Video Article

A Rapid and Chemical-free Hemoglobin Assay with Photothermal Angular Light Scattering

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Abstract

Photo-thermal angular light scattering (PT-AS) is a novel optical method for measuring the hemoglobin concentration ([Hb]) of blood samples. On the basis of the intrinsic photothermal response of hemoglobin molecules, the sensor enables high-sensitivity, chemical-free measurement of [Hb]. [Hb] detection capability with a limit of 0.12 g/dl over the range of 0.35 - 17.9 g/dl has been demonstrated previously. The method can be readily implemented using inexpensive consumer electronic devices such as a laser pointer and a webcam. The use of a micro-capillary tube as a blood container also enables the hemoglobin assay with a nanoliter-scale blood volume and a low operating cost. Here, detailed instructions for the PT-AS optical setup and signal processing procedures are presented. Experimental protocols and representative results for blood samples in anemic conditions ([Hb] = 5.3, 7.5, and 9.9 g/dl) are also provided, and the measurements are compared with those from a hematology analyzer. Its simplicity in implementation and operation should enable its wide adoption in clinical laboratories and resource-limited settings.

Video Link

The video component of this article can be found at http://www.jove.com/video/55006/

Introduction

A blood test is commonly performed to evaluate overall human health and to detect biomarkers related to certain diseases. For example, the cholesterol concentration in blood serves as a criterion for hyperlipidemia, which is closely related to cardiovascular diseases and pancreatitis. The blood glucose contents should be measured frequently, as the glucose level is associated with complications such as diabetic ketoacidosis and hyperglycemic hyperosmolar syndrome. Serious illnesses such as malaria, human immunodeficiency virus and acquired immune deficiency syndrome are diagnosed by blood examinations, and quantification of blood components including erythrocytes, thrombocytes, and leukocytes enables screening of pancreatic and renal diseases.

Hemoglobin (Hb), a critical component of blood, makes up about 96% of erythrocytes, and transports oxygen to human organs. Significant alteration of its mass concentration ([Hb]) may indicate metabolic changes, hepatobiliary disease, and neurological, cardiovascular and endocrinological disorders[1]. [Hb] is therefore routinely measured in blood tests. In particular, anemic patients, dialysis patients, and pregnant women are strongly recommended to monitor [Hb] as a vital task[2].

Various [Hb] detection methods have thus been developed. The hemoglobin cyanide method, one of the most common techniques for [Hb] quantification, employs potassium cyanide (KCN) to destroy the lipid bilayer of erythrocytes[3]. The cyanide hemoglobin produced by the chemical exhibits high absorption around 540 nm; hence, [Hb] measurements can be made via colorimetric analysis. This method is widely employed owing to its simplicity, but the employed chemicals (*e.g.*, KCN and dimethyllaurylamine oxide) are toxic to humans and the environment. The hematocrit scheme measures the volume ratio of red blood cells compared to the total blood volume through centrifugal separation; however it requires a relatively large blood volume (50-100 µl)[4]. Spectrophotometry methods measure [Hb] precisely without any chemicals, but measurements at multiple wavelengths and a large blood volume are required[5,6]. Similarly, several optical methods for measuring [Hb] have been proposed including detection methods based on light-scattering, but their measurement accuracies depend strongly on the accuracy of the theoretical blood model.

To overcome these limitations, [Hb] detection methods based on the photothermal (PT) effect of Hb have recently been proposed[7]. Hb, which is composed mainly of iron oxides, absorbs light at 532 nm and converts the light energy into heat[8-10]. This PT temperature increase can be detected optically by measuring a change in the refractive index (RI) of blood samples. Yim *et al.* employed spectral-domain optical coherence reflectometry to measure the PT optical path-length change in a blood-containing chamber[11]. Although the method enables chemical-free and direct [Hb] measurement, the use of a spectrometer and an interferometric arrangement may hinder its miniaturization. We recently presented an alternative [Hb] detection method, termed photo-thermal angular light scattering (PT-AS) sensor, which is more suitable for device

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miniaturization[12]. The PT-AS sensor exploits the high RI sensitivity of the back-scattering interferometry (BSI) to measure PT changes in the RI of a blood sample inside a capillary tube. BSI have been utilized to measure RI of various solutions[13-15] and to monitor biochemical interactions in free solution[16]. The PT-AS sensor employs similar optical arrangement as in BSI, but combines photothermal excitation setup to measure PT increase of RI in blood samples. Operating principles of the BSI and the PT-AS sensors are described in detail elsewhere[12,15]. PT-AS sensor demonstrated high-sensitivity [Hb] measurement over a wide detection range (0.35-17.9 g/dl) and is capable of operating with sample volumes of <100 nl. No preconditioning of blood sample is required, and the measurement time is only ~5 sec. Here, the experimental setup and a detailed measurement protocol are described. Representative PT-AS results are provided using blood samples from anemic patients, and the results are compared against those from a hematology analyzer to assess the accuracy of the PT-AS sensor.

