

Tumor Perfusion Shows Substantial Hour-Scale Temporal and Spatial Dynamics in Superresolution Ultrasound Measurements

Alexandra Raab

Universitätsklinikum Aachen

Jasmin Baier

Universitätsklinikum Aachen

Céline Porte

Universitätsklinikum Aachen

Thomas Lisson

Ruhr University Bochum

Palma Emese Inczeffy

Universitätsklinikum Aachen

Rahaf Mihyar

Universitätsklinikum Aachen

Andreas Schuppert

Universitätsklinikum Aachen

Renée Michèle Girbig

Universitätsklinikum Aachen

Anne Rix

Universitätsklinikum Aachen

Susanne Koletnik

Universitätsklinikum Aachen

Jochen Maurer

Universitätsklinikum Aachen

Stefanie Dencks

Ruhr University Bochum

Georg Schmitz

Ruhr University Bochum

Fabian Kiessling

fkiessling@ukaachen.de

Universitätsklinikum Aachen

Article

Keywords: ultrasound localization microscopy, microvascular heterogeneity, tumor perfusion, temporal heterogeneity, functional perfusion, hour-scale vascular dynamics

Posted Date: July 16th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9900073/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: Competing interest reported. The authors declare the following competing financial interest(s): A.Rix and F.K. are among the co-founders of the SonoMac GmbH that produces polymeric microbubbles that were used in these experiments.

Abstract

Tumor perfusion is highly heterogeneous, primarily due to the abnormal structure and function of the tumor-associated vasculature. While the spatial heterogeneity of tumor perfusion is well recognized, temporal changes over short time intervals remain poorly understood, as most studies rely on single time point measurements or longer observation periods spanning from days to weeks. In this study, we used motion model ultrasound localization microscopy (ULM) to investigate hour-scale changes in both tumor microvascular architecture and perfusion in two breast cancer xenograft models, MCF-7 (an ER+/PR+ human breast cancer cell line) and BCSC1 (a patient-derived triple-negative breast cancer model). Experiments were conducted in female BALB/cAnNRj-nu and NOD/SCID mice at 6–8 weeks of age. The tumors were imaged longitudinally at identical positions over a 9-hour period, with repeated acquisitions every 3 hours following intravenous microbubble injections, allowing for the extraction and quantification of microvascular parameters. Across all analyzed parameters, no systematic temporal trends or tumor subtype-specific differences were detected at the group level. However, intra-tumoral heterogeneity and temporal fluctuations were consistently observed in individual tumors, highlighting that tumor perfusion is dynamic and spatially heterogeneous even over short time intervals. Our findings indicate that tumor perfusion is temporally unstable on an hour scale, which may critically influence the efficiency and consistency of vascular drug delivery and contribute to variability in treatment response depending on the timing of therapeutic intervention. Rapidly changing and inconsistent tumor perfusion may further impact experimental outcomes, underscoring the importance of appropriate study design and timing of measurements.

Introduction

Tumor growth and progression depend on the formation of new blood vessels that supply oxygen and nutrients to the expanding tissue [1]. However, tumor-associated vasculature is fundamentally different from that of healthy tissue, as it lacks the hierarchical organization of network structures that ensures regulated blood flow under physiological conditions [2–4]. Instead, vessels within tumors form a chaotic and irregular network, characterized by heterogeneous vessel diameters, excessive branching, blind ends, and uneven vessel distribution [5]. In addition, tumor endothelial cells are often immature and insufficiently covered by pericytes with discontinuous basement membrane [6]. These structural abnormalities result in hyperpermeable, tortuous, and dilated vessels with aberrant blood flow patterns, leading to spatially heterogeneous perfusion [7]. Importantly, tumor perfusion is also modulated by the tumor microenvironment. Continuous tumor expansion, extracellular matrix remodeling, and stromal accumulation generate biomechanical forces that can compress intratumoral vessels and reduce luminal diameter and thereby limit blood flow [8]. At the same time, elevated interstitial fluid pressure, arising from increased vascular permeability and impaired lymphatic drainage, alters transvascular pressure gradients and may further restrict effective blood flow [9, 10]. As these pressure-related factors are spatially heterogeneous and dynamically evolving, transient vessel compression and reopening may occur, contributing to short-term changes in microvascular perfusion, independent of angiogenic remodeling [11–13].

Such spatial and temporal heterogeneity of tumor perfusion has important implications for therapeutic efficacy, as the majority of anticancer therapies depend on efficient vascular delivery. The distribution of blood flow within a tumor critically influences the uptake of chemotherapeutic, targeted therapies, and nanomedicines, which are particularly dependent on vascular permeability and perfusion [14, 15]. If perfusion fluctuates over short time scales, the effective exposure of tumor cells to systematically administered agents may vary depending on the timing of drug administration [10, 16]. Thus, understanding the temporal stability of tumor perfusion is essential for interpreting therapeutic outcomes and for optimizing perfusion-dependent treatment strategies.

Although tumor angiogenesis and vascular morphology have been extensively characterized in breast cancer and other malignancies, most studies assess vascular parameters at single time points or over longer intervals spanning days to weeks [17–20]. However, the vascular dynamics occurring on the minute-to-hour scale have not been evaluated systematically. In this context, it is known that a significant fraction of tumor vessels is not continuously perfused and that rapid fluctuations may occur [21]. To assess the functional heterogeneity and dynamics of the tumor vasculature, imaging approaches with high spatial and temporal resolution are required [22, 23].

Super-resolution ultrasound imaging (i.e. motion model Ultrasound Localization Microscopy (ULM)) has emerged as a powerful tool for detailed assessment of tumor microvascular morphology and perfusion [24, 25]. By localizing and tracking intravenously injected microbubbles (MB) and reconstructing high-resolution vascular maps, ULM enables the quantification of parameters such as flow velocity and vessel density beyond conventional ultrasound resolution limits [26, 27]. Recent advances suggest that this technique enables the capturing of second-to-hour scale microvascular and hemodynamic variability that was previously unresolvable [28, 29].

In this study, the short-term dynamic changes of tumor perfusion were systematically investigated in two phenotypically different breast cancer xenograft models: the ER+/PR+ hormone-receptor-positive MCF-7 model and the patient-derived triple-negative BCSC1 (Breast Cancer Stem Cell 1) model. Both exhibit irregular vascular networks, typical of solid tumors, but differ in tumor biology and growth kinetics [30–34]. At the identical position structural and functional features of the tumor microvasculature were assessed by ULM at multiple time points over 9 hours. In addition to in vivo imaging, histological analysis was performed to further assess vessel distribution and maturity. We hypothesized that physiologically relevant structural and perfusion-related fluctuations, including transient flow redistribution and alterations in microvascular profiles may occur that have strong implications on diagnostic approaches, drug delivery and drug administration schemes.

Experimental section

Animal experiments

All animal experiments were performed in accordance with relevant guidelines and regulations. The experimental protocol was reviewed and approved by the German State Office for Nature, Environment and Consumer Protection (LANUV) North Rhine-Westphalia (animal proposal number (AZ): 81-02.04.2022.A173) [35]. Five BALB/cAnNRj-nu mice and five NOD/SCID mice (female mice, age 6–8 weeks; Janvier Labs, Saint Berthevin, France) were group-housed under specific pathogen free conditions with a 12h light/dark cycle, controlled temperature (20–24°C) and humidity (45–65%), according to the guidelines of the “Federation for Laboratory Animal Science Associations” (FELASA, www.felasa.eu). All experiments are reported in adherence to the ARRIVE guidelines [36]. Mice had ad libitum access to standard pellets (Sniff GmbH, Soest, Germany) and acidified water. Animal welfare was monitored regularly throughout the study using a predefined clinical score sheet assessing general condition and signs of distress (Table S1). A priori sample size calculation was performed based on literature research to determine the required number of animals and to ensure sufficient statistical power. The present study did not involve human participants, human tissue samples, or human-derived materials. Therefore, institutional approval for human studies and informed consent were not applicable.

Study design

Subcutaneous tumor inoculation

Tumor blood vessel perfusion was characterized in two breast cancer xenograft models. For the establishment of the triple-negative BCSC1 model, five NOD/SCID mice were anesthetized with isoflurane, and the right thigh was prepared for tumor cell inoculation. For this purpose, mice were shaved with a small animal razor, followed by the application of VEET sensitive hair removal cream (Reckitt Benckiser, Slough, UK) for 3 minutes. BCSC1 cells (no RRID available) (5×10^5 cells in 100 μ L of a 1:1 mixture of Mammary Stem Cell culture medium [30] and Matrigel® (Corning, Corning, New York, USA)) were co-injected subcutaneously with human fibroblasts (1×10^6) into the right thigh. Before the inoculation of MCF-7 cancer cells (RRID: CVCL_0031), estradiol pellets (17 β -Estradiol, 1.5 mg/pellet, 60-day release, Innovative Research of America, Florida, USA) were implanted into the neck of five female Balb/cAnNRj nude mice via a precision trocar (10G). One week after pellet implantation, MCF-7 cancer cells (Cell Lines Service GmbH (CLS) Eppenheim, Deutschland) were injected subcutaneously into the right thigh (1×10^7 cells in 100 μ L mixture of high glucose DMEM (11965092, Gibco, Waltham, Massachusetts, USA) and Matrigel). Tumor sizes were assessed daily using caliper measurements and tumors with a size of at least 6x6 mm were used for ultrasound measurements.

