MULTISPECTRAL IMAGING AND MINIATURIZED ELECTROPHORESIS BASED POINT-OF-CARE NEWBORN SCREENING FOR SICKLE CELL DISEASE

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List of Abbreviations

Cellulose Acetate	CA
Deionized	DI
Ethylenediaminetetraacetic Acid	EDTA
Hemoglobin	Hb
Hemoglobin Variant Multispectral	Hb VM
Hemoglobin Variant	Hb V
High Performance Liquid Chromatography	HPLC
Isoelectric Focusing	IEF
Institutional Review Board	IRB
In Vitro Diagnostic	IVD
Limit of Detection	LoD
Microchip Electrophoresis	MCE
Nationwide Newborns Screening	NBS
Negative Predictive Value	NPV
Point-of-Care	POC
Pearson-product-moment Correlation Coefficient	PCC
Positive Predictive Value	PPV
Polymerase Chain Reaction	PCR
Red Blood Cell	RBC
Region of Interest	ROI
Sickle Cell Disease	SCD
Sickle Cell Anemia	SCA
Sickle Cell Trait	SCT
Tris/Borate/EDTA	TBE
Tandem Mass Spectrometry	MS/MS
Ultraviolet	UV
World Health Organization	WHO

Abstract

Multispectral Imaging and Miniaturized Electrophoresis Based Point-of-Care Newborn Screening for Sickle Cell Disease

By

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Globally, hemoglobin (Hb) disorders affect nearly 7% of the world's population, especially around 400,000 newborns annually with sickle cell disease (SCD) suffering from a high mortality rate. Even though treatments are available for Hb disorders, screening, early diagnosis, and monitoring are not widely accessible due to technical challenges and cost, especially in low-income countries. We hypothesized that multispectral imaging would enhance sensitive Hb variant identification in existing affordable paper based Hb electrophoresis. In this thesis, the first integrated point-of-care Multispectral Hb variant test (Hb VM) including the related algorithm qualification method was initially proposed and verified. Furthermore, the validation of this new multispectral approach via testing multiple samples with known Hb variant including Hb A2/C, Hb A, Hb F, and Hb S from Korle-Bu hospital. Hb VM demonstrated high sensitivity and specificity identifying all 4 Hb variants, which can enhance detection sensitivity for low-level Hb level variant enabling, for sensitive nationwide newborn screening for Hb disorders.

1. Introduction

1.1 Sickle Cell Disease

Hemoglobin (Hb) is an oxygen-containing transport metalloprotein in red blood cells (RBC) consisting of two α and two β protein chains, which can carry oxygen to tissues and transports carbon dioxide back to the lungs. Hb disorders are among the world's most common monogenic diseases. Nearly 7% of the world's population carry Hb gene variants [1-3]. SCD consists of a group of hereditary blood disorders which are characterized by the presence of an abnormal hemoglobin known as sickle hemoglobin. 'Sickle' anemia phenomenon was first clinically demonstrated in the USA during 1910 [4], and the mutated, inheritable sickle Hb molecule was discovered in 1949 [5]. SCD is caused by a point mutation in the sixth codon of the beta (β) globin chain that produces healthy, normal hemoglobin A (Hb A). This substitution of hydrophilic glutamic acid with hydrophobic valine will generate Hemoglobin S (Hb S). As a result of this substitution in the β-globin chain, Hb S forms long and insoluble polymers in low oxygen conditions. Red blood cells that contain Hb S become less deformable and take the characteristic "sickle" shape. This abnormal polymerization of sickle hemoglobin affects the membrane properties, shape, density, and lifespan of red blood cell (RBC). The sickle cell can cause vascular complications and critical changes in inflammatory response and endothelial cell function [6, 7].

Even though SCD is led by a single hemoglobin mutation, it is a complicated disorder that affects different parts of the body including legs, arms, abdomen, chest, and lower back. The clinical expression of SCD can be summaries as painful crises, widespread organ damage, and early mortality [8, 9]. The clinical consequences of SCD can be categorized into four groups: hemolysis and hematological complications, vaso-occlusion, infection, and organ dysfunction [10]. Though the sickle gene in the molecular level is a single point mutation, sickle cell disease is multiple phenotypic expressions, which constitute the various complications in clinical aspect, especially

sickle cell anemia [11].

Patients who inherit two copies of the sickle mutated beta chain (Hb SS) develop sickle cell anemia (SCA), which is the most prevalent and one of the most severe forms of SCD. However, if patients who inherit one copy of the sickle mutated beta chain and one copy of the normal beta chain will have the sickle cell trait (SCT, Hb AS) and are considered healthy carriers that do not display the severe symptoms of SCD [12, 13]. Patients who carry one copy of the sickle chain and one copy of the abnormal beta chain C have compound heterozygous Hb SC, also known as Hemoglobin SC disease. Hb SC disease confers a milder but still debilitating form of SCD [14]. Roughly two-thirds of SCD patients have homozygous Hb SS, and one-quarter have compound heterozygous Hb SC [15]. The remaining SCD population is comprised of subjects with Hb S-beta thalassemia, in which Hb S, fetal hemoglobin (Hb F), and small (or no) amounts of Hb A are produced [16]. In addition, a high concentration of non-beta chains containing Hb F is present in newborns, declining over 6 months [17].

1.2 Importance and Challenges of Nationwide Newborn Screening

Hemoglobin disorders are the most common single-gene diseases in global. SCD will perform when Hb variant mutations are inherited homozygously (Hb SS) or paired with another β-globin gene mutation [18, 19]. Globally, an estimated 400,000 babies are born annually with SCD, and 70%-75% are in sub-Saharan Africa [20]. The mortality of SCD is especially high in Africa amongst the under-fives, though it is worth noting that 70% of these deaths are preventable [21-23]. Effective management of SCD revolves around genetic counselling, early diagnosis, and, importantly, newborn screening (NBS) [24-27]. NBS performed in centralized laboratories has dropped SCD mortality in high-resource countries [23, 28]. NBS requires sensitive detection of relatively low levels of hemoglobin A (Hb A) and hemoglobin S (Hb S) in the presence of high levels (up to 90%) of fetal hemoglobin (Hb F) [29].

SCD NBS in centralized laboratories has dramatically dropped SCD mortality in resource-rich countries [30, 31]. However, in sub-Saharan Africa and central India, where > 90% of annual SCD births occur, newborn screening programs have not been implemented universally, if at all, due in large part to the cost and logistical burden of laboratory diagnostic tests [32]. For example, among newborns, normal hemoglobin A (Hb A) and sickle hemoglobin S (Hb S) are at lower levels, while high levels of fetal hemoglobin (Hb F) holds up to 90% of total Hb [17].

SCD NBS is challenging in low- and middle-income countries, where a heavy SCD burden exists due to a lack of lab infrastructure and skilled personnel [3, 33]. World Health Organization (WHO) has mentioned that hemoglobin testing as one of the most important in vitro diagnostic (IVD) tests primary care use in low- and middle-income regions during the 2019 report [31]. Furthermore, hemoglobin electrophoresis has recently been added to the WHO essential list of IVDs for diagnosing SCD and sickle cell trait [1]. As a result, there is a need for a point-of-care (POC) test device which are affordable, portable, easy to use, accurate to accelerate decentralized hemoglobin testing in low-resource settings to enable nationwide NBS [33].

The current centralized tests used for hemoglobin NBS are high-performance liquid chromatography (HPLC) and isoelectric focusing (IEF) that require specialized instruments and highly trained personnel. In addition, these tests rely on state-of-the-art facilities, which are lacking in most low resource settings where SCD is most prevalent [34, 35]. Due to the high cost and scarce resources, NBS is relatively new and has not yet become routine in low resource settings [22].

1.3 Principles of Electrophoresis Diagnosis and Challenges in newborn screening

The hemoglobin electrophoresis and isoelectric focusing (IEF) technologies are two key technologies for Hb variant diagnosis. Both technologies are based on the principle that different Hb types migrate with different velocities when placed in a constant electric field due to their different net charges and mobility. The velocities (v) with which different Hb migrate can be

defined as

$$v = \mu E$$

$$\mu = \frac{q}{6\pi nr}$$

$$E = \frac{V}{L}$$

Where μ is the electrophoretic mobility of the specific Hb type and E is the electric field strength. Electrophoretic mobility is determined by the Hb charge (q), the buffer viscosity (n), and the Hb radius (r). The electric field strength is determined by the voltage difference (V) and the distance (L) between the electrodes.

Hb electrophoresis is a common lab technique which can be used to sieve variety of materials including gel and paper under alkaline or acidic conditions. All the Hb types have net negative charges in alkaline conditions and move towards the positively charged electrode during the electrophoresis process. There are many variables, including the ionic concentration of the buffer solution, charges of the Hb, and the pore size of the medium, which can determine the distance of different Hb types migrate in a certain time. The separation of different Hemoglobin types forms visible bands which can be used to identify and quantify different Hb disease. Hb electrophoresis under alkaline conditions has a lower quality of resolution between Hb S and Hb F, especially in patients with high Hb F levels.

Similar to Hb electrophoresis, IEF separates Hb based on their isoelectric points. This technique uses an applied electric field across a medium with a fixed pH gradient. As it migrates through the pH gradient, each Hb type becomes immobilized once it reaches its specific isoelectric points. At the specific isoelectric points, the Hb has a zero net charge and no longer migrates. Although IEF is more expensive than Hb electrophoresis, it can identify more Hb variants.