Protocol

Experiments with blood samples were performed in compliance with the relevant laws and institutional guidelines. The samples were the residual blood samples that had been acquired and processed in clinical tests at the institution.

1. PT-AS Optical Setup

NOTE: One may use an empty micro-capillary tube for an initial PT-AS setup.

1. Mount an empty micro-capillary tube with inner and outer diameters of 200 and 330 µm, respectively, and a length of greater than ~5 cm on a capillary tube fixture. Commercially-available fiber fixtures can be used as the tube fixture.
2. Securely anchor a 650 nm laser pointer, *i.e.*, probe light source, to illuminate the capillary tube. The probe beam should be larger than the capillary tube. Place a screen (*e.g.*, white paper) behind the capillary tube to observe an angular periodic pattern.
3. For the detection part, remove any lenses in a webcam to directly capture the scattering pattern. Position the webcam behind the capillary tube at an angle of 25-35° relative to the probe beam direction. Ensure that the angular periodic pattern produced by the capillary tube can be measured with the detector (**Figure 1**). Observe the angular periodic pattern in the middle of the image sensor when the image sensor is properly positioned.
4. Position a 532-nm PT excitation light source to illuminate the capillary tube. Position the PT light source at any angle, as long as the PT excitation light overlaps with probe beam on the capillary tube and does not reach the detector directly. PT excitation of blood samples using high optical power typically improves the PT-AS sensitivity, as it leads to a larger change in the RI.
    1. Use the highest optical power of the employed PT excitation light source. In addition, ensure that the PT excitation light overlaps the probe light on the capillary tube. Use a beam size of the PT excitation light at least twice that of the probe light to heat the entire probe volume.
5. Place a long-pass filter in front of the detector to block the 532-nm light and measure only the 650-nm probe light.
6. Install an optical chopper in the path of the PT excitation light before illuminating the capillary tube. The optical chopper is employed to modulate the PT excitation light intensity.

2. Blood Sample Preparation

1. Draw 6 ml of fresh whole blood in anemic condition into ethylenediaminetetraacetic acid blood sampling tubes, and mix the samples well. No other processing is required.
2. Measure the blood samples using the PT-AS sensor within 24 hr of extraction to prevent coagulation.

3. PT-AS Measurement Protocols

1. Load a micro-capillary tube with a blood sample to measure. Fill the capillary tube with the blood through capillary action by placing the tube into the blood sample. The minimum sample volume required for measurement is determined by the inner diameter of the capillary tube and the probe beam size.
    1. Employ a tube with an inner diameter of 200 µm. The probe beam size was 2 mm in the representative results, suggesting that the measurement can be performed with a sample volume of >63 nl.
2. Mount the capillary tube at the designated position in the fixture.
3. Turn on the 650 nm probe laser to illuminate the blood-loaded micro-capillary tube. The angular periodic pattern should be observed with the webcam.
4. Turn on the 532-nm PT excitation laser to illuminate the tube.
5. Run the optical chopper to modulate the intensity of the PT excitation light at 2 Hz.
   NOTE: The rationale for the selection of this operating condition is described in Discussion and Kim *et al.*[12].
    1. Mount a chopper wheel in the motor head assembly of the optical chopper system.
    2. Turn on the chopper control box, and use the control knob in the console to set the modulation frequency.
    3. Run the chopper using the control knob.
6. Record the fluctuating scattering pattern via the webcam for 5 sec in MPEG-4 (mp4) format.

4. Signal Processing

NOTE: PT-AS signal processing was performed using a lab-developed MATLAB code.

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1. Load the video file to extract the images. For each image [see **Figure 2(a)** for a representative image], obtain the averaged scattering pattern by computing the mean of the pixel values along the vertical direction [**Figure 2(b, c)**].
2. Evaluate the Fourier transform of the averaged scattering pattern, and compute the phase at the peak spatial frequency. Perform these operations for all the frames of all the recorded images.
3. Using the phase values obtained from all the images, plot the temporal phase fluctuation [**Figure 2(d)**]. Note that the phase fluctuates at the PT modulation frequency. Take the Fourier transform of the phase fluctuation in the time domain, and obtain the magnitude at the modulation frequency. This signal is referred to as the PT-AS signal [**Figure 2(e)**].
4. Measure the [Hb] of a blood sample by converting its PT-AS signal into the corresponding [Hb] using the calibration curve that is obtained in Protocol 5.