Ultrasound imaging and post-processing

For ultrasound imaging, PBCA-MB (SonoMac, Inden-Lamersdorf, Germany) were administered intravenously via the tail vein at a dose of 1×10^7 MB in 50 μ L sterile 0.9% NaCl solution. Ultrasound imaging was performed using a Vevo 3100 ultrasound device equipped with an MX250 linear array transducer (FUJIFILM VisualSonics, Toronto, Canada). For every measurement, three consecutive acquisitions were recorded, each consisting of approximately 670–700 frames with a transmit frequency of 18 MHz, power of 10%, and a frame rate of 10 frames per second. Acquired ultrasound videos were analyzed using a ULM framework based on previously established methodologies with specific modifications to the preclinical dataset [37]. To account for breathing and cardiac motion, MATLAB's *imregdemon.m* was applied to SVD-filtered background B-mode images. For microbubble localization, a matched filter was employed on rank-filtered contrast data. The resulting MB positions were tracked using the MCMCDA algorithm and only tracks with a minimum length of three positions were retained for further analysis [38]. Finally, ULM parameter maps were reconstructed with a pixel resolution of 10 μ m. The degree of reconstruction was calculated according to Dencks et al. and had an average of 58.68% across all measurements [39].

In addition, flow dynamics were semi-quantitatively assessed using the Vevo Lab 5.7.1 software (FUJIFILM VisualSonics, Toronto, Canada). Regions of interest (ROIs) were consistently placed over tumor tissue and adjacent muscle tissue. Time-intensity curves were generated based on the CEUS videos in contrast mode within these ROIs. Peak Enhancement (PE) was defined as the maximum signal intensity between the onset of signal increase, after the wash-in (baseline signal prior to MB arrival) and the plateau phase of the time-intensity curve. For Area Under the Curve (AUC) analysis, the wash-in signal was subtracted and the area under the time-intensity curve was calculated over a fixed time window 40 seconds. This analysis was performed in a subset of three animals, where reliable acquisition of the muscle tissue could be achieved.

Microfil perfusion

Following the final imaging time point at 9 hours, mice were anesthetized with ketamine/xylazine in 0.9% NaCl (120 mg/kg body weight ketamine and 16 mg/kg body weight xylazine; 30 μ L/10 g body weight intraperitoneal injection). For vascular casting of perfused vessels, mice were subjected to transcardial perfusion with Microfil® (Flow Tech, Carver, Massachusetts, USA), resulting in euthanasia by exsanguination, in accordance with the approved animal protocol. Briefly, mice received 250 IU of heparin intraperitoneally prior to perfusion. Animals were fixed in the dorsal position, followed by a midline laparotomy to expose the thoracic cavity. A peripheral venous catheter (27G) was inserted into the left ventricle, and the inferior vena cava was incised. Initially, 10 mL PBS were perfused at a pump-controlled flow rate of 100 mL/h to clear the vascular system. This was followed by a perfusion with 10 mL of a 4% paraformaldehyde solution at the same flow rate for fixation. Finally, 5 mL of Microfil® were injected at 100 mL/h for vascular casting. After injection, the contrast agent was left to polymerize for 25 min, before tumors were harvested for histological analysis. Microfil signal was imaged using the green fluorescence channel at an excitation wavelength of 488 nm.

Immunohistology

Paraffin-embedded tumors were sectioned into 4 μm -thick slices. Sections were deparaffinized in xylene and rehydrated through a graded ethanol series to distilled water. Heat-induced antigen retrieval was performed in epitope retrieval solution (1:10 dilution in distilled water, pH 9.0; Leica Biosystems, Wetzlar, Germany) at 95°C for 30 minutes, followed by cooling to room temperature. Endogenous enzyme activity was blocked using BLOXALL® (Vector Laboratories, Newark, California, USA) for 1 hour. Primary antibodies were diluted in TNT buffer (TRIS-NaCl-Tween) and incubated for 1 hour at room temperature. Blood vessels were detected using anti-CD31 (ab182981, Abcam, Cambridge, UK) and pericyte coverage as a marker of vessel maturity was assessed using anti- α SMA (ab124964, Abcam, Cambridge, UK). Following washes in TNT buffer, samples were incubated with appropriate secondary antibodies. For CD31, HRP-conjugated secondary antibody (ab97080, Abcam, Cambridge, UK) was used, followed by signal amplification using TSA Plus Working Solution (1 x Plus Manual Amplification Diluent, FP1135, Akoya Biosciences, Marlborough, Massachusetts, USA) together with a Cyanine 5 fluorophore (TSA Plus Cyanine 5, NEL745001KT, Akoya Biosciences, Marlborough, Massachusetts, USA) according to the manufacturer's instructions. α SMA was detected using the according fluorescent secondary antibody (#111-155-003, Dianova, Hamburg, Germany). Slides were mounted with fluorescence mounting medium (S302380-2, Agilent Dako, Santa Clara, California USA). Whole-slide images were acquired using a Keyence fluorescence microscope (BZ-X1000, Keyence, Osaka, Japan) with a PanFluor 10x objective (NA 0.30). Vessel density, vascular area fraction, and vessel maturity via CD31 and α SMA colocalization were quantified by whole-slide analysis using QuPath 7.0 software.

Statistical analysis

Longitudinal changes in microvascular parameters (vessel coverage, mean flow velocity, mean vessel tortuosity, mean distance to closest track, mean track length, mean track counts, and mean track-wise velocity) and differences between the two tumor models were assessed using linear mixed-effect models in GraphPad Prism10 (GraphPad Software, v10.6.1, San Diego, California, USA) and SPSS (BM Corp., v31.0, Armonk, New York, USA). Linear mixed-effects models were chosen, as they provide flexibility for modelling longitudinal data. Moreover, they allow explicit modelling of within-subject correlations and are robust to potential missing readouts. Each parameter was analyzed separately as the dependent variable. The fixed-effects structure included the four imaging time points (0h, 3h, 6h, and 9h) and the tumor models (BCSC1 and MCF-7), as well as their interaction term (time point x tumor model) to evaluate model-specific temporal patterns. Mouse identity was included as a random effect to account for repeated measurements within the same animal. Within-mouse temporal correlation was modelled using a first-order autoregression covariance structure (AR(1)) that allows for correlation between adjacent time points, while accommodating for variabilities between individual animals. Models were estimated using restricted maximum likelihood (REML) with Satterthwaite approximation for denominator degrees of freedom. F-statistics and corresponding p-values for fixed effects, as well as covariance estimates were reported for each parameter (Table S2).

Results

To investigate temporal changes within the tumor microvascular architecture and function, longitudinal ULM was performed in BCSC1 and MCF-7 tumor-bearing mice over a 9-hour observation period, enabling repeated measurement and quantification of microvascular parameters (Fig. 1). Due to the extended duration of the imaging protocol, 6 of the 10 mice completed the full 9-hour period. Additionally, in rare cases, individual time points could not be acquired, resulting in partially incomplete longitudinal datasets for some mice. Across all analyzed parameters, a consistent pattern emerged where no systematic temporal trends or tumor subtype-specific differences were found (Table S2). However, each parameter revealed marked intra-tumoral heterogeneity, which emerged as a defining feature of the observed microvascular dynamics.

Microvascular vessel coverage, representing the relative perfused vessel area, remained stable throughout the observation period and showed no significant difference between the tumor models (Fig. 2A). Mixed-effects modelling did not reveal significant effects of time ($F(3,13.52) = 1.67$, $p = 0.221$), tumor model ($F(1,8.36) = 0.98$, $p = 0.349$), or the interaction between both time and tumor model ($F(3,13.52) = 0.49$, $p = 0.693$) (Table S2). Despite this stability at the group level, individual tumor profiles revealed marked fluctuations in vessel coverage over time (Fig. 2B,C), indicating highly dynamic and spatially heterogeneous perfusion patterns within single tumors. This was further supported by variability analysis, showing substantial intra-tumoral heterogeneity with maximum deviations from the mean ranging from - 67.26% to 173.04% in BCSC1 and - 47.15% to 59.85% in MCF-7 tumors (Fig. 2D). Notably, vessel coverage represented the most variable parameter across all the analyzed metrics, suggesting that tumor perfusion is highly dynamic even over short time intervals. Representative binary track maps further illustrate these dynamics, revealing local and temporally evolving perfused regions within the same tumors (Fig. 2E, F), while the analysis of non-normalized values and absolute deviations from baseline yielded comparable results, further confirming the absence of systematic temporal trends (Fig. S1A, S2A).

To assess potential confounding effects of prolonged anesthesia on microvascular perfusion, PE and AUC were quantified in adjacent muscle tissue using contrast-enhanced ultrasound (Fig. S3). In contrast to tumor tissue, muscle perfusion remained largely stable over time. Similarly, physiological parameters including heart rate and breathing rate remained within a stable range throughout the isoflurane anesthesia (Fig. S3A,B). Together, these findings indicate that the observed temporal variability predominantly reflects tumor-intrinsic microvascular dynamics rather than a systemic physiological drift.

The spatial organization of the tumor microvasculature was further assessed by quantifying the distance to the closest MB track (Fig. 3). This parameter complements vessel coverage by capturing the spatial organization of perfused vessels, as comparable coverage values may arise from either numerous small vessels or fewer larger vessels, resulting in different distances to the closest MB track. Mixed-effects modelling revealed no significant effects of time ($F(3,13.88) = 0.17$, $p = 0.917$), tumor model ($F(1, 8.47) = 2.80$, $p = 0.131$), or the interaction between time and tumor model ($F(3,13.88) = 0.28$, $p = 0.838$). Despite this overall stability, individual tumor profiles demonstrated heterogeneous changes in vessel spacing over time with baseline-normalized values ranging from 9% to 328% for BCSC1 and 18% to 372% for MCF-7 (Fig. 3B,C). This was supported by the analysis of non-normalized values and absolute deviations from the baseline, which confirmed the absence of systematic temporal trends despite pronounced local variability (Fig.