To summaries, the diagnosis of SCA, SCT, Hb SC disease, and Hb S-β thalassemia is based

on the varying percentages and combinations of Hb S, Hb A, Hb F, Hb C, and HbA2 present in RBC's electrophoresis screening. However, these screenings tests cannot be performed accurately on newborns (Age<6 weeks) due to the predominance of Hb F (Fetal hemoglobin) at birth. Newborn who suffers from SCD or SCT will generate a vast amount of Hb S after they born for several months. If used at birth, the tests may give a false-negative result if Hb S is less than 10% of the total Hb.

Secondly, standard clinical laboratory technology, including high-performance liquid chromatography (HPLC) and isoelectric focusing (IEF) are typically used in testing Hb variant, which requiring unaffordable ($15k - $35k) specialized instruments, highly trained personnel, and sample transport. However, advanced laboratory techniques require trained personnel and state-of-the-art facilities [36, 37]. These tests rely on laboratory facilities, which are lacking in low resource settings where SCD is most prevalent. IEF is a less expensive central test option but lacks quantification capacity, misses some Hb variants and requires skilled interpretation. Major hospitals in low-resource settings may have a manual electrophoresis device, but these devices are time-consuming to run, need a laboratory setting, and require expertise to read.

To address these concerns, we have developed and extensively tested a point-of-care (POC) microchip electrophoresis hemoglobin variant testing system, Hemoglobin Variant (Hb V, previously called 'HemeChip'), which is being commercialized under the product name "Gazelle" [36, 38] (**Figure.1**). Hb V has been extensively tested and validated in the US, Africa, India, and Southeast Asia for hemoglobin variants, including SCD and hemoglobin E disease, and thalassemia but is only available for testing people over six weeks of age [39]. Infants below six weeks of age can have very low percentages of abnormal Hb, and so an improvement in the limit of detection (LoD) is needed. By decreasing the LoD from 10% to <4% newborns can be screened.

1.4 Objective of This Study

Here, we propose to add multi-spectral imaging capability to the previous Gazelle product and develop Gazelle-multispectral system to accurately screen newborns for common Hb variants. The proposed system is the first and only POC platform that is portable, affordable, and accurate enough for identification and quantification of hemoglobin variants in newborns.

Specifically, we firstly performed a proof-of-concept study of white field and UV imaging of sickle cell and C trait phenotypes of the platform in a laboratory setting then initially provided their hemoglobin variants identification and quantification for different illuminative conditions. Secondly, we determined the Hb S detection limit of platform using a single blood sample or a mixture of blood samples that continuously provided by University Hospital's Hematology and Oncology Division, including SCD patient blood or transfused SCD patient blood containing high concentration sickled hemoglobin (Hb S > 60%), purchased cord blood containing high concentration fetal hemoglobin (Hb F>80%), healthy donor blood with high concentration Hb A (Hb A > 90%). Additionally, we describe a study for evaluating the diagnostic performance of this platform for screening Hb SS, Hb SC disease, and the related carrier states (Hb S trait and Hb C trait) using whole blood at the POC in 321 completed tests in Ghana, a location selected for its high prevalence of both the Hb S and Hb C variants. Based on previous limit of detection experiment, the limit of detection of Gazelle-Multispectral is determined to be 4%, which is sufficient for detecting low percentage of Hb S typically found in newborn samples for newborns with sickle cell trait and SCD. The clinical test result demonstrate Gazelle-Multispectral detects both disease vs normal and disease vs trait at 100% sensitivity and 100% specificity, as well as trait vs normal at 98.2% sensitivity and 97.1% specificity. Overall, the ability to achieve low limit of detection and the ability to obtain rapid and accurate results on newborn samples suggest that Gazelle-Multispectral is suitable for large-scale newborn screening and potentially for accurate diagnosis.

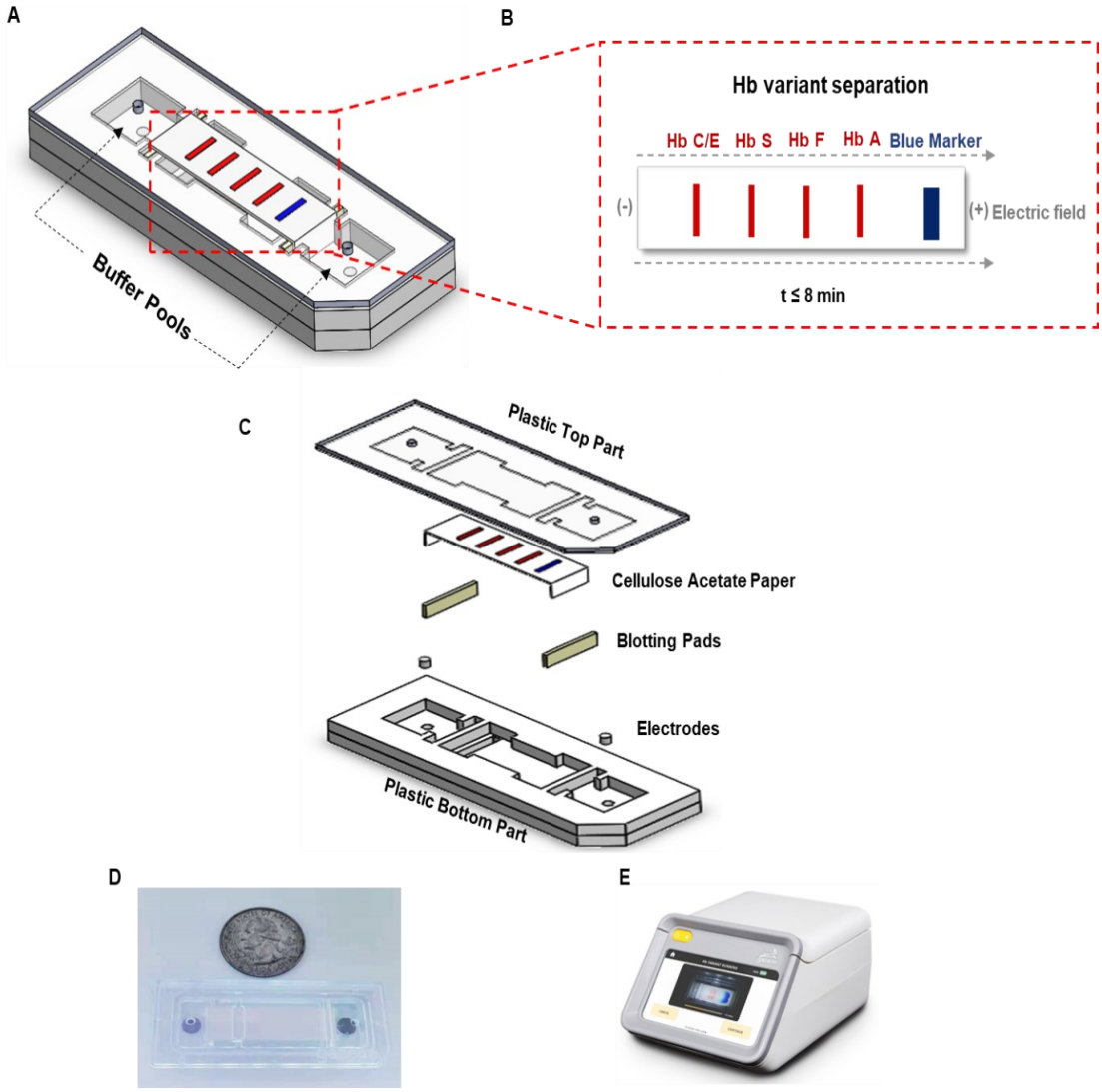


Figure 1. Commercialized Gazelle platform and designed sample cartridge can be used for individual patient Hb variant identification and quantification. (**A**) The schematic diagram of the final assembly Gazelle cartridge during the experiment running. (**B**) Detailed view of Hb variant separation on cellulose acetate paper (left to right: Hb C/E, Hb S, Hb F, Hb A and blue marker). (**C**) Explosive view of the designed cartridge (top to bottom: Plastic top part, cellulose acetate paper, blotting pads, electrodes, plastic bottom part). (**D**) Gazelle single-use cartridge. (**E**) The Gazelle system is placed inside a solid POC device with an embedded GPS designed for clinics and remote settings.

2. Materials and Methods

2.1 Study Design and Oversight

This diagnostic test accuracy study was conducted using the Gazelle-Multispectral, a miniaturized electrophoresis test, to detect hemoglobin variants including Hb A, Hb F, Hb S, and Hb C/E. Besides, a 1x Tris/Borate/EDTA (TBE) with saponin buffer solution (pH 8.3) was made from a 10x TBE Buffer solution, diluted with deionized (DI) water, and cellulose acetate (CA) paper will also be included. All these materials and Gazelle platform with Ultraviolet (UV) and White imaging light was designed and provided by Hemex Health, headquartered in Portland, Oregon, USA. The laboratory standard test used in the study was high-performance liquid chromatography (HPLC) at Korle Bu Teaching Hospital.

2.2 Blood Preparation

During the concept proving process, discarded and de-identified patient blood samples were obtained, under Institutional Review Board (IRB) approval, from University Hospital's Hematology and Oncology Division (Cleveland, OH). Patient blood samples will be placed into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulants and stored at 4 ºC. When the formal test starts, the whole blood sample will be placed into a vortex machine to fully mix the whole blood. To lyse whole blood directly on the chip, a 0.1% (w/v) lysing buffer solution was made by combining saponin (S4521, Sigma-Aldrich, St. Louis, MO) and a 1x TBE buffer solution, which can reduce the necessary time and materials required to lyse whole blood.

