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Super-resolution functional photoacoustic microscopy via label-free cell tracking

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Abstract

Microvascular function and oxygen metabolism are central to tissue and organ health. However, label-free methods for imaging oxygen dynamics in three-dimensional (3D) microvascular networks at the level of single red blood cells (RBCs)—the fundamental units of oxygen transport *in vivo*—remain lacking. Here, we introduce super-resolution functional photoacoustic microscopy (SR-fPAM), which spatiotemporally tracks RBC movements under dual-wavelength excitation. SR-fPAM reconstructs super-resolved 3D microvascular architecture comparable to two-photon microscopy while providing quantitative measurements of RBC flow and oxygenation. In live mice, SR-fPAM revealed redistribution of oxygen and hemodynamics across 3D microvascular networks following a single-vessel stroke. These findings establish SR-fPAM as an enabling tool that bridges a critical gap in oxygen-metabolism imaging and opens new avenues for studying microvascular health and disease with unprecedented functional insights.

Introduction

The functional integrity of the brain relies on a delicate balance between the energy demand of neuronal activity and the oxygen supply through the microvasculature^{1,2}. While two-photon microscopy (TPM) enables volumetric imaging of neuronal activity at single-cell resolution³, imaging the coevolving hemodynamics and oxygen supply at a comparable spatial scale remains challenging. This limitation hampers our understanding of neurovascular function and brain metabolism, which underlie modern neuroimaging technologies⁴ and play a vital role in various brain diseases⁵.

Fluorescence-based techniques have revolutionized *in vivo* imaging of the brain microvasculature. Confocal light-field microscopy⁶ and wide-field fluorescence microscopy⁷ can resolve microvascular structure and flow down to single capillaries by tracking fluorescently labeled red blood cells (RBCs) or beads injected into the bloodstream. However, these methods cannot measure blood oxygenation. TPM

with phosphorescent dyes can measure partial pressure of oxygen (pO_2) in microvessels in addition to blood flow^{8–10}, but it requires at least tens of milliseconds per pixel to collect sufficient photons for accurate measurements¹⁰. As a result, pixel-by-pixel pO_2 mapping at high resolution across the 3D microvasculature is impractical.

Photoacoustic microscopy (PAM), which is highly sensitive to the optical absorption of hemoglobin, enables label-free, high-speed imaging of blood oxygenation (sO_2) and flow^{11,12}. Therefore, it holds promise to overcome the limitations of fluorescence-based methods. Nevertheless, while the lateral resolution of PAM can reach single-RBC levels, its axial resolution remains mesoscopic due to the limited bandwidth of detected acoustic signals^{13,14}. This pronounced resolution anisotropy, often more than an order of magnitude, limits PAM's ability for functional microvascular imaging in 3D¹⁵.

Several strategies have been developed to mitigate the resolution anisotropy in PAM. Grüneisen-relaxation PAM¹⁶ and photo-imprint PAM¹⁷ achieve sub-diffraction axial resolution via nonlinear effects, but the required temperature rise or photo-bleaching raises safety concerns, limiting *in vivo* applications. Moreover, these methods have not demonstrated functional imaging of sO_2 and blood flow. Localization PAM improves axial

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resolution to the single-cell level by identifying static RBC positions in individual frames and then superimposing them¹⁸. However, it lacks the ability to dynamically track RBC movements across frames and thus cannot reliably quantify blood flow. Also, sO₂ measurement has not been demonstrated with localization PAM.

Inspired by super-resolution ultrasound imaging, which tracks microbubble flow in blood vessels^{19,20}, localization photoacoustic tomography has been developed. This technique achieves 3D imaging of microvascular structure and flow at a resolution of less than 10 μm by tracking optically absorbing dye droplets^{21–23}. Incorporating multi-spectral imaging allows sO₂ quantification²³, but the accuracy is limited by depth- and wavelength-dependent light fluence²⁴. Moreover, the use of exogenous contrast agents prolongs imaging time due to the need for low agent concentrations, limits performance in microvascular imaging, and raises concerns about adverse effects on the microcirculation^{22,23,25}.

Here, we present the first implementation of spatio-temporal RBC tracking across optical wavelengths in PAM, enabling label-free imaging of sO₂ and blood flow at 3D-isotropic, single-RBC resolution in vivo. This technique, termed super-resolution functional PAM (SR-fPAM), combines a high-speed functional PAM system for dynamic RBC tracking and a multi-step data processing strategy for accurate extraction of RBC trajectories. The high-speed PAM system uses a nanoimprinted micro-ring resonator (MRR) for high-sensitivity, wide-field acoustic detection²⁶. Laser scanning through the optically transparent MRR allows volumetric imaging at 15 Hz and depth-resolved B-scans at 1 kHz, enabling spatiotemporal tracking of individual RBCs. Unlike localization PAM, which superimposes static RBC positions extracted from single frames, SR-fPAM tracks RBC movement trajectories across frames—not only improving structural image quality but also enabling measurements of flow speed and direction. Furthermore, simultaneous tracking of RBCs at two wavelengths enables spectroscopic measurements of sO₂.

We validated the resolution and fidelity of SR-fPAM through a side-by-side comparison with TPM and demonstrated its in vivo application by imaging the functional responses of the mouse brain microvasculature to a single-vessel stroke. SR-fPAM clearly resolved oxygen-hemodynamic redistribution across 3D microvascular networks, highlighting its potential to provide novel insights into microvascular function and oxygen metabolism under both physiological and pathological conditions.

Results

High-speed functional PAM system based on a transparent micro-ring resonator

A PAM system capable of high-speed functional imaging has been developed for SR-fPAM. The system uses a

nanosecond-pulsed laser (PAM laser in Fig. 1a) and stimulated Raman scattering (SRS)-based wavelength conversion for high-speed sO₂ imaging¹². The dual-wavelength laser pulses are optically scanned by a 2D galvanometer (GM in Fig. 1a), achieving a 15-Hz volumetric rate and a 1-kHz B-scan rate. The laser beam is focused into the mouse brain through a transparent MRR, gets partially absorbed by hemoglobin, and induces ultrasonic emission (inset, Fig. 1a). A portion of the emitted ultrasound back-propagates to the MRR, modulating its resonance spectrum (Fig. 1b). The output of a narrow-band tunable continuous-wave (CW) laser (MRR laser in Fig. 1a) is coupled into the MRR, with its wavelength tuned near the resonance. Ultrasound-induced resonance shifts are detected as intensity modulations by a high-speed photodetector (PD) to derive the waveform of the photoacoustic signal. An adiabatically tapered waveguide is used to improve the in-coupling efficiency of the MRR and, thus, the sensitivity of acoustic detection.

The acoustic field of view (FOV) of the MRR was experimentally determined by imaging a neutral optical absorber (black tape in this case) at different depths. As shown in Fig. 1c, the detected photoacoustic amplitude decreased with depth (i.e., distance from the MRR). To balance the FOV and sensitivity, the optical focus was positioned 550 μm from the MRR (dashed line in Fig. 1c) for all in vivo studies reported herein. To maintain the MRR's stability for precise RBC tracking over time, a proportional-integral-derivative (PID) controller dynamically locked the interrogation wavelength of the CW laser to the MRR's resonance, compensating for drifts due to environmental temperature fluctuations or laser-induced thermal accumulation. The closed-loop control markedly improved the MRR's stability, as confirmed by time-lapse monitoring of the black tape (Fig. 1d). The stability of MRR for in vivo mouse brain imaging was also confirmed, with the image quality remaining consistent throughout the entire 6-min imaging session (Fig. S1). Further details about the high-speed PAM system and the MRR design and fabrication are provided in the “Materials and methods”.

