Quantitative imaging of red blood cell velocity *in vivo* using optical coherence Doppler tomography

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We present particle counting ultrahigh-resolution optical Doppler tomography (pc- μ ODT) that enables accurate imaging of red blood cell velocities (ν_{RBC}) of cerebrovascular networks by detecting the Doppler phase transients induced by the passage of a RBC through a capillary. We apply pc- μ ODT to image the response of capillary ν_{RBC} to mild hypercapnia in mouse cortex. The results show that ν_{RBC} in normocapnia ($\nu_{\text{N}} = 0.72 \pm 0.15$ mm/s) increased $36.1\% \pm 5.3\%$ ($\nu_{\text{H}} = 0.98 \pm 0.29$ mm/s) in response to hypercapnia. Due to uncorrected angle effect and low hematocrit (e.g., $\sim 10\%$), ν_{RBC} directly measured by μ ODT were markedly underestimated ($\nu_{\text{N}} \approx 0.27 \pm 0.03$ mm/s, $\nu_{\text{H}} \approx 0.37 \pm 0.05$ mm/s). Nevertheless, the measured ν_{RBC} increase (35.3%) matched that ($36.1\% \pm 5.3\%$) by pc- μ ODT. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4726115>]

Cerebrovascular networks play a vital role in regulating brain oxygenation and energy supplies and thus serve as the basis for most functional neuroimaging approaches (e.g., positron emission tomography (PET), functional MRI (f-MRI)).¹ However, studying the physiology of cerebral microvascular hemodynamics requires quantitative imaging tools that can provide both high spatiotemporal resolutions and a large field of view (FOV). For instance, multiphoton microscopy (MPM),² which enables imaging of red blood cell (RBC) velocities ν_{RBC} in capillaries at up to ~ 300 μm of depth, is very useful for studying neurovascular physiology *in vivo*; however, the measurements are limited to capillary vessels and require administering fluorescence dyes, thus limiting its use in studies that require repeated imaging as may be needed when monitoring dynamic vascular responses. Recent advances in photoacoustic microscopy³ and coherence-domain optical techniques (e.g., optical microangiography) (Refs. 4–6) have permitted label-free 3D visualization of the cerebral networks with unprecedented neurovascular details. Although quantitative 3D imaging of cerebral blood flow speeds (CBF) has been reported using optical coherence Doppler tomography (ODT), the flow sensitivity and spatial resolution are insufficient to fully resolve the capillary CBF in brain cortices. Here, we present a technique, termed "particle-counting ultrahigh-resolution ODT" (pc- μ ODT) that allows accurate imaging of capillary ν_{RBC} without the need for fluorescence labeling. In addition, we demonstrate the capability of pc- μ ODT for functional neuroimaging studies on a mouse model of cerebral hypercapnia.

pc- μ ODT operates in a similar manner to MPM for imaging RBC velocity (i.e., RBCs passing through a capillary per unit time) in that MPM counts the intensity transient of a fluorescently labeled RBC whereas pc- μ ODT counts the resultant Doppler phase transient of a moving RBC. The ultra-sensitive detection of individual RBC-induced phase

transients is made possible by the combination of ultrahigh-resolution OCT (μ OCT) with a phase-intensity-mapping (PIM) algorithm recently developed in our lab to enhance the sensitivity of Doppler flow detection.⁷ Fig. 1 illustrates the principle of pc-ODT, in which a broadband spectral-domain OCT was illuminated by a sub-8 fs laser ($\lambda = 800$ nm, $\Delta\lambda \approx 128$ nm). A high axial resolution (i.e., coherence length $L_c = 2(\ln 2)/\pi \cdot \lambda^2/\Delta\lambda_{\text{cs}}$) of 1.8 μm was achieved in brain by maximizing the bandwidth (e.g., $\Delta\lambda_{\text{cs}} \geq 154$ nm) of the cross spectrum ($S_{\text{cs}}(\lambda) \equiv [S_s(\lambda) \cdot S_r(\lambda)]^{1/2}$, where $S_s(\lambda)$, $S_r(\lambda)$ are the sample and reference power spectra) and compensating the dispersion mismatch between sample and reference arms.⁸ The collimated light in the sample arm was transversely scanned (x/y-axes) by a high-precision servo mirror and focused with a f16 mm/NA0.25 objective (lateral resolution: $\sim \phi 3$ μm) onto the capillary beds through a cranial window, enabling the resolution of individual capillaries (e.g., $\phi 3$ – 7 μm) in the cortex of a mouse brain. The backscattered light from the mouse cortex of different depths (along z-axis) was collected back to the sample arm and recombined with the reference light in the

