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Letter

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Label-Free Transient Absorption Microscopy for Red Blood Cell Flow Velocity Measurement *in Vivo*

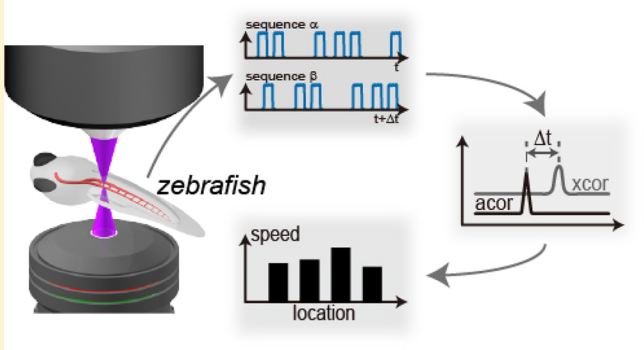
Tao Chen[†] and Yanyi Huang*[,†,‡]

[†]Biodynamic Optical Imaging Center (BIOPIC), and College of Engineering, Peking University, Beijing 100871, China

[‡]Beijing Advanced Innovation Center for Genomics (ICG), Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China

Supporting Information

ABSTRACT: Red blood cells have intrinsic transient absorption property at the near-infrared region, allowing for label-free detection and imaging. We present a new approach to measure the blood flow velocity *in vivo* with a transient absorption microscope and correlation analyses of signal sequences. With specific scan modes, we have quantitatively obtained the flow velocity in capillaries, arteries, and veins in a live zebrafish with accuracy of about 30 μm/s. In addition, a high-resolution three-dimensional vessel network can be reconstructed through this approach with spatial resolution of 1 μm.



It is in strong demand to track blood flow for inspecting and monitoring the cardiovascular system since the proper functionality of cardiovascular system maintains the life of many organisms. Blood flow, as the main motion form in cardiovascular system, drove different cells and various molecules throughout the organism, to provide oxygen, nutrients, and signaling. The blood flow can also generate shear stress in vessels, hence affect the morphology and functionality of related cells.[1,2] Additionally, cancer research had revealed the role of cardiovascular system in transporting cancer cells and signaling molecules.[3−5] The optical Doppler effect probes the blood flow in the Fourier domain with a specific dual beam configuration.[6] Ultrasound is also an effective approach in blood flow measurement, and it has achieved spatial resolution around 10 μm, which is far shorter than acoustic waves used.[7] Practically, the flow of cells inside the blood vessels is directly connected to physiological conditions. Hence, red blood cells can be suitable candidate to inspect blood flow, since they are great in number and their motion velocity equals the plasma and other cells. Red blood cells can provide spatially quasi-continuous tracking of blood flow, and their small size allows them to reach the tiniest capillary in the body. In addition, the morphology of red blood cells is unique and stable, ensuring easy identification.

Red blood cells can be detected through various techniques. Autofluorescence,[8] or fluorescent labeling including chemical and genetic approaches,[9,10] can be used for fluorescence detection. However, for most applications *in vivo*, label-free methods are preferred. The photophysical processes that involve two photons, such as photothermal effect[11] and two-color two-photon absorption,[12,13] can be applied to directly observe red blood cells through targeting hemoglobin in the cells without labeling. Photoacoustic microscopy is another choice widely used to image red blood cells *in vivo*.[14] Combined with the optical Doppler effect, optical coherence tomography can measure the blood flow velocity as well.[15]

Here, we presented a new approach to quantitatively assess the blood flow in live zebrafish (*Danio rerio*) based on label-free imaging of red blood cells by transient absorption microscopy. We chose zebrafish because it is a widely used model organism in cardiovascular research.[16] Transient absorption, short-lived, is the secondary absorption of electrical excitation states of specific molecules upon the excitation through primary absorption (Figure 1b). Unlike the primary absorption, which is often buried in high background, the secondary absorption undergoes with little interference from the environment. From spectroscopy to microscopy, it has presented the high ability in imaging nanomaterials.[17−19] Using the pump−probe scheme, the red blood cells can be directly imaged with two ultrafast laser pulse trains. With specific scanning modes, we have measured the flow velocity of red blood cells in vessels in live zebrafish and reconstructed the three-dimensional vessel network with high resolution.

