MULTIMODALITY IMAGING OF THE VASCULATURE USING THE VASCUVIZ PROTOCOL

Here we describe a detailed protocol for multimodality imaging of the vasculature in a healthy murine brain. It includes a step-by-step procedure for: (A) preparation of the GalRh-BVu contrast agent combination for one animal; (B) intravascular labeling; (C) sample fixation; followed by a protocol for multimodality imaging of vasculature with (D) MRI, and (E) CT. Also included are protocols for: (F) immunofluorescent labeling; (G) H&E Staining; (H) tissue clearing; (I) epi-fluorescence microscopy; (J) multiphoton microscopy; (K) and light-sheet microscopy of the same brain sample. These protocols are also applicable for multimodality vascular imaging of other murine tissues and preclinical disease models.

(A) GalRh-BVu mixture preparation protocol (Total time: 45 min)

i. Heat 25 ml of distilled water to 40-45°C on a hot plate an hour prior to animal perfusion.
ii. Add 0.1 ml of the BriteVu Enhancer® (Scarlet Imaging, UT) to the distilled water while stirring it for 1 minute.
iii. Next add 7 g of the BriteVu® CT contrast agent (BVu, Scarlet Imaging, UT) while stirring the mixture until its temperature reaches 60-70°C (~10 minutes).
iv. Remove the beaker from the hot plate and add 1 ml of the Galbumin™-Rhodamine MRI contrast agent (GalRh, BioPAL Inc., MA) to the mixture and stir it for 2-3 minutes. We recommend starting with the original concentration (25 mg/ml) of GalRh to achieve high CNR in MRI and optical images. This results in a final concentration of 0.96 mg/ml of GalRh in the final GalRh-BVu mixture.
v. Stir the final GalRh-BVu mixture again using a vortex mixer for 1 minute.
vi. Keep the GalRh-BVu mixture warm at ~40-50°C using a temperature-controlled water bath until ready for perfusion.

(B) Vascular labeling protocol (Total time: 20 min)

i. Anesthetize the animal using i.p. administration of ketamine/xylazine at 0.1 ml/10 g body weight.
ii. Transcardially perfuse 10-15 ml of heparinized phosphate-buffered saline (PBS) at 0.1 ml/s using a syringe pump.
iii. Then perfuse 10-15 ml of 10% buffered formalin at 0.1 ml/s for tissue fixation using the syringe pump.
iv. Next, perfuse 15-20 ml of the warm GalRh-BVu mixture (~40-50°C) at 0.03 ml/s using the syringe pump.
v. Excise the brain and store it in cold 10% buffered formalin in the dark at 4°C.

(C) Sample fixation (Overnight)

i. Place the brain inside a 15 ml conical tube filled with 10% buffered formalin for overnight fixation.

(D) MRI imaging protocol (Total time: 7-8 hours)

i. Forty-eight hours prior to imaging, rinse the brain sample thoroughly with PBS.
ii. Place the brain in a 10 mm diameter NMR tube filled with Fomblin® (Solvay Solexis, Milano, Italy).
iii. Place the brain-bearing NMR tube inside a vacuum pump for 15 min to remove any air bubbles.

iv. All samples were imaged on a 9.4T vertical bore MRI scanner (Bruker BioSpin Corp, Billerica, MA) using a birdcage RF volume coil and ParaVision (v5.1) MRI software.

v. Acquire T1-weighted (T1W) images using a 3D-FLASH sequence with flip angle=30°, TE/TR=4.2/40 ms, 4 averages, and 40 µm isotropic spatial resolution.

vi. Acquire diffusion tensor images (DTI) using a 3D diffusion-weighted (DW) GRASE sequence[1] with 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 image reconstruction.

