Supplementary Information

Rapid Measurement of Hemoglobin-Oxygen Dissociation by Leveraging Bohr Effect and Soret Band Bathochromic Shift

Zoe Sekyonda[1], Ran An[2], Utku Goreke[2], Yuncheng Man[2], Karamoja Monchamp[2,3], Allison Bode[2,3], Qiaochu Zhang[2], Yasmin El-Gammal[4], Cissy Kityo[5], Theodosia A. Kalfa[4,6], Ozan Akkus[1,2,7], Umut A. Gurkan[1,2,7,8*]

Affiliations:
[1]Department of Biomedical Engineering, Case Western Reserve University; Cleveland, OH, USA.
[2]Department of Mechanical and Aerospace Engineering, Case Western Reserve University; Cleveland, OH, USA.
[3]Division of Hematology and Oncology, University Hospitals Cleveland Medical Center; Cleveland, OH, USA.
[4]Division of Hematology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center; Cincinnati, OH, USA.
[5]The Joint Clinical Research Center; Kampala, Uganda.
[6]Department of Pediatrics, University of Cincinnati College of Medicine; Cincinnati, OH, USA
[7]Department of Orthopedics, Case Western Reserve University; Cleveland, OH, USA.
[8]Case Comprehensive Cancer Center, Case Western Reserve University; Cleveland, OH, USA.

***Corresponding author:**
Umut A. Gurkan, PhD
Wilbert J. Austin Professor of Engineering
Case Western Reserve University
Address: 10900 Euclid Ave., Glennan Building 616B, Department of Mechanical & Aerospace Engineering, Cleveland, OH, 4416.
Phone: +1 (216) 368-6447
E-mail: umut@case.edu

Keywords:
Red blood cells, hemoglobin, hemoglobin-oxygen dissociation, optical absorption, heme-globin interaction.

Number of Supplementary Tables: 3

Number of Supplementary Figures: 3

Supplementary Results

Hb light absorption in oxygen dissociation assay

Net light extinction is a function of absorption and scattering, and light scattering is particularly sensitive to particle size and aggregation. While we cannot rule out scattering completely in the hemoglobin oxygen dissociation assay (Hb-O_2), its involvement is not likely to be as pronounced as absorption. The wavelength shift that we report holds regardless of Hb-O_2 dissociation measurements are conducted on Hb samples solubilized in solution (~5 nm particles), as RBCs (<10-micron particles), or whole blood (<10 microns and > 10-micron particles). On the other hand, the magnitude of the shift was different between RBCs, whole blood, and purified Hb (**Table S2**), (**Fig. S3**), indicating that light scattering may affect the intensity of the wavelength shift phenomenon because Hb is at high concentrations in red cells, where sickling is possible upon deoxygenation. To ascertain that the effects we report mainly stem from absorption, we sought the wavelength shift in samples by gradually increasing the concentration of Hb. We have seen that the shifting phenomenon, along with the magnitude of the shift, did not change with increasing concentration (**Fig. S1**), implying that scattering is not a significant contributor to Hb-O_2 dissociation when the Hb is dissolved in the aqueous environment.

Rigor and reproducibility

The repeatability of the bathochromic shift was established by comparing variations between two users in repeated measurements of the same whole blood samples at ~20 mmHg pO_2 **(Fig. S2).** Two samples were used in this study (one HbAA and one HbSS). Each sample was evaluated five times, and the same sample was analyzed in three microwells throughout each test, resulting in 15 tests for each user. The peak wavelength shifts between the two users showed good repeatability ((Mean± SEM) (User1: HbSS 421.6 ± 0.24, HbAA; 414.9 ± 0.11; User2: HbSS 421.1

± 0.26, HbAA; 415.1 ± 0.05) and coefficient of variance (COV) = 0.05 % for sickle and 0.02 % for normal). Bathochromic shifts between User 1 and User 2 for both HbAA and HbSS whole blood ($p = 0.48$) and ($p = 0.48$) were not significantly different. These results indicate reproducibility and good precision of the assay.

Supplementary Tables

Table S1: Comparison of $Na_2S_2O_5$ molecules, Hb molecules, and O_2 in buffer.