S1B, S2B). Variability analysis further highlighted pronounced intra-tumoral heterogeneity, with deviations from the mean ranging from – 88.87% to 103.97% for BCSC1 and – 67.42% to 78.24% for MCF-7 xenografts (Fig. 3D). Notably, the distance to the closest track represented the second most variable parameter across all analyzed metrics, suggesting that, in addition to dynamic changes in perfusion, the spatial distribution of the vascular supply within tumors is highly heterogeneous and temporally unstable.

As the final ULM-derived parameter, microvascular flow velocity was analyzed to assess functional changes in tumor perfusion dynamics (Fig. 5). Unlike structural parameters, flow velocity reflects the speed of blood flow within perfused vessels and is therefore largely independent of vessel density. Mixed-effects modelling revealed the absence of significant effects of time ($F(3,16.06) = 2.5$, $p = 0.090$), tumor model ($F(1,8.11) = 0.51$, $p = 0.494$), or their interaction ($F(3,16.06) = 0.16$, $p = 0.921$), indicating comparable flow velocities across tumor types and time points (Table S2). In line with previously described parameters, it was observed that despite the absence of systematic differences, individual tumors displayed temporal variability in flow velocity over time (Fig. 5B,C). However, in contrast to vessel coverage and other spatial parameters, the variability in flow velocity was relatively low with deviations from the mean ranging from – 16.64% to 17.42% in BCSC1 and – 9.72% to 7.75% for MCF-7 tumor-bearing mice (Fig. 5D-F). Non-normalized values and absolute deviations from the baseline yielded consistent results, further supporting the overall stability of flow velocity, despite localized fluctuations (Fig. S1D, S2D).

To complement longitudinal ULM measurements and provide structural validation of the observed microvascular dynamics, endpoint histological analysis using immunofluorescence imaging was performed on tumor sections stained for CD31 (endothelial cells) and α SMA (vascular maturity and perivascular coverage). In addition, tumors were perfused with Microfil prior to tissue collection to visualize the perfused fraction of the vascular network (Fig. 6A). Consistent with the *in vivo* observations, histological analysis revealed a heterogeneous vascular architecture in both tumor models, characterized by variable vessel density, perfusion, and maturation states across individual tumors. While strong trends between BCSC1 and MCF-7 could be observed, quantitative analysis did not reveal significant differences between tumor types across the assessed histological parameters. Specifically, vessel density, defined as the number of CD31⁺ vessels per area, was higher in MCF-7 tumors with 136.64 ± 36.19 vessels/mm² compared to BCSC1 tumors with 102.94 ± 26.48 vessels/mm². Similarly, the density of perfused vessels, identified by Microfil⁺ vessels, was higher in MCF-7 tumors with 61.07 ± 29.69 vessels/mm² compared to BCSC1 with 41.19 ± 18.22 vessels/mm². The fraction of perfused vessels relative to the total CD31⁺ vessels further indicated that only a subset of the vascular network was functionally perfused in both tumor models ($39.73 \pm 12.49\%$ in BCSC1 and $45.13 \pm 17.37\%$ in MCF-7), consistent with the heterogeneous perfusion patterns observed in ULM-derived coverage maps (Fig. 2E,F). Assessment of vessel maturity, reflected by the fraction of α SMA⁺/CD31⁺ double-positive vessels, revealed $41.41 \pm 26.29\%$ for BCSC1 and $47.22 \pm 20.42\%$ for MCF-7 tumors, indicating heterogeneous stabilization states of the tumor vasculature. Notably, despite the higher total and perfused vessel density in MCF-7 tumors, the relative fraction of perfused vessels was similar between both models. This suggests that the increased vascularization in MCF-7 tumors is accompanied by a proportionally similar fraction of vessels that are not perfused. Thus, these tumors have a higher total but not relative functional reserve.

Discussion

In this study, longitudinal ULM imaging revealed that the tumor microvascular architecture and function are characterized by pronounced intra-tumoral heterogeneity and rapid temporal fluctuations even within short observation intervals of only a few hours. BCSC1 and MCF-7 tumors were selected as moderately vascularized models with comparable baseline vascular characteristics, allowing for the assessment of temporal microvascular dynamics under similar starting conditions. While no consistent temporal trends, tumor subtype-specific and interaction effects were observed in the analyzed parameters, individual tumors exhibited highly variable and non-uniform changes over time, indicating that the tumor microvasculature is inherently dynamic and undergoes continuous remodeling. This dynamic behavior was most pronounced for vessel coverage and the distance to closest track, which emerged as the most variable parameters. The marked fluctuations observed in both highlight that not only the extent of perfusion, but also perfusion-driven changes in spatial organization undergo rapid and heterogeneous variation over time. Importantly, these changes occurred in the absence of any systematic trends, suggesting that microvascular remodeling within tumors follows a stochastic and chaotic pattern, rather than a predictable trajectory [40–43]. Furthermore, this dynamic behavior occurs on the background of inherently limited baseline perfusion, with only a fraction of vessels being functionally perfused at any given time [21]. Histological analysis additionally revealed that, while both tumors exhibit similar relative perfusion, differences in absolute perfusion and vascularization may indicate variations in the overall functional capacity of the tumor vasculature between the models.

The clinical relevance of these findings becomes particularly apparent in the context of imaging-based perfusion assessment, which is routinely used to guide diagnostic and therapeutic decision making in oncology. For instance, in breast cancer and other solid malignancies, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is widely used to evaluate tumor vascularization, permeability, and treatment response [44, 45]. However, these measurements are typically acquired at a single time point or at different days, and it is assumed that they reflect a stable vascular state [46]. While preclinical MRI-based studies have shown that tumor perfusion can fluctuate over short time periods ranging from 30 minutes to a few hours, the limited spatial resolution hinders the detailed assessment of microvascular behavior [47–49]. Our ULM-based findings suggest that this assumption may be over-simplistic, as tumor perfusion may vary even within short time intervals. Temporal instability in tumor perfusion may therefore contribute to the variability seen in DCE-MRI and other measurements and could potentially influence clinical decision-making [50]. If perfusion fluctuates so significantly, the measured enhancement characteristics may depend not only on underlying tumor biology, but also on the specific timepoint of acquisition. This could help explain inconsistencies observed in longitudinal imaging studies and highlights the importance of considering temporal heterogeneity when interpreting perfusion-based imaging biomarkers [51].

Beyond diagnostic imaging, the dynamic and spatially heterogeneous nature of the tumor microvasculature also has direct implications for therapeutic strategies. Many anticancer treatments, including conventional therapeutics, antibody-based therapies, and nanoparticle-based drug delivery systems, rely on sufficient and temporally stable vascular perfusion to achieve effective target accumulation and intratumoral distribution [52, 53]. In both experimental and clinical settings, tumor perfusion is commonly assessed at a single time point and subsequently used as a baseline to guide therapeutic interventions [54–56]. However, our data indicate that such single time point measurements may not adequately reflect the dynamic nature of the tumor microvasculature. If perfusion states, including vessel coverage and spatial accessibility, can vary substantially within a matter of hours, the actual microvascular environment at the time of drug administration may differ markedly from the initially measured baseline. This becomes particularly relevant for anti-cancer drugs with short systemic half-lives, where the availability is limited to a narrow time frame [50, 57]. Under these conditions, temporal fluctuations in tumor perfusion could lead to substantial variability in drug delivery efficiency, depending on whether the administration coincides with periods of higher or lower perfusion. In contrast, therapeutic strategies involving prolonged systemic circulation times or sustained drug exposure may be less susceptible to these transient changes in microvascular dynamics, potentially leading to more consistent delivery. This is likely due to an increased probability of alignment with phases of improved vascular access, particularly in tumor regions that present high heterogeneity.

The observed heterogeneity and partial perfusion of tumor vasculature are consistent with previous reports that describe the abnormal and inefficient nature of tumor blood vessels, which are often poorly perfused and irregularly distributed [58, 59]. These characteristics have been a central motivation for the concept of vascular normalization, which aims to transiently improve perfusion and enhance drug delivery [60, 61]. However, our findings suggest that tumor vasculature is not just structurally abnormal, but also temporally unstable. Notably, mean perfusion was limited to approximately 40–45%, as indicated by both histological analysis (~42%) and ULM-derived vessel coverage (~45%) across tumors and time points. This implies that, at any given time, a substantial fraction of the tumor vasculature remains non-functional, thereby imposing a fundamental limitation on effective drug delivery.

Furthermore, several limitations of this study should be discussed. First, the number of animals included in the experiments was limited, reflecting the technical complexity and duration of the longitudinal imaging protocol. In addition, not all animals completed the full imaging period due to the extended duration of anesthesia required for continuous monitoring. Nevertheless, the observed intra-tumoral variability was consistent across multiple tumors and parameters, indicating that the identified patterns are robust despite the limited remaining cohort size at 9 hours. Importantly, continuous physiological monitoring and comparison to muscle tissue as an internal reference for perfusion stability throughout the imaging time points, allowed us to largely exclude systemic anesthesia-related effects as a driver of the observed vascular fluctuations. Furthermore, it should be noted that ULM-based analysis relies on MB detection and tracking and may therefore be influenced by factors such as incomplete sampling of the vascular network, false track reconstruction, or artefacts related to ultrasound acquisition. However, the high density of reconstructed tracks and the consistency of the findings across different parameters, support the reliability of the observed patterns [39, 62]. In addition, while the ultrasound transducer was mechanically stabilized and coupled to the mice to maintain a constant imaging plane, minor deviations over the prolonged imaging period cannot be fully excluded. However, given the reproducibility of the measurements and the absence of systematic trends, such effects are unlikely to have mislead our conclusions.

