Supplementary Information for

# "High Throughput Assessment of Hemoglobin Polymer in Single Red Blood Cells from Sickle Cell Patients under Controlled Oxygen Tension"

## Supplementary Methods

*Optical measurement system*

Cells are imaged using a 40×, 0.75 NA, Olympus fluorite objective lens onto a color CMOS VGA camera, running at 200 frames per second. Illumination is triggered to alternate between the two blue LEDs, 410 and 430 nm, in consecutive frames. The two blue LEDs are combined using a 50-50 non-polarizing beam splitter in transmission mode.

*Calculation of single-RBC oxygen saturation*

Images of optical intensity were captured at each wavelength, 410 and 430 nm. Samples were diluted such that images were captured of single RBCs in flow. Custom segmentation algorithms were written in MATLAB (Natick, MA) to mask individual RBCs. Algorithms were based on those used and validated for non-polymerizing HbA-containing RBCs in prior publications (1, 2). The presence of a cell in a captured frame was detected by observing the variation in time of the absorption signal in a window of the field of view. At these selected frames the cells were segmented by first applying an intensity threshold determined by the MATLAB *graythresh* function which uses Otsu's method (3). A morphologic area-opening operation was performed on these binarized images, and any small artifacts were removed by flood-filling using the MATLAB *imfill* function. Segmented cells were then tracked to the following frame and re-segmented with the same approach to provide two-color spectroscopy. Optical density was calculated by integrating all pixel intensity values inside the segmented cell masking function. The integrated cellular absorption values at each wavelength were then used to solve for the oxygenated and deoxygenated hemoglobin mass of each cell using hemoglobin reference spectra (4). To evaluate the noise level for cellular measurements, total mass of individual cells was compared across consecutive camera exposures and yielded a standard deviation of 75 fg (0.4%). This standard deviation was found to be similar for both stationary and flowing cells. Mean and standard deviations of mass distributions were validated for non-polymerizing RBCs in a prior study by comparison with a Siemens Advia 2120i clinical hematology instrument (2). For this study investigating hemoglobin polymerization, we compared single-RBC hemoglobin mass distributions for RBCs at high and low oxygen to provide evidence for mass measurement accuracy even when polymer was present. See Supplementary Results for more detail. Two-point calibration of raw saturation calculations was performed for each experiment using measurements at 0% $ppO_2$, assumed to produce 0% saturation, and 21% $ppO_2$, assumed to produce 100% saturation.

## Supplementary Results

*Stability of RBC mass distributions before and after hemoglobin polymerization*

Means and standard deviations of mass distributions were validated for non-polymerizing RBCs in a prior study by comparison with a Siemens Advia 2120i clinical hematology instrument (2). For this study investigating hemoglobin polymerization, we compared single-RBC hemoglobin mass distributions for RBCs at high and low oxygen to investigate whether light scattering from hemoglobin polymer might be contributing significant noise or bias to our measurements. Figure S1 shows for both SS specimens studied that there is no marked difference in the mean and variance of the single-RBC mass distribution inferred from the optical measurements. If the presence of polymer were causing enough scatter to alter our results, we would expect a systematic difference between the mass distributions at high oxygen tension where little or no polymer is present and low oxygen tension where the greatest amount of polymer is present. While these results are consistent with the conclusion that light scattering is not significantly altering our results, future work will explore the use of additional illumination wavelengths to provide an opportunity to reduce noise even further.

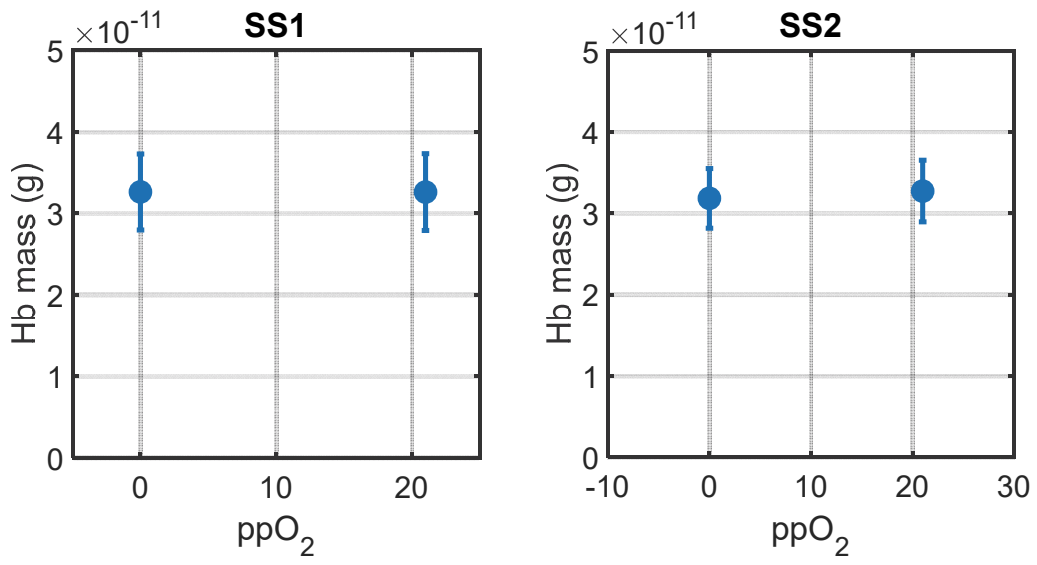


**Figure S1: Single-RBC mass distributions are similar at low and high oxygen tension**. Means and standard deviations are shown for single-RBC mass measured for the two SS study samples at high and low oxygen tension. Each distribution includes about 1000 single-RBC measurements. The mean RBC masses determined independently for each specimen on a Sysmex XN-9000 clinical hematology instrument were $3.41 \times 10^{-11}$g (SS1) and $3.46 \times 10^{-11}$g (SS2). Single-RBC mass was determined by summing the oxygenated and deoxygenated masses for each RBC. If hemoglobin polymer were causing enough scattering to alter measurements, the distribution at the lowest oxygen where significant polymer is present would be expected to differ markedly from the distribution at the highest oxygen tension. These results provide some reassurance that the scattering caused by hemoglobin polymer is not significantly altering the single-RBC oxygen

1. Di Caprio G, Stokes C, Higgins JM, & Schonbrun E (2015) Single-cell measurement of red blood cell oxygen affinity. *P Natl Acad Sci USA* 112(32):9984-9989.
2. Schonbrun E, Malka R, Di Caprio G, Schaak D, & Higgins JM (2014) Quantitative Absorption Cytometry for Measuring Red Blood Cell Hemoglobin Mass and Volume. *Cytom. Part A* 85(4):332-338.
3. Otsu N (1979) THRESHOLD SELECTION METHOD FROM GRAY-LEVEL HISTOGRAMS. *Ieee Transactions on Systems Man and Cybernetics* 9(1):62-66.
4. Prahl S (1998) Tabulated Molar Extinction Coefficient for Hemoglobin in Water. https://omlc.org/spectra/hemoglobin/summary.html. Accessed 9/22/2019.