In vivo flow speed measurement of capillaries by photoacoustic correlation spectroscopy

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We recently proposed photoacoustic correlation spectroscopy (PACS) and demonstrated a proof-of-concept experiment. Here we use the technique for *in vivo* flow speed measurement in capillaries in a chick embryo model. The photoacoustic microscopy system is used to render high spatial resolution and high sensitivity, enabling sufficient signals from single red blood cells. The probe beam size is calibrated by a blood-mimicking phantom. The results indicate the feasibility of using PACS to study flow speeds in capillaries.

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The smallest of a body's blood vessels are capillaries, serving the exchange of water, oxygen, carbon dioxide, and nutrients between blood cells and surrounding tissues. The flow speeds of red blood cells (RBCs) through capillary networks are affected by various factors such as metabolic demand and heart rate [1,2]. Analysis of capillary flow benefits disease diagnosis and treatment. For example, study of the alteration of retinal capillary flow velocity may help to identify patients at high risk for cerebrovascular diseases [3].

Current blood velocimeters such as laser Doppler velocimetry and optical/ultrasound particle image velocimetry employ the scattering properties of tracer particles to provide imaging contrast [4]. This dependence on scattering by tracers may limit the sensitivity, resolution, and detection depth due to a high optical scattering coefficient in tissue and much stronger ultrasonic reflection from tissue boundaries than ultrasonic scattering from tracer particles. Recently, photoacoustic (PA) blood flow speed measurements have received growing attention [5–8]. Previously, PA correlation spectroscopy (PACS) was proposed based on the fluctuation of detected PA signals, analyzed using temporal correlation [7]. A low-speed flow measurement of tracer beads by PACS shows its potential for measuring flow speeds in capillaries.

PACS uses the endogenous light-absorbing tracer particles, RBCs, which can absorb light 100 times more than the background if no other absorbers are within the excitation volume. In the previous work, a pulsed laser without focusing was used, which limited the spatial resolution and sensitivity. Besides, the range of measurable flow speeds was restricted due to low pulse repetition rate. In this Letter, we use laser-scanning PA microscopy (PAM) [9] to meet the demanding requirements for speed measurement in capillaries, which, for the first time to our knowledge, facilitated study on biological samples *in vivo*.

In the laser-scanning optical-resolution PAM system, the ultrasonic detector was kept stationary while the laser was raster scanned by an x–y galvanometer scanner. We used an Nd:YAG laser (Spot-10-200-532, Elforlight Ltd., UK) working at 532 nm wavelength with a pulse duration of 2 ns. A highly sensitive custom-built needle hydrophone with a center frequency of 35 MHz and a −6 dB bandwidth of 100% was used for ultrasonic detection and was aligned to the scanned region of laser light. For flow speed measurement, the laser light was positioned and stayed at a designated point on a capillary by controlling the galvanometer fixed at a corresponding angle.

In PACS, PACS strength, $P(t)$, is used, which is defined in Eq. (1) in [7]. As shown in Fig. 1, when the light-absorbing particles pass through the illuminated volume, PA signals are generated. Because the PA signal A line (the PA signal plotted on screen as a function of depth) is not exactly the same as $P(t)$, the information of $P(t)$ should be extracted from the measured PA signals. Because of the small probe volume from the tight optical focus in the PAM system, the average particle number in the probe volume, N, is small, equal or less than 1. It is appropriate as a simple start, therefore, to use the peak-to-peak PA signal amplitude as $P(t)$, avoiding integration of noise. The calculation of the normalized autocorrelation function (ACF), $G(\tau)$, from the $P(t)$ fluctuation has been described in Eq. (2) in [7]. The decay profile of the $G(\tau)$ reveals the particles' dwell time in the probe volume, and the magnitude of $G(0)$ is related to the number density of the beads in the probe region [10].

Considering that the focused laser light has a Gaussian distribution in all directions, $I(x,y,z) = I_0 \exp(-2x^2/r_0^2 - 2y^2/r_0^2 - 2z^2/z_0^2)$, where I_0 is the peak intensity, $x(y)$ and z are radial and axial position of the laser beam, respectively, and r_0 and z_0 are the radial and axial radii.

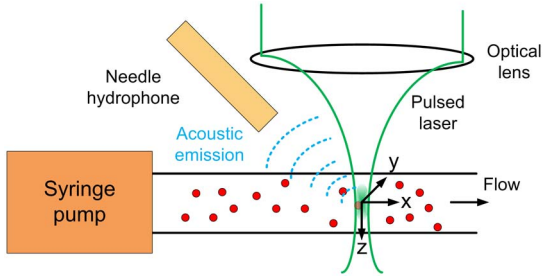

Fig. 1. (Color online) Schematic of PACS flow speed measurement using the PAM system.