2.3 Confirmatory Laboratory Procedures

Blood samples that were stored at -80°C were retrieved and thawed. 5 µl of the sample was pipetted and diluted with 1500 µl of distilled water. Diluted hemolysates were arranged on racks and loaded into the Bio-Rad D-10 HPLC system. Each sample was tested for approximately 6 minutes. The results reported for each blood sample included the relative percentages of each

hemoglobin type present.

2.4 Test Operation

After the whole blood sample was placed into vortex machine to mix fully, the whole blood sample should be lysed by 0.1% (w/v) lysing saponin by combining blue marker solution (0.0625g Xc-marker in 10ml ultra-pure water) with 1:2 ratio (20 μL of whole blood: 40 μL of saponin marker solution). Next, inversing the microtube that contains coating solution, coat the stamping applicator tip (dip in the solution, then shake 5 times, and then dry for 5-7 min). Once the coating solution dries on stamper, slightly inclined angle the cartridge (45 degrees) and put 50 μL of running buffer into the hole, mildly tilt and rotate the cartridge to fully wet the paper.

Allowing the cartridge to sit vertically for 1 min to make sure the cellulose acetate paper is fully submerged into running buffer. While putting 20 μL of whole blood +marker fluid sample on a piece of glass slide, load the sample onto the applicator (make sure to 45-degree angle the applicator and load the sample from one side from the middle of the sample droplet on the glass slide). Then applying the applicator that including whole blood +marker fluid sample into the cartridge and press for 5 seconds by using two fingers from one hand. Finally, filling the buffer pool on each side of the cartridge with 200 μL of running buffer and placing the cartridge in the Gazelle reader. The detailed procedure can refer to **Appendix 1 and 2.**

2.5 Multi-Spectral Imaging

Our integrated point-of-care Multispectral Hb variant test (Hb VM) system which contains 4 different illuminations (white, UV, blue and green) enables real-time tracking, identification and quantification of low concentration hemoglobin variants during electrophoresis. In the Hb VM system (**Figure 2**), the white light field can detect hemoglobin at high concentration levels. The acquired data under the white light field demonstrates a natural red color of hemoglobin, thus validating Hb VM tests (**Figure 2C**). In addition to high concentration hemoglobin variants, data

acquired under UV field further enables detection of low concentration hemoglobin variants which are not detectable under the white light field. What is more, data acquired under UV field holds enhanced limit of detection and higher signal to background ratio than white light field data (**Figure 2D** and **Figure 3**).

In this Hb VM system, images are collected every 4 seconds during electrophoresis and the software analyses these data in real-time. The UV wavelength of 410nm was chosen to be close to the peak hemoglobin absorbance. The image analysis leading to identification and quantification of hemoglobin will be extended to run on the UV images in addition to the current white light images. Lab-based testing will be used to tune the system for accurate identification and quantification (**Figure 2C** and **Figure 2D**). Combining both white light and UV detection spectrums, Hb VM tracks, detects, identifies and quantifies electrophoretically separated low concentration hemoglobin variants. Hb VM automatically validates electrophoresis separation, identifies low concentration hemoglobin, and determines their relative percentages based on multi-spectrum imaging system.

Figure 3 shows that UV light made the Hemoglobin A, F and S bands crisper and discrimination among them easier. The concentration of Hb S below 8% which were not visible with white light were visible with UV down around 4%. **Figure 3** demonstrates the added value of multispectral detection (UV) in the comparison of the current microchip electrophoresis (MCE) product in white light compared to the bands shown with UV imaging on a Hb SF sample and Hb AF sample where white light missed bands below 8%. Preliminary results demonstrated that with the use of UV multispectral imaging, the Gazelle system could detect low concentrations of hemoglobin (Hb F in **Figure. 3C**, as well as Hb S, 6.4% and Hb A, 12.5% in **Figure. 3D**). In contrast, this low concentration hemoglobin was not detected when using the current white light imaging system. The preliminary test results demonstrate the feasibility of detection and quantification of low concentration hemoglobin variants. The needed LoD for newborn screening is

4% for Hb A, S, or C, which was consistently achieved over these clinical and lab samples.

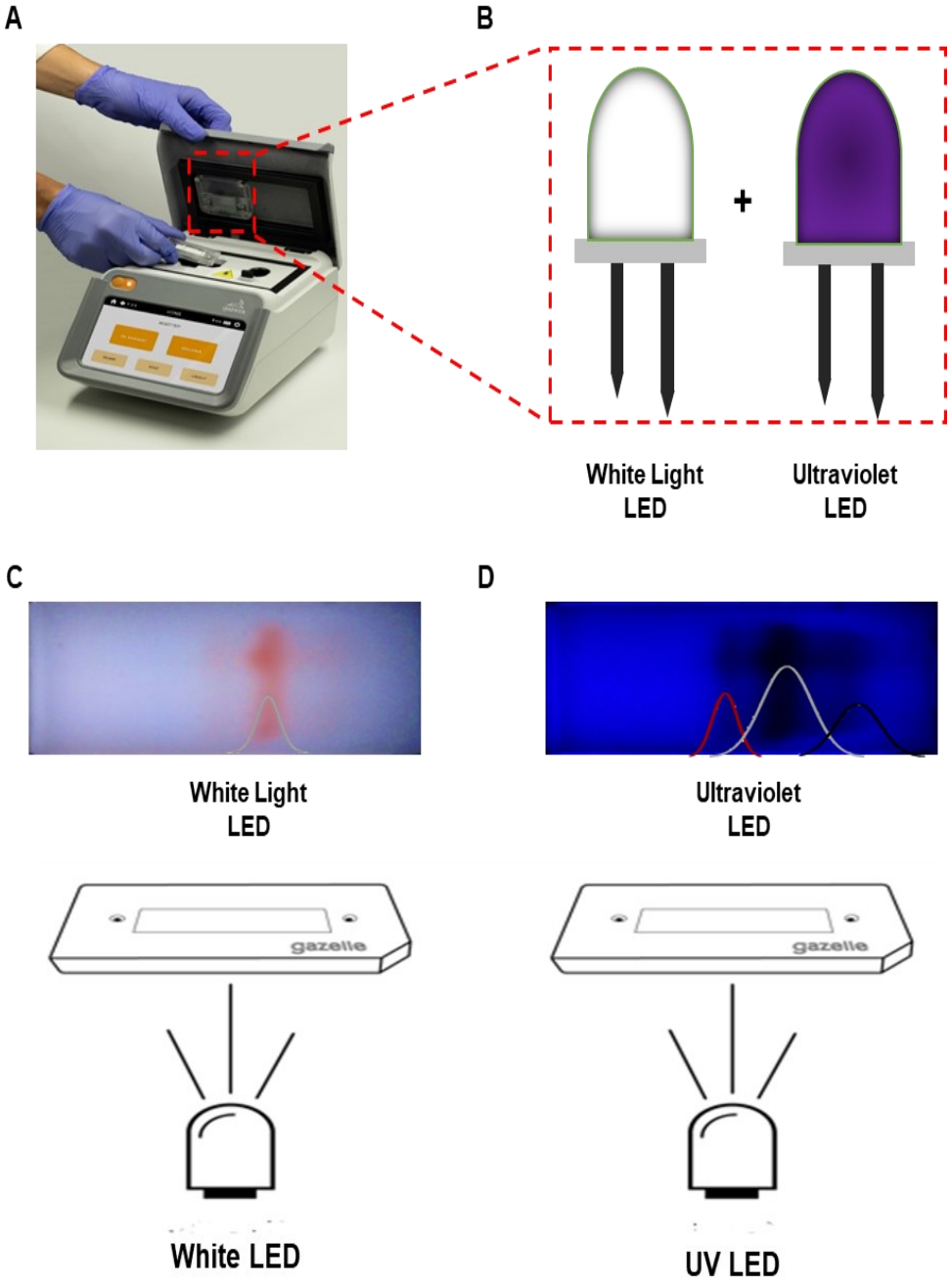


Figure 2. The schematic diagram of Hb VM system and related captured images. (A, B) White and ultraviolet light inside the Gazelle test device. **(C, D)** Peak intensity from original Gazelle images under different illumination conditions