To extend observations made in this study, the integration of complementary assessments of tumor hypoxia may provide additional insights regarding the spatial heterogeneity of perfusion. Changes in parameters such as vessel coverage and distance to the closest track may reflect variations in local oxygen supply, where increased distances to perfused vessels are indicative of regions with reduced perfusion and potentially increased hypoxia within the tumor microenvironment [63]. Additionally, while other factors relevant to dynamic changes in the tumor vasculature such as interstitial fluid pressure were not directly assessed in the present study, future investigations combining high-resolution vascular imaging with measurements of intra-tumoral pressure may provide further mechanistic insight into vascular changes within tumors. Such multimodal approaches could help to understand whether transient vascular fluctuations are linked to changes in local pressure gradients or mechanical vessel compression within the tumor microenvironment. In addition, extending this approach to tumor models with differing baseline vascular characteristics, such as more highly vascularized tumors or those with increased vessel maturity, could provide further insight into the underlying mechanisms. This may help determine whether the observed temporal instability varies with the degree of tumor vascularization.

Conclusion

In conclusion, our findings demonstrate that the tumor microvasculature and its functionality cannot be considered as static but rather represents a highly dynamic and heterogeneous system. The combination of limited baseline perfusion and rapid, unpredictable temporal fluctuations would imply a continuously evolving vascular landscape, in which both the fraction of perfused vessels and their spatial distribution change over short time scales. This intrinsic variability over time emerges as a critical factor that can substantially influence drug delivery and therapeutic efficacy, representing a barrier for efficient and reliable treatment. Incorporating dynamic vascular behavior into experimental design, imaging interpretation, and therapeutic strategies may therefore improve the understanding of the heterogeneity of tumor perfusion and help to explain variability in treatment responses across both preclinical and clinical settings.

Declarations

Author contributions:

F.K. conceptualized and supervised the study. F.K., A.Rix, and C.P. designed the experiments. J.B., R.G., A.Rix, and S.K. performed the in vivo experiments and ultrasound acquisition. J.M. provided tumor cells necessary for the experiment. T.L. performed ULM processing, G.S. and S.D. supervised ULM processing, and C.P. and P.E.I. performed ULM parameter extraction and analysis. A.Raab and R.M. performed histological analysis. A.Raab, J.B., and

A.S. analyzed the data. A.Raab designed the figures and drafted the original manuscript. All authors read, revised, and approved the final version of the manuscript.

Acknowledgements

The authors gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft (DFG) project number 321137804 and the DFG research training group RTG 2375: project number: 331065168.

Source of funding:

This research was funded by the Deutsche Forschungsgemeinschaft (DFG) project number 321137804 and the DFG research training group RTG 2375: project number: 331065168.

Declaration of competing interest:

The authors declare the following competing financial interest(s): A.R. and F.K. are among the co-founders of the SonoMac GmbH that produces polymeric microbubbles that were used in these experiments.

Data availability statement:

The datasets generated and / or analyzed during the current study can be made available upon reasonable request to the corresponding author.

References

1. Folkman, J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol.* **29**, 15–18. <https://doi.org/10.1053/sonc.2002.37263> (2002).
2. Forster, J., Harriss-Phillips, W., Douglass, M. & Bezak, E. A review of the development of tumor vasculature and its effects on the tumor microenvironment. *Hypoxia* **5**, 21–32. <https://doi.org/10.2147/HP.S133231> (2017).
3. Baluk, P., Hashizume, H. & McDonald, D. M. Cellular abnormalities of blood vessels as targets in cancer. *Curr. Opin. Genet. Dev.* **15**, 102–111. <https://doi.org/10.1016/j.gde.2004.12.005> (2005).
4. Ribatti, D., Nico, B., Crivellato, E. & Vacca, A. The structure of the vascular network of tumors. *Cancer Lett.* **248**, 18–23. <https://doi.org/10.1016/j.canlet.2006.06.007> (2007).
5. Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249–257. <https://doi.org/10.1038/35025220> (2000).
6. De Palma, M., Biziato, D. & Petrova, T. V. Microenvironmental regulation of tumour angiogenesis. *Nat. Rev. Cancer.* **17**, 457–474. <https://doi.org/10.1038/nrc.2017.51> (2017).
7. Guelfi, S., Hodivala-Dilke, K. & Bergers, G. Targeting the tumour vasculature: from vessel destruction to promotion. *Nat. Rev. Cancer.* **24**, 655–675. <https://doi.org/10.1038/s41568-024-00736-0> (2024).
8. Liu, Z. L., Chen, H. H., Zheng, L. L., Sun, L. P. & Shi, L. Angiogenic signaling pathways and anti-angiogenic therapy for cancer. *Signal. Transduct. Target. Ther.* **8**, 198. <https://doi.org/10.1038/s41392-023-01460-1> (2023).
9. Goel, S. et al. Normalization of the vasculature for treatment of cancer and other diseases. *Physiol. Rev.* **91**, 1071–1121. <https://doi.org/10.1152/physrev.00038.2010> (2011).
10. Jain, R. K. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. *J. Clin. Oncol.* **31**, 2205–2218. <https://doi.org/10.1200/JCO.2012.46.3653> (2013).
11. Michiels, C., Tellier, C. & Feron, O. Cycling hypoxia: a key feature of the tumor microenvironment. *Biochim. Biophys. Acta Rev. Cancer.* **1866**, 76–86. <https://doi.org/10.1016/j.bbcan.2016.06.004> (2016).
12. Padera, T. P. et al. Cancer cells compress intratumour vessels. *Nature* **427**, 695. <https://doi.org/10.1038/427695a> (2004).
13. Brurberg, K. G., Gaustad, J. V., Mollatt, C. S. & Rofstad, E. K. Temporal heterogeneity in blood supply in human tumor xenografts. *Neoplasia* **10**, 727–735. <https://doi.org/10.1593/neo.08388> (2008).
14. Stylianopoulos, T., Munn, L. L. & Jain, R. K. Reengineering the tumor vasculature: improving drug delivery and efficacy. *Trends Cancer.* **4**, 258–259. <https://doi.org/10.1016/j.trecan.2018.02.010> (2018).
15. May, J. N. et al. Histopathological biomarkers for predicting the tumour accumulation of nanomedicines. *Nat. Biomed. Eng.* **8**, 1366–1378. <https://doi.org/10.1038/s41551-024-01197-4> (2024).
16. Dewhirst, M. W. & Secomb, T. W. Transport of drugs from blood vessels to tumour tissue. *Nat. Rev. Cancer.* **17**, 738–750. <https://doi.org/10.1038/nrc.2017.93> (2017).
17. Hu, X. et al. Microvascular heterogeneity exploration in core and invasive zones of orthotopic rat glioblastoma via ultrasound localization microscopy. *Eur. Radiol. Exp.* **9**, 30. <https://doi.org/10.1186/s41747-025-00555-4> (2025).
18. McCall, J. R. et al. Longitudinal 3-D visualization of microvascular disruption and perfusion changes in mice during the evolution of glioblastoma using super-resolution ultrasound. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control.* **70**, 1401–1416. <https://doi.org/10.1109/TUFFC.2023.3320034> (2023).