Real-time en-face imaging of the live mouse brain at single-RBC lateral resolution

By 2D galvo scanning of dual-wavelength laser pulses across the acoustic FOV of the MRR, our PAM system achieves near-video-rate functional imaging of microvascular sO₂ and blood flow. For simplicity, we refer to this mode as real-time functional PAM (RT-fPAM).

We first tested the in vivo performance of RT-fPAM in the mouse brain. A cranial window was created and covered by the MRR, with water in between for acoustic coupling (inset, Fig. 1a). The en-face image of the brain vasculature and simultaneously measured sO₂ map



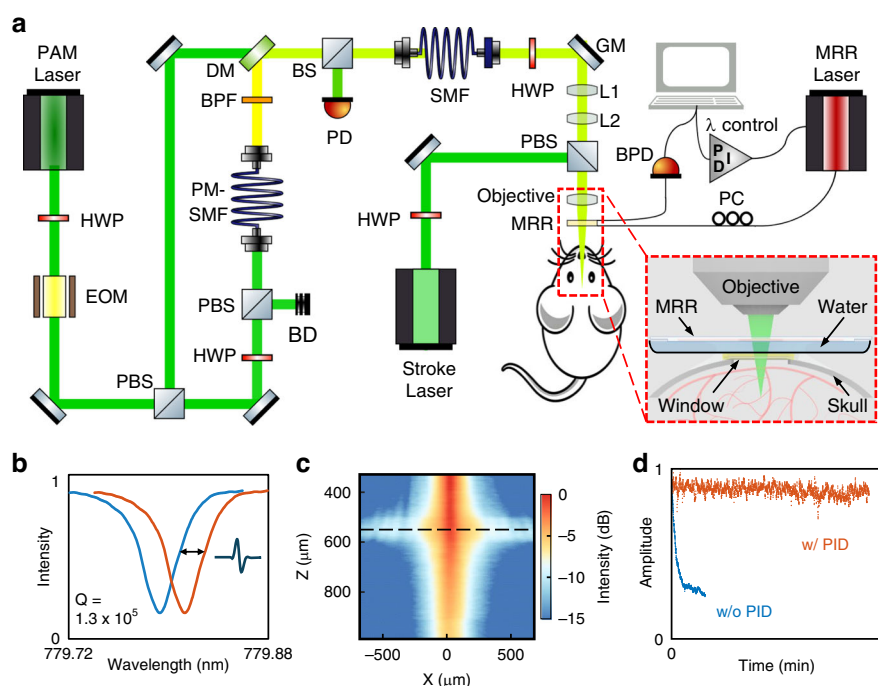


Fig. 1 Configuration of MRR-based high-speed functional PAM system. **a** Schematic of the PAM system. HWP half-wave plate, EOM electro-optic modulator, PBS polarizing beam splitter, BD beam dump, PM-SMF polarization-maintaining single-mode fiber, BPF band-pass filter, DM dichroic mirror, BS beam sampler, PD photodetector, SMF single-mode fiber, GM galvanometer, L1, L2 lens, MRR micro-ring resonator, BPD balanced photodetector, PC polarization controller, PID proportional-integral-derivative controller, λ wavelength. The inset illustrates the MRR-based PAM for mouse cerebrovascular imaging through a cranial window. Briefly, the nanosecond laser pulses pass through the transparent MRR, a thin layer of water for acoustic coupling, and the cranial window covered with a thin acrylic film to excite the cortical microvasculature and generate ultrasonic waves. **b** Shift in MRR's resonance due to the perturbation by an ultrasound wave. The spectral shift can be interrogated in real-time by the MRR laser in (a) for optical recording of the ultrasonic signal. **c** Ultrasonic sensitivity map of the MRR at different lateral (X) and axial (Z) locations. The dashed line highlights the axial location where an optimal tradeoff between the detection sensitivity and FOV is achieved. The optical focus was set at this axial location for all in vivo studies. **d** Stabilization of the MRR's detection sensitivity by a PID controller. A comparison of the photoacoustic amplitude of a black tape monitored by the PAM system over time, with and without the PID, shows much-improved stability with real-time feedback control

confirmed that RT-fPAM provided not only a detailed visualization of the microvascular structure but also a quantitative assessment of blood oxygenation, both at single-RBC lateral resolution (Fig. 2a, b). A side-by-side comparison with our established PAM system^{27,28}, which uses a tightly focused piezoelectric transducer, showed comparable image quality (Fig. S2).

Then, we demonstrated the ability of RT-fPAM for high-speed functional imaging of the brain microvasculature by monitoring the dynamic responses of sO_2 and blood flow to a micro-stroke. For this purpose, an additional nanosecond laser (stroke laser in Fig. 1a) was integrated into the PAM system to enable targeted occlusion of a single microvessel²⁹. Further details about the stroke model are provided in the “Materials and methods”.

Video-rate volumetric imaging was performed before, during, and after the single-vessel occlusion for 360 s (Fig. 2c and Movie S1). Quantitative analysis revealed distinct responses in the parallel branch (P1) and upstream branch (P3) relative to the targeted branch (P2) (Fig. 2d).

Specifically, P2 lost blood perfusion immediately, precluding further measurements of sO_2 and flow. P1 showed an increase in blood flow and a slight decrease in sO_2 , which eventually returned to the pre-occlusion level. By contrast, P3 showed a biphasic response—starting with an immediate and pronounced drop in the flow speed, followed by a reversal in the flow direction about 200 s later (inset, Fig. 2d). The stroke-induced flow reversal is consistent with a previous TPM observation³⁰. The significant flow reduction during the first phase was accompanied by a gradual decline in sO_2 , indicating an increased oxygen release from these RBCs due to prolonged stalling. As the flow reversed in the second phase, sO_2 rapidly recovered to the pre-occlusion level, suggesting a hemodynamic redistribution in response to ischemia, a phenomenon previously observed in the peripheral microvasculature³¹.

SR-fPAM of the live mouse brain enabled by RBC tracking

While RT-fPAM provides dynamic insights into microvascular function, its limited axial resolution (Fig.