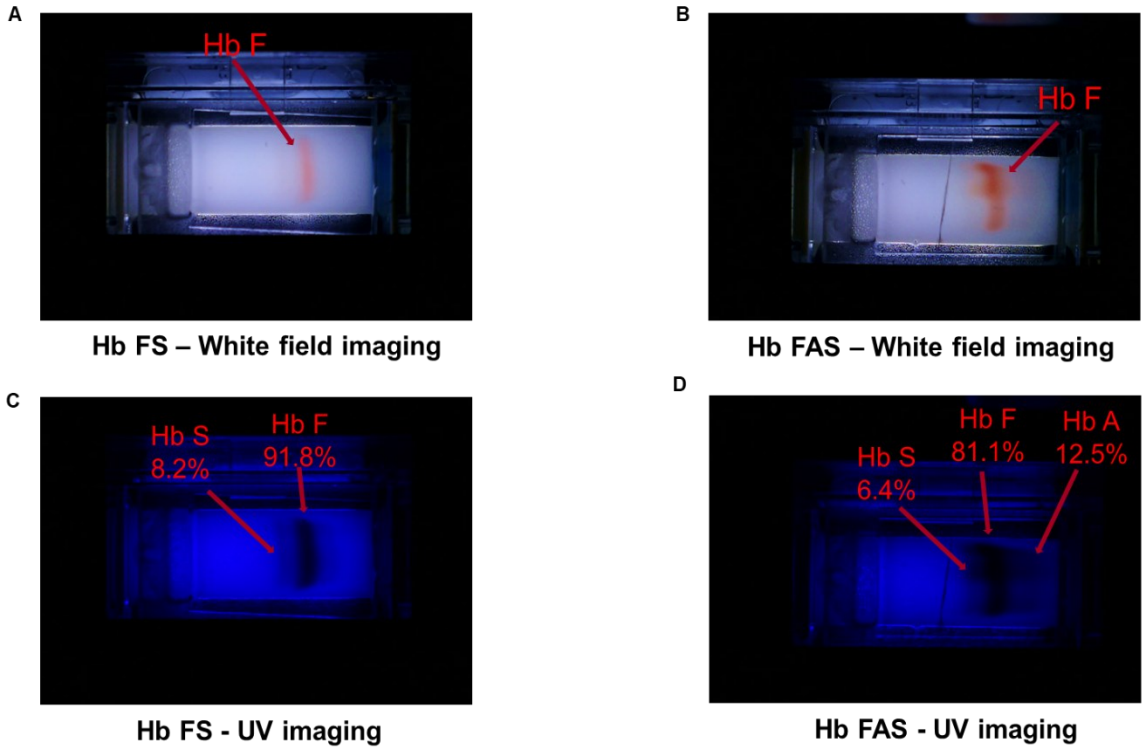


Figure 3. Comparison of imaging with white light and UV with two representative patient samples. Hb FS (A, C) and Hb FAS (B, D). UV detected low level hemoglobin bands missed with white light (C, D) (Hb S – 8.2% in **Hb FS**; Hb S – 6.4%, Hb A – 12.5% in **Hb FAS**). The HPLC percentage: 7.2% S and 92.8% F in **Hb FS**; 5.1% S, 83.2% F and 11.7% A in in **Hb FAS.**

2.6 Determination of Limit of Detection

Infants below 6 weeks of age can have very low percentages of abnormal Hb and so an improvement in the limit of detection (LoD) is needed. By decreasing the LoD from 10% to <4%, newborns can be screened. Hence, NBS requires sensitive detection of relatively low levels of hemoglobin S (Hb S) in the presence of high levels (up to 90%) of fetal hemoglobin (Hb F) [29]. In our Hb VM system, multispectral imaging will help identify and quantify hemoglobin variants in newborns. One of the milestones is to achieve accurate identification and quantification of hemoglobin variants in newborns using a drop of blood in less than 10 minutes and further demonstrate that the LoD for Hb% has decreased from the current 10% to 4% for Hb S in the

presence of high Hb F (up to 90%) percentages.

Based on this goal, an LoD experimental plan was proposed and tested by the Gazelle system (**Appendix 3**). In this plan, Gazelle Multispectral lower limit of detection for sickle Hb (Hb S) was determined using artificially created samples by mixing a heathy donor blood with known Hb A level (Hb AA: 96.3% Hb A, 0.4% Hb F, 3.3% Hb A2, predetermined by HPLC) and a blood sample from a patient with SCD undergoing transfusion therapy with known levels of Hb A and Hb S (Hb AS: 59.1% Hb A, 39.3% Hb S, 0.2% Hb F, 1.4% Hb A2, predetermined by HPLC). Hb S levels in artificially created samples were obtained by HPLC as 39.3%, 19.2%, 12.8%, 9.6%, 6.4%, 3.2%, 1.6%, and 0.8%. Each artificially created sample was tested 3 times using the Gazelle-Multispectral system and then reported results were compared with Hb S levels determined by HPLC.

2.7 Clinical Study Populations

To validate our Hb VM system can be used in clinical application, the Hb VM system was used to test at Korle Bu Hospital in Ghana and 321 subjects younger than 6 weeks will be tested. In these tests, 297 out of 321subjects will be used to performed assessment (Refer to **Section 3.4**). The population in the clinical study was from the Korle Bu Teaching Hospital in Korle Bu, Ghana. Newborn babies were enrolled from the newborn clinic, and children were enrolled from the vaccination clinic. The Institutional Review Board approved this clinical investigation at the site and informed consent was obtained from each participant's parent or guardian. A blood sample was obtained from each participant using a finger prick at the vaccination clinic or heel prick at the newborn clinic. Any blood samples not tested immediately on Gazelle-Multispectral were refrigerated until use. After a Gazelle test was conducted, the remaining blood from the blood collection tube was saved and frozen at -20 °C. HPLC tests were performed at Korle Bu Teaching Hospital on the frozen samples using the D-10 HPLC system (Bio-Rad Laboratories, Hercules, CA,

USA). The Hemex data viewer based on our preliminary algorithm will quantify all the hemoglobin percentages in each patient sample and then compare these quantified percentages with HPLC results.

2.8 Image Processing

In summary, the image processing is to find the intensity of each band and use fitting tool to find the percentage of each band. A Nikon D3200 camera was used to capture Gazelle cartridge results' close-up images under different illumination conditions. Both UV and White Images were processed using the RGB method in MATLAB (MathWorks, Natick, Massachusetts) with no additional plugins. In each frame picture, only the tested CA paper was cropped as the region of interest (ROI) and used for analysis. Relative pixel intensities along the paper were used to identify the peaks corresponding to each type of hemoglobin band (**Appendix 4 A-C and H-J**). Based on the Gaussian fitting function, each function peak associate with original intensity represents different types of Hb regions. The area under each peak was calculated to obtain the relative hemoglobin percentages (**Appendix 4 D-F and K-M**).

2.9 Statistical Analysis

The correlation and agreement between the measured hemoglobin concentrations for the Gazelle device and standard clinical method (HPLC) were evaluated using Pearson Correlation Coefficient (PCC) and Bland-Altman analysis. For the limits of agreement in the Bland-Altman graph, limits of agreement in this graph were defined as the mean difference ± 1.96 times the standard deviation of the differences.

3. Results

3.1 Hemoglobin Variants Identification and Quantification

The quantification of different hemoglobin variants was demonstrated as the percentage relative to the sum of overall hemoglobin intensity peak value in the whole blood sample. To obtain

the final percentage value of each Hb, we do the following computational steps. Firstly, we should normalize the targeted image to reference image: normalized [x, y] = reference [x, y]-frame [x, y]. Here, [x, y] means the pixel value at position x and y; frame is the targeted image frame; reference is the reference image frame, which is just the first frame when the cartridge is just placed into the machine. Secondly, the median value of each column of the normalized image will be picked and transfer the 2D matrix into the 1D array: Collapse 2D to 1D: collapsed[x] = median (rescaled [x,:]); here [x,:] means image column at index x. Thirdly, plotting the Gaussian fitting curve of this array. Finally, calculating normalized ratios for each blood band by using the area of each peak curve to divide the sum of all the peak areas respectively.

In **figure 4**, each of the curve peaks is calculated and the relative percentages are reported under the white field and UV image conditions respectively, for these two examples, the Hb FAS (**Figure. 4 A-G**), Hb FSC (**Figure. 4 B-H**) blood containing sickle hemoglobin (Hb S), and hemoglobin C trait. Hb A, Hb S, Hb F and Hb C separation are all significantly clear by comparing the Gaussian fitting curve and original images from the system in both white field and UV images, which can be used to distinguish different Hb percentages. Furthermore, this figure preliminarily proves UV image are more sensitive to detect Hb variant than white field for Newborn patients.