19. Lowerison, M. R., Huang, C., Lucien, F., Chen, S. & Song, P. Ultrasound localization microscopy of renal tumor xenografts in chicken embryo is correlated to hypoxia. *Sci. Rep.* **10**, 2478. <https://doi.org/10.1038/s41598-020-59338-z> (2020).
20. Hoyt, K. Super-resolution ultrasound imaging for monitoring the therapeutic efficacy of a vascular disrupting agent in an animal model of breast cancer. *J. Ultrasound Med.* **43**, 1099–1107. <https://doi.org/10.1002/jum.16438> (2024).
21. Fisher, D. T. et al. Intraoperative intravital microscopy permits the study of human tumour vessels. *Nat. Commun.* **7**, 10684. <https://doi.org/10.1038/ncomms10684> (2016).
22. O'Connor, J. P. B. et al. Imaging intratumor heterogeneity: role in therapy response, resistance, and clinical outcome. *Clin. Cancer Res.* **21**, 249–257. <https://doi.org/10.1158/1078-0432.CCR-14-0990> (2015).
23. Lin, Q., Choyke, P. L. & Sato, N. Visualizing vasculature and its response to therapy in the tumor microenvironment. *Theranostics* **13**, 5223–5246. <https://doi.org/10.7150/thno.84947> (2023).
24. Porte, C. et al. Ultrasound localization microscopy for breast cancer imaging in patients: protocol optimization and comparison with shear wave elastography. *Ultrasound Med. Biol.* **50**, 57–66. <https://doi.org/10.1016/j.ultrasmedbio.2023.09.001> (2024).
25. Dencks, S. et al. Super-resolution ultrasound: from data acquisition and motion correction to localization, tracking, and evaluation. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control.* **72**, 408–426. <https://doi.org/10.1109/TUFFC.2025.3543322> (2025).
26. Dencks, S. & Schmitz, G. Ultrasound localization microscopy. *Z. Med. Phys.* **33**, 292–308. <https://doi.org/10.1016/j.zemedi.2023.02.004> (2023).
27. Opacic, T. et al. Motion model ultrasound localization microscopy for preclinical and clinical multiparametric tumor characterization. *Nat. Commun.* **9**, 1527. <https://doi.org/10.1038/s41467-018-03973-8> (2018).
28. Renaudin, N. et al. Functional ultrasound localization microscopy reveals brain-wide neurovascular activity on a microscopic scale. *Nat. Methods.* **19**, 1004–1012. <https://doi.org/10.1038/s41592-022-01549-5> (2022).
29. Chavignon, A. et al. 3D transcranial ultrasound localization microscopy in the rat brain with a multiplexed matrix probe. *IEEE Trans. Biomed. Eng.* **69**, 2132–2142. <https://doi.org/10.1109/TBME.2021.3137265> (2022).
30. Metzger, E. et al. KDM4 inhibition targets breast cancer stem-like cells. *Cancer Res.* **77**, 5900–5912. <https://doi.org/10.1158/0008-5472.CAN-17-1754> (2017).
31. Li, X., Strietz, J., Bleilevens, A., Stickeler, E. & Maurer, J. Chemotherapeutic stress influences epithelial–mesenchymal transition and stemness in cancer stem cells of triple-negative breast cancer. *Int. J. Mol. Sci.* **21**, 404. <https://doi.org/10.3390/ijms21020404> (2020).
32. De Lorenzi, F. et al. Engineering mesoscopic 3D tumor models with a self-organizing vascularized matrix. *Adv. Mater.* **36**, e2303196. <https://doi.org/10.1002/adma.202303196> (2024).
33. Wall, A. et al. Differentiation of angiogenic burden in human cancer xenografts using a perfusion-type optical contrast agent (SIDAG). *Breast Cancer Res.* **10**, R23. <https://doi.org/10.1186/bcr1875> (2008).
34. Dall, G., Viesusseux, J., Unsworth, A., Anderson, R. & Britt, K. Low dose, low cost estradiol pellets can support MCF-7 tumour growth in nude mice without bladder symptoms. *J. Cancer.* **6**, 1331–1336. <https://doi.org/10.7150/jca.10890> (2015).
35. European Union. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. (2010). <https://eur-lex.europa.eu/eli/dir/2010/63/oj/eng>
36. Percie et al. Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biol* **18**:e3000411. (2020). <https://doi.org/10.1371/journal.pbio.3000411>
37. Porte, C. et al. Ultrasound localization microscopy for breast cancer imaging in patients: protocol optimization and comparison with shear wave elastography. *Ultrasound Med. Biol.* **50**, 57–66. <https://doi.org/10.1016/j.ultrasmedbio.2023.09.001> (2024).
38. Ackermann, D. & Schmitz, G. Detection and tracking of multiple microbubbles in ultrasound B-mode images. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control.* **63**, 72–82. <https://doi.org/10.1109/TUFFC.2015.2500266> (2016).
39. Dencks, S., Piepenbrock, M. & Schmitz, G. Assessing vessel reconstruction in ultrasound localization microscopy by maximum likelihood estimation of a zero-inflated Poisson model. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control.* **67**, 1603–1612. <https://doi.org/10.1109/TUFFC.2020.2980063> (2020).
40. Fukumura, D., Duda, D. G., Munn, L. L. & Jain, R. K. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. *Microcirculation* **17**, 206–225. <https://doi.org/10.1111/j.1549-8719.2010.00029.x> (2010).
41. Brown, E. B. et al. In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nat. Med.* **7**, 864–868. <https://doi.org/10.1038/89997> (2001).
42. Matsumoto, S., Yasui, H., Mitchell, J. B. & Krishna, M. C. Imaging cycling tumor hypoxia. *Cancer Res.* **70**, 10019–10023. <https://doi.org/10.1158/0008-5472.CAN-10-2821> (2010).
43. Pries, A. R. et al. Structural adaptation and heterogeneity of normal and tumor microvascular networks. *PLoS Comput. Biol.* **5**, e1000394. <https://doi.org/10.1371/journal.pcbi.1000394> (2009).
44. Frankhouser, D. E., Dietze, E. C., Mahabal, A. & Seewaldt, V. L. Vascularity and dynamic contrast-enhanced breast magnetic resonance imaging. *Front. Radiol.* **1**, 735567. <https://doi.org/10.3389/fradi.2021.735567> (2021).
45. Kim, H. Variability in quantitative DCE-MRI: sources and solutions. *J Nat Sci* **4**(1). PMID: 29527572. (2018).

46. Shukla-Dave, A. et al. Quantitative imaging biomarkers alliance (QIBA) recommendations for improved precision of DWI and DCE-MRI derived biomarkers in multicenter oncology trials. *J. Magn. Reson. Imaging*. **49**, e101–e121. <https://doi.org/10.1002/jmri.26518> (2019).
47. Nagaraja, T. N. et al. Imaging acute effects of bevacizumab on tumor vascular kinetics in a preclinical orthotopic model of U251 glioma. *NMR Biomed*. **34**, e4516. <https://doi.org/10.1002/nbm.4516> (2021).
48. Brurberg, K. G., Benjaminsen, I. C., Dørum, L. M. R. & Rofstad, E. K. Fluctuations in tumor blood perfusion assessed by dynamic contrast-enhanced MRI. *Magn. Reson. Med*. **58**, 473–481. <https://doi.org/10.1002/mrm.21367> (2007).
49. Baudelet, C. et al. Physiological noise in murine solid tumours using T2*-weighted gradient-echo imaging: a marker of tumour acute hypoxia? *Phys. Med. Biol.* **49**, 3389–3411. <https://doi.org/10.1088/0031-9155/49/15/006> (2004).
50. RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307**, 58–62. <https://doi.org/10.1126/science.1104819> (2005).
51. O'Connor, J. P. B., Jackson, A., Parker, G. J. M. & Jayson, G. C. DCE-MRI biomarkers in the clinical evaluation of antiangiogenic and vascular disrupting agents. *Br. J. Cancer*. **96**, 189–195. <https://doi.org/10.1038/sj.bjc.6603515> (2007).
52. Famta, P. et al. Amelioration of breast cancer therapies through normalization of tumor vessels and microenvironment: paradigm shift to improve drug perfusion and nanocarrier permeation. *Drug Deliv Transl Res*. **15**, 389–406. <https://doi.org/10.1007/s13346-024-01669-9> (2025).
53. Jordan, B. F. & Sonveaux, P. Targeting tumor perfusion and oxygenation to improve the outcome of anticancer therapy. *Front. Pharmacol.* **3**, 00094. <https://doi.org/10.3389/fphar.2012.00094> (2012).
54. Sridharan, N. et al. Measuring repeatability of dynamic contrast-enhanced MRI biomarkers improves evaluation of biological response to radiotherapy in lung cancer. *Eur. Radiol.* **35**, 664–673. <https://doi.org/10.1007/s00330-024-10970-7> (2025).
55. Weber, J. P. D. et al. Reproducibility of dynamic contrast enhanced MRI derived transfer coefficient K_{trans} in lung cancer. *PLoS One*. **17**, e0265056. <https://doi.org/10.1371/journal.pone.0265056> (2022).
56. Tao, X. et al. DCE-MRI perfusion and permeability parameters as predictors of tumor response to CCRT in patients with locally advanced NSCLC. *Sci. Rep.* **6**, 35569. <https://doi.org/10.1038/srep35569> (2016).
57. Troendle, E. P., Khan, A., Searson, P. C. & Ulmschneider, M. B. Predicting drug delivery efficiency into tumor tissues through molecular simulation of transport in complex vascular networks. *J. Control Release*. **292**, 221–234. <https://doi.org/10.1016/j.jconrel.2018.11.010> (2018).
58. Nagy, J. A., Chang, S. H., Dvorak, A. M. & Dvorak, H. F. Why are tumour blood vessels abnormal and why is it important to know? *Br. J. Cancer*. **100**, 865–869. <https://doi.org/10.1038/sj.bjc.6604929> (2009).
59. Martin, J. D., Seano, G. & Jain, R. K. Normalizing function of tumor vessels: progress, opportunities, and challenges. *Annu. Rev. Physiol.* **81**, 505–534. <https://doi.org/10.1146/annurev-physiol-020518-114700> (2019).
60. Jain, R. K. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307**, 58–62. <https://doi.org/10.1126/science.1104819> (2005).
61. Stylianopoulos, T., Munn, L. L. & Jain, R. K. Reengineering the tumor vasculature: improving drug delivery and efficacy. *Trends Cancer*. **4**, 258–259. <https://doi.org/10.1016/j.trecan.2018.02.010> (2018).
62. Dencks, S. et al. Clinical pilot application of super-resolution US imaging in breast cancer. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control*. **66**, 517–526. <https://doi.org/10.1109/TUFFC.2018.2872067> (2019).
63. Kabir, A. U., Subramanian, M., Kwon, Y. & Choi, K. Linking tumour angiogenesis and tumour immunity. *Nat. Rev. Immunol.* **26**, 35–51. <https://doi.org/10.1038/s41577-025-01211-z> (2026).