5. PT-AS Calibration

1. Prepare blood samples, having [Hb] values that are uniformly distributed in the detection range of the PT-AS sensor (*e.g.*, 0 - 18 g/dl).
2. Before calibration, quantify the [Hb] values of the samples using a reference hematology analyzer. Measure the PT-AS signals of the samples.
3. Derive a calibration curve relating [Hb] to the PT-AS signal by performing a linear least squares fit, [Hb] = A[PT-AS Signal] + B, of the experimental results. For the operating conditions specified in **Table 1**, the relationship between [Hb] and the PT-AS signal was found to be [Hb] = 5.13 [PT-AS signal] - 0.09. Use MATLAB code to perform the linear fit.

Representative Results

A hemoglobin assay was performed using the PT-AS sensor, and its measurements were compared with those from a hematology analyzer. The experiment was conducted with a PT excitation light intensity of 1.4 W/cm^2, PT modulation frequency of 2 Hz, and measurement time of 5 sec. **Table 1** summarizes the experimental conditions. The beam sizes of the probe and PT excitation light were 5.5 and 2 mm, respectively. The webcam recorded the images at a frame rate of 30 fps. For measurement, anemic blood samples with three different Hb concentrations were employed. Before the PT-AS measurements, the [Hb] values of the samples were first measured as 5.3, 7.5, and 9.9 g/dl by the hematology analyzer.

Figure 3(a) shows representative time-lapse phase fluctuations of the angular scattering patterns under the modulated PT light illumination. This information was obtained by taking the Fourier transform of the angular scattering pattern and measuring the temporal phase fluctuations at the peak spatial frequency. Note that blood samples with a higher [Hb] exhibit larger phase shifts. The corresponding PT-AS signals were evaluated and converted into [Hb] values. Eleven measurements were performed for each sample, and the mean [Hb] values were found to be 5.46, 7.23, and 9.85 g/dl, respectively. The results agreed well to those obtained using the hematology analyzer [**Figure 3(b)**]. The [Hb] measurement precision of the PT-AS sensor was found to be <0.89 g/dl. This fluctuation may be in part accounted for by the number fluctuation of erythrocytes in the probe volume and the intensity fluctuations of the employed light sources. **Table 2** presents a detailed comparison of the PT-AS measurements against those from the hematology analyzer.

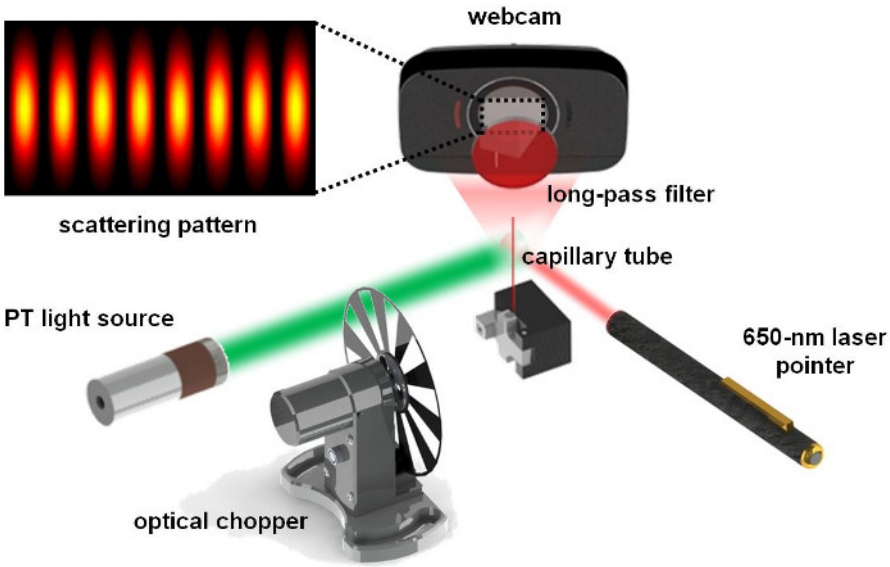

Figure 1: Schematic of PT-AS sensor. 650-nm probe light from a laser pointer is directed to a blood-loaded capillary tube. The light is then scattered by the blood-containing tube, generating a periodic pattern on a webcam. Upon illumination with 532-nm light, at which Hb molecules exhibit high absorption, Hb molecules absorb the light energy and convert it into heat. The resultant temperature rise changes the RI of the blood. Because the angular periodic pattern varies with the RI and the physical size of the tube, [Hb] in the blood is quantified by measuring this PT shift in the angular periodic pattern. An optical chopper is employed to achieve [Hb] measurement with a high signal-to-noise ratio. A low-cost plastic long-pass filter is located directly in front of the webcam to detect only the probe light. Please click here to view a larger version of this figure.