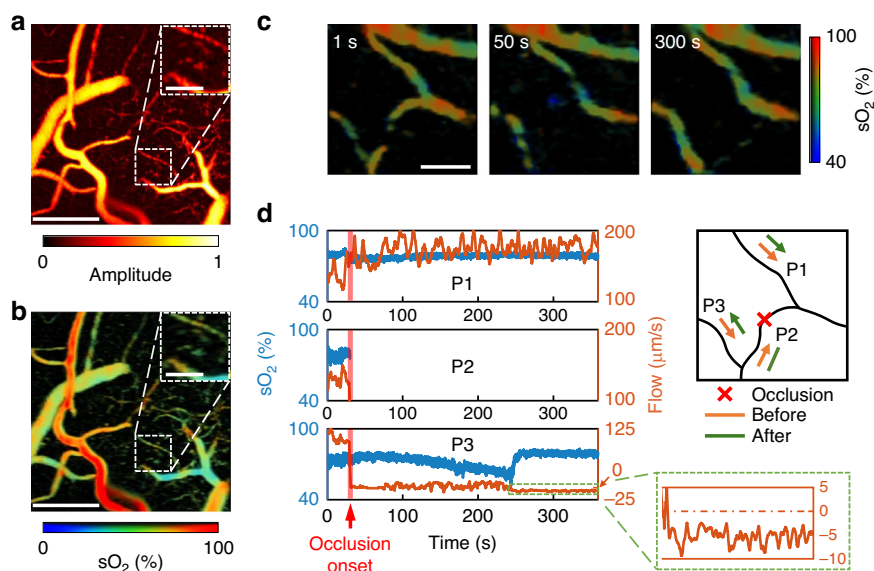


Fig. 2 Real-time en-face PAM of mouse cerebrovascular responses to single-vessel stroke. **a, b** Structural (**a**) and sO_2 (**b**) images of cerebral vessels in a live mouse brain. The insets are close-up views of the boxed region, showing individual RBCs traversing the capillaries and their sO_2 values. Scale bars, 200 μm and 20 μm (inset). **c** PAM images of dynamic changes in microvascular perfusion and sO_2 in response to a single-vessel occlusion. The three images are three frames taken from Movie S1 at 1, 50, and 300 s, respectively. Scale bar, 50 μm . **d** Quantitative changes in the sO_2 and flow speed of the parallel branch (P1), the targeted branch (P2), and the upstream branch (P3) before, during, and after the micro-stroke. The map of vessel segments at the upper-right corner illustrates the occlusion site (red cross) and the blood flow direction of individual segments (green and orange arrows) before and after the single-vessel stroke. The inset at the lower-right corner is a close-up view of the flow reversal in P3 at ~200 s after the onset of stroke. The negative value of flow speed indicates a reversed blood flow direction

S3) precludes 3D imaging at the single-RBC level. To overcome this, we advanced RT-fPAM into the super-resolution regime via spatiotemporal RBC tracking, leading to the development of SR-fPAM. The performance comparison between SR-fPAM and RT-fPAM is summarized in Table S1.

In SR-fPAM, the spatiotemporal tracking of RBCs is achieved through repeated B-scan acquisitions. The kHz B-scan rate allows us to track the movements of individual RBCs across sequentially acquired frames. As shown in Fig. 3a, the positions of three RBCs (indicated by the green, yellow, and blue arrows) change between frame 1 and frame 5, revealing their movements in a microvessel. By compiling RBC trajectories across frames, SR-fPAM reconstructs super-resolved microvascular networks (Fig. 3b). Moreover, blood flow direction is determined by the trajectory orientation (Fig. 3c), while flow speed is calculated based on the RBC displacement between frames and the known frame rate (Fig. 3d). The detailed method for spatiotemporal RBC tracking is provided in the “Materials and methods”.

Similar to ultrasound localization microscopy²⁰, image reconstruction fidelity in SR-fPAM improves with B-scan dwell time. A longer dwell time yields more frames for tracking, leading to more accurate reconstruction but lower acquisition speed. To balance the image quality and

speed, we tested multiple dwell times in two different animals (Fig. 3e, f, and Movie S2). Microvascular diameters measured at shorter dwell times were benchmarked against those from the longest dwell time (i.e., 3000 frames). According to Fig. 3f, 800 repeated B-scans (i.e., 0.8 s dwell time) gave the optimal tradeoff between reconstruction fidelity and imaging speed. Furthermore, decorrelation-based resolution analysis³² confirmed that the SR-fPAM achieved a nearly isotropic spatial resolution of ~1.3 μm in both the lateral and axial directions after 800 frames (Fig. S4). Further details on the dwell time optimization are provided in the “Materials and methods”.

Benchmarking SR-fPAM against TPM

To validate the resolution enhancement of SR-fPAM, we performed a side-by-side comparison with conventional PAM and TPM, where TPM served as the gold standard owing to its high resolution in all three dimensions (Fig. 4 and Movie S3).

The 2D projections of conventional PAM and SR-fPAM (the left and middle columns in Fig. 4a, respectively) revealed a marked improvement in resolution, particularly in the axial direction. Direct comparison with the TPM image of the same cortical region perfused with fluorescein isothiocyanate-dextran (the right column in Fig. 4a) confirmed the fidelity of the super-resolved microvasculature

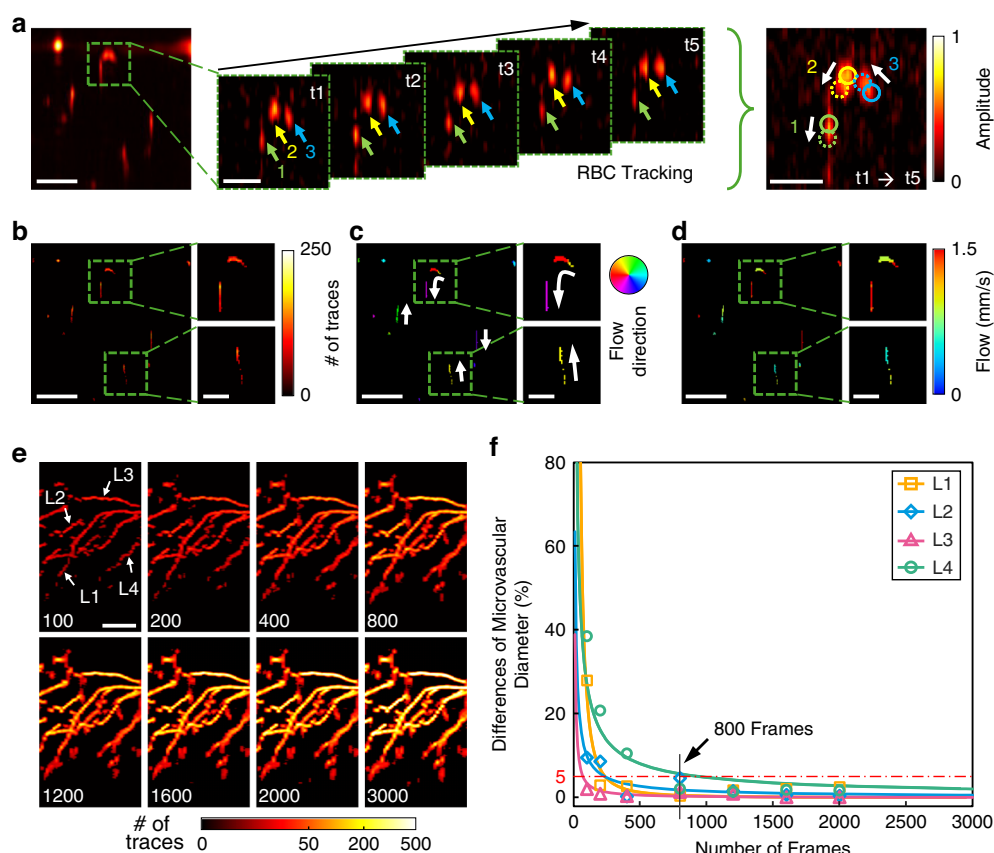


Fig. 3 Reconstruction of super-resolved mouse cerebral vasculature based on label-free RBC tracking. **a** RBC tracking across adjacent B-scans. Right: overlapping frames reveal RBC displacement. Scale bars, 50 μm and 20 μm (close-up views). **b–d** Super-resolved microvascular structure (**b**), flow direction (**c**), and flow speed (**d**) cross-sectional images generated via RBC tracking in 800 sequentially acquired B-scans. Scale bars, 50 μm and 20 μm (close-up views). **e** Side-by-side comparison of the SR-fPAM images (in X–Z projected view) reconstructed using different numbers of repeated B-scans. Scale bar, 50 μm . **f** Differences in the microvascular diameters of L1–L4 while the number of repeated B-scan frames increases, showing that the reconstructed image converges (i.e., the difference is less than 5%) when the number of dwell B-scan frames reaches 800