The Gaussian form in deriving the ACF in flow speed measurement has been studied in fluorescence correlation spectroscopy (FCS) [11]. The PACS concept follows from FCS, and thus we can simply apply the formula in the PACS velocimeter. At the flow speed V_f of tracer particles, the ACF can be expressed as

$$G(\tau) = 1/N \times \exp -(\tau/\tau_f)^2, \quad (1)$$

where $\tau_f = r_0/V_f$ and $N = 1/G(0)$.

The PACS probe volume using the PAM setup was calibrated by a phantom experiment with known flow speeds, designed from 0.51 to 3.64 mm/s, and particle concentration of $\phi = 0.2\%$. Suspended red-dyed polybeads (mean diameter 6.0 μm, Polysciences, Inc.) dispersed in distilled water were used. Beads were flowing, driven by a syringe pump, in a tubing (inner diameter 250 μm, TSP250350, Polymicro Technologies, Phoenix, Arizona). We first studied the dependence of the autocorrelation decay curves on the flow speeds. Figure 2(a) plots the flow time, τ_f, calculated from the fitted autocorrelation curves, versus the designed flow speeds. The flow time becomes shorter as the speed increases. As for extracting τ_f, we acquired the sequential A-line PA signals (repetition rate 2048 Hz for 2 s) and the fluctuation of $P(t)$. One example at $V_f = 1.9$ mm/s is plotted in Fig. 2(b). Then, the ACFs were calculated and fitted using Eq. (1), as shown in Fig. 2(c). To analyze our data properly, the mean and standard deviation of τ_f are obtained from at least four reliable measurements (the coefficient of determination R^2 in the fitting being larger than 0.9) at each flow speed. It is assumed that the small R^2 may result from the fact that both one and more than one beads are passing through the probe volume during one measurement, which may cause erroneous estimation of τ_f. Large variations in τ_f at slower speeds might be due to less chance for beads passing through the probe volume in each measurement compared to that in the high-speed case.

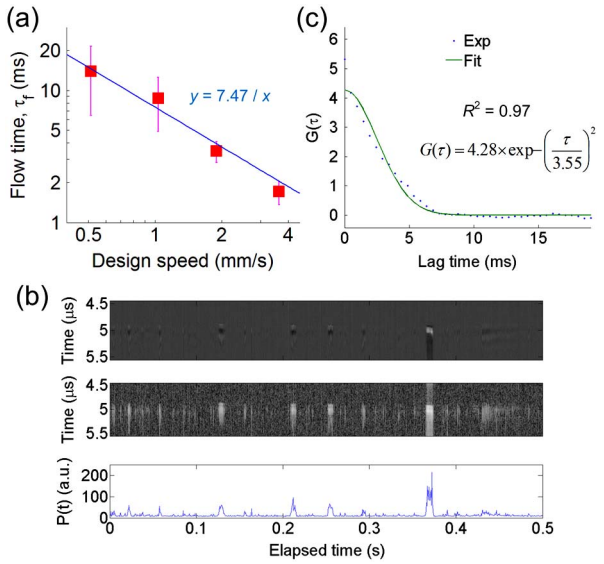

Fig. 2. (Color online) (a) Dependence of the flow time on the design flow speed. The square symbols and the error bars represent the mean flow time and the standard deviations, respectively. The solid line is the curve fitting. (b) (Top) A-line signals, (middle) its Hilbert transform displayed over a 50 dB dynamic range for better contrast, and (bottom) the $P(t)$. Only the first 0.5 s are plotted. (c) Calculated and fitted autocorrelation curves in dotted and solid lines, respectively.

To obtain the probe radial beam radius, Fig. 2(a) is fitted using $\tau_f = r_0/V_f$. The extracted r_0 is 7.47 μm. For the axial beam radius, the average value and standard deviation of $G(0)$ from all valid measurements is 7.18 ± 1.87, and thus $N = 0.14$. Note that, in ACF calculation, the noise in the $P(t)$ estimate was offset to zero for more accurate estimation of $G(0)$ [12]. The noise in $P(t)$ after zero-baseline processing results in underestimation of τ_f and N, which can be alleviated by improving the signal-to-noise ratio. With known sample concentration ϕ, calculated N, and calibrated r_0, the z_0 can be estimated as 33.7 μm using $\phi = (N \times V_{\text{bead}})/V_{\text{probe}}$, where V_{bead} is the volume of one bead and the probe volume $V_{\text{probe}} = 4/3 \times \pi r_0^2 z_0$. Compared to a Gaussian beam calculation, the z_0 is smaller, which could be due to a limited directivity of the hydrophone. In the flow measurement, the Brownian motion can be neglected because flow time τ_f is much shorter than the diffusion time due to Brownian motion, about several minutes, obtained by a similar analysis in [7].