Figures

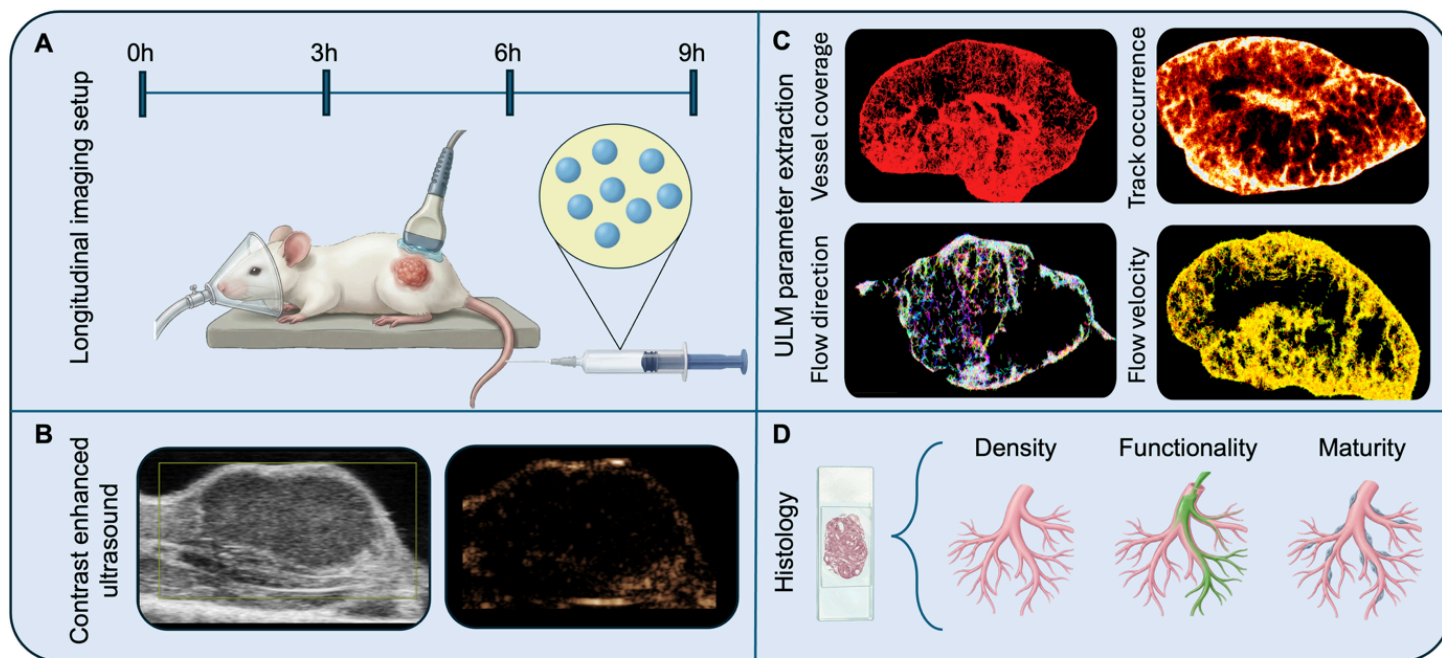


Figure 1

Longitudinal workflow for the analysis of tumor microvascular dynamics. (A) Schematic overview of the longitudinal imaging setup. Tumor-bearing mice were kept under continuous isoflurane anesthesia for 9 hours with PBCA-MB being administered via tail vein injection every 3 hours (0h, 3h, 6h, 9h), for repeated vascular imaging at an identical imaging plane. (B) Representative CEUS images acquired at each time point, including simultaneous B-mode acquisition (left) and contrast-mode enhanced acquisition (right). (C) Representative ULM parameter maps used for the extraction of key vascular parameters. These included binary vessel maps (top left), track occurrence maps (top right), flow direction maps (bottom left), and flow velocity maps (bottom right). (D) Endpoint histological analysis of tumor tissue was performed to assess vessel density, functionality and maturity through pericyte coverage. (Icons in the figure were produced with Illustrae.co)

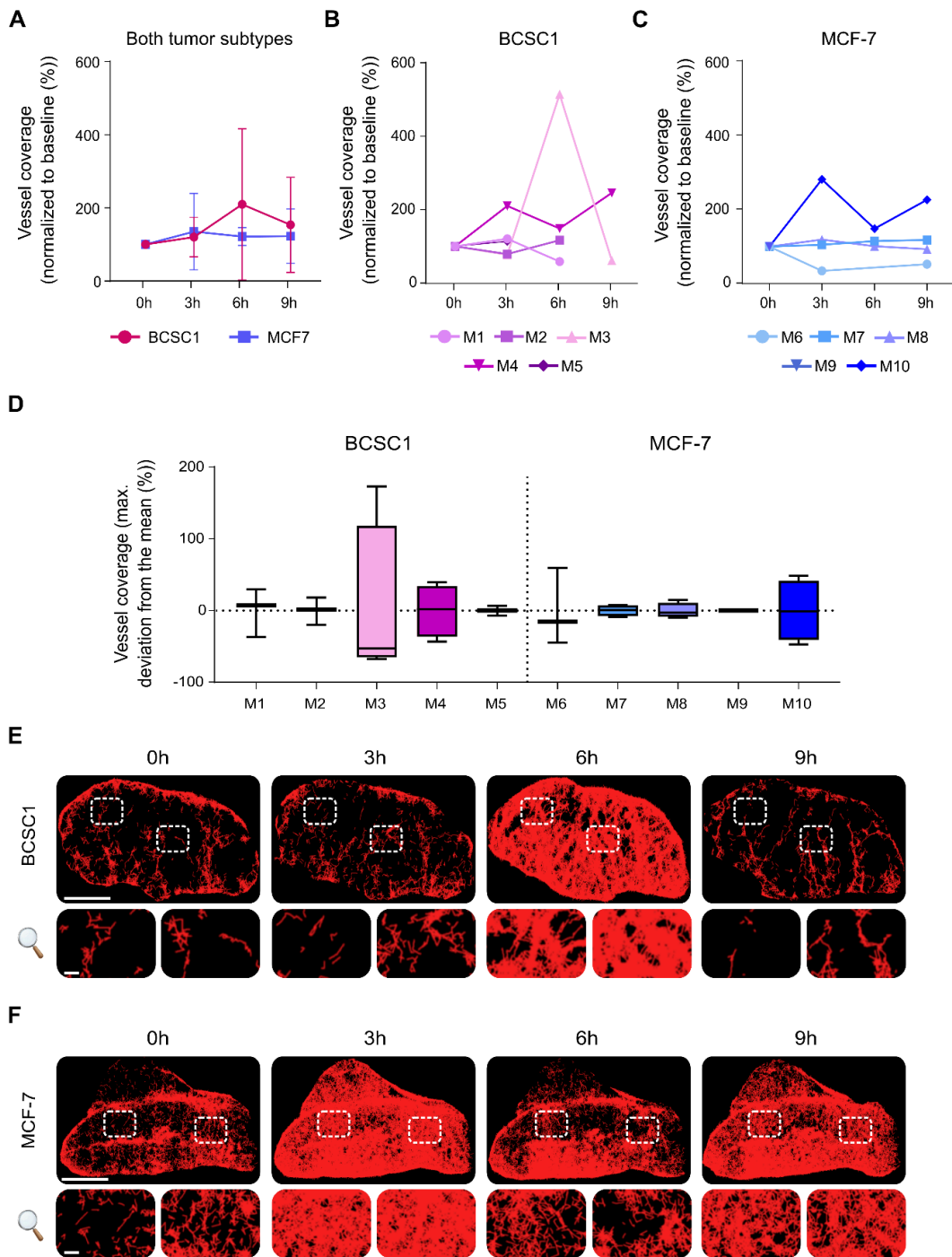


Figure 2

Microvascular vessel coverage shows no systematic time- or subtype-dependent changes despite intra-tumoral variability. (A) Vessel coverage over monitored time points (0h, 3h, 6h, 9h) in BCSC1 and MCF-7 xenografts quantified by ULM. Values were normalized to baseline (0h = 100%) and are presented as mean $\pm$ SD. Across the monitored time points, no consistent temporal trend or subtype-specific differences were observed. (B,C) Individual tumor profiles of vessel coverage for (B) BCSC1 and (C) MCF-7 xenografts across individual time points. The letter “M” represents individual mice (1-10). Although the coverage fluctuated within individual tumors, these variations did not follow a uniform time-dependent pattern. (D) Magnitude of vessel coverage variability across all monitored time points in all mice of both tumor models. Data are expressed as the maximum percentage deviation from the mean, reflecting the extent of intra-tumoral variability during the 9-hour observation period. Box-and-whisker plots display the median as well as the interquartile range with the full range of the data. (E,F) Representative binary ULM maps of (E) BCSC1 (Mouse 3) and (F) MCF-7 (Mouse 10) at 0h, 3h, 6h, and 9h, respectively. Binary segmentation illustrates the spatial distribution of perfused microvascular structures within the tumor area. While no uniform

temporal pattern is evident at the tumor-subtype level, substantial variability between individual tumors and time points can be observed. White dashed boxes indicate regions that are shown as magnified views below each respective tumor map, highlighting changes in the tumor microvasculature over time at the same location. The left and right magnified panels correspond to the respective boxes in the image above. Scale bars of maps in the top rows: 2 mm. Scale bars of zoomed-in maps: 200 μ m