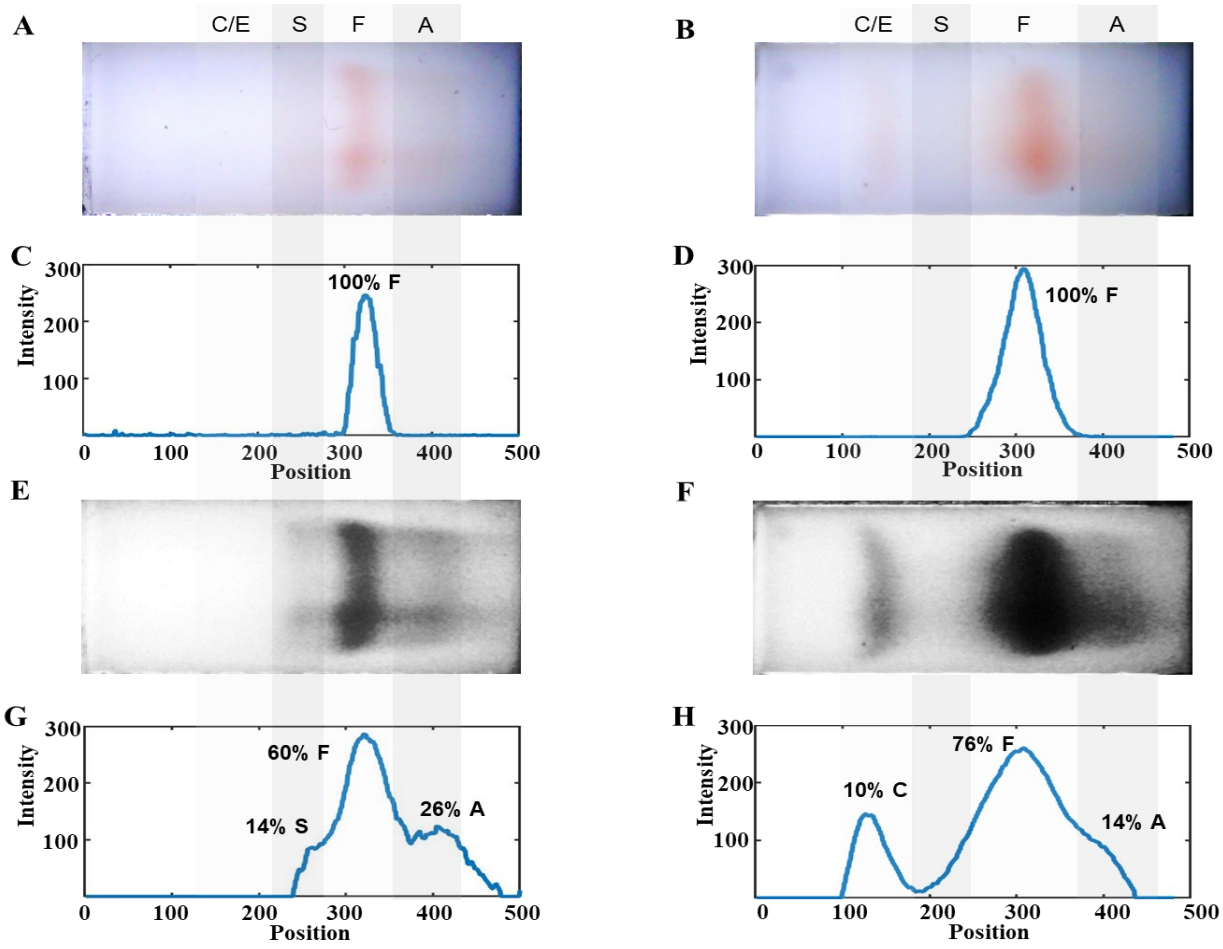


Figure 4. Hb variants separation and identification of two representative cases from White and UV image to Gaussian fitting curve: Hb FAS sickle cell trait (A-G), Hb FAC Hb C trait (B-H). (A-C) Hb variants separation and identification of Hb FAS sickle cell trait and its initial Gaussian fitting profile in white field image. (B-D) Hb variants separation and identification of Hb FAC Hb C trait and its initial Gaussian fitting profile in white field image. (E-G) Hb variants separation and identification of Hb FAS sickle cell trait and its initial Gaussian fitting profile in UV image. (F-H) Hb variants separation and identification of Hb FAC Hb C trait and its initial Gaussian fitting profile in UV image. (The HPLC percentage in **Hb FAS** is 25% A, 63% F and 12%S respectively; The HPLC percentage in **Hb FAC** is 14% A, 78% F and 8%C respectively)

3.2 Analysis of Limit of Detection of Hb S

During the initial analytical assessment (**Figure 5**), the limit of detection (LOD) was

determined as the lowest concentration for which all three replicates scored positive. Gazelle-Multispectral consistently identified Hb S in 3 out of 3 replicates for artificially created samples with Hb S levels at 3.2%, 6.4%, 9.6%, 12.8%, 19.2%, and 39.3%. However, Gazelle-Multispectral identified Hb S in 0 out of 3 replicates for artificially created samples with Hb S levels at 1.6% and 0.8%. As a result, the lower LOD of Gazelle-Multispectral for identifying Hb S was determined to be around 4% in UV spectrum and 8% in white spectrum. This 4% lower LOD is sufficient for detecting low percentage of Hb S typically found in newborn samples for newborns with sickle cell trait (SCT, Hb FAS, 6.5 ± 2.8%) and SCD (Hb FS, 10.2 ± 3.9%)[40]. Furthermore, the Gazelle-Multispectral imaging system results of two representative newborns clinical cases in Ghana including Hb FAS (Newborn with sickle cell disease trait, A 9%, F 87%, S 4% in HPLC), and Hb FAC (hemoglobin C disease, A 8%, F 88%, C 4% in HPLC) have further indicate that LoD of 4% in UV spectrum and 8% in white spectrum (**Figure 6**).

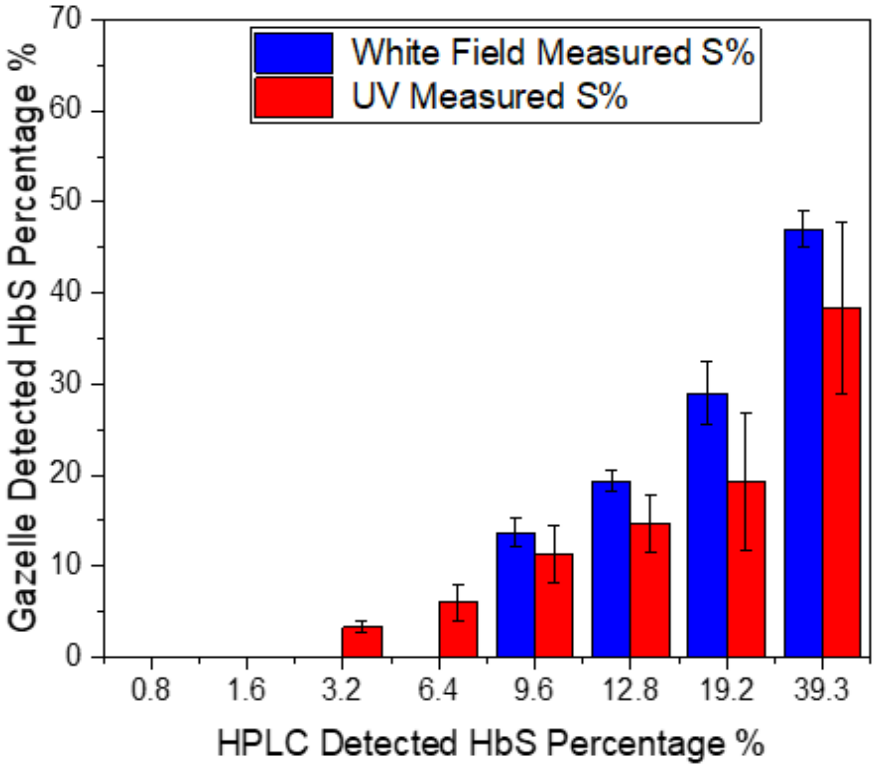


Figure 5. The average Hb S detection percentage of 3 repeated tests under the white and UV

illuminations for artificially created samples with Hb S levels at 0.8%, 1.6%, 3.2%, 6.4%, 9.6%, 12.8%, 19.2%, and 39.3% cases respectively. X-axis from left to right: The detected percentage of White field and UV are 0% and 0% respectively when HPLC detected percentage is 0.8%; The detected percentage of White field and UV are 0% and 0% respectively when HPLC detected percentage is 1.6%; The detected percentage of White field and UV are 0% and 3.3% ± 0.58% respectively when HPLC detected percentage is 3.2%; The detected percentage of White field and UV are 0% and 4.3% ± 2.1% respectively when HPLC detected percentage is 6.4%; The detected percentage of White field and UV are 13.7% ± 1.5% and 12.7% ± 6.7% respectively when HPLC detected percentage is 9.6%; The detected percentage of White field and UV are 19.3% ± 1.2% and 16.7% ± 7.8% respectively when HPLC detected percentage is 12.8%; The detected percentage of White field and UV are 29.0% ± 1.2% and 19.3% ± 7.5% respectively when HPLC detected percentage is 19.2%; The detected percentage of White field and UV are 47.0% ± 2.0% and 38.3% ± 9.5% respectively when HPLC detected percentage is 39.3%;