We have built a transient absorption microscope (Figure 1a) on a scanning multiphoton microscope base, using two collinearly aligned picosecond lasers with wavelengths at 1064 nm (pump) and 810 nm (probe).[20,21] Fast imaging of transient signal requires a high signal-to-background ratio which is

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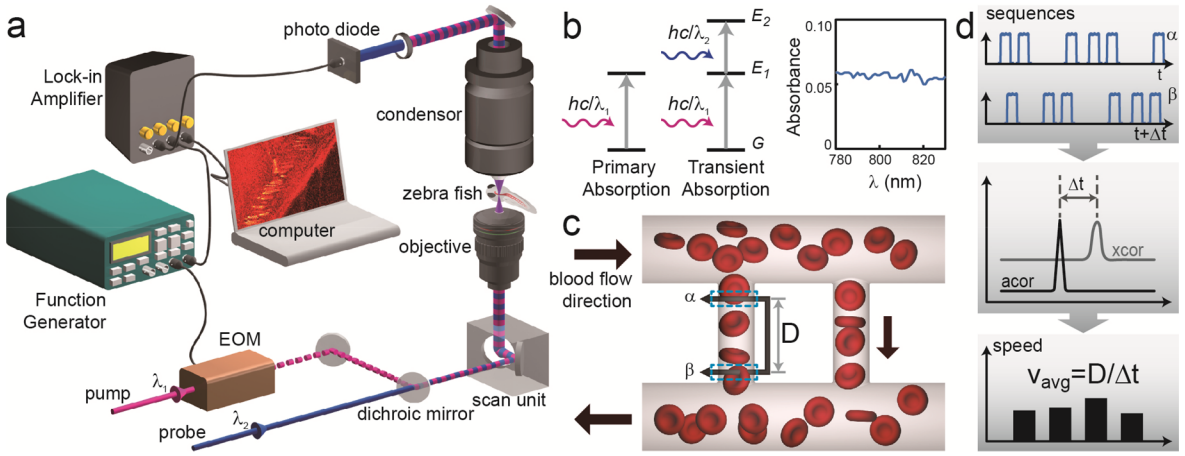

Figure 1. Schematic illustration of transient absorption microscope and the principle of blood flow measurement. (a) Transient absorption microscope setup. (b) Energy diagram of primary absorption and transient (secondary) absorption and the absorption spectrum of hemoglobin between 780 and 830 nm. (c) Scanning paths and the blood cells in vessels. (d) Basic procedure and analysis of blood flow.