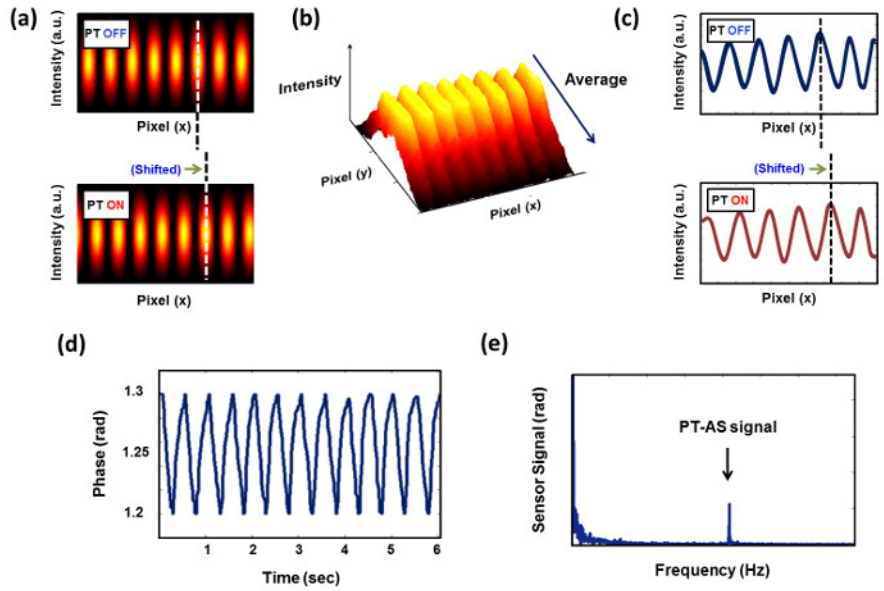

Figure 2: PT-AS signal processing procedures. (**a**) Representative webcam images with PT excitation light on and off. The angular scattering pattern shifts because of the PT response of Hb molecules. (**b**) Each image is averaged along the vertical (y) direction to obtain the averaged pattern. (**c**) Representative averaged periodic patterns with PT excitation on and off. (**d**) The averaged periodic pattern is then Fourier transformed, and the phase at the peak spatial frequency is examined as a function of time. Under the modulated PT light illumination, the phase of the periodic pattern fluctuates at the modulation frequency. (**e**) The measured phase fluctuation is Fourier-transformed, and its magnitude evaluated at the modulation frequency, referred to as the PT-AS signal, is converted into [Hb]. Please click here to view a larger version of this figure.

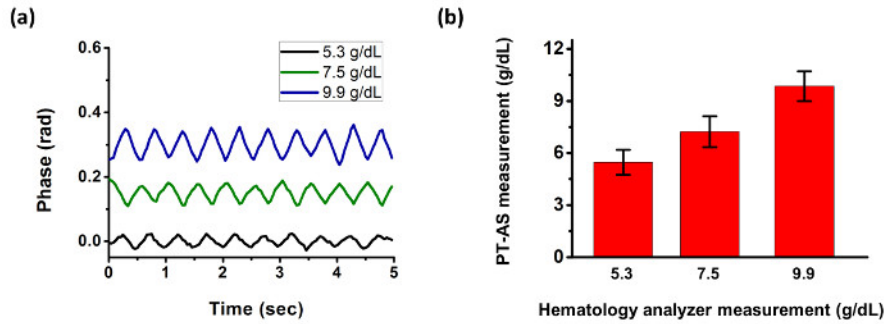

Figure 3: PT-AS measurement of anemic blood samples. (**a**) Representative phase fluctuations of the angular scattering patterns measured for three blood samples in anemic conditions ([Hb] = 5.3, 7.5, and 9.9 g/dl). The blood samples with higher [Hb] values produce larger phase variations. (**b**) Comparison of [Hb] values measured using the PT-AS sensor with those from the reference hematology analyzer. Eleven PT-AS measurements were performed for each sample. The error bar denotes the standard deviation. Please click here to view a larger version of this figure.

Experimental conditions	
PT modulation frequency	2 Hz
PT light intensity	1.4 W/cm²
PT beam size	5 mm
Probe beam size	2 mm
Measurement time	5 sec
Frame acquisition rate	30 fps

Table 1: Experimental conditions.