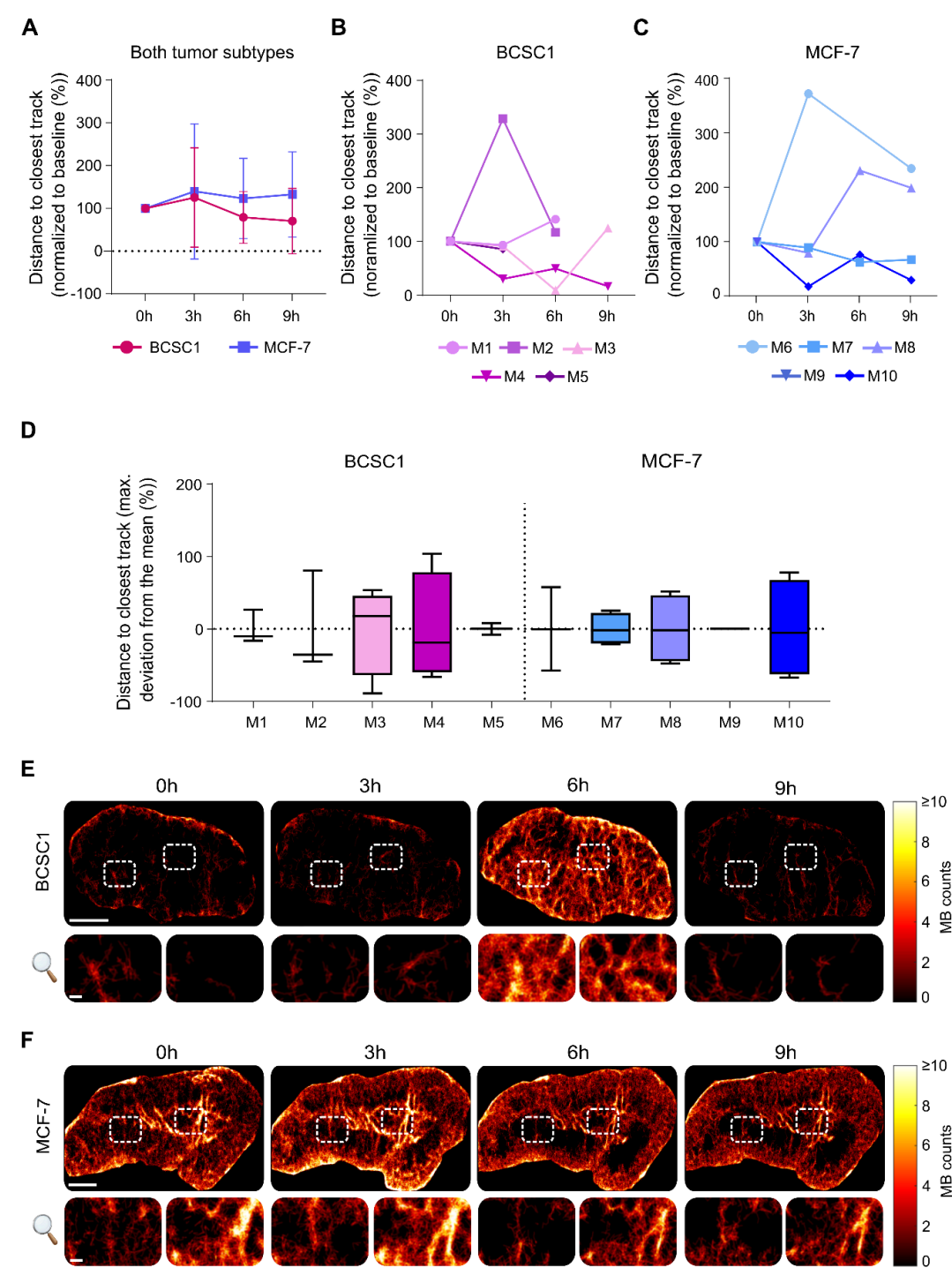


Figure 3

Spatial organization of microvascular networks assessed by distance to the closest track shows heterogeneous variability over time in tumors. (A) Mean distance to closest vascular track quantified by ULM in BCSC1 and MCF-7 xenografts over 9 hours. Values are displayed relative to baseline (0h = 100%). Despite moderate temporal variability, the spatial relationship between tissue regions and the nearest vascular tracks remained similar between the two tumor types. Data are presented as mean $\pm$ SD. **(B,C)** Individual tumor profiles show changes in the distance to the closest vascular track over

time in (B) BCSC1 and (C) MCF-7 xenografts, highlighting considerable intra-tumoral variability. The letter “M” represents individual mice (1-10). (D) Distribution of distance to closest track values across all time points for BCSC1 and MCF-7 tumors. Data represent the maximum percentage deviation from the mean distance measured within each tumor during the 9-hour period, indicating temporal variability in vascular spacing, driven by changes in perfusion and MB-track detection. Box-and-whisker plots display the median as well as the interquartile range with the full range of the data. (E,F) Representative ULM-derived MB occurrence maps illustrating the spatial distribution of the distance to the closest vascular track in (E) BCSC1 (Mouse 3) and (F) MCF-7 (Mouse 8) xenografts at the monitored time points (0h, 3h, 6h, 9h). The intensity of the colors represents the local MB occurrence, reflecting the frequency of MB passage and detection through the vasculature. Regions with higher signal intensity correspond to areas that are closer to perfused vascular tracks, whereas lower intensities indicate regions with larger distances to the nearest detected vessel. White dashed boxes indicate regions that are shown as magnified views below each respective tumor map, highlighting changes in microvascular structures within singular tumor compartments. Scale bars of maps in the top rows: 2 mm. Scale bars of zoomed-in maps: 200 μ m

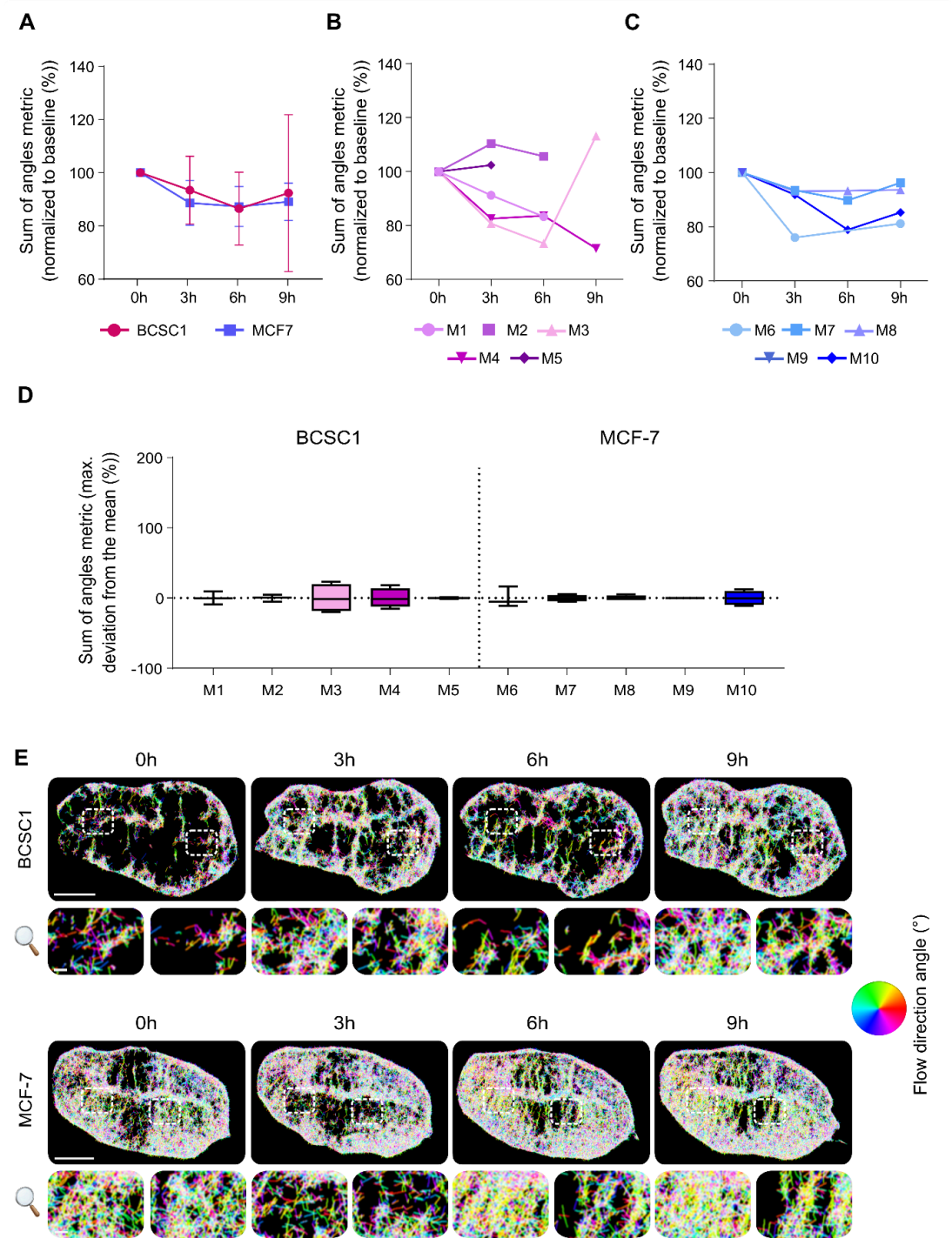


Figure 4

Microvascular tortuosity exhibits heterogeneous temporal dynamics independent of tumor subtype. (A) Mean microvascular tortuosity quantified using the sum of angles metric derived from MB-based ULM tracks in BCSC1 and MCF-7 xenografts over the 9-hour observation period. Values are displayed relative to baseline (0h = 100%). While minor temporal fluctuations were observed, no consistent differences between the two tumor subtypes were detected. Data are presented as mean $\pm$ SD. **(B,C)** Individual tumor profiles show changes in vessel tortuosity over time in (B) BCSC1 and (C) MCF-7 xenografts, highlighting considerable variability between individual tumors, while overall trends remain comparable between models. The letter “M” represents individual mice (1-10). **(D)** Distribution of relative tortuosity values across all time points for BCSC1 and MCF-7 tumors. Data represent the maximum percentage deviation from the mean tortuosity within each tumor over the 9-hour imaging period, reflecting intra-tumoral variability in vascular geometry. Box-and-whisker plots display the median as well as the interquartile range with the full range of the data. **(E,F)** Representative ULM-derived flow direction maps illustrating microvascular architecture in (E) BCSC1 (Mouse 4) and (F) MCF-7 (Mouse 7) xenografts at the monitored time points (0h, 3h, 6h, 9h). Colors indicate the local direction of individual MB trajectories within the vascular network, as illustrated by the direction color wheel. Color transitions along individual vessel tracks reflect directional shifts and correspond to angular deviations used for tortuosity quantification. White dashed boxes indicate regions that are shown as magnified views below each respective tumor map, highlighting local vessel profiles and temporal changes at the same location within the tumor. Scale bars of maps in the top rows: 2 mm. Scale bars of zoomed-in maps: 200 μ m