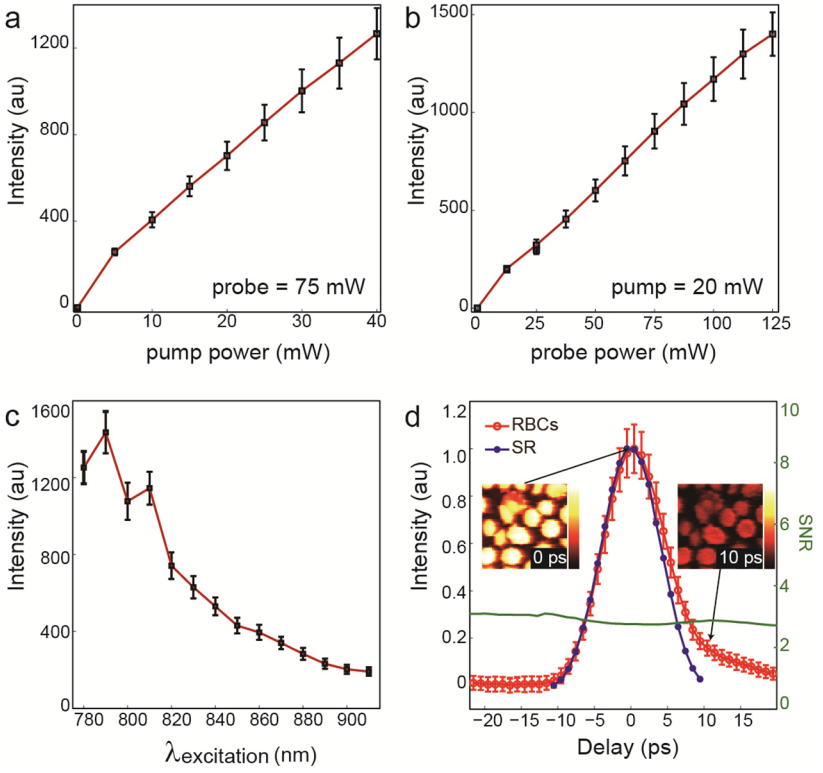

Figure 2. *Ex vivo* characterization of transient absorption properties in red blood cells. (a, b) Signal strength against pump and probe beam powers. (c) Spectrum of probe beam. (d) Time-resolved measurement of transient absorption signal. The green curve showed the change of SNR as a function of delay. SR: system response.

achieved using a lock-in detection scheme. We modulated the pump beam intensity at frequency f = 9.825 MHz, and the primary absorption occurred periodically at frequency f.

The secondary absorption at 810 nm only happened in the presence of primary absorption, hence resulted in a periodic probe beam intensity variation at f as well. The probe beam was selected with a short-pass filter and then collected by a fast-speed photodiode. The transient absorption signal, proportional to the intensity variation of probe beam, was demodulated by a lock-in amplifier and then reconstructed into images.

The wavelengths in our system are selected to facilitate the blood flow monitoring through the transient absorption of red blood cells. The red blood cells presented a homogeneous absorption in the near-infrared region (Figure 1b). Laser at such wavelengths will have better penetration depth for monitoring the live animals. To quantitatively assess the velocity of red blood cells in the blood vessels, we designed a U-shape repetitive scanning path (Figure 1c), through which the same vessel could be monitored twice (positions α and β) in each scanning period, with distance D apart. At each position, the repetitive scan produced a time sequence of transient absorption signal of red blood cells flowing through

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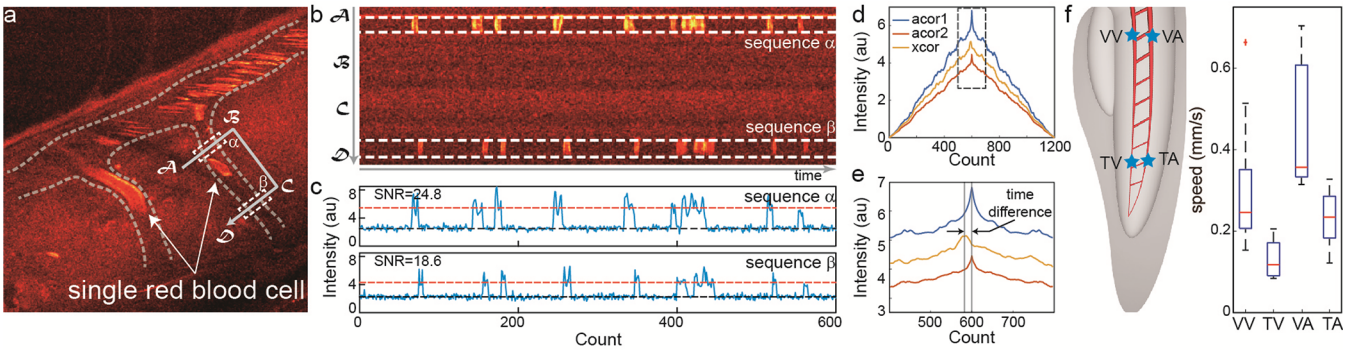

Figure 3. Blood flow velocity measurement. (a) Transient absorption image of part of a zebrafish, dashed line shows the boundary of blood vessels. (b) Line scan by U-shaped path image. (c) Extracted sequences from part b, the red and gray dash lines represent the mean value of signal and background. (d, e) Calculation of Δt based on autocorrelation and cross-correlation of sequences in part c. (f) Statistics of blood flow velocity in 9 zebrafish. Four locations (about 120 μm below surface) were illustrated in the cartoon. VV, vein at ventral side near yolk; VA, aorta at ventral side near yolk; TV, vein at tail; TA, aorta at tail.