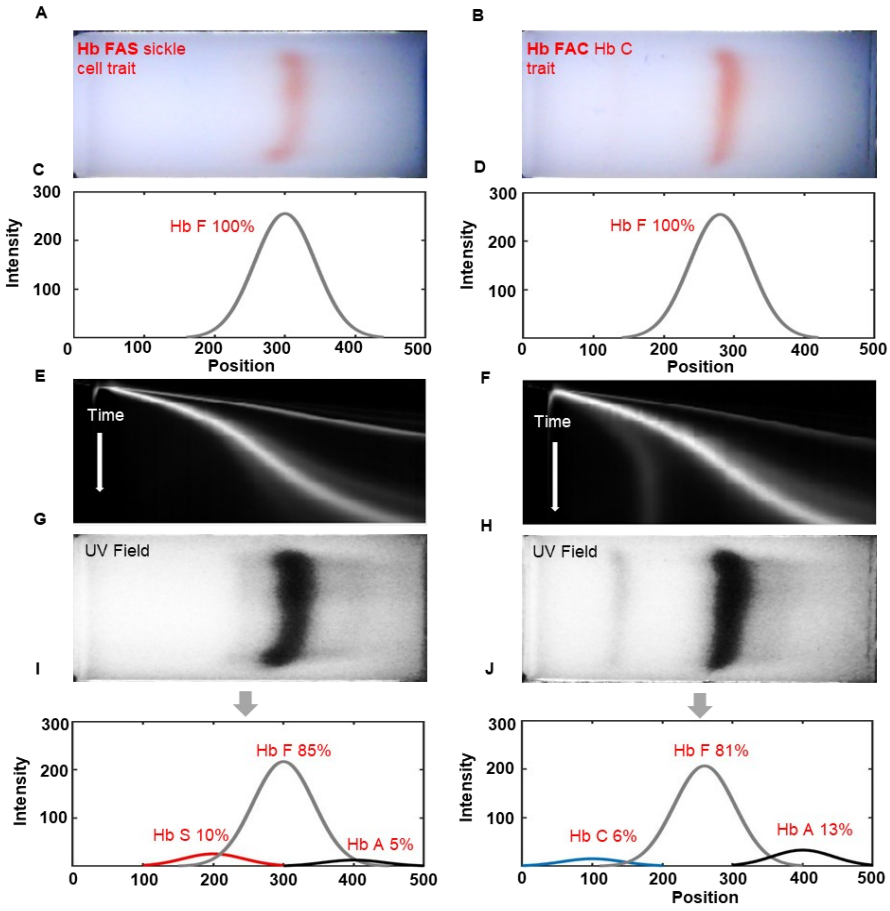


Figure 6. The limit of detection of Gazelle-Multispectral imaging system in clinical testing of two representative newborns cases in Ghana. (A-B) The first row shows images captured with white light. (C-D) The second row shows electropherograms generated based on the white light image using the white data analysis algorithm. (E-F) The third row illustrates the 2D representation of Gazelle-Multispectral space-time plots of band migration in UV imaging mode used with the machine learning algorithm. (G-H) The fourth row shows images captured with ultraviolet light. (I-J) The fifth row shows electropherograms generated based on the UV light image using the UV data analysis algorithm. Compared with related HPLC results, the limit of detection of Hb S under white (8%) and UV (4%) algorithm were further validated in both clinical tests. Column 1 - 2: Multispectral test result for the sample with Hb FAS (Newborn with sickle cell disease trait, A 9%, F 87%, S 4% in HPLC), and Hb FAC (hemoglobin C disease, A 8%, F 88%, C 4% in HPLC).

3.3 The imaging and intensity of different newborn phenotypes based on system

Gazelle-Multispectral image tracking and data analysis method is similar to its previous generation, Gazelle, as described in our previous publication [37]. Briefly, the Gazelle-Multispectral algorithm recognizes the initial application point of the mixture containing a blue control marker and hemoglobin. The algorithm then distinguishes and tracks the blue marker and hemoglobin according to their naturally distinct blue and red colors within the white light field during separation. The tracked blue marker and red hemoglobin migration pattern is analyzed by the image processing and decision algorithm to determine test validity (**Figure. 7 A-H**). A validated test will be further analyzed utilizing data acquired under UV field (**Figure. 7 I-T**). A space-time plot was generated to illustrate the entire band migration on the paper (x-axis, from left to right) within the entire time (y-axis, from top to bottom, **Figure. 7 I-L**). Four representative tests with different Hb variants were demonstrated in **Figure. 7** (Column 1: Healthy newborn, Hb FA; Column 2: Newborn with SCD, Hb FS; Column 3: Newborn with Sickle Cell Trait, Hb FAS; and Column 4: Newborn Hemoglobin C Trait, Hb FAC). Gazelle-Multispectral sensitively and accurately identified Hb variants agreeing with HPLC reported results in all these 4 representative samples (Gazelle-Multispectral vs. HPLC: Hb FA: Hb A: 8% vs. 6%, Hb F: 92% vs. 94%; Hb FS: Hb S 17% vs. 11%, Hb F 83% vs. 89%; Hb FAS: Hb S 16% vs. 16%, Hb F 57% vs. 55%, Hb A 27% vs. 29%; and Hb FAC: Hb C 20% vs. 20%, Hb F 45% vs. 45%, Hb A 35% vs. 35%).

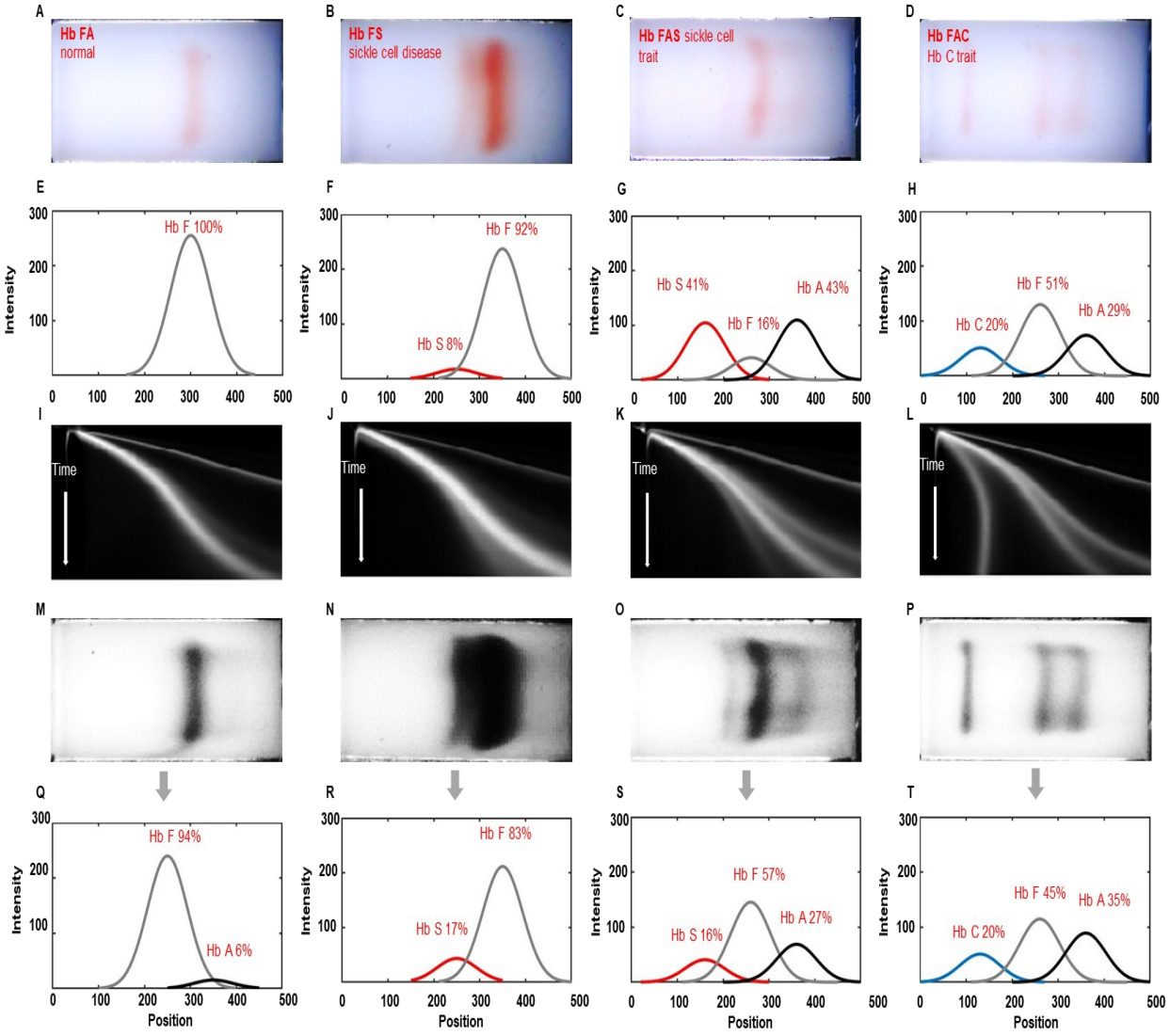


Figure 7. Identification of Hb variants and quantification of Hb percentages by Gazelle-Multispectral System. (A-D) The first row shows images captured under the white light field. (E-H) The second row shows electropherograms generated based on the white light images using the white data analysis algorithm. (I-L) The third row illustrates the 2D representation of Gazelle-Multispectral space-time plots of band migration in UV imaging mode used with the machine learning algorithm. (M-P) The fourth row shows images captured under the UV light field. (Q-T) The fifth row shows electropherograms generated based on the UV light images using the UV data analysis algorithm. The Gazelle-Multispectral UV data analysis algorithm sensitively and accurately identified Hb variants agreeing with HPLC reported results. Column 1 – 4: Multispectral

test results for samples with different phenotypes. Column 1: Hb FA (Healthy newborn, Hb A: 8% vs. 6%, Hb F: 92% vs. 94%, Gazelle-Multispectral vs. HPLC); Column 2: Hb FS (Newborn with sickle cell disease, Hb F 83% vs. 89%, Hb S 17% vs. 11%, Gazelle-Multispectral vs. HPLC); Column 3: Hb FAS (Newborn with sickle cell trait, Hb S 16% vs. 16%, Hb F 57% vs. 55%, Hb A 27% vs. 29%, Gazelle-Multispectral vs. HPLC); and Column 4: Hb FAC (Newborn with Hb C disease, Hb C 20% vs. 20%, Hb F 45% vs. 45%, Hb A 35% vs. 35%, Gazelle-Multispectral vs. HPLC). Gazelle-Multispectral enabled identification and quantification of low concentration Hb variants with higher sensitivity (I–T) compared to white light imaging mode (A-H).