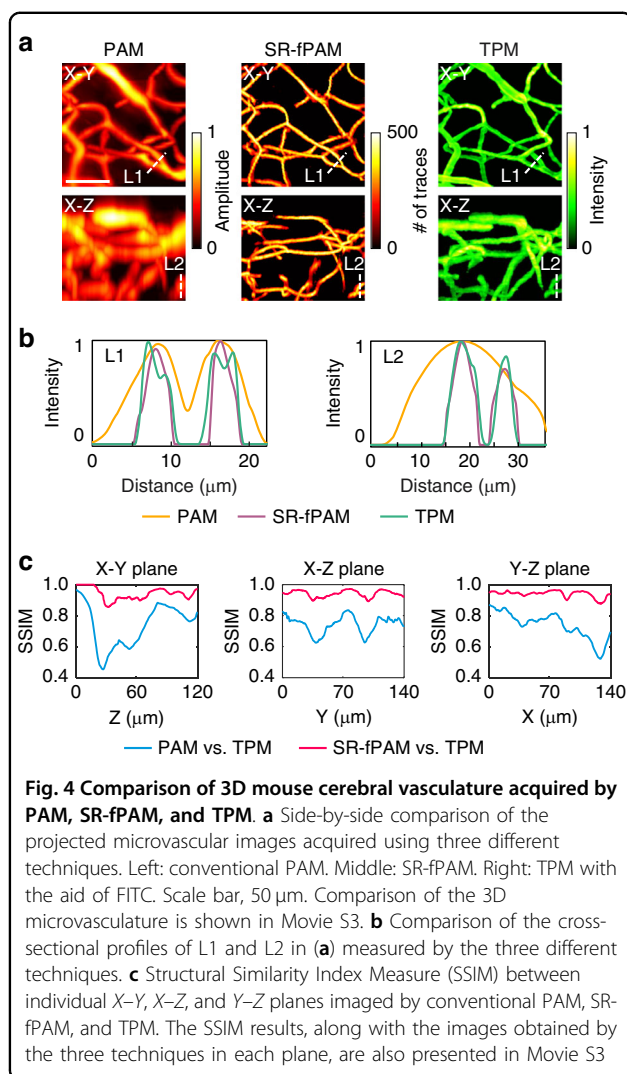
generated by SR-fPAM. Cross-sectional profiles of two selected microvessels (the dashed lines in Fig. 3a) in both X–Y and X–Z projections further demonstrated that SR-fPAM achieved TPM-level 3D resolution (Fig. 4b). Quantitative analysis of the structural similarity between individual sagittal, frontal, and horizontal planes of the 3D microvascular images acquired by conventional PAM, SR-fPAM, and TPM showed a consistently high similarity index (>0.9) between SR-fPAM and TPM, far exceeding that between conventional PAM and TPM (Fig. 4c and Movie S3). Together, these results establish that SR-fPAM achieves true 3D single-RBC resolution, generating super-resolved images that faithfully represent native vascular architecture with minimal reconstruction artifacts.

3D functional imaging of the mouse brain microvasculature by SR-fPAM

With dual-wavelength excitation, SR-fPAM enables 3D mapping of microvascular $s\text{O}_2$. Figure 5a shows

representative X–Y and X–Z projections of the 3D $s\text{O}_2$ distribution in a live mouse brain measured by SR-fPAM. By tracking RBC movements in high-frame-rate B-scans acquired along both lateral directions, SR-fPAM can also measure the speed and direction of blood flow across the 3D microvasculature. Specifically, we collected two series of B-scans along the X and Y directions, performed RBC tracking in each of them to quantify the velocity components in the X–Z and Y–Z planes (i.e., v_x , v_y , and v_z), respectively, and combined them by vector addition to obtain the 3D resultant velocity. Figure 5b illustrates the 3D flow quantification strategy and the decomposition of flow velocity along all three axes. The final 3D images of super-resolved microvascular $s\text{O}_2$ and flow speed are shown in Fig. 5c and Movie S4. By combining high-resolution measurements of vascular structure and flow velocity, we quantified the volumetric blood flow at multiple bifurcation sites. The agreement between inflow and outflow rates confirmed the conservation of blood





flow (Fig. S5), thereby validating the quantitative accuracy of SR-fPAM for both structural and flow measurements. More details about the functional quantification are provided in the “Materials and methods”.

Functional imaging of cerebral microvascular responses to a micro-stroke by SR-fPAM

Finally, we demonstrated the utility of SR-fPAM by imaging the functional responses of the 3D cortical microvasculature to the occlusion of a single pial arteriole in the mouse brain (Fig. 6). SR-fPAM of a $\sim 170 \times 170 \times 170 \mu\text{m}^3$ region centered around the occlusion site (white cross in Fig. 6) before and 10 min after the occlusion revealed that a single-arteriole stroke can induce widespread hemodynamic and oxygen-metabolic changes across the 3D microvascular network in the cortex. Vessel segmentation of the super-resolved 3D vasculature enabled quantitative analysis of individual microvessels, capturing loss of blood perfusion, reversal of

flow direction (Fig. 6a), change in flow speed (Fig. 6b), and attendant alterations in blood oxygenation (Fig. 6c). Together, these responses indicated an oxygen-hemodynamic redistribution. Specifically, vessels near the occlusion site either lost blood perfusion or exhibited reduced sO_2 compared to the baseline (Fig. 6b, c). In contrast, parallel vessels (e.g., L1 in Fig. 6) showed elevated flow speed, and downstream vessels (e.g., L2 in Fig. 6) reversed flow direction and increased flow speed. This redistribution reflects the recruitment of collateral circulation to restore oxygen supply to the ischemic tissue³¹.

Discussion

SR-fPAM enables label-free imaging of 3D microvascular structure, blood oxygenation, and flow at single-RBC resolution. This capability arises from spatiotemporal tracking of unlabeled RBCs, extraction of the RBC movement trajectories across frames to form super-resolved microvascular networks, assessment of blood flow speed and direction based on the RBC trajectories, and spectroscopic measurement of hemoglobin oxygen saturation with dual-wavelength excitation. Direct validation against TPM in the same animal confirms that SR-fPAM achieves comparable resolution while extending image contrasts into the functional regime. Application to a mouse model of micro-stroke further demonstrated SR-fPAM's ability to reveal oxygen-hemodynamic redistribution across cortical microvascular networks. Compared with existing super-resolution photoacoustic imaging techniques^{18,21–23}, SR-fPAM enables label-free quantification of microvascular blood flow and sO_2 with 3D-isotropic resolution (Table S2). These capabilities position SR-fPAM as an enabling tool for mechanistic studies of microvascular function and metabolism.