(Figure 1d). The sequences recorded from positions α and β were similar but temporally shifted by a time span Δt, which can be quantitatively calculated through correlation analyses between these two sequences. Therefore, the average flow velocity of the red blood cells can be easily deduced by $v_{avg} = D/\Delta t$ (Figure 1d). In fact, such an approach can be applied to any vessel to measure the local blood flow velocity.

We also characterized the transient absorption property of red blood cells. We measured the dependency between the transient absorption signal and the intensity of pump and probe laser beams (Figure 2a,b). With one beam's power fixed, the signal ascended when the other beam's power increased. We also inspected the wavelength dependence on the probe beam (Figure 2c) between 780 and 920 nm, which is the maximum span of the tuning rage of our optical setup. Within this span, the strongest transient absorption signal was found around 790 nm. There is, however, a shoulder peak around 810 nm, probably containing partial contribution of the stimulated Raman scattering from the methyl groups in the biochemicals inside the cells.[22]

Such a finding also reflected that, for red blood cells, with the identical laser power, the signal of transient absorption is much stronger than that of stimulated Raman scattering occuring simultaneously. Additionally, we chose 810 nm serving as the probe beam to achieve a high transient absorption signal and record tissue morphology by SRS signal simultaneously. Although at certain wavelengths both transient absorption and stimulated Raman scattering will happen, experimentally we can still isolate two phenomena by time gating. One of the intrinsic differences between these two phenomena is that the transient absorption has its characteristic decay[23] due to the lifetime of the excited states (Figure 2d). The system response curve can be obtained through measuring SRS signal of dodecane. Deconvolution of the transient absorption time-dependence curve with such temporally symmetric system response curve gave us a lifetime of signal decay about 7 ps. Hence when necessary, gating the right time difference between the pump and probe pulse trains will improve the image quality by increase the signal-to-background ratio.

We then used live zebrafish as the model animal and tested the applicability of our technology *in vivo*. We first monitored the capillary vessels (Figure 3a, Figure S1), which are narrow and only allow red blood cells to flow as discrete single-cell streams (Figure 3b). With the D determined by the scanning path, as well as the Δt deduced from the cross-correlation of the signal-time sequences obtained from the two intersecting positions on the vessel, we should be able to accurately calculate the blood flow velocity. If projecting the pixels to temporal axis, we could have the profiles of red blood cells' motions in the time domain (Figure 3c). The signal-to-background ratio of such profiles was high enough to identify each single red blood cell.

The accuracy of Δt determination is essential in calculating the flow velocity. We first calculated the autocorrelation of each sequence, and the cross-correlation between them (Figure 3d,e). The highest peaks represented the overlap of each calculated pair. Autocorrelations of the two sequences (acor1, acor2) shared the peak position, meanwhile, the peak of cross-correlation (xcor) dislocated from autocorrelation peaks, with the displacement in time as Δt (Figure 3e). We measured the blood flow at four positions (Figure 3f) in zebrafish including two positions in the artery and two in the vein. The average blood flow velocity among these four locations were around 294 ± 170 μm/s, which is in accordance with results from other methods.[24,25] Not surprisingly, for the artery and vein around the same body-position of the zebrafish larva, the blood flow velocity in the artery (458 ± 156 μm/s near yolk, 232 ± 71 μm/s at tail) is higher than that in the vein (312 ± 167 μm/s near yolk, 131 ± 48 μm/s at tail). In addition, the blood flow velocities at the tail were slower than those near the larva's yolk. The phenomenon could be explained with fluid mechanics in consideration of pulse wave propagation.[26] Simply, the blood flow experienced a pressure drop along vessels after pumped out by the heart.