3.4 The system Hb variant quantification indicates high correlation with HPLC

A total of 321 completed tests from both Gazelle-Multispectral and HPLC acquired at Korle Bu Teaching Hospital in Korle Bu, Ghana was included in this study. We defined ‘Valid’ and ‘Inconclusive’ tests according to published recommendations in literature [41] and the STARD guidelines [42]. A ‘Valid’ test was defined as a test that performed as expected according to objective standards and the test result was reported properly from the data analysis algorithm. An 'Inconclusive' test was a test that performed adequately according to an objective set of standards. However, an 'Inconclusive' test has quantification confidentiality value automatically evaluated by the algorithm that is lower than the preset threshold value, which can be recognized at the end of the test. Reasons for 'Inconclusive' tests include the appearance of a band or bands at or close to the borderline region between two adjacent detection windows. In this study, 297 out of 321 (92.5%) tests were recognized as 'Valid', while 24 out of 321 (7.5%) tests were recognized as 'Inconclusive'., $p<0.001$, PCC = 0.95).

Pearson correlation analysis and Bland-Altman analysis were performed on the 297 tests recognized as 'Valid'. The correlation plots include the Gazelle-Multispectral determined Hb variant levels (y-axis) versus the Hb variant levels reported by the HPLC including Hb A, Hb F, Hb S, and

Hb C/E (**Figure 8, First column**). The Bland-Altman analysis plots demonstrate the difference between Gazelle-Multispectral determined Hb variant levels (y-axis) at the entire range of Hb levels detected (x-axis). The results from Pearson correlation analysis demonstrate Pearson correlation coefficient (PCC) of 0.97, 0.97, 0.89, and 0.96 for Hb A, Hb F, Hb S, and Hb C/E, revealing a strong association between Gazelle-Multispectral determined Hb variant levels and HPLC reported Hb variant levels (**Figure 8 A, C, E, G**). As a result, the correlations between Gazelle-Multispectral and HPLC were determined at Pearson correlation coefficient (PCC) of 0.84, $p < 0.05$ for Hb A; PCC = 0.95, $p < 0.05$ for Hb F; PCC = 0.92, $p < 0.05$ for Hb S; and PCC = 0.96, $p < 0.05$ for Hb C/E. These results reveal a strong association between Gazelle-Multispectral determined Hb variant levels and HPLC reported Hb variant levels (**Figure 8, First column**). Bland-Altman analysis showed Gazelle-Multispectral determines blood Hb variant levels with mean bias of 2.3% for Hb A (Limits of agreement, LOA: -9.2% to 13.8%); -2.7% for Hb F (LOA: -16.0% to 10.5%); 0.7% for Hb S (LOA: -5.5% to 7.0%), and -0.3% for Hb C/E (LOA: -3.0% to 2.4%, **Figure 8, Second Column**).

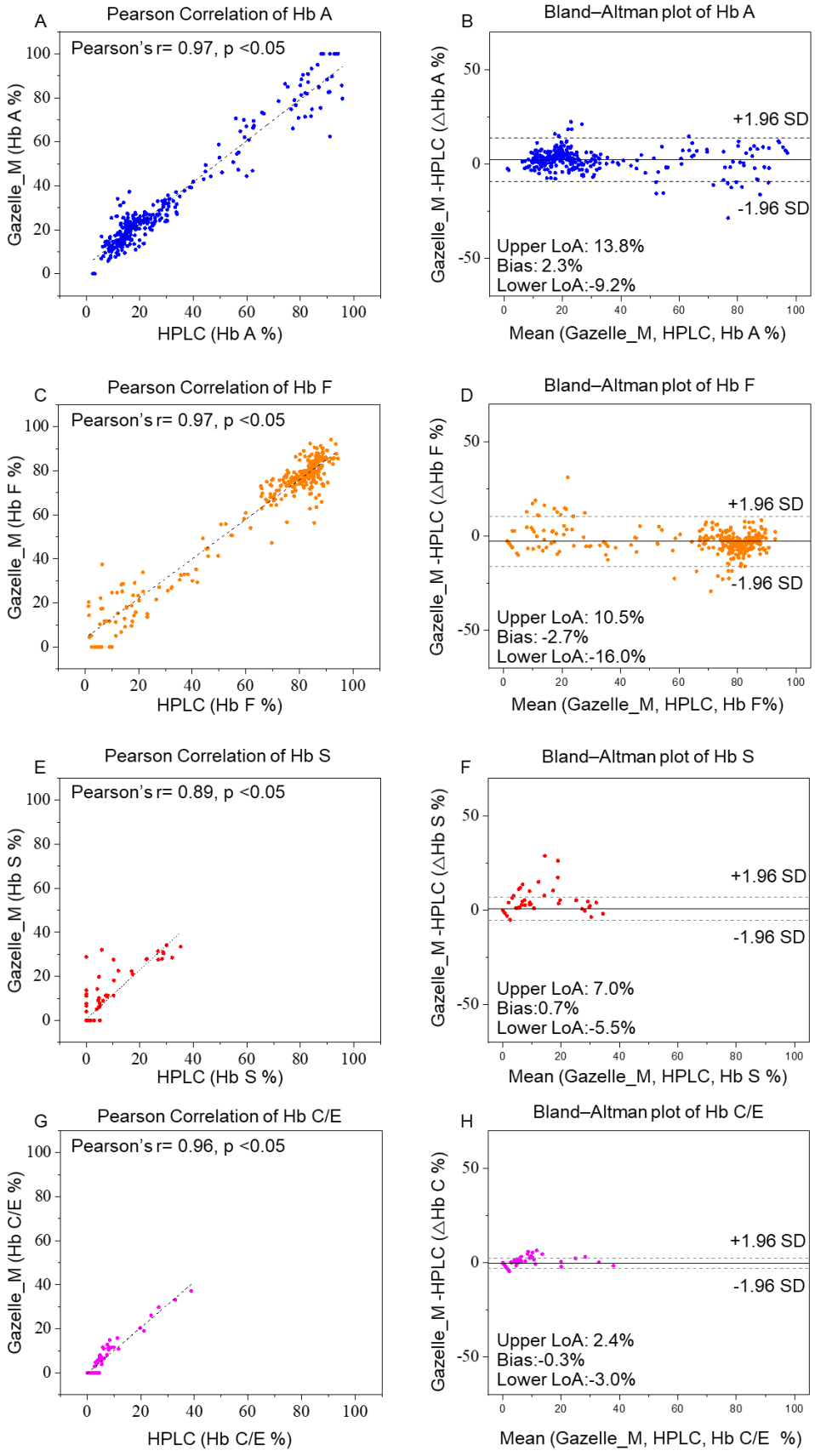

A
Pearson Correlation of Hb A
Pearson's r= 0.97, p <0.05
Gazelle_M (Hb A %)
HPLC (Hb A %)
B
Bland–Altman plot of Hb A
+1.96 SD
-1.96 SD
Upper LoA: 13.8%
Bias: 2.3%
Lower LoA:-9.2%
Gazelle_M -HPLC (ΔHb A %)
Mean (Gazelle_M, HPLC, Hb A %)
C
Pearson Correlation of Hb F
Pearson's r= 0.97, p <0.05
Gazelle_M (Hb F %)
HPLC (Hb F %)
D
Bland–Altman plot of Hb F
+1.96 SD
-1.96 SD
Upper LoA: 10.5%
Bias: -2.7%
Lower LoA:-16.0%
Gazelle_M -HPLC (ΔHb F %)
Mean (Gazelle_M, HPLC, Hb F%)
E
Pearson Correlation of Hb S
Pearson's r= 0.89, p <0.05
Gazelle_M (Hb S %)
HPLC (Hb S %)
F
Bland–Altman plot of Hb S
+1.96 SD
-1.96 SD
Upper LoA: 7.0%
Bias:0.7%
Lower LoA:-5.5%
Gazelle_M -HPLC (ΔHb S %)
Mean (Gazelle_M, HPLC, Hb S %)
G
Pearson Correlation of Hb C/E
Pearson's r= 0.96, p <0.05
Gazelle_M (Hb C/E %)
HPLC (Hb C/E %)
H
Bland–Altman plot of Hb C/E
+1.96 SD
-1.96 SD
Upper LoA: 2.4%
Bias:-0.3%
Lower LoA:-3.0%
Gazelle_M -HPLC (ΔHb C %)
Mean (Gazelle_M, HPLC, Hb C/E %)

Figure 8. Gazelle-Multispectral Hb variant identification and quantification in 297 clinical newborns samples. Pearson correlation (Column 1) and Bland-Altman analysis (Column 2) showed Gazelle-Multispectral identified and quantified Hb A (**A&B**, Pearson coefficient correlation (PCC) = 0.97, $p < 0.05$, Mean bias ± 1.96 × Standard Deviation (SD) = 2.3% ± 11.5%), Hb F (**C&D**, PCC = 0.97, $p < 0.05$, Mean bias ± 1.96SD = -2.7% ± -13.3%), Hb S (**E&F**, PCC = 0.89, $p < 0.05$, Mean bias ± 1.96 SD = 0.7% ± 6.3%), and Hb C/E levels (**G&H**, PCC = 0.96, $p < 0.05$, Mean bias ± 1.96SD = -0.3% ± 2.7%) agree with the ones reported by laboratory standard HPLC. In Column 2, the solid black lines indicate the mean biases, and the dashed gray lines represent 95% limits of agreement

3.5 The system performs SCD newborn screening at high sensitivity and specificity

We investigated the sensitivity and specificity of Gazelle-Multispectral SCD screening based on the identified and quantified Hb variant levels comparing to the ones reported by HPLC. In this study, Gazelle-Multispectral test results included the following (**Table 1**): SCD (Hb SS/Hb SF), Hemoglobin C Disease (Hb SC/Hb SFC), Sickle Cell Trait (Hb AS/Hb ASF), and normal (Hb AA/Hb AF). Seven subjects with normal Hb (Hb AA/Hb AF) were identified as Sickle Cell Trait (Hb AS/Hb ASF, Table 1). One subject with Sickle Cell Trait (Hb AS/Hb ASF) was identified as normal Hb (Hb AA/Hb AF, Table 1). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 100% for disease vs. normal and disease vs. trait. Sensitivity, specificity, PPV, and NPV were 98.2%, 97.1%, 88.7%, and 99.6% for trait vs. normal.