A critical innovation enabling SR-fPAM is the implementation of an MRR-based high-speed functional PAM system. Conventional PAM typically employs tightly focused PZTs, which provide high sensitivity but only within a narrow focal zone, limiting their capability for wide-field, rapid imaging required for spatiotemporal RBC tracking. In SR-fPAM, high-speed laser scanning generates photoacoustic signals throughout the entire acoustic FOV, necessitating detectors with both broad coverage and uniform, sustained sensitivity. Weakly focused or unfocused PZTs can expand the detection range but at the cost of reduced sensitivity^{33,34}. Alternative strategies using water-immersible MEMS or polygon-mirror scanners enable rapid scanning of optical-acoustic dual foci but may suffer from distortions or instability caused by water damping and facet misalignment, impeding precise RBC tracking across frames^{35–37}. In contrast, the MRR in SR-fPAM combines high sensitivity with a substantial FOV, achieving image contrast



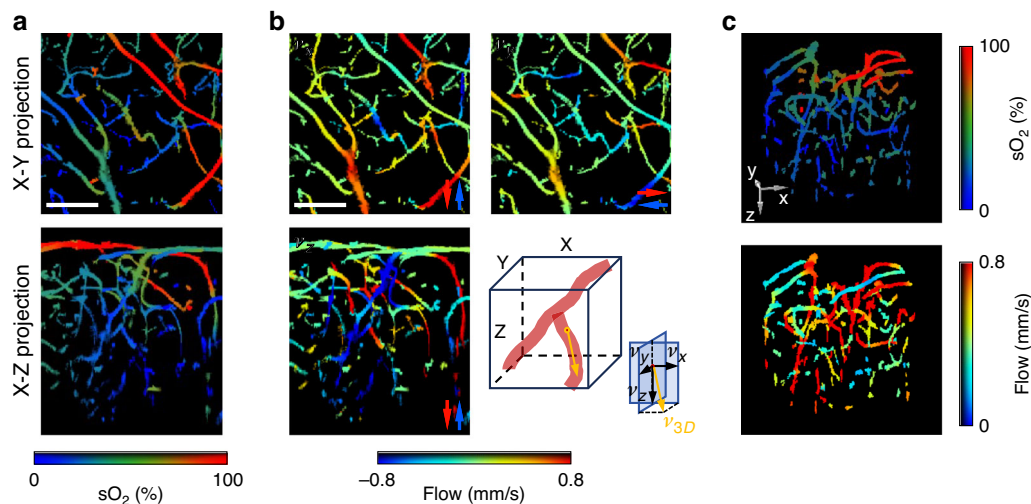


Fig. 5 3D super-resolution imaging of mouse cerebrovascular function by SR-fPAM. **a** X-Y and X-Z projected super-resolved sO_2 images of mouse cerebral vessels. Scale bar, 50 μm . **b** X-Y and X-Z projected super-resolved flow velocity images of mouse cerebral vessels. v_x , v_y , and v_z represent the flow velocity components along the X, Y, and Z axes, respectively. Flow directions are marked by the red and blue arrows. The bottom-right inset illustrates the combination of individual velocity components to reconstruct 3D microvascular flow. Scale bar, 50 μm . **c** Blood sO_2 (top) and flow speed (bottom) in the 3D super-resolved microvasculature. The corresponding 3D renderings are shown in Movie S4

comparable to tightly focused PZTs (Figs. 2a, b and S2). Its high sensitivity lowers the laser pulse energy requirement, enabling the use of high laser repetition rates to achieve frame rates needed for robust RBC tracking in biological tissue.

Another major innovation of SR-fPAM lies in the spatiotemporal tracking of individual RBCs, which advances beyond previously reported RBC localization strategies¹⁸. The localization-based approaches generate super-resolved vasculature by identifying static RBC positions in individual frames and then accumulating them over time. However, they lack the ability to follow dynamic RBC movements across frames, which hinders functional quantification of blood flow. SR-fPAM overcomes this by tracking RBC trajectories continuously across sequential B-scans, enabling simultaneous reconstruction of high-fidelity 3D vascular structures and measurement of flow speed and direction. This approach not only enhances structural resolution, particularly along the axial dimension, but also integrates important functional information such as blood flow and oxygenation—providing a unified framework for label-free, high-resolution, 3D oxygen-hemodynamic imaging that is not achievable with earlier localization-based methods.

Similar to optical and ultrasound localization microscopy^{19,38}, the spatial resolution of SR-fPAM is influenced by multiple factors, including the intrinsic system resolution, signal-to-noise ratio, sampling density, tracking algorithm accuracy, and reconstruction effects³⁹. The high intrinsic contrast of PAM, together with the high sensitivity of the MRR, plays a critical role

in achieving the high spatial resolution of SR-fPAM. The decorrelation-based resolution analysis shows that the SR-fPAM achieves a nearly isotropic resolution of $\sim 1.3 \mu m$ in both the lateral and axial directions. Further, we benchmarked SR-fPAM against TPM through side-by-side comparison in the same animal in vivo (Fig. 4 and Movie S3). This is, to our knowledge, the first direct in vivo benchmarking of localization-based super-resolution photoacoustic imaging against a gold standard—an essential step in validating these emerging technologies^{39,40}. Our results confirm that SR-fPAM significantly enhances the spatial resolution of PAM, both laterally and axially, reaching the level of TPM. It is worth noting that this side-by-side comparison is enabled by the optical transparency of the MRR, which significantly simplifies the integration of SR-fPAM with TPM by allowing light to pass undisturbed.

The bi-directional scanning scheme of SR-fPAM, collecting B-scans along both lateral directions (Fig. 5), enables comprehensive quantification of the velocity components along all three dimensions and the combination of them to obtain the 3D resultant velocity on a single-pixel basis. We demonstrated in vivo super-resolution 3D imaging of functional changes in microvascular blood oxygenation and flow in response to a single-arteriole stroke, revealing novel oxygen-hemodynamic redistribution patterns (Fig. 6). The ability to simultaneously quantify these parameters in the 3D microvasculature with endogenous contrast makes SR-fPAM a powerful tool for studying cerebrovascular dysfunction in animal models, including but not limited to ischemic stroke^{23,41–44}.