Furthermore, such a label-free transient absorption imaging technique can also been used to reconstruct the three-dimensional vessel network in the body (Figure 4, Figures S2−S4). For example, in Figure 4, the intrinsic optical sectioning capability of transient absorption allows for high-resolution, two-dimensional imaging of the blood cell-containing positions, and by stacking of such images the vessel network near the zebrafish heart is obtained. To have better spatial resolution, the dwell time of each pixel during imaging is longer than our previous line-scan experiment. For such a small volume with complex vessels entangled together in the living organism, transient absorption microscopy shows promising advantages over other approaches.

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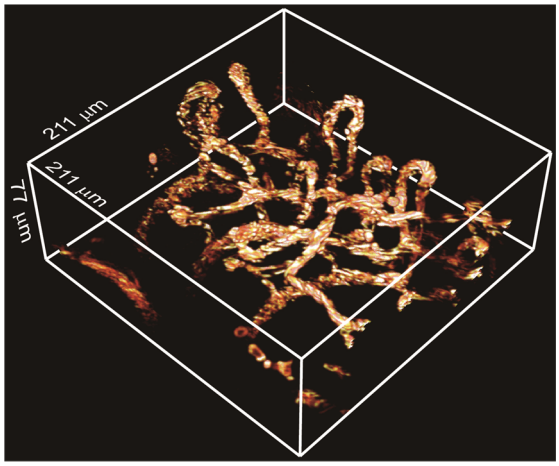

Figure 4. Reconstruction of three-dimensional vessel network through transient absorption microscopy of red blood cells.

Through development of this method, we have demonstrated that label-free blood flow monitoring can be easily achieved through the intrinsic transient absorption of red blood cells with an optical microscope. Such an approach provides great penetration depth for *in vivo* studies and maintains the finest resolution using optical microscopy. Further improvement includes an increase in the penetration depth with higher power lasers without causing photodamage. The velocity deduced from the correlation calculation can be applied to any size of blood vessels and is independent of cell density, as long as the signal can be detected with a satisfactory signal-to-background ratio.

Blood flow measurement through transient absorption microscopy also has its limitations. First, the obtained velocity is the averaged velocity over the two intersection points between scan path and vessel. It is still challenging to provide velocity distribution along the vessel with high spatial resolution. Besides, blood flow velocity at structures like a T-junction was also difficult to measure with this approach. Second, this method cannot be used when the flow velocity is higher than the scanning speed.

In summary, we have developed an *in vivo* blood flow measurement approach by the transient absorption of red blood cells. We have demonstrated precise measurement in live zebrafish without exogenous labeling. It can also be applied to reconstruct the three-dimensional fine vessel network. This technique offers a new option for live animal functional imaging and measurement of the cardiovascular system in model animals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01952.

TAM system setup, experiment parameters, and data analysis (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yanyi@pku.edu.cn. Phone: +86-10-62744058.

ORCID

Yanyi Huang: 0000-0002-7297-1266

Author Contributions

T.C. and Y.H. conceived the project. T.C. performed the experiment and analysis. T.C. and Y.H. prepared the manuscript.

Notes

The authors declare no competing financial interest.

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Electrical Supplementary Information

Label-free transient absorption microscopy for red blood cell flow velocity measurement *in vivo*

Tao Chen[a], Yanyi Huang[b*]

[a] *Biodynamic Optical Imaging Center (BIOPIC),Peking University, Beijing, China*
[b] *Beijing Advanced Innovation Center for Genomics (ICG), Peking-Tsinghua Center for Life Science, and College of Engineering, Peking University, Beijing, China.*
[*]E-mail: yanyi@pku.edu.cn
[*]Phone: +86-10-62744058 (O)

This supporting information includes supplementary figures of 3D reconstructed vessels (Figure S-1 to S-4), and parameter details of the TAM system and experiments.