Hemoglobin (Hb) and oxygen (O_2) molecules	
Total Hb molecules used in phosphate buffer	= 0.1g/ml
Each Hb molecule weights	= 64500 g/mol
Thus, Hb moles per ml of phosphate buffer (0.1/64500)	= 1.55×10^{-6} mol/ml
Number of hemoglobin molecules per mole	= 6.02×10^{23} mol^{-1}
Thus, total Hb molecules per ml of buffer ($6.02 \times 10^{23} * 1.55 \times 10^{-6}$)	= 9.34×10^{17} molecule/ml
Each Hb molecule contains 4 O_2 molecules.	
Thus, total O_2 molecules per ml in buffer (9.34×10^{17} *4)	= 3.78×10^{18} molecule/ml
$Na_2S_2O_5$ molecules	
Total $Na_2S_2O_5$ molecules used in phosphate buffer (0.053M)	= 0.02g/ml
Each $Na_2S_2O_5$molecule weights	= 190.107g/mol
Thus, $Na_2S_2O_5$ moles per ml of buffer (0.02/64500)	= 1.05×10^{-4} mol/ml
Number of $Na_2S_2O_5$ molecules per mole	= 6.02×10^{23} mol^{-1}
Thus, total $Na_2S_2O_5$ molecules per ml of buffer ($6.02 \times 10^{23} * 1.05 \times 10^{-4}$)	= 6.34×10^{19} molecule/ml
Ratio of $Na_2S_2O_5$ molecules: oxygen molecules per ml = 17	

Table S2: Peak wavelengths of hemoglobin analytes at pH 6.9 and pO_2 = 75 mmHg.

Peak wavelength			
	HbAA (Sample size)	**HbSS** (Sample size)	**P-value**
Purified hemoglobin	417.2 ± 0.78 (n=23)	420.3 ± 0.52 (n=25)	P= 0.009
RBCs	418.0 ± 0.56 (n=15)	422.9 ± 0.63 (n=15)	P= 0.002
Whole blood	415.3 ± 0.23 (n=15)	419.6 ± 0.48 (n=20)	P= 0.001

HbAA: healthy hemoglobin.
HbSS: homozygous sickle cell disease hemoglobin.
Mann Whitney Non-parametric test was used to calculate p-values.
Data is reported as mean ± SEM.

Table S3: Correlations between optical variables and physiological relevance

	Peak wavelength	**Peak intensity**	**Area under the curve**	**peak FWHM**	**Physiological Parameter Correlated**	**Physiological Relevance**
Peak wavelength	1				Percentage of variant hemoglobin SS	Identification of Variant SS
Peak intensity	PCC = - 0.46 p = 0.000 Negative	1			Blood concentration of Hemoglobin (g/dl)	Determine the level of Hemoglobin level and Anemia
Area under the curve	PCC = - 0.76 p = 0.000 Negative	PCC = 0.94 p = 0.000 Positive	1		Anemia (low hemoglobin detection)	Identification of Anemia
Peak FWHM	PCC = - 0.22 p = 0.006 Negative	PCC = - 0.41 p = 0.000 Negative	PCC = - 40.0 p = 0.000 Negative	1	Sample homogeneity	Variation in samples

**PCC: Pearson Correlation Coefficient, p-values were obtained from linear regression model.*

Supplementary Figures

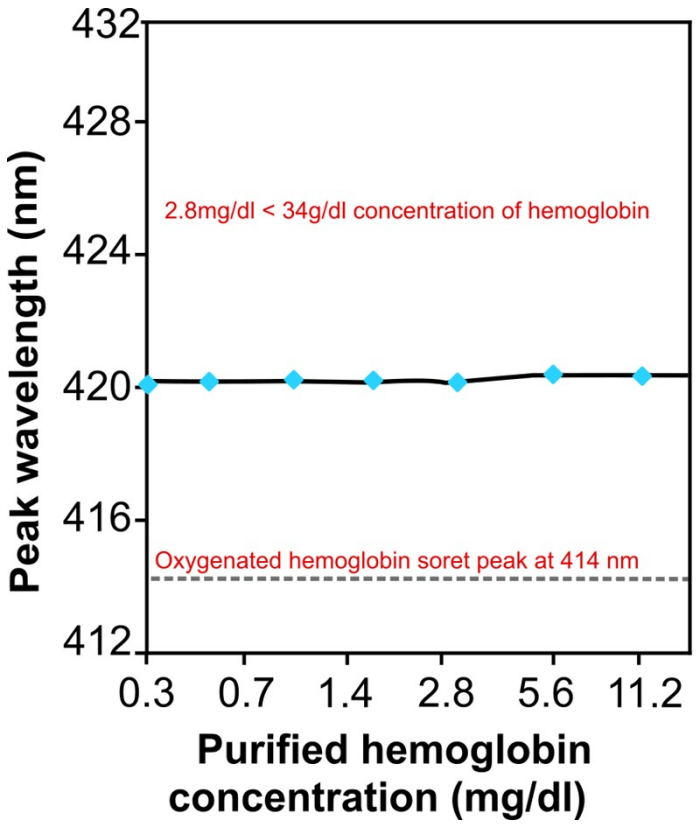

Figure S1: Increasing the hemoglobin concentration (mg/dl) did not affect the shifting phenomenon in the oxygen-hemoglobin dissociation assay. Increasing the concentration of Hb from 0.3-11.2 mg/dl did not affect the peak wavelength shift. For all Hb concentrations below the critical polymerization concentration of 34g/dl, the peak wavelength of purified Hb was 420 ± 0.2.