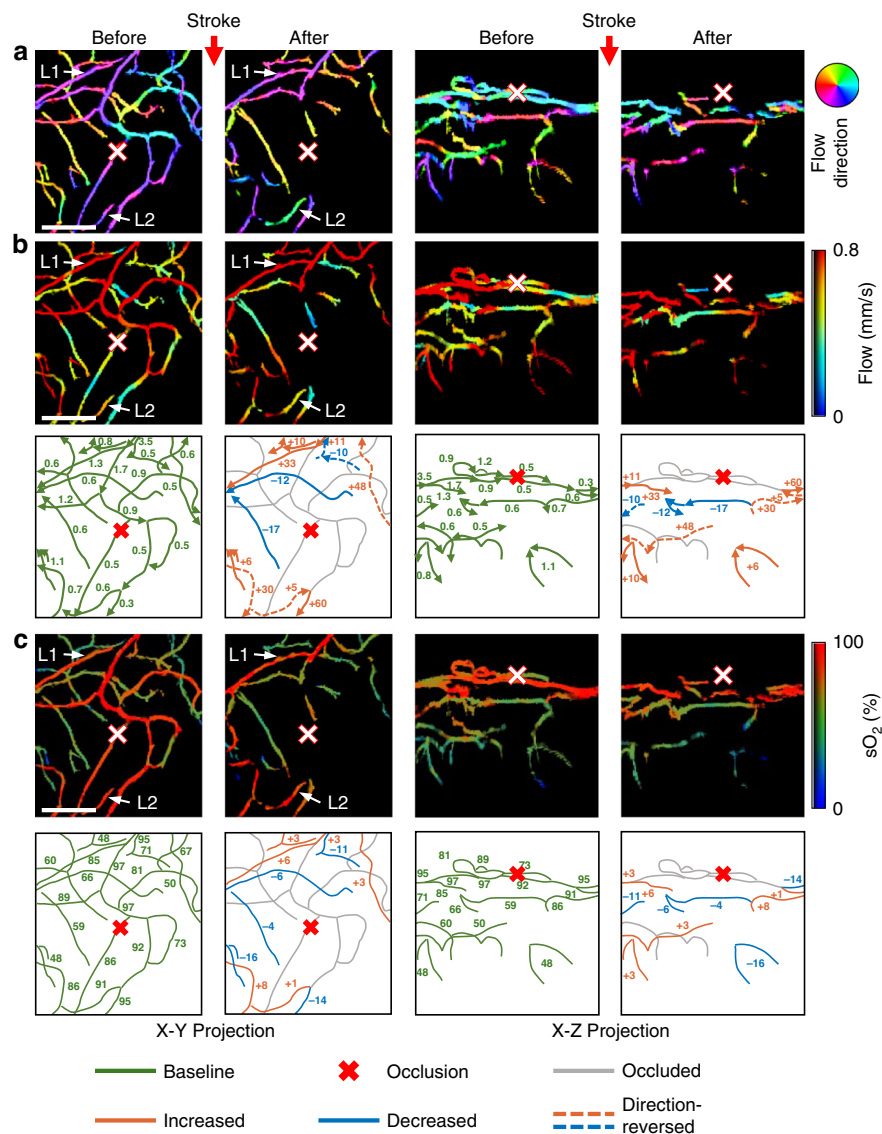


Fig. 6 Oxygen-hemodynamic redistribution in response to single-vessel stroke revealed by SR-fPAM. **a** X-Y and X-Z projected super-resolved images of blood flow direction before and after the single-vessel stroke. Scale bar, 50 μm . **b** X-Y and X-Z projected super-resolved images of flow speed before and after the single-vessel stroke. Scale bar, 50 μm . **c** X-Y and X-Z projected super-resolved images of sO_2 before and after the single-vessel stroke. Scale bar, 50 μm . White cross: the occlusion site; L1: a representative parallel vessel; L2: a representative downstream vessel. Baseline and stroke-induced changes in flow direction, average flow speed, and average sO_2 in each microvessel are shown in (b, c) with vessel segmentation

Future work integrating SR-fPAM and TPM for simultaneous volumetric imaging of neuronal activity and microvascular oxygen-hemodynamics is expected to advance our understanding of neurovascular coupling and its implications in neurological diseases at a more fundamental level^{45,46}. Additionally, SR-fPAM holds the potential to elucidate cortical layer-specific microvascular functions, building on recent findings of layer-dependent oxygen metabolism and hemodynamics^{47,48}. Achieving this requires further enhancement in imaging speed and tissue penetration. The former can be reduced with the

use of deep learning^{49–51}, while the latter can be extended by incorporating near-infrared⁵² or needle-shaped⁵³ laser excitation for PAM imaging.

Materials and methods

PAM system

The system configuration is shown in Fig. 1a. The excitation source is a nanosecond-pulsed laser (PAM laser; VGEN-G-20, Spectra-Physics). A half-wave plate (HWP; WPH10M-532, Thorlabs), an electro-optic modulator (EOM; 350-50-01-RP, Conoptics), and a polarizing



beam splitter (PBS; PBS121, Thorlabs) work together as a high-speed switch to distribute individual laser pulses between two optical paths. When a low voltage is applied to the EOM, the laser pulse passes through the PBS. Then, the energy of the pulse is adjusted by a second HWP (WPH10M-532, Thorlabs) and a second PBS (PBS121, Thorlabs) before being coupled into a 10-meter-long polarization-maintaining single-mode fiber (PM-SMF; HB450-SC, Fibercore) for wavelength conversion based on the SRS effect. Finally, a band-pass filter (BPF; ZET561/10X, Chroma) is applied to the output of the PM-SMF to isolate the 558-nm component. When a high voltage is applied to the EOM, the polarization of the laser pulse is rotated by 90°. As a result, it is reflected by the PBS to a different path, where no wavelength conversion occurs. The laser pulses coming out of the two paths have different wavelengths (i.e., 558 nm and 532 nm), which are combined using a dichroic mirror (FF552-Di02-25 × 36, Semrock) and coupled into a single-mode fiber (SMF; P1-460B-FC-1, Thorlabs). Before the SMF, a beam sampler (BS; BSF-05-A, Thorlabs) and a photodetector (PD; PDA10A2, Thorlabs) are used to record the temporal jitter and energy fluctuation of the laser pulses for offline compensation. The dual-wavelength output of the SMF is scanned by a 2D galvanometer (GM; GVS202, Thorlabs) and relayed by a lens pair (L1; AC508-80; L2; AC508-400, Edmund optics). A second nanosecond-pulsed laser performs the single-vessel occlusion (stroke laser; GLPM-10, IPG Photonics). Two HWPs (WPH10M-532, Thorlabs) separately adjust the polarization states of the laser pulses used for PAM imaging and vessel occlusion, and a PBS (PBS121, Thorlabs) combines the two types of pulses with orthogonal polarization states. The combined beam is then focused by an objective (MY-5X-802, Thorlabs) through the transparent MRR to the target to be imaged. A tunable CW laser (MRR laser; TLB-6712, Newport) is used for MRR interrogation, and a manual polarization controller (PC; FPC022, Thorlabs) adjusts the polarization state of the interrogation light. The output of the MRR is detected by a balanced photodetector (BPD; PDB465A-AC, Thorlabs) and acquired by a field-programmable gate array (FPGA; PCIe-7842, National Instruments). The DC output of the MRR is input into a closed-loop control in LabVIEW (see the “Proportional-integral-derivative (PID) control” section below for details), while the radio-frequency output is digitized by a high-speed waveform digitizer board (DAQ; ATS9350, AlazarTech) for PAM image formation.

Micro-ring resonator

An 80- μm -diameter polymer MRR and a matching waveguide are fabricated on a 250- μm -thick quartz plate using the soft nanoimprinting lithography method, similar to that previously reported²⁶. A 6- μm -thick polymer layer

(MY-131-MC, MY Polymer) is spin-coated to fully encapsulate the MRR on the quartz substrate. Then, the MRR is coupled to an SMF (S630-HP, Thorlabs) as the input port and a multi-mode fiber (MMF, GIF625, Thorlabs) as the output port, on an 8-mm-diameter glass coverslip. In comparison to the previous MRR²⁶, the MRR used in this study features two major design innovations to improve its sensitivity for ultrasonic detection. First, we have changed the cladding materials from polydimethylsiloxane (PDMS) to MY-131-MC, which has a lower refractive index of 1.31. It results in an improved Q-factor of the MRR (from 4.2×10^4 to 1.3×10^5) and thus higher ultrasonic detection sensitivity. Second, we have improved the coupling efficiency from the SMF to the MRR by integrating an adiabatically tapered waveguide. The wider opening of the tapered waveguide reduces its mode mismatch with the SMF and improves the coupling efficiency from 5 to 19%, which results in an increased signal-to-noise ratio of recorded photoacoustic signals. More detailed information about the design and fabrication of MRR can be found in the Supplementary Information.