(E) CT imaging protocol (Total time: 2-3 hours)

i. Immediately before imaging, prepare 4% w/v agarose solution (A9539 Sigma-Aldrich).

ii. Fill a 10 mm diameter Eppendorf tube with the warm agarose solution.

iii. Place the brain sample inside the tube using tissue forceps and wait for the agarose to solidify (~2 min).

iv. The brain sample was imaged on a Skyscan 1275 CT scanner (Bruker, USA).

v. Acquire a 3D scan with the following settings: 0.5 mm aluminum filter, 55 kVp, 145 µA, 335 ms exposure time, 0.2 rotation step, 3 averages and 7.5 µm isotropic spatial resolution.

vi. Reconstruct 3D images using the NRecon software (v1.7.0.4).

vii. After CT imaging, take the brain out of the agarose embedding gel and store it in PBS in the dark at 4°C.

(F) Immunofluorescent staining of GalRh-BVu labeled tissue (Total time: 0.5 day for 10 µm sections, 7-10 days for 1-2 mm sections)

i. Rinse the brain sample thoroughly with PBS.

ii. Next cryoprotect the sample by immersing it in a 30% sucrose solution (CAS-57-50-1 Fisher Scientific) for 48-72 hours, or until it sinks.

iii. After cryoprotection, freeze the sample using liquid nitrogen before embedding it in the optimal cutting temperature (OCT) compound (Tissue-Tek®, Sakura Finetech, CA) using a cryomold.

iv. Cut 10-2000 µm sections (as required) using a cryostat (Leica CM-3050), and mount them on silanized slides for immunofluorescent labeling.

v. Gently rinse the slide twice with PBS and then PBS mixed with 0.1% TritonX-100 (T8787 Sigma-Aldrich).

vi. Perform antigen retrieval. For example, heat inducted antigen retrieval with 1× citrate buffer solution (C-9999 Sigma-Aldrich) was used for brain sections to be labeled with anti-glial fibrillary acidic protein antibody (GFAP).

vii. Incubate the tissue sections with Carbo-free™ blocking solution (SP-5040 Vector Laboratories) for 10 min at room temperature.

viii. Incubate tissue sections with the primary antibody (e.g. FITC-conjugated rabbit anti-GFAP antibody at 1:50, Cell Signaling, Danvers, MA) using cover wells and store them overnight at 4°C in a humidity-controlled chamber.

ix. Gently rinse tissue sections with PBS and incubate with secondary antibody (e.g. Goat anti-rabbit IgG Alexa Fluor 488 at 10 µg/ml, ThermoFisher, MA) for 30-45 minutes at room temperature.
x. Gently rinse tissue sections with PBS, cover tissue sections with an aqueous mounting medium VectaMount® (H-5501 Vector Laboratories) and then place the cover slip .
xi. For immunofluorescent labeling of thick tissue sections (i.e. 1-2 mm), incubate the sample in blocking solution for 1 day (step vii), primary antibody solution (step viii) for 5 days and secondary antibody (step ix) for 3 days on an orbital shaker at 4°C.

(G) Hematoxylin and Eosin (H&E) staining of GalRh-BVu bearing sections (Total time: 15 min)

i. Gently rinse tissue sections with tap water with the help of a transfer pipette.
ii. Apply hematoxylin solution (H-3404, Vector Laboratories) for 1 min.
iii. Gently rinse tissue sections again with PBS until the solution runs colorless.
iv. Apply Eosin-Y solution (R-7111 Thermo Scientific™) for 30 seconds and rinse sample with PBS.
v. Apply VectaMount® aqueous mounting medium (H-5501 Vector Laboratories) and then place the cover slip on the tissue sections.

(H) (Optional) Optical clearing of 1-2 mm GalRh-BVu bearing sections using the PEGASOS[2] method (Total time: 3-4 days)

i. Cut 1-2 mm tissue sections using an acrylic tissue slicer (TM S12 Braintree Scientific, Inc., MA).
ii. Follow the immunofluorescent staining protocol for thick sections (please see step xi under **F**).
iii. Decolorize the tissue with Quadrol (Sigma-Aldrich 122262) for 1-1.5 days at 37°C while shaking.
iv. Delipidate tissue with tert-Butanol (tB) (Sigma-Aldrich 360538) for 2-4 hours at 37°C while shaking.
v. Dehydrate the tissue using a polyethylene glycol (PEG) methacrylate Mn 500 (PEGMMA500) solution for 1-1.5 days at 37°C while shaking.
vi. Optically clear the tissue using a benzyl benzoate (BB) PEG solution (Sigma-Aldrich B6630) for 1-1.5 days at 37°C while shaking.
vii. Store the tissue in the benzyl benzoate-PEG clearing medium at room temperature until ready for MPM or LSM imaging.