Hematology analyzer (g/dl)	PT-AS Sensor	
	Mean (g/dl)	SD (g/dl)
5.3	5.46	0.72
7.5	7.23	0.89
9.9	9.85	0.84

Table 2: Comparison of [Hb] measurements by the PT-AS sensor with those by the hematology analyzer.

Discussion

The PT-AS sensor represents an all-optical method capable of direct [Hb] measurement of unprocessed blood samples. The method quantifies [Hb] in blood using the intrinsic PT response of hemoglobin molecules in erythrocytes. Under illumination by 532-nm light, Hb molecules absorb the light energy and produce heat. The resultant temperature rise changes the RI of the blood sample. The high RI sensitivity of BSI was exploited to measure this RI change in blood. Previously, we demonstrated that the PT-AS sensor enables [Hb] measurement with a detection limit of 0.12 g/dl over the range of 0.35-17.9 g/dl, which is comparable to that of commercial [Hb] sensors on the market.

A notable feature of the PT-AS sensor is that it does not require any preconditioning of blood samples or chemicals. Hence, the sensor enables direct, rapid (<5 sec), and environment-friendly measurement. The use of glass-based micro-capillary tubes as a sample container enabled [Hb] assay at a low operating cost. The minimum sample volume in the PT-AS sensor is determined by the inner diameter of the capillary tube and the measurement beam size on the capillary tube. It is estimated to be ~63 nl in the representative results. In comparison to the sample volumes required in the commercial instruments (*e.g.*, 50-200 µl for the reference hematology analyzer), the PT-AS sensor enables [Hb] measurement with a significantly reduced sample volume. Several rapid and low-cost [Hb] detection techniques have been reported[11,17,18] but still require the sample volumes of 2-10 µl for operation.

Several features of the PT-AS sensor implementation should be noted. One should ensure that the size of the PT excitation light beam is at least twice that of the probe light beam on the capillary tube. The two light beams should overlap on the capillary tube, as no or partial overlap of the two light beams on the tube will result in either no or a smaller PT-AS response. One should also make sure that the angular scattering pattern is not saturated on the detector. Adjustment of the scattering pattern orientation along the horizontal or vertical direction may be necessary; otherwise, the acquired image should be rotated in the signal processing stage. Note that scattering of 532-nm PT excitation light by the tube also generates an angular scattering pattern on the detector. Thus, a long-pass filter is required to block the 532-nm light. Larger image sensor captures more angular periodic patterns. Fourier transform of the angular pattern would thus produce higher signal at the corresponding spatial frequency, which allows phase measurement with higher precision. Moreover, a higher frame rate would typically result in a PT-AS measurement with an improved SNR, as it enables more sampling of the temporal phase fluctuation. Therefore, the use of a large, high-speed image sensor with a high pixel density is advantageous.

Some comments should also be made on the measurement time and PT modulation frequency. As described in Kim *et al.*[12], the PT-AS signal refers to the magnitude of the Fourier transform of the phase fluctuations of the angular scattering pattern measured at the PT modulation frequency. The noise is defined as the peak amplitude of the Fourier transform of the phase measurement before PT excitation[12]. The SNR of the PT-AS signal is evaluated by dividing the magnitude of the PT-AS signal by the noise. A longer measurement time typically yields measurements with a higher SNR, but increases the total [Hb] assay time. The measurement time was set to be 5 sec to achieve an SNR greater than 3 even for blood samples of [Hb] < 1 g/dl. The optimal PT modulation frequency can be found by examining the SNR of the PT-AS sensor as a function of the PT modulation frequency. The optimal modulation frequency for the representative results was found to be 2 Hz. Operation with a PT modulation frequency less than 2 Hz did not produce a high SNR owing to low-frequency noise such as excessive motion of the optical chopper and vibration.

In this demonstration, the PT-AS sensor was demonstrated in a benchtop configuration using a commercial laser pointer and webcam. The optical setup is straightforward, and, because no chemicals are involved, the measurement procedures are simple. On the other hand, it should be emphasized that the sensor can potentially be packaged in a compact handheld device. The light sources for probe and PT excitation can be replaced by low-cost laser diodes or light-emitting diodes. A miniaturized complementary metal-oxide-semiconductor image sensor with built-in computational power can also be utilized as a detector. Integrating these components in a small form factor would generate a new portable, chemical-free, and inexpensive platform for [Hb] assay. In addition to [Hb] assay, the detection principle of the PT-AS sensor may be extended to sensing various biomarkers and chemicals that exhibit PT responses. For example, PT assay of organophosphates and pesticides has also been demonstrated[19], and can be readily realized with the PT-AS scheme.

Disclosures

No conflict of interest is declared.

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