FIG. 1. A sketch to illustrate pc- μ ODT for accurate imaging of capillary ν_{RBC} , which was based on spectral-domain 3D μ OCT. ν_{RBC} was measured by counting the number of frames (n) across a RBC, where $f = 1.1$ kHz is the frame rate for pc- μ ODT.

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detection fiber to be detected by a spectral imager (a 2048-pixel linear CCD camera, Atmel) synchronized with sequential x/y-scans at 20 kHz to acquire 2D/3D μ OCT images. The raw image data were stream-lined via camera link to Raid-0 hard disks (300 MB/s) of a PC for image processing by FFT analysis to reconstruct and display the Doppler flow (μ ODT) images.

PIM for high-sensitivity 3D μ ODT reconstruction⁷ is based on linearly projecting the flow-induced Doppler frequency shift ν_x , Δz to its intensity in the Fourier domain (in x-axis)

$$P(\Delta z, x) = \frac{2\pi FFT^{-1}[\nu_{xi} I_i(k, x)|_k]}{FFT^{-1}[I(k, x)|_k]} = 2\pi \nu_{xi} \Delta z, \quad (1)$$

where $P(\Delta z, x)$ is a linear intensity projection to ν_x , Δz and $I(k, x)$ is the detected spectral μ OCT signal. $k = 2\pi/\lambda$ is wave number and i refers to the i -th flow ν_{xi} , Δz . As PIM requires high-capacity data acquisition (e.g., a $0.05 \mu\text{m}$ -pitch sampling over 1 mm or 20 k points in x-axis) and intensive computation, 3D μ ODT was reconstructed by post-image processing (e.g., ~ 4 min for a 3D μ ODT of $20 \text{ k} \times 0.5 \text{ k} \times 2 \text{ k}$ voxels). A pre-scan with simple phase-subtraction method (PSM) (Ref. 9) was applied to permit real-time flow display, so that the parameters for cortical flow imaging such as the focus onto the capillary beds and the field of view of interest could be optimized. With the coordinates (z, x, y) of individual capillaries accurately located by 3D μ ODT, pc- μ ODT is then performed to quantitatively image capillary ν_{RBC} . In this study, cross-sectional μ ODT scans perpendicular to a capillary of interest were implemented. Assume that a capillary is of $\sim 7 \mu\text{m}$, a minimal of $7.6 \mu\text{m}$ lateral scanning range is needed to fully cover the cross section of the capillary. And as a high pitch density such as $\Delta x \approx 0.32 \mu\text{m}$ is required by PIM to detect ν_{RBC} -induced minute Doppler

phase changes, 24 A-lines ($x_N = 7.6/0.32 \approx 24$) per 2D μ ODT were adopted. Meanwhile, as a RBC is of $D_{\text{RBC}} \approx 7 \mu\text{m}$ and capillary $\nu_{\text{RBC}} \approx 1 \text{ mm/s}$, an μ ODT scanning rate of $f \approx 1 \text{ kHz}$ (i.e., $\sim 1 \text{ kfps}$) is needed to ensure $n \approx 5$ time-lapsed μ ODT frames to be captured during the crossing of a RBC through the capillary in order to provide sufficient temporal resolution for accurate ν_{RBC} quantification. Both requirements were fulfilled by running the μ ODT system at its full A-scan rate (27 kHz), which provided a frame rate of $f \approx 1.1 \text{ kHz}$ for pc- μ ODT. The image reconstruction of pc- μ ODT was post-processed by PIM, similar to that of μ ODT. An oversampling across the vessel wall provided sufficient points ($x_N = 24$) in the lateral direction for PIM to calculate its phase image $\phi(x, z, t)$, and phase subtraction $\Delta\phi(x, z, t)$ between 2 adjacent 2D phase images was used to retrieve the instantaneous phase change profile, by which the background phase noise (ϕ_n) caused by the micromotion of the vessel wall was largely removed with a dynamic mask generated based on phase thresholding (e.g., $\Delta\phi_T \geq 1.2\phi_n$). This was because an abrupt phase transient was generated upon the arrival ("+") or the departure ("-") of a RBC across the small imaging section. ν_{RBC} could be assessed by