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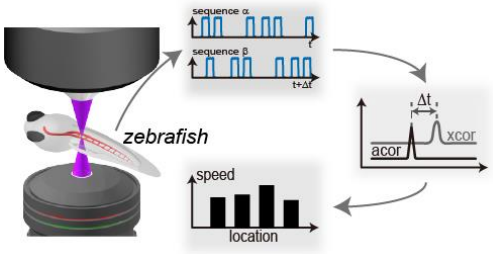

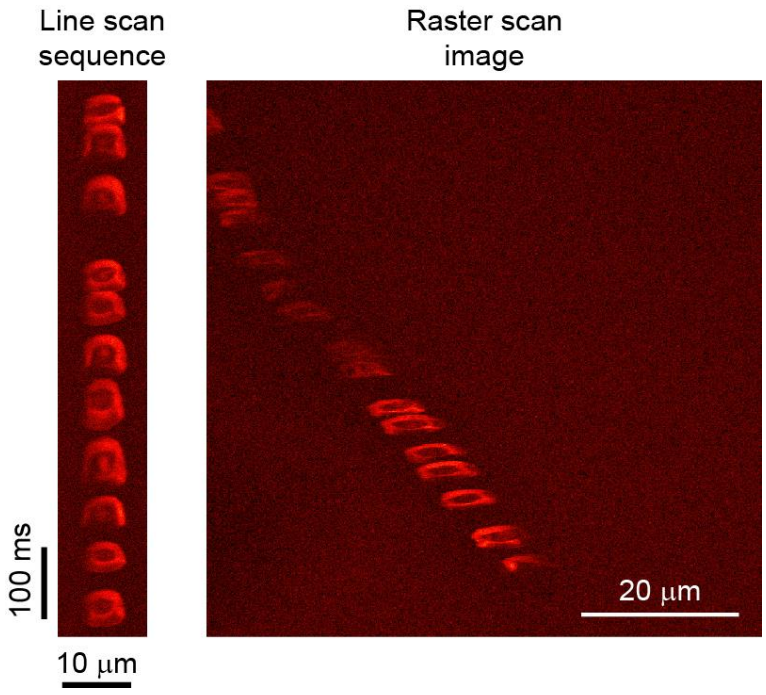

Figure S-1. High resolution image showing red blood cell sequence in capillary vessel.

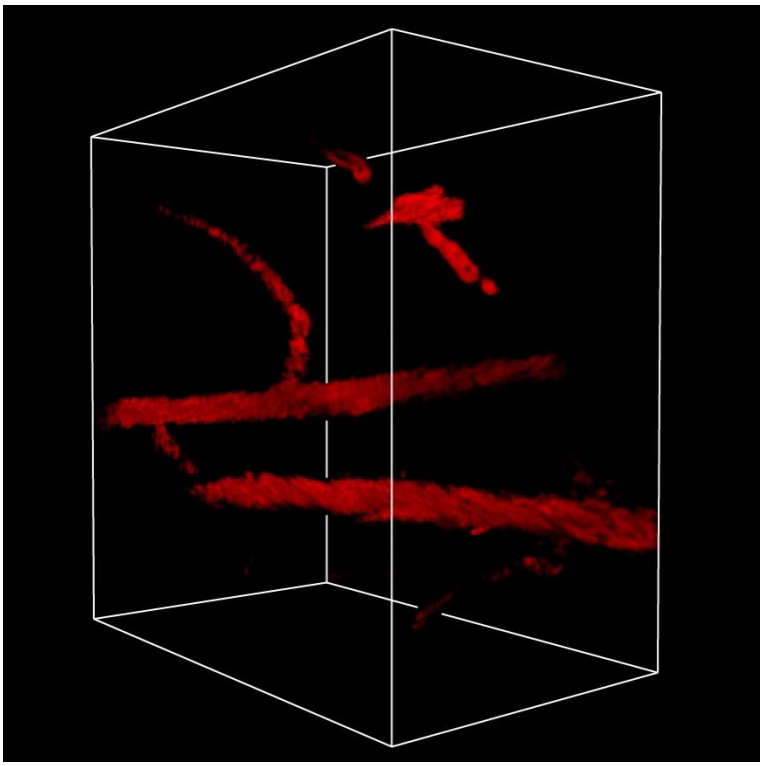

Figure S-2. Reconstructed image showing parallel artery and vein, and capillary vessel branching out from them.