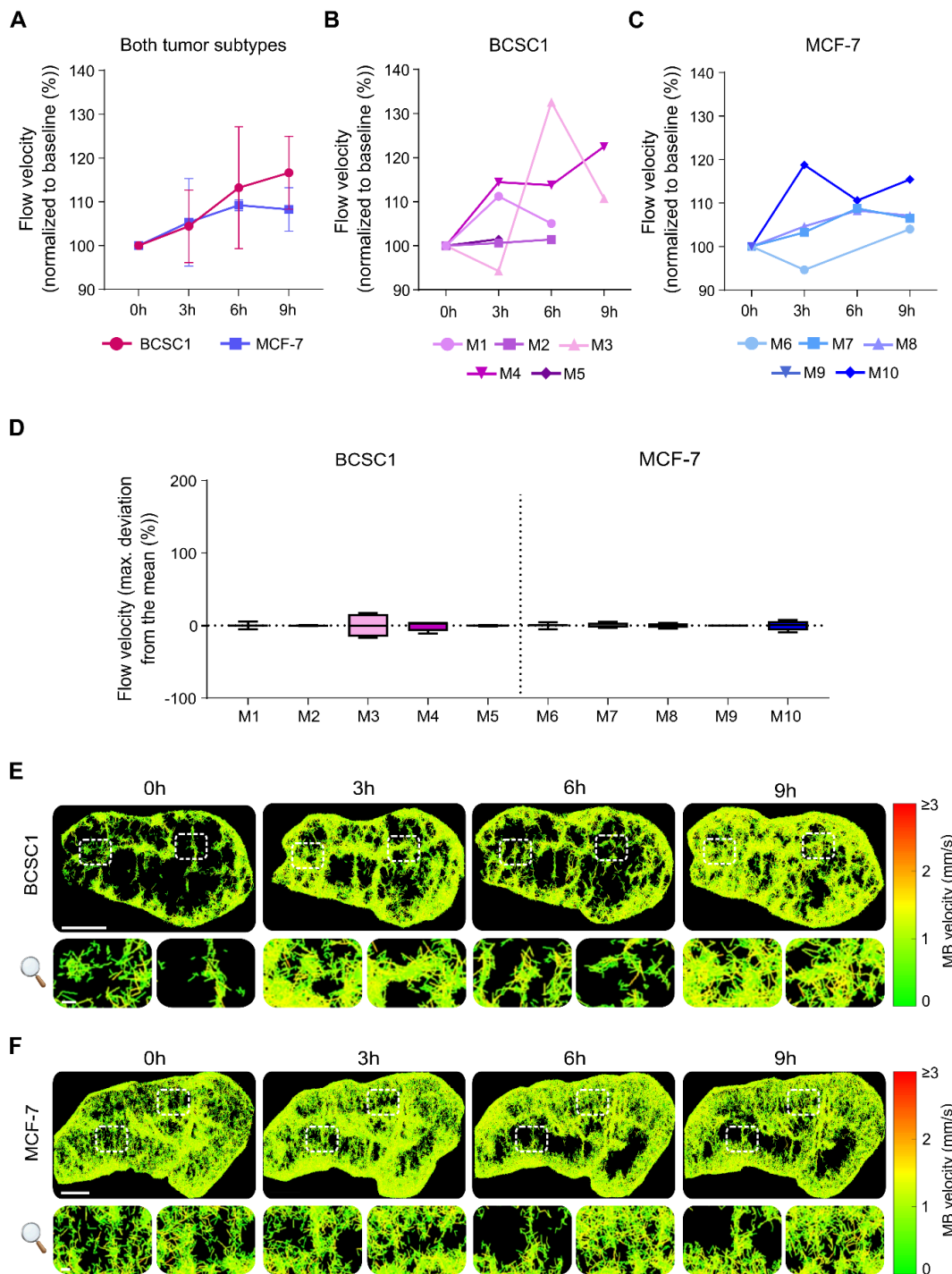
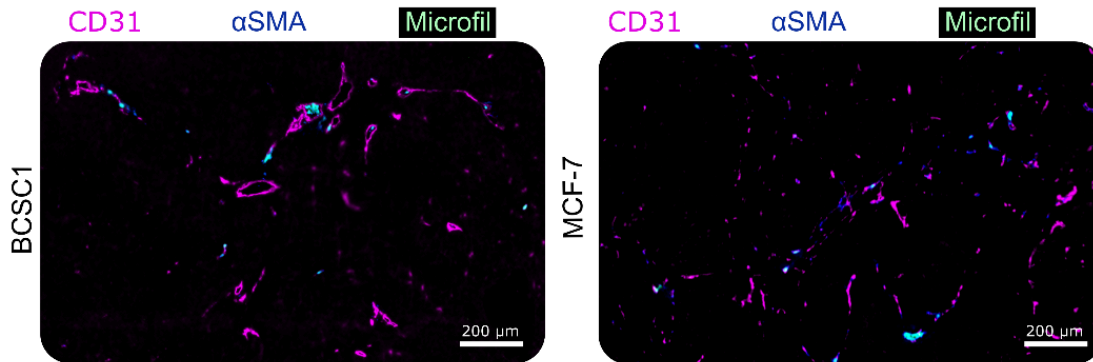


Figure 5

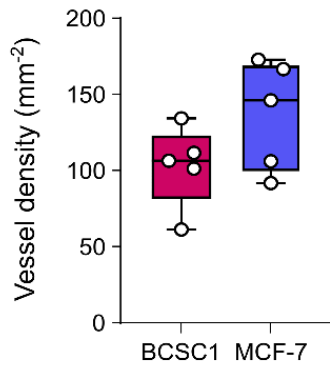
Longitudinal assessment of microvascular flow velocity reveals temporal variability independent of tumor subtype in breast cancer xenografts. (A) Mean microvascular flow velocity (measured in mm/s) over time in BCSC1 and MCF-7 tumors quantified by ULM. Values are expressed as percentage changes relative to the baseline (0h = 100%). No significant differences were observed between tumor models, whereas time-dependent variations were evident across monitored time points. Data are displayed as mean $\pm$ SD. (B,C) Individual tumor profiles illustrate intra-tumoral variability of flow velocity over the 9-hour observation period in (B) BCSC1 and (C) MCF-7 xenografts. The letter "M" represents individual mice (1-10). (D) Distribution of relative flow velocity across all time points in BCSC1 and MCF-7 xenografts. Data are expressed as the maximum percentage deviation from the mean, reflecting the magnitude of variability of intra-tumoral velocity over 9 hours. Box-and-whisker plots display the median as well as the interquartile range with the full range of the data. (E,F) Representative ULM-derived velocity maps of (E) BCSC1 (Mouse 4) and (F) MCF-7 (Mouse 8) at monitored time points (0h, 3h, 6h, 9h). Color coding indicates the relative microvascular flow velocity within the detected vascular tracks. White dashed boxes indicate regions that are

shown as magnified views below each respective tumor map, highlighting local vascular structures and temporal changes at the same position within the tumor. Scale bars of maps in the top rows: 2 mm. Scale bars of zoomed-in maps: 200 μm

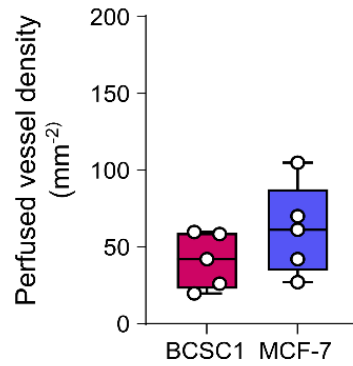
A



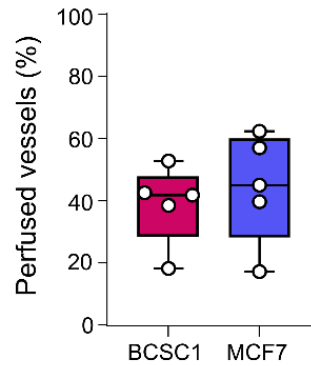
B



C



D



E

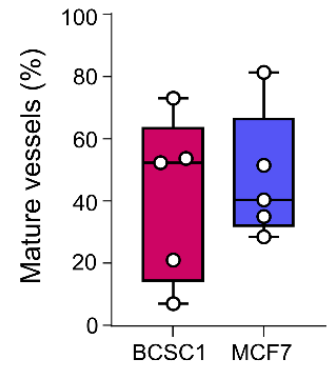


Figure 6

Histological analysis reveals structurally heterogeneous, partially perfused and variably matured tumor vessels in BCSC1 and MCF-7 xenografts. (A) Representative immunofluorescence images of BCSC1 and MCF-7 tumor stained for CD31 and αSMA with Microfil indicating perfused vessels. **(B)** Quantification of total vessel density, defined as the number of CD31^+ vessels per tumor section area (mm^{-2}), showing a trend towards higher vessel density in MCF-7 compared to BCSC1 tumors. **(C)** Quantification of perfused vessel density, defined as the number of Microfil^+ vessels per area, indicating a trend towards increased functional vessel perfusion in MCF-7 tumors compared to BCSC1 tumors. **(D)** Fraction of perfused vessels relative to total CD31^+ vessels, reflecting the proportion of functionally perfused vascular structures, with similar values observed between both tumor models. **(E)** Fraction of mature vessels, defined as $\alpha\text{SMA}^+/\text{CD31}^+$ double-positive vessels, representing perivascular stabilization, with no differences between BCSC1 and MCF-7 tumors. (D-E) Data are represented as box plots indicating the median and interquartile ranges with whiskers representing the full range of the data. Individual data points correspond to individual tumors ($n = 5$ tumors per group). Scale bars: 200 μm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterialsTumorPerfusionshowSubstantialHourScaleTemporalandSpatialDynamicsinSuperresolutionUltrasoundMeasurements.docx](#)