$$\nu_{\text{RBC}} = D_{\text{RBC}} \times (f/n), \quad (2)$$

where n is the total number of sampling cross-sections over a RBC. As the length (D_{RBC}) and ν_{RBC} are in the same direction, Eq. (2) can be approximated to a more general situation where the capillary flow has angles with x-/y-axes. To demonstrate the capability of pc-ODT for accurate capillary ν_{RBC} measurement *in vivo*, we chose a mouse model of cerebral hypercapnia as it has been extensively validated and widely used as a means to increase CBF.¹⁰ Female CD-1 mice (~ 8 wk of age) were used in the experiments during which they were anesthetized with inhalational 2% isoflurane mixed with 100% O_2 and mounted on a custom

FIG. 2. 3D μ ODT of mouse cortical CBF networks *in vivo*. (d), (i): Quantitative CBF images with enhanced sensitivity (0–3 mm/s) by phase unwrapping. (k), (l): Hypercapnia-elicited CBF changes in vessels of different caliper. Red, blue arrows in (a): arteriolar and venous flows. Image size: $1.6 \times 1 \times 1 \text{ mm}^3$, CV in (d), (i): a capillary vessel chosen to perform pc- μ ODT in Fig. 3.

stereotaxic frame. A $\sim\varphi 5$ mm cranial window was created on the mouse brain with the dura left intact. The exposed brain surface was then covered with 2% agarose gel under a glass coverslip. Mild hypercapnia was induced by switching the gas from 100% O₂ to a mixture with 5% CO₂. All the animal procedures were approved by the Institutional Animal Care and Use Committee of the Stony Brook University.

Fig. 2 shows quantitative 3D μ ODT images of cortical CBF networks in normocapnia vs hypercapnia. As CBFs in large branch vessels and capillaries are vastly different, μ ODT acquired at 10 kHz (c), (h) was temporally down binning (i.e., to prolong Δt) to 5 kHz (b), (g) and 1 kHz (a), (f) and then recombined (d), (i) by phase-unwrapping to extend the dynamic range to 0–3 mm/s for enhancing flow detection. Owing to significantly improved flow detection sensitivity of PIM and spatial resolution of μ OCT, the 3D projection images (e), (j) over a small region ($0.4 \times 0.4 \times 0.6$ mm³) show abundant microcirculatory CBF networks, in particular, the high turnout of the capillary CBF. Although the CBF increase elicited by hypercapnia was clearly visualized by comparing the corresponding images (e.g., d vs i or e vs j), their ratio image (k = d/i) exhibits the heterogeneous CBF responses to hypercapnia in the cerebrovascular networks. The statistical figures by Student's t tests in Fig. 2(l) indicate that CBF increased $61\% \pm 30\%$ in capillaries vs $42\% \pm 14\%$ in small and $22\% \pm 12\%$ in large branch vessels.