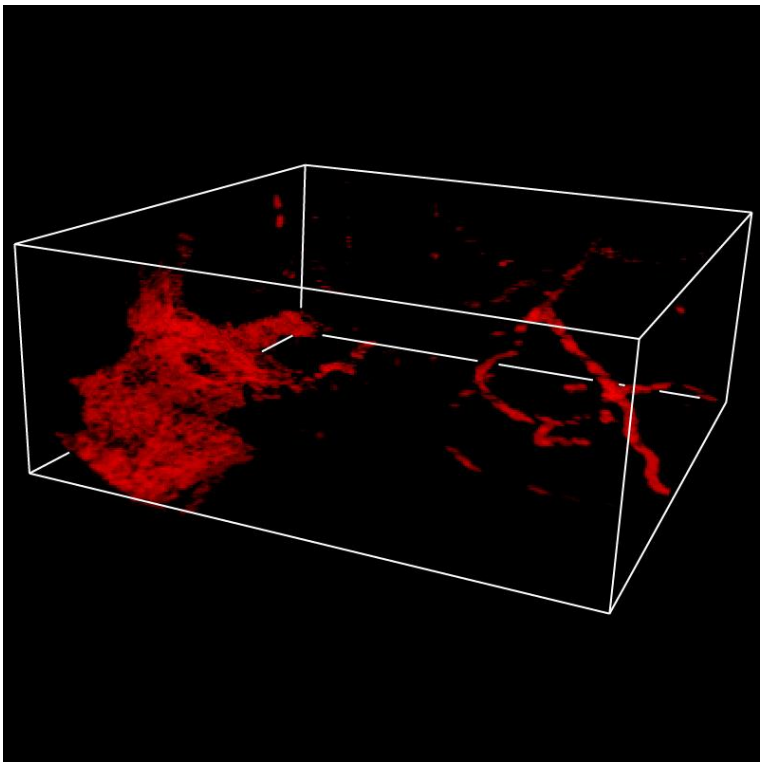

Figure S-3. Reconstructed image showing capillary vessel near the surface of the body.

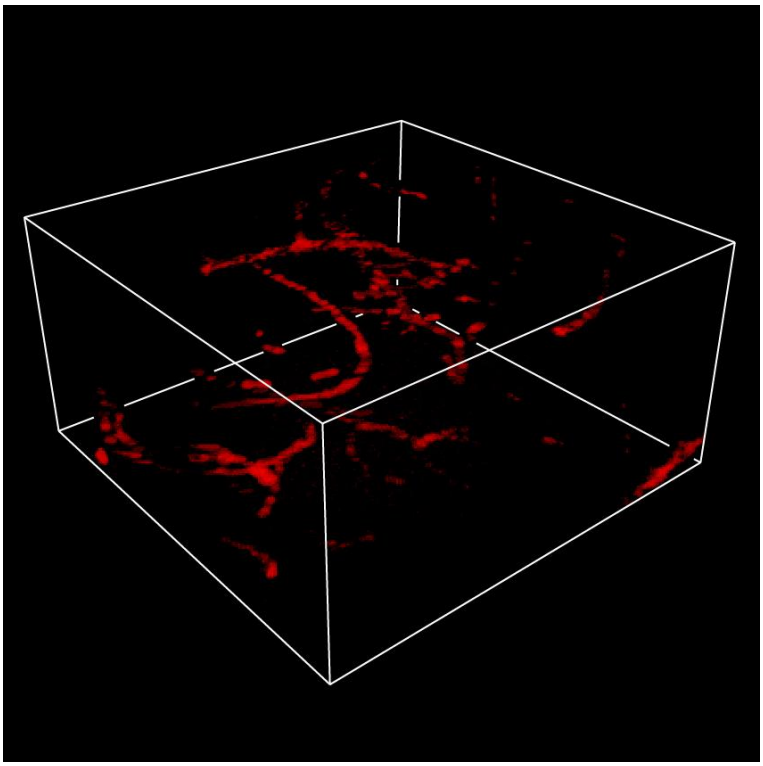

Figure S-4. Reconstructed image showing the capillary vessels.