Proportional-integral-derivative control

Laser-induced thermal accumulation during PAM imaging can shift the resonant frequency of the MRR, thereby impairing its sensitivity. To address this issue, a closed-loop PID controller is used to dynamically lock the interrogation light at the MRR's resonant wavelength. First, the wavelength of the MRR laser is set to the MRR's resonant wavelength. Then, the PID control module in the customized LabVIEW program is activated. The fast-monitoring output of the BPD (i.e., the DC output of the PDB465A-AC) is captured by the FPGA and fed into the PID control module, based on which a feedback analog signal is generated by the DAQ to fine-tune the MRR laser wavelength. By optimizing the PID parameters (typically, proportional gain: 0.0005; integral gain: 0.005; derivation gain: 0.05; filter coefficient: 0.2), the DC output from the MRR can be locked at a point where the sensitivity is maximized by tuning the interrogation wavelength of the MRR laser. Time-lapse photoacoustic signals of a black tape with and without PID control are shown in Fig. 1d, showing that the feedback control effectively maintains the MRR's sensitivity at the optimal level.

System characterization

The original lateral and axial resolution of the PAM system is characterized using a resolution target (R1DS1P, Thorlabs). Specifically, the edge spread function was experimentally measured by scanning the light focus across the sharp edge of a bar pattern on the target (the red curve in Fig. S3a), based on which the line spread function was derived (the pink curve in Fig. S3a). Then,



the lateral resolution was estimated to be $3.2\ \mu\text{m}$, based on the full-width-at-half-maximum (FWHM) value of the line spread function. The axial resolution was estimated to be $12\ \mu\text{m}$, based on the FWHM value of an A-line photoacoustic signal of the resolution target after Hilbert transform (Fig. S3b). Note that the axial resolution significantly degraded in vivo due to the strong attenuation of high-frequency ultrasound in tissues, as evidenced in Fig. 4. The cross-sectional profile of L2 shows that conventional PAM completely fails to resolve the two microvessels along the axial direction.

Scanning schemes

The high frame rate provided by the PAM system relies on the high-speed laser scanning within the acoustic FOV of the MRR. Our PAM system has two different scanning schemes: 2D real-time imaging (Fig. 2 and Movie S1) and 3D super-resolution imaging (Figs. 3–6 and Movies S2–S4). Specifically, to achieve 2D real-time imaging (i.e., RT-fPAM), the PAM laser is operated at 150 kHz for 2D galvo scanning over a $150 \times 150\ \mu\text{m}^2$ region with a frame rate of 15 Hz (each frame contains 100×100 pixels). To achieve 3D super-resolution imaging (i.e., SR-fPAM), the pulse repetition rate of the PAM laser is set to 200 kHz, and the galvanometer's line rate is set to 1 kHz. Before advancing to the next B-scan position, 800 repeated B-scans are acquired for RBC tracking. In addition, to achieve blood flow quantification in 3D, two series of B-scans along both lateral directions (i.e., *X* and *Y*) are collected, and RBC tracking is performed on each of them and finally combined.

Functional imaging

We have previously reported simultaneous quantification of microvascular blood oxygenation (sO_2) and flow using spectroscopic and correlation analyses in conventional PAM^{27,28}. In this study, sO_2 is measured as before, which employs dual-wavelength (i.e., 532 nm and 558 nm) laser pulses for excitation to distinguish oxy- and deoxy-hemoglobin based on differences in their optical absorption spectra. It is worth noting that the SRS output at 558 nm exhibits high stability (fluctuation <2%), and the power fluctuations are compensated by normalizing the A-line signal to the PD-recorded light intensity. As for blood flow speed quantification, 2D or 3D tracking of RBCs is performed. Detailed methods for the flow quantification are described below.

Flow quantification by Hough transform in RT-fPAM

In RT-fPAM, the high volumetric imaging rate (i.e., 15 Hz) enables RBC tracking in sequentially acquired en-face images. As shown in Fig. S6a, a kymograph can be generated for a particular vessel of interest. To quantify the blood flow speed, we first normalize the kymograph

and extract RBC trajectories via edge detection to form a binary image (Fig. S6b). Then, the Hough transform⁵⁴ is applied to individual sliding windows ($3\ \text{s} \times 100\ \mu\text{m}$) in the binary image to quantify the flow speed at a desired time and location. The horizontal axis (θ) of the resulting Hough transform matrix represents the slope of the RBC trajectory (Fig. S6c). Next, the ten matrix elements with the highest intensity values are identified (highlighted by the green box in Fig. S6c), and the mean value of their corresponding angles is calculated to estimate the mean slope of RBC trajectories. Finally, the blood flow speed in the vessel of interest is quantified based on its inverse relationship with the slope of the RBC trajectory.

Red blood cell tracking for 3D functional imaging in SR-fPAM

To achieve SR-fPAM, we are inspired by the ultrasound localization microscopy²⁰. Hundreds of B-scans are acquired with an extremely high frame rate (i.e., 1 kHz), and RBC movements in them are tracked to realize super-resolution vascular imaging. Fig. S7 shows the step-by-step flow diagram of the RBC tracking algorithm that enables 3D label-free super-resolution imaging of microvasculature and vascular functions.

Firstly, each B-scan is Hilbert-transformed after being compensated for jitter using the PD data. Then, motion and reverberation artifacts are removed from individual B-scans. Next, the pre-processed B-scans are rescaled using bicubic interpolation, and the correlation between the interpolated B-scans and the 2D point spread function (PSF) of the PAM system is performed, with the assumption that the RBC centroids are highly correlated with the PSF. The PSF is generated based on experimentally measured spatial resolutions of the MRR-based PAM system ($3.2\ \mu\text{m}$ laterally and $12\ \mu\text{m}$ axially, respectively, as shown in Fig. S3). To suppress noise, in the 2D correlation coefficient map of each frame, coefficients below 0.35 or falling outside the vascular regions are excluded. The vascular regions are identified based on the signal intensity of the raw PAM images averaged over 800 frames, where pixels with normalized intensities greater than 0.02 are classified as vessel areas. After the correlation coefficient map is thresholded, the RBC centroids are identified by detecting local maxima. To pair centroids corresponding to the same RBC or RBC cluster in adjacent frames, a nearest-neighbor matching strategy is applied. Specifically, for each centroid, all candidate centroids in the next frame within a predefined search range are evaluated, and the one with the minimum spatial distance is selected and linked. Given the dense distribution and average size of RBCs, the maximum search range for centroid linking is set to $6\ \mu\text{m}$ in both the lateral and axial directions. To further reduce misconceptions, only RBC centroids that can be continuously tracked and



move in a consistent direction for at least 3 ms (i.e., three successive frames) are retained. Trajectories are then formed by linking the positions of RBC centroids across adjacent frames, enabling the calculation of flow velocity from the centroid displacement and the frame rate. Finally, the structural image of the microvasculature and the blood flow map can be generated based on the number of trajectories and the average flow speed measured at each pixel, respectively.

