Electronic Supplementary Information:

A simple, rapid, low-cost diagnostic test for sickle cell disease

Xiaoxi Yang[1], Julie Kanter[2,3], Nathaniel Z. Piety[1], Melody S. Benton[3], Seth M. Vignes[1], Sergey S. Shevkoplyas[1,*]

[1] Department of Biomedical Engineering, Tulane University, New Orleans, LA 70118

[2] Department of Pediatrics, Section of Hematology/Oncology, Tulane University School of Medicine, New Orleans, LA 70112

[3] Sickle Cell Center of Southern Louisiana, Tulane University School of Medicine, New Orleans, LA 70112

* Corresponding author: Sergey S. Shevkoplyas (shevkop@tulane.edu)

Materials and Methods

Blood samples

Normal human venous blood (Hb AA) was collected from healthy consenting volunteers; SCD (Hb SS, SC and $S\beta^{+}$) blood samples were obtained with informed consent at the Sickle Cell Center of Southern Louisiana (New Orleans, LA) into 4 mL Vacutainer tubes (K_2EDTA, BD, Franklin Lakes, NJ). Blood samples from SCD patients who received blood transfusions in the previous three months were excluded. The Hb A, F, C, and S content of SCD samples was quantitatively determined via hemoglobin electrophoresis as part of standard patient care. SCT blood samples (Hb AS) were collected from biological parents (usually mothers) of SCD patients;[1] SCT samples with hematocrit values lower than 25% were excluded.[2]

Hb solubility assay

The Hb solubility assay (SickleDex™, Streck, Omaha, NE) used in this study is a commercially available test kit that consists of two components: (i) saponin and sodium hydrosulfite supplied as dry reagent powder, and (ii) 2.3M potassium phosphate solubility buffer. The contents of one vial containing the reagent powder were added to one bottle of the solubility buffer (as instructed by the manufacturer) and dissolved completely. The Hb solubility assay was mixed with blood at 20:1 ratio by volume.

Paper substrate patterning

The paper-fluidic substrate was fabricated using a previously published method.[3, 4] The pattern of the hydrophobic barriers was drawn in black lines on white background using illustration software (Canvas 11, ACD Systems International Inc, Seattle, WA) and then printed on sheets of chromatography paper (No. 1, Whatman**,** Piscataway, NJ) using a solid-ink printer (Phaser 8560N, Xerox, Norwalk, CT). The printed chromatography paper was heated on a hot plate (150°C, 3 min) above the melting point of the wax to enable the formation of hydrophobic barriers through the full thickness of the paper.[3] The melting process resulted in widening of the printed lines, which was accounted for when originally designing the pattern of the microfluidic paper-based analytical device (μPAD).[4]

Many identical μPADs arranged in a 5×4 array were fabricated on sheets of chromatography paper. The square pattern of the hydrophobic barriers of a μPAD was designed to limit the spread of blood form one device to another, thus preventing the potential cross-contamination of samples (**Fig. 2a**). The 45º alignment lines in each corner of a μPAD provided the operator with visual guides for depositing the sample droplet in the center of the device (**Fig. 2a**). The simplistic design of the μPAD also significantly simplified the automated image analysis algorithm we used to analyze the blood stain patterns. We selected the size of the μPAD and the volume of the droplet such that the outermost margin of the stain could not reach the alignment lines and device outline on the periphery of the μPAD (**Fig. 2a**).

Quantification of the blood stain patterns

The sheets of chromatography paper containing arrays of μPADs with developed blood stains were digitized using a portable flatbed scanner (CanoScan LiDE110, Canon USA Inc, Lake Success, NY), and the scanned images were analyzed with an image analysis algorithm

(MATLAB, The Math Works Inc, Natick, MA). The quantitative analysis of blood stain patterns was based entirely on the RGB values of the digitized images, and was performed automatically and completely independently from the user, particularly from the subjective definition of "red" (i.e., even a color blind individual could use the assay).

Each component in the RGB color model varies from 0 to 255, with "white" corresponding to (255, 255, 255), "black" corresponding to (0, 0, 0), and various other combinations of the three RBG components each producing a different outcome in terms of color, brightness or both. In this representation, the variation of the red color intensity from pure bright "red" (255, 0, 0) down to "white" (255, 255, 255) is achieved by adjusting the values of the G and B components equally from 0 to 255 (for example, "pink" is (255, 128, 128)). We used MATLAB function *impixel* to extract the RGB values for each pixel of the scanned blood stain images. Because of the inverse relationship between the G and B values and the intensity of "red" color, we used the B component to quantify the transition from the dark red spot in the center to the light pink ring on the periphery of the blood stain (**Fig. 2a**). We defined the intensity of red color as <color intensity> = 255 – B (**Fig. 2b**, values on the Y-axis). The natural symmetry of the blood stains significantly simplified the quantification of the blood stain patterns. The algorithm automatically detected the geometric center of the stain, and the image was rotated (with a 1° step) about its center to collect 360 independent one-pixel-wide horizontal line scans of the blood stain (along the dashed line shown in **Fig. 1a, iii**). These line scans were averaged to obtain a single curve representing the pattern of the red color intensity change from the center of the blood stain to its periphery (**Fig. 2b**).

Normalization of the color intensity profiles / SCD index

The differences in hematocrit among subjects and imperfect metering of the volume of blood used to prepare the sample introduced a significant variation in the total amount of Hb present in the sample droplet being deposited onto the µPAD. Droplets with different amount of Hb produced blood stains with different overall brightness, which interfered with the ability of the assay to distinguish between normal, SCT and SCD samples.

To account for these variations in the overall brightness of the blood stains, we normalized the color intensity profiles by the total area under the curve (**Fig. 3a**). To accomplish that for a given color intensity profile curve (e.g. **Fig. 2b**), we calculated its area under the curve through integration (trapezoid rule with unit spacing), and then divided each value on the profile curve by the calculated area. Finally, we scaled each normalized profile by a common scaling factor equal to the absolute maximum of color intensity over all normalized color intensity profiles. This final operation did not change the relative position of the curves, but changed the scale on the Y-axis such that the values of normalized color intensity would fall within the [0 1] interval. On this scale, the point of the brightest red among all blood stain profiles corresponded to 1, and dry paper corresponded to 0 (**Fig. 3a**).

The *SCD index* was defined as the normalized color intensity at 5 mm from the center of the blood stain. Data were presented as results of individual experiments and as a mean ± SD. The values of the *SCD index* for samples from each Hb genotype were compared using a paired two-tailed *t*-test. Two levels of statistical significance were defined as*($p<0.001$) and **($p<0.05$) versus another Hb genotype.

References:

1. S. D. Roseff, *Immunohematology*, 2009, **25**, 67-74.

2. G. Tsaras, A. Owusu-Ansah, F. O. Boateng and Y. Amoateng-Adjepong, *Am J Med*, 2009, **122**, 507-512.

3. E. Carrilho, A. W. Martinez and G. M. Whitesides, *Anal Chem*, 2009, **81**, 7091-7095.

4. X. Yang, O. Forouzan, T. P. Brown and S. S. Shevkoplyas, *Lab Chip*, 2012, **12**, 274-280.