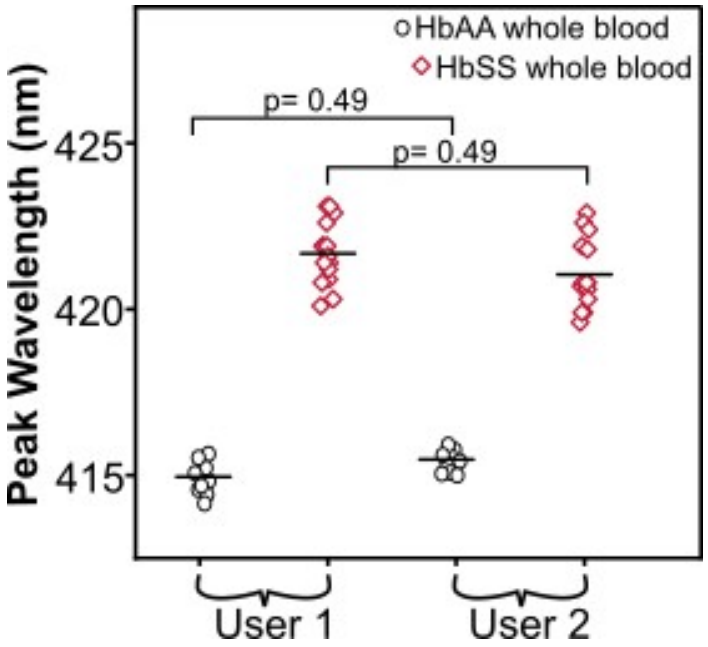

Figure S2: Robustness and repeatability of the oxygen-hemoglobin dissociation assay. Repeatability was determined from 20 tests comparing variances between 2 users. The peak wavelength shifts between the two users demonstrated strong repeatability (User1: HbSS: 421.6 ± 0.24, HbAA: 414.9 ± 0.11; User2: HbSS: 421.1 ± 0.26, HbAA: 415.1 ± 0.05) and coefficient of variance (COV) = 0.05 % for HbSS and 0.02 % for HbAA).

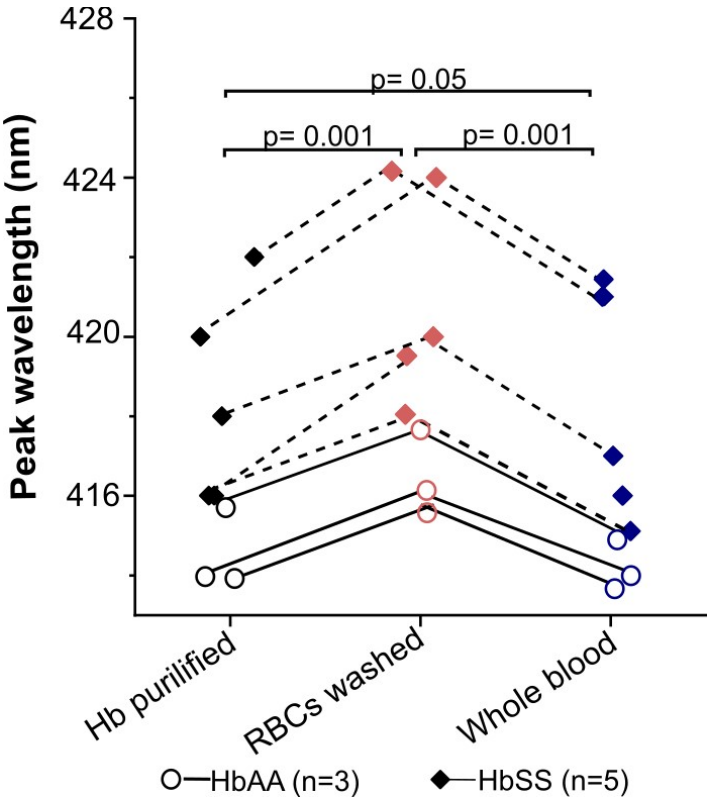

Figure S3: Peak wavelength comparative analysis of the purified Hb, RBCs, and Whole blood upon deoxygenation. Comparative analysis is shown for 5 individuals with HbSS (sickle hemoglobin) and 3 individuals with HbAA individual (healthy hemoglobin) in deoxygenated state. The peak wavelength shifts for RBCs were found to be higher than purified Hb ($p = 0.001$) and Whole blood ($p = 0.001$) and purified Hb was higher than whole blood for the same patient ($p= 0.05$). Peak wavelength shifts for HbAA are lower than that of HbSS.