Recognizing that RBC tracking in repeated B-scans only allows the quantification of flow velocity within the 2D imaging plane, bi-directional scanning along both lateral directions (i.e., X - Z and Y - Z) is necessary to collect orthogonal B-scan series along both lateral directions. From the X - Z B-scans, we obtain the velocity components along the X and Z axes (i.e., $v_X(x, y, z)$ and $v_Z(x, y, z)$). For vessels that are not fully parallel to the X - Z plane, the lateral flow velocity contains a Y -direction component (i.e., $v_Y(x, y, z)$), which cannot be recovered from X - Z scans alone and is instead obtained from RBC tracking in the Y - Z B-scans. After obtaining the three orthogonal velocity components, they are combined by vector addition in 3D (i.e.,

$$v_{3D}(x, y, z) = \sqrt{v_X(x, y, z)^2 + v_Y(x, y, z)^2 + v_Z(x, y, z)^2}.$$

Owing to our unique system design, changing the scanning direction is simple and straightforward: the fast-scanning axis of the galvanometer is switched by adjusting the driving voltage, and no modification of the acoustic sensor is required. Figure 5b shows the flow velocity maps along all X , Y , and Z directions, which are combined into the 3D flow speed distribution shown in Fig. 5c and Movie S4.

As for the blood oxygenation measurement, information obtained through the dual-wavelength excitation is incorporated to produce a 3D visualization of the vascular sO_2 , since we can quantify the sO_2 inside the super-resolved 3D microvasculature. 2D projections and 3D visualization of the microvascular sO_2 are shown in Fig. 5a, c and Movie S4.

Dwell time optimization for RBC tracking

Increasing the dwell time for repeated B-scan acquisitions allows more RBC trajectories to be recorded, but the associated time cost also increases. Thus, the dwell time needs to be optimized for the best tradeoff between image reconstruction fidelity and time expense. To this end, a small region in the mouse cortex was imaged with different dwell times, each corresponding to a different number of repeated B-scans for tracking (Fig. 3e, f and Movie S2). Microvascular diameters of four representative vessels (L1–L4; indicated by the white arrows in Fig. 3e) generated using different dwell times were calculated and compared with the diameter generated using the longest dwell time (i.e., 3000 frames). As shown in Fig. 3f, once the number of repeated B-scans reaches 800 (i.e., 0.8 s

dwell time), further increasing the frame number for tracking does not yield a significant change (<5%) in the measured diameter. This optimization study suggests that a 0.8 s B-scan dwell time is sufficient for RBC tracking-based super-resolution image reconstruction and thus is used in SR-fPAM.

Validation of the localization-based blood flow quantification

A phantom experiment was performed to assess the accuracy of the tracking-based flow speed measurement. In this experiment, a syringe pump (NE-300, Pump System Inc.) was used to pump 3- μ m-diameter black polystyrene microspheres (24292-15, Polysciences) through a transparent plastic tube at flow speeds ranging from 0.05 to 6 mm/s. As shown in Fig. S8, the flow speeds measured using the spatiotemporal RBC tracking show a good linear relationship ($R^2 = 0.999$) with the preset values across the entire flow range.

Two-photon microscopy

To benchmark SR-fPAM, we have performed a side-by-side comparison of SR-fPAM and TPM since TPM can serve as a gold standard for high-resolution vascular imaging. A TPM image of the same mouse brain region is acquired using a homemade TPM system with a 1.05 NA objective lens (XLPLN25XWMP2, Olympus). The TPM can be easily integrated into the PAM system and utilized to collect image data of the same FOV on the mouse brain due to the optical transparency of MRR. During TPM imaging, fluorescein isothiocyanate-dextran (FITC; FD-2000S, Sigma) was intravitreally injected to label the vasculature. The brain region was scanned with a lateral step size of 0.5 μ m and an axial step size of 0.625 μ m for volumetric two-photon imaging. Side-by-side comparisons of the 2D projection images and 3D renderings acquired using conventional PAM, SR-fPAM, and TPM are shown in Fig. 4a and Movie S3, respectively. Quantitative assessments were performed on the cross-sectional profiles of selected microvessels (Fig. 4b) and each plane of the 3D microvasculature (Movie S3), both illustrating high similarity between SR-fPAM and TPM.

Depth compensation

In PAM, depth information is obtained based on time-resolved ultrasonic detection. Due to the small size, MRR can be regarded as a point detector, so both the depth and lateral distances between the photoacoustic source and the MRR contribute to the time delay. Leveraging the geometric relationship shown in Fig. S9a, we developed a delay compensation algorithm to accurately retrieve the true depth information. To evaluate its performance, we acquired the B-scan images of a piece of flat black tape at three different depths. Before the compensation, all three



B-scans showed arc-shaped delays, with the curvature diminishing as the black tape was positioned further away from the MRR (top row of Fig. S9b). After compensation, the additional delay arising from the lateral displacement was successfully eliminated (bottom row of Fig. S9b).

Single-microvessel occlusion

We used short laser pulses to directly generate blood clots in targeted microvessels without the aid of photothrombotic dye²⁹. Specifically, a 5-s pulse train (150 kHz repetition rate, 300 nJ energy, 1.5 ns width) was launched onto a microvessel near the center of the FOV. The location of the stroke is precisely controlled by the laser induction. Disappeared photoacoustic signals at and around the occlusion location indicate a successful occlusion (Figs. 2c, d and S10, and Movie S1; successful occlusion was repeated in five different animals).

Crosstalk elimination

The nanosecond laser pulses used to induce micro-strokes have relatively high energy, which can excite considerable photoacoustic signals. By synchronizing the two lasers used for imaging and occlusion (i.e., PAM laser and stroke laser in Fig. 1a), we can precisely control the timing of the two types of photoacoustic signals and the delay between them. With proper time-gating, the crosstalk induced by the stroke laser can be completely removed from each B-scan, as shown in Fig. S10.

Animal preparation

C57BL/6J mice (male, 9–10 weeks, The Jackson Laboratory) were used for all in vivo experiments herein. Following craniotomy, a 100- μ m-thick acrylic film (Emco Industrial Plastics) was used to seal the open-skull window. After the procedure was completed, the mice were returned to their home cages and administered buprenorphine at a dosage of 0.1 mg/kg for pain management. Throughout the post-operative period, the mice were monitored for signs of distress or pain. Two weeks later, imaging experiments were performed under general anesthesia with 1.5% isoflurane, and the mice were kept at 37 °C using a heating pad. All experimental procedures were carried out in conformity with the animal protocol approved by the Institutional Animal Care and Use Committee at Washington University in St. Louis.

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Author contributions

F.Z., Z.W., and S.H. conceived the project idea. F.Z., Z.W., J.H., S.Y., and S.J. designed the experiments. F.Z., Z.W., and J.H. performed the experiments. Y.L., H.F.Z., and C.S. designed, fabricated, and optimized the packaged MRR-based ultrasound detectors. F.Z., Z.W., and N.S. processed and analyzed the data. F.Z. and Z.W. performed the animal preparation for in vivo experiments. All authors contributed to the writing of the manuscript. All authors have given approval to the final version of the manuscript.

Data availability

The data and code are available from the corresponding author upon reasonable request.

Conflict of interest

H.F.Z. and C.S. have financial interests in Opticent Inc., which did not support this work. All other authors declare no competing interests.

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