Fig. 3 shows pc- μ ODT images across a $\varphi 5.6$ μ m capillary (CV) in Figs. 2(d) and 2(i). The two subsets on top of panel (a) show the microangiographic (μ OCA) and Doppler phase (μ ODT) images at time points without and with a RBC flowing through the capillary, indicating that the pas-

sage of a RBC elicited a predominant phase transient compared to the phase noise induced by the motion artifacts of the vessel wall and of plasma. Thus, by analyzing time-lapse pc- μ ODT images, important microcirculatory parameters such as RBC flux (RBC counts/s), and ν_{RBC} and hematocrit (i.e., fractal volume of RBCs) can be quantified. For instance, by averaging the pc- μ ODT images within the masked vessel wall over two 0.48 s traces, the erythrocyte flux, mean ν_{RBC} , and hematocrit in normocapnia (a) were $\sim 19/\text{s}$, $\nu_{\text{N}} = 0.72 \pm 0.15$ mm/s and 8.80% during hypercapnia (b), the flux increased 52.6% to 29/s and the mean ν_{RBC} increased 36.1% to $\nu_{\text{H}} = 0.98 \pm 0.29$ mm/s (i.e., $\Delta\nu_{\text{H/N}} = 36.1\%$) while the hematocrit remained 9.86%. But it is noteworthy that high ν_{RBC} fluctuations were evident in most capillaries; therefore, measurements over a longer period of time (e.g., >1 min) should have been made. Interestingly, based on Figs. 2(d) and 2(i), ν_{CBF} in the same capillary (CV) measured directly by μ ODT (Doppler CBF speed) were $\nu_{\text{N}} \approx 0.27 \pm 0.03$ mm/s and $\nu_{\text{H}} \approx 0.37 \pm 0.05$ mm/s, respectively. Taking into account the angle effect of light incidence (e.g., $\theta = 57.6^\circ$, $\cos 57.6^\circ \approx 0.54$), the values could be corrected to $\nu_{\text{N}} = 0.51 \pm 0.06$ mm/s and $\nu_{\text{H}} = 0.69 \pm 0.09$ mm/s. The results indicate that the capillary CBF rates can be markedly underestimated by errors resulting from both uncorrected angles of Doppler measurement (more severe for horizontally oriented flows) and low hematocrit (e.g., $<50\%$). Nevertheless, the relative change ($\Delta\nu_{\text{H/N}} = 36.1\% \pm 5.3\%$) by pc- μ ODT matched that ($\Delta\nu_{\text{H/N}} = 35.3\%$) by μ ODT.

In summary, we present a method pc- μ ODT based on Doppler phase thresholding technique that enables accurate measures of cerebral capillary RBC velocity, and flux and hematocrit with no need of fluorescence labeling (e.g., tracker free). While the Doppler-based μ ODT measures average velocity of all moving scatterers (RBCs) over the camera exposure, pc- μ ODT accurately tracks the velocity of individual RBC (ν_{RBC}). For laminar flow (e.g., arterioles or venules in Fig. 2), μ ODT provides sufficient accuracy to retrieve their continuous parabolic flow profiles. However, for discrete flow in capillaries, the low hematocrit (e.g., $<50\%$) leads to severe underestimation of ν_{RBC} (averaged over time), as confounded by the angle effects. Similar to MPM, pc- μ ODT—although accurate—is unable to quantify large laminar flows. Interestingly, our results in Fig. 3 show that, despite underestimation, the hypercapnia-elicited CBF increase ($\Delta\nu_{\text{H/N}}$) measured by μ ODT matched that by pc- μ ODT, suggesting that μ ODT can be suited for quantitative imaging of the CBF changes (ΔCBF) in the cerebrovascular vessels of different calibers (including capillaries). This is crucial for brain functional studies, because μ ODT is a unique neuroimage tool that enables quantitative imaging of changes in CBF networks (both branch vessels and capillaries) at high spatiotemporal resolutions, over a FOV, and without fluorescence dye loading. It is noteworthy that pc- μ ODT may not able to separate the contributions of RBCs from other moving particles such as white blood cells in the blood stream. In addition, pc- μ ODT at a higher frame rate (e.g., 140 kHz by employing new CMOS linescan cameras) and by employing other scanning scheme is being performed to further enhance the temporal resolution for ν_{RBC} detection

FIG. 3. Capillary ν_{RBC} measured by pc- μ ODT. (a), (c): Time-lapse pc- μ ODT images; (b), (d): their RBC traces. Top 2 sublets show the Doppler phase transient with the passage of a RBC.

or to extend the lateral scanning region to allow for simultaneous pc- μ ODT detection of ν_{RBC} in multiple capillaries.

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