Online Methods

Animal models and cell lines

We implanted mammary fat pad windows or cranial windows into 8-10 week old Tie2-GFP mice and nude mice respectively[14]. Small mammary carcinoma (MCaIV) or glioma (U87 and GL261 used for intussusceptive angiogenesis analysis) tumor fragments (0.2-0.3 mm diameter) were implanted into the mammary fat pad or the left cerebral cortex respectively. To spatially locate the tumor within the brain, GFP was stably transfected into U87 using a retroviral construct. All cell lines were maintained in DMEM medium with 10% FBS. All experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Red Blood Cells Fluorescent labeling

We labeled red blood cells ex-vivo with 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine perchlorate (Invitrogen), a far red lipophilic fluorescent die. Blood was collected through cardiac puncture from old donor males of the same strain used in the experiment. RBCs were separated from plasma and leukocytes using centrifugation. RBCs were diluted 1:100 in PBS (10 ml) and were incubated for 20 minutes with 100ul 1mg/ml DID (dissolved in 95% Ethanol). We washed RBCs with PBS and mixed with saline at 50% Hematocrit. We injected 150-200 µl of labeled RBCs through tail veins at one to five days before imaging. We monitored fluorescent RBC fraction *in vivo* through analysis of the normal brain capillaries. Non-fluorescent RBCs are detected in small caliber capillaries (in the brain or the mammary fat pad) through their ability to exclude TAMRA-BSA. The ratio of fluorescent to total number of RBCs is calculated at every imaging time point.

Intravital multiphoton laser scanning microscopic (MPLSM)

In vivo multiphoton laser scanning microscopic analysis of glioblastoma and mammary carcinoma vessels was performed as described previously [14, 15]. The MPLSM consisted of a MilleniaX pumped Tsunami Ti:Sapphire laser (Spectra-Physics). Two photon excitation of the used fluorophores (TAMRA, GFP and DID) was achieved using 840 nm light. Power at the sample was estimated to be 1–5 mW. MPLSM microscope consisted of a Olympus Fluoview FV300 system customized for multiphoton imaging. We identified the tumor area by analysis of GFP constitutively expressed by U87 (Supplementary Fig. 5). We performed vessel angiography after i.v. injection of 0.1 ml 10 mg/ml TAMRA-BSA (Invitrogen). We imaged two adjacent areas in the tumor and one are in the contralateral brain by acquiring 3-D stacks (dimension: 630 x 630 x 250 um; Resolution: 2.4 x 2.4 x 2.5 µm/pixel). We segmented tumor volume using a semi-automated algorithm that is based on a user specified threshold (Matlab, Mathworks). Vessels were segmented using a tracing algorithm previously described[16].

Residence time line scan imaging

We performed RTLS imaging by scanning along a line intersecting the vessels. In our analyses of gliomas (Fig. 1c), 20 to 40 line scans were manually positioned at various depths. To generate cross-sectional flow profiles, scanning was performed along a manually positioned line scan (blue line in Fig. 2) at depths ranging from 0 to 50 μm with 2.5 μm step size. At each depth 3000-4000 lines were scanned at a resolution of 2.5 μm/pixel in the xy plane with one line per 1.3 to 1.5 ms. We performed phenotypic clustering of glioma vessels by scanning along a pseudo grid which randomizes vessel selection. While collecting the line scans, a snapshot of the analyzed plane was imaged and used to determine the orientation of the intersected vessels (Fig. 1a and Supplementary Fig. 1). We segmented RBC and extracted residence times using a computer assisted semi-automated in-house algorithm (coded in Matlab). The velocity of each RBC was calculated based on the equation presented in Fig. 1b. We analyzed vessel profiles by determining the RBCs flowing at each lane within the cross-section of the vessel. RBC flux reflects the total number of RBCs per second. Hematocrit was measured as the fraction of RBC pixels over the total number of pixels within the lane. RBC z resolution was measured by imaging stationary RBCs within 0.5mm microchannels (RBC z resolution = 10 μm).

Relative velocity field scanning

The imaging protocol for RVFS consists of imaging the same field at various scanning velocities. Using a conventional MPLSM system, we performed repetitive 3D imaging of the vessels at variable scanning velocities (1.53, 1.16, 0.78 and 0.39 mm s^{-1}) and at various resolutions (1.3 and 2.5 μm/pixel). Since vessels have various orientations the projected scanning velocity over the vessel axis is different for each vessel and is higher than the vertical velocity. To ensure all vessels were scanned along the RBC flow direction, imaging was performed at 4 rotation angles 0, 90, 180 and 270 degrees. To align the volumes and correct for microscope-related drift, a rigid registration algorithm was implemented based on 3D normalized cross-correlation. Vessel networks were traced and subdivided into single vessel segments of equal length (50 μm). Within each vessel, we determined the residence time for segmented RBCs and fit to the scanning velocity adjusted to the vessel angle. RBC tracks representing a traveled distance greater than four times the length of a RBC (28 μm) were directly identified and analyzed to extract travel distance and calculate velocity.

Shear rate calculation

We calculated vessel shear rate based on the gradient of RBC velocities in adjacent lanes within the blood vessel. The lanes are defined by image pixels. For each lane, we calculated RBC velocity as the mean velocity of all the RBCs passing through. Thus, in general, each RBC is present in more than one lane– most often 2 lanes– and therefore contributes to the average flow, hematocrit and flux of those lanes.

For the cross-sectional analysis of flow (Fig. 2 and Supplementary Fig. 4), shear rate maps were generated by calculating the average shear rate for each pixel at the resolution of 2.5 μm/pixel. Shear rate is equal to the average difference in velocity between the two adjacent pixels, divided by the distance. For the clustering

experiment (Fig. 3 and Supplementary Fig. 5), we calculated shear rate based on two approaches. Mean shear rate is the velocity gradient in the entire vessel cross-section along the RTLS divided by distance (Data not Included). Wall shear rate is the velocity gradient at the vessel wall calculated as the velocity in the lane closest to the vessel wall divided by the distance (assuming that the velocity at the vessel wall is 0 mm s^{-1}).

Statistical analysis

Data are expressed as mean ± s.e.m. The principal statistical test was the Student's t-test (two tailed with unequal variance). We analyzed the experiments involving multiple comparisons using multivariate analysis of variance followed by post-hoc within and between groups hypothesis testing (SYSTAT 12 - SYSTAT Inc). We considered a *P* value less than 0.05 to be statistically significant.