(I) Epifluorescence microscopy protocol (Total time: 30-45 min)

i. Tissue sections (< 20 µm thickness) were imaged on a Nikon ECLIPSE-TS100 microscope (Nikon Instruments Inc., NY) with the appropriate filters for detecting immunofluorescence.
ii. Regions-of-interest (ROI) were imaged at 2×, 10× and 40× magnification using a SPOT INSGHT™ CCD camera and its associated software (v5.6) (Diagnostic Instruments Inc., MI).

(J) Multiphoton Microscopy (MPM) protocol (Total time: 2-3 hours)

i. Tissue samples (25-50 µm) were imaged on an Olympus laser scanning FV1000 MPE multiphoton microscope running the FV10-ASW viewer software (Olympus Corp., Center Valley, PA).

ii. Select 860 nm laser illumination for two-photon excitation of GFAP-FITC labeled astrocytes, and rhodamine fluorescence in blood vessels. The GFAP-FITC fluorescence was detected at 495-540 nm, and rhodamine fluorescence at 575-630 nm, respectively.

iii. Acquire 200 µm x 200 µm fields-of-view with a 25× objective at 0.1 µm spatial resolution with a z-step size of 3 µm.

(K) Light Sheet Microscopy (LSM) of cleared tissue (Total time: 2-3 hours)

i. Optically cleared tissue samples (> 1 mm thickness) were imaged on a light sheet microscope (Ultramicroscope II, LaVision BioTec, Germany) running the ImSpector (v5.1.328) data acquisition software, and using BB-PEG as the tissue mounting medium.

ii. Excitation wavelengths of 488 nm for the Alexa 488 fluorophore and 561 nm for the GalRh-BVu polymer. The corresponding emission wavelengths detected were between 525-550 nm and 620-660 nm, respectively.

iii. Acquire multichannel 3D fields-of-view at 0.65-5 µm spatial resolution and 1 µm z-step size.

(L) 3D data integration of multiscale, multimodality brain images (Total time: 1-2 weeks)

i. Enhance the vascular contrast-to-noise ratio (CNR) of the CT/optical images prior to vessel segmentation. For optical imaging data, the global background signal was first removed by subtracting a Gaussian smoothed image from the 3D image stack. For CT imaging data including bone, a 3D bone mask generated using intensity-based thresholding was first subtracted from the 3D image stack. Then the 3D image histogram was normalized to 0.1% of the overall dynamic range followed by the application of a 3D median filter to the image stack.

ii. Perform vessel segmentation using the using the Interactive Learning and Segmentation toolkit ilastik (v 1.3). Typically, 20-50 images were employed to train the classifier within Ilastik to distinguish between blood vessel and background pixels. Image features at all spatial scales were used for classification and to automate the segmentation process. In the next step, small isolated vessel segments (< 5 voxels) were removed and a 3D gaussian filter with a standard deviation of 1 voxel applied to reduce noise. The resulting binary vascular maps were assessed via a slice-by-slice comparison with the underlying grayscale 3D data in FIJI (v2.1).

iii. Finally, vessel centerlines and radius were estimated using the "auto-skeleton" function in Amira™ (v5.4) (Visage Imaging, San Diego, CA, USA). Finally, the 3D skeleton was visualized as a spatial graph object in Amira™, and saved as an ASCII file for eventual vascular density calculations and blood flow simulations.

iv. Co-register 3D MRI/CT/optical imaging data using vascular fiducials[3] visible in each imaging modality using Amira™ (v5.4) software (Visage Imaging, San Diego, CA, USA). Briefly, 30-40 landmark pairs were placed on large blood vessels (i.e. diameter > 40 µm) visible in both MRI and CT images or (CT and optical images) and image co-registration was achieved using the thin spline-based Bookstein algorithm module in Amira™.