An *in vivo* experiment was performed on an 8 d old chick embryo, as shown in Fig. 3(a). The 8 d old check embryo was used for its mature capillaries [13]. An *ex ovo* chick embryo culture method [14] was used for easy experiments. To maintain the life of chick embryo, an IR lamp was used as a heating resource during the experiment. The membrane around the center of the embryo was first imaged by the PAM system. From the acquired PA image, the positions of capillaries can be

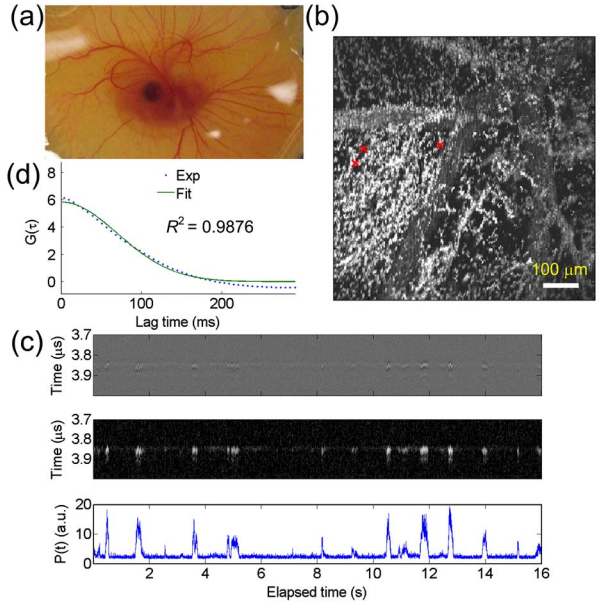

Fig. 3. (Color online) (a) Photograph of an 8 d old chick embryo. (b) Maximum amplitude projection image on the x–y plane of the chick embryo. Red crosses, three different positions for flow speed evaluation. (c) (Top) A-line signals, (middle) its Hilbert transform displayed over a 25 dB dynamic range, and (bottom) the $P(t)$. (d) Calculated and fitted autocorrelation curves in dotted and solid lines, respectively. The fitted $\tau_f = 99$ ms.

recognized. Then, the scanner was positioned so that the probe beam stayed on targeted capillaries for PACS flow speed measurements. The 8192 sequential A lines were obtained at a repetition rate of 512 Hz for 16 s. For the statistical analysis, six reliable measurements were used. The data were acquired 40–80 min after the embryo was removed from the incubator. Since RBCs must deform to enter the capillaries, the RBCs length in the capillaries was considered as 16 μm [15], and thus an effective radial beam radius of $10.5[= (7.47^2 - (6/2)^2 + (16/2)^2)^{0.5}]\,\mu$m [6] was used for speed calculation. The PACS measured flow time is 53 ± 28 ms. Thus, the calculated flow speed using the effective beam radius is 199 μm/s [$\sim = (10.5\,\mu\text{m})/(53\,\text{ms})$], which is close to those reported in the literature [13,15]. The results confirmed that the PACS velocimeter is plausible. Note that, without the assumption of RBC deformation, the calculated speed is 141 μm/s, which is slightly slower but is still reasonable considering the widely ranged flow speeds in capillaries [16]. Verification of RBC deformation is under investigation.

To evaluate the variations of capillary blood flow speeds at different capillary positions, the same embryo was used for measurements. Similarly, we first took the PA image. The maximum amplitude projection image is shown in Fig. 3(b). Three different positions, marked by crosses in Fig. 3(b), were chosen for evaluation. One measured sequential A-line PA signal and its $P(t)$ is shown in Fig. 3(c). The ACFs are shown in Fig. 3(d). The measured flow times are 177 ± 70, 188 ± 37, and 136 ± 53 ms, which are averages from at least four valid measurements for each point. The corresponding flow speeds are 59, 56, and 77 μm/s. Results show that the variation of flow speeds in different capillaries is not obvious. Mildly reduced speeds may be due to the fact that the data were taken ~150 min after removal of the embryo from the incubator, mainly because of the time used for realignment of optical focus for different imaging area. The temperature control by the IR lamp may not be uniform. Thus, as time elapsed, flow speeds could decrease due to changes of physiologic parameters such as blood pressure.

In summary, PACS for *in vivo* flow measurement of capillaries in an 8 d old chick embryo has been demonstrated. We calibrated the probe beam size in the PAM system by a blood-mimicking fluid. PACS is suitable for measuring the capillaries blood speed, which might be unattainable by other PA flow speed measurement methods. Combing with the unique ability of laser-scanning PAM in three-dimensional mapping of microvasculature, PACS provides a promising tool to monitor the disease process. We are also interested in exploring other applications by PACS.

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