validation of all primary antibodies. Relevant citations reported on vendor websites were consulted for the successful use of these antibodies in frozen and FFPE tissue sections. Examples of studies reporting independent validation for each primary antibody are listed below:
1. Alexa Fluor® 488 conjugated monoclonal GFAP (GAS) mouse antibody (Cell Signaling, 36555): Heyangzi Li, et. al. Theranostics, 2019; Kuniyuki Nakamura, et. al. Journal of Cell Biology, 2019
### Eukaryotic cell lines

| Policy information about: cell lines | Both cell lines were sourced from American Type Culture Collection (ATCC). |
|-------------------------------------|---------------------------------------------------------------------------|
| Cell line source(s)                | MDA-MB-231 breast cancer cell line, 4T1 breast cancer cell line Culture Collection (ATCC). |
| Authentication                     | American Type Culture Collection (ATCC). Original cell lines were obtained from ATCC and confirmed to be negative for mycoplasma contamination. |
| Mycoplasma contamination            | Negative                                                                  |
| Commonly misidentified lines (See [IGAC register](#)) | N/A No commonly misidentified cell lines were used in the study. |

### Animals and other organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research.

#### Laboratory animals
- The mouse types described below were used:
  1. For mouse brain imaging experiment (in vivo): Athymic Nude, Male, 26 g 6 weeks
  2. For mouse brain, hind limb imaging experiments: Athymic Nude, Female, 26 g 6 weeks
  3. For mouse kidney imaging experiment: Athymic Nude, Female, 25 g 6 weeks
  4. For tumor imaging experiment: Athymic Nude, Female, 27 g 6 weeks
  5. For IHC experiments: BALB/c, Female, 25 g 4T1-BALB/c, Female, 25 g, 6 weeks

#### Wild animals
- N/A No wild animals were used in the study.

#### Field-collected samples
- N/A No field collected samples were used in the study.

#### Ethics oversight
- All animal experiments were conducted in accordance with an approved Johns Hopkins University Animal Care and Use Committee (JHU AUC) protocol. The JHU animal facility is accredited by the American Association for the Accreditation of Laboratory Animal Care, and meets the National Institutes of Health (NIH) standards as set forth in the “Guide for the Care and Use of Laboratory Animals”.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Magnetic resonance imaging

**Experimental design**

| Design type | N/A Experiment entailed ex vivo imaging of murine samples using T1-weighted and diffusion-weighted MR pulse sequences. |
|-------------|------------------------------------------------------------------------------------------------------------------|
| Design specifications | N/A T1-weighted images were acquired using a 3D-FLASH sequence. Diffusion weighted images were acquired using a 3D GRASE sequence. |
| Behavioral performance measures | N/A Since our goal was to conduct ex vivo imaging of tissue samples, we did not measure animal behavior in this study. |

**Acquisition**

| Imaging type(s) | T1-weighted (i.e. structural) and diffusion-weighted (i.e. diffusion) MRI were used. |
|-----------------|--------------------------------------------------------------------------------------|
| Field strength  | 9.4                                                                                 |
| Sequence & imaging parameters | T1-weighted images were acquired using a 3D-FLASH sequence with the following parameters: FOV = whole-brain, flip angle = 30°, TE/TR = 4.2/40 ms, 4 averages and 40 μm isotropic spatial resolution. |
| Area of acquisition | Whole-brain scans were acquired. |
| Diffusion MRI   | X Used                                                                                |
| Parameters      | Diffusion-weighted images were acquired using a 3D GRASE sequence with the following parameters: FOV = whole-brain, TE/TR = 32/800 ms, 12 echoes per excitation, 2 averages, diffusion gradient duration/separation = 2.8/10 ms, and 16 directions with a b-value = 1700 s/mm² and 100 μm isotropic spatial resolution, zero-padded to 50 μm during reconstruction. |

### Preprocessing

| Preprocessing software | MRI Studio was used for computing the fractional anisotropy (FA), apparent diffusion coefficient (ADC) and 3D direction-encoded color (DEC) maps as well as for the generation of 3D brain region labels. Amira v5.4 software was used for region validation, segmentation and data visualization. |
Normalization
- Normalization was not used.

Normalization template
- We did not apply a normalization template.

Noise and artifact removal
- Noise and artifact removal was not applied.

Volume censoring
- Volume censoring was not applied.

Statistical modeling & inference
- We did not apply any statistical modeling and inference tools in the study.

Model type and settings
- No effects were tested.

Effect(s) tested

Specify type of analysis:  
- Whole brain
- ROI-based
- Both

Statistic type for inference
(See Klund et al. 2016)
- We did not apply any statistical modeling in this study.

Correction
- Statistical correction was not applicable.

Models & analysis
- Involved in the study

- Functional and/or effective connectivity
- Graph analysis
- Multivariate modeling or predictive analysis