v. Generate 3D maps of different vascular metrics (e.g. blood volume, vascular fractional area, simulated blood flow) in MATLAB® (vR2017A) using the binarized (i.e. segmented) CT/LSM vascular data from step ii.

vi. Compute 3D maps of complementary contrasts (e.g. fractional GFAP volume derived from LSM data, fractional anisotropy derived from DW-MRI data) using DTI-Studio (www.mristudio.org) and MATLAB® (vR2017A).

vii. Co-register 3D maps computed from steps iv-v to respective imaging data according to step iii.

viii. Visualize multiscale, multimodality 3D integrated data using the Amira software (v 5.4).

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Supplementary Table 1: Multimodality imaging with VascuViz and its comparison with other imaging workflows using commercially available intravascular contrast agents/dyes.

Reference	Commercial intravascular label	Imaging modalities			Preparation time for imaging[ŧ]	Complementary tissue workflows			Non-destructive imaging	Commercially available imaging equipment	Tissue type
		MRI	CT	Optical microscopy		Tissue clearing	IHC	H&E			
Mayerich et al.	India Ink			✓	1-3 weeks						Brain
Xue et al.	Gelatin-India Ink			✓	3-4 days						Brain
Krucker et al.	PU4ii		✓	✓**	3-4 days					✓	Brain
Schaad et al.	µAngiofil®		✓	✓**	4 days		✓		✓	✓	Hind limb
Di Giovanna et al.	Gel-BSA-FITC			✓	N/A	✓			✓	✓	Brain
Erturk et al.	Lectin-FITC			✓	N/A	✓	✓		✓	✓	Brain, Spinal Cord, Tumor
Xie et al.	BriteVu®		✓		~ 1 hour			✓	✓	✓	Kidney
Pathak et al.	Microfil®	✓*	✓		~ 1 hour				✓	✓	Brain
VascuViz (current study)	GalRh-BVu	✓	✓	✓	~ 1 hour	✓	✓	✓	✓	✓	Breast tumor, Brain, Hind limb, Kidney

* Negative contrast from the contrast agent.

**Autofluorescence from the contrast agent.

[ŧ]Interval between final fixation step and first imaging session. Subsequent tissue processing steps such as clearing, IHC, H&E are not included.

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Supplementary Table 2: Comparison between hemodynamic parameters from this study and those reported in the literature for the murine brain.

Hemodynamic parameter	Case I (V_s) Mean ± SD	Case II (T_s) Mean ± SD	Literature (murine brain) Min – Max
Pressure (mmHg)	21 ± 5.1	20.5 ± 4.8	10 – 60 [2, 3]
Blood flow rate (nl/min)	2.8 ± 18.9	7.0 ± 57.4	0 – 780 [1, 2, 3, 4]
Velocity (mm/s)	0.4 ± 2.4	0.7 ± 4.1	0.5 – 8 [2]
Shear stress (dyne/cm^2)	57.2 ± 254	56.5 ± 243	0 – 400 [3]

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Supplementary Table 3: Summary statistics for hemodynamic variables in large (≥ 8 μm) and small blood vessels (< 8 μm) for Case I (V_s) and Case II (T_s).

Hemodynamic parameter	Large vessels (≥ 8 μm) Mean ± SD		Small vessels (< 8 μm) Mean ± SD	
	V_s	T_s	V_s	T_s
Diameter (μm)	12.6 ± 5.1	13.2 ± 6.3	3.1 ± 1.5	3.8 ± 1.5
Pressure (mmHg)	27 ± 11.4	24.2 ± 9.6	20.6 ± 4.1	19.9 ± 3.4
Blood flow rate (nl/min)	86.7 ± 250.6	131.8 ± 509	0.2 ± 0.9	0.23 ± 1.3
Velocity (mm/s)	6.7 ± 18	6.6 ± 20.36	0.2 ± 0.8	0.21 ± 1.0
Shear stress (dyne/cm^2)	159.6 ± 452.5	127.5 ± 384	49.5 ± 237.5	45.6 ± 216.6