Methods

Transient absorption microscope

A pump integrated synchronized OPO (picoEmerald, APE, Germany) is applied to offer pump and probe beams. Also known as 'one box laser', this OPO can provide two synchronized infrared pulse trains simultaneously, with repetition rate of 80MHz. One beam is tunable, ranging from 780nm-990nm, used as the probe beam. The other is fixed at 1064nm, as the pump beam. Pulse durations are 6ps for probe beam and 8ps for the pump beam. The two beams are overlapped both in space and time. The pump beam was modulated by an electro-optical modulator (EO-AM-NR-C2, Thorlabs, USA) at the frequency of 9.825MHz. The overlapped beams were directed into an inverted two-photon microscope (IX81/FV1000, Olympus, Japan). Focused by a water immersed 60X objective (UPLSAPO60XW, Olympus, Japan), or silicone oil immersed 30X objective (UPLSAPO30X, Olympus, Japan), overlapped laser beams were shone on sample. The power density, taking both pump and probe into consideration, on focal plane was about 12 MW/cm^2 (or 150 mJ/cm^2 per pulse). The power density is under photo ablation threshold and coagulation threshold for our wavelength.[1]

Transmitted light was collected by an water immersed condenser (N.A. 0.9, Olympus, Japan). Collected light was then filtered by a band pass filter, (890/220m, Chroma, USA), and relayed to a large area photo diode (FDS1010, Thorlabs, USA) reverse biased at 60 V. After conversion, the photo diode output was sent into a RF lock-in amplifier (designed by Dr. Brian Saar), and demodulated. Output of lock-in amplifier was sent into Olympus software (FV10ASW, Olympus, USA) for image reconstruction.

Absorption spectrum measurement

The absorption spectrum was acquired in a desktop spectrometer (D30, Eppendorf, Germany). Blood sample were collected and dispersed in deionized water with concentration of 1:200 in cuvette. Dionized water was used as reference. Raw data was redrew with matlab.

Sample preparation

4 dpf fish was raised in water with PTU, a chemical to prevent pigmentation formation. Pigmentation can contribute to MMA signal via absorption, hence to complicate the acquired images. Fish was anaesthetized by 1/20 tricaine solution right before experiment each time. After anaesthesia, fish was transferred onto a piece of coverslip, and then covered with low melting point agarose. When argarose turned to gelly after cooling down to room temperature, the fish was then immobilized and ready for observation.

Blood flow measurement

After localizing the vessel under bright field, and double-checked with TA image, the U-shaped scan path was drew by hand on the image. For each measurement, the laser scanned 5000 times to collect long enough sequence for calculation. The raw images of line scan were processed with Matlab (Mathwork, USA). Each image was cropped into two sequence with only RBCs, and then analyzed as described.

Blood flow rate accuracy estimation

The velocity accuracy was determined by the average distance (105 μm) over average Δt (160 ms), with consideration of the Δt variation (30 ms), since the distance measurement is much more precise than time in this approach. Hence the variation of velocity measurement was estimated to be 105 μm/(160 ms-30 ms)-105 um/(160 ms+30 ms) ~ 30 μm/s. The spatial resolution is the same as our previous transient absorption studies.[2,3] It is diffraction limited, hence about half micron. We relaxed the resolution to be 1 μm, as fair spatial sampling for the vessels.

Tomography of blood vessels

Three dimensional imaging were performed to map vessel structure. The pixel dwell time was 40 μs. Image size was 256×256×78.

Reference:

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