Table 1: Gazelle-Multispectral screening sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) in comparison to reference standard method.

	Disease vs. Normal[a]	**Disease vs. Trait** [b]	**Trait vs. Normal** [c]
True positive, TP	3	3	55
True negative, TN	231	55	231
False Positive, FP	0	0	7
False negative, FN	0	0	1
Sensitivity, TP/ (TP + FN)	100.0%	100.0%	98.2%
Specificity, TN/ (TN + FP)	100.0%	100.0%	97.1%
PPV, TP/ (TP + FP)	100.0%	100.0%	88.7%
NPV, TN/ (TN + FN)	100.0%	100.0%	99.6%

[a] Hb SS/Hb SC/Hb SF/Hb SFC vs. Hb AA/Hb AF

[b] Hb SS/Hb SC/Hb SF/Hb SFC vs. Hb AS/Hb AC/Hb ASF/Hb SFC

[c] Hb AS/Hb AC/Hb ASF/Hb SFC vs. Hb AA/Hb AF

4. Discussion

4.1 Advantages of Gazelle-Multispectral System

Gazelle-Multispectral implements multispectral imaging into a point-of-care hemoglobin electrophoresis test. Hb F expression represents up to 90% of total Hb expression in newborn babies [17, 43]. Gazelle-Multispectral allows sensitive detection and quantification of low concentration Hb variants with a limit of detection at 4%, thus enables SCD screening for Hb variants among newborns having low levels of Hb A and Hb S. Gazelle-Multispectral algorithm automatically provides Hb variant identification and quantification results of relative Hb percentages and does not require users to perform result interpretation (**Figure 9**). The Korle Bu results showed high sensitivity and specificity percentage regarding disease vs normal, disease vs trait and trait vs normal. Due to rapid results, the use of this system device can facilitate the early diagnosis of sickle newborn and that the reliability of this test is comparable to HPLC.

Secondly, the system is easy to operate and can be administered by an entry-level healthcare worker after a short (5 minute) video training. Only a finger-stick or heel prick drop of blood is needed and stamped into a cartridge inserted into the reader, then the test procedures can be completed.

Additionally, Gazelle-Multispectral uses low-cost disposable cartridges, and does not require a dedicated lab environment and can be operated based on battery power. The price to the end-user per test is around 2 dollars, which can be reduced further when manufactured in high volumes. These critical features distinguish Gazelle-Multispectral from currently available laboratory methods and other emerging POC technologies and enables affordable, simple, and rapid newborn screening for Hb variant at the point of care.

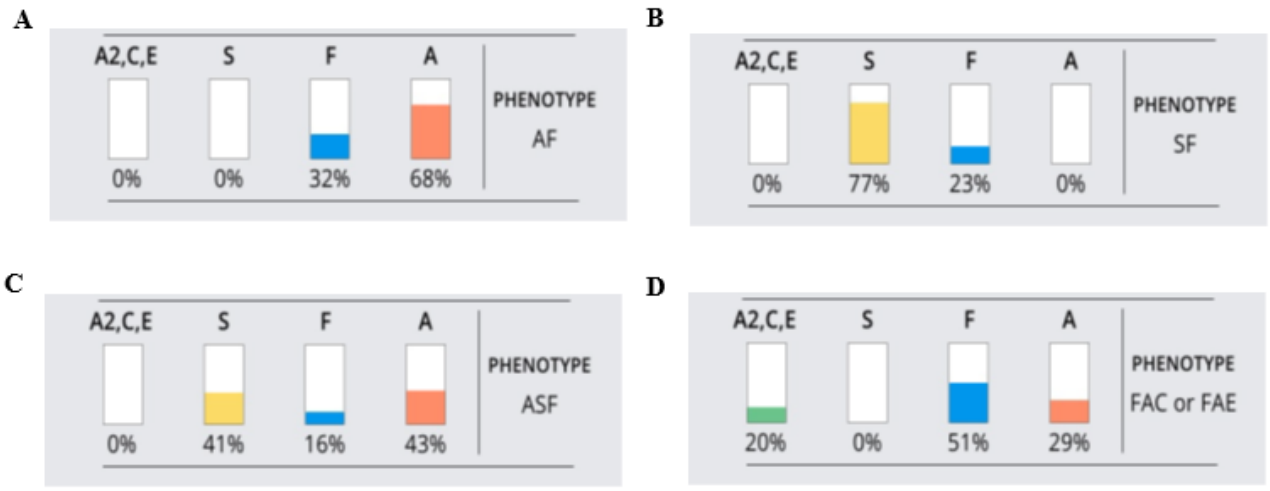


Figure 9. The Hb variant identification and quantification results of relative Hb percentages from the Gazelle-Multispectral System based on different patient types. (**A**) The Hb interpreted results of AF phenotype patient. (**B**) The Hb interpreted results of SF phenotype patient. (**C**) The Hb interpreted results of ASF phenotype patient. (**D**) The Hb interpreted results of FAC or FAE phenotype patient.

4.2 Previous Technologies

For normal SCD diagnosis, other techniques have been developed including density-based separation [44], Raman spectroscopy [45], polymerase chain reaction (PCR) [46], and locked nucleic acid (LNA) probing [47]. However, these developed techniques are time-consuming, costly, and require special equipment. Particularly for newborn SCD diagnosis, a paper-based technique has been used for distinguishing hemoglobin variant types by evaluating the trait of blood stain patterns produced on a paper substrate [48]. When the blood is dropped on a paper, the sickle Hb remains entrapped by the paper substrate at the center of the stained paper because of the insolubility and polymerization of deoxy-Hb S in saponin and sodium hydrosulfite. Other forms of Hb remain soluble and transport laterally to the periphery of the stain by capillary action. The key to developing this method for newborn is to optimize the test to increase its sensitivity for Hb S<20% and investigating whether Hb F>60%-70% have any effect on the diagnostic accuracy of

the Hb S test. However, the limitation of this method can only detect Hb S and Hb A for diagnosing normal blood (Hb AA), Sickle cell trait (Hb AS), and pure SCD patients (Hb SS), whereas the detection of other Hb disorders, such as Hb SC is not attainable [49].

Secondly, investigation of blood spot tryptic peptides performed by using tandem mass spectrometry (MS/MS) is another common method to identify Hb variant disease. This instrument is sensitively enough to diagnose the various peptides by using single ion transitions in blood spots from premature infants and Hb S peptides in blood spots at concentrations 1% of the total Hb present. Although such technology can successfully identify infants with the disease states of SCD, many sickle cell carrier infants are unable to be detected which results in unnecessary follow-up testing and referral for genetic counseling [50, 51].

Isoelectric focusing (IEF) is a current practice of classical laboratory methods and semi-automated systems for the Hb variant, which is able to run several gels that entail need labor-intensive steps. Easier work-up and documentation of gels are achieved using correlative scanning software. Controls for internal quality assurance contain Hb F, Hb A, Hb S and Hb C [52, 53]. However, compared with our gazelle system, this lab method requires a large amount of labor and a high price. It is not easy to be popularized in poor areas with multiple SCD.

Compared to other methods, our current gazelle device offers a rapid, cost-efficient, accurate, and reliable approach for diagnosing SCD in infants. Compared to laboratory-scale assays, the gazelle technology is cheaper, faster, and more accurate (**Table 2**).

Table 2: Summary of newborn SCD diagnosis and competitive advantages of Gazelle-Multispectral system								
Technology	**Test Time**	**Cost / Test**	**Quantitative Results**	**Automated Interpretation**	**Digital Record**	**Hb Types**	**NBS**	**Other Limitations**
Paper-based technique [48]	20 min	$0.5	No	No	No	A, S	No	- Does not distinguish Hb SC. - Hb SS, Hb AS, Hb AA only
Tandem mass spectrometry (MS/MS) [50, 51]	2 min	$15	No	No	No	A, F, S, C	Yes	- Unable to detect sickle cell carrier infants
Isoelectric focusing [52, 53]	>20 min	About $6	No	No	No	A, F, S, C	Yes	- Large amount of labor work
Gazelle-Multispectral system	**<8 min**	**$2**	**Yes**	**Yes**	**Yes**	**A, F, S, C/E/A_2**	**Yes**	

4.3 Gazelle Point of Care Device vs. HPLC

Our Gazelle-multispectral system provides a simple and quick solution to POC diagnosis of SCD and other Hb disorders. The system is a cheaper, easy-to-use, and disposable mobile device that can identify and quantify newborn hemoglobin variants based on the finger-prick volume of blood. It identifies and quantifies newborn Hb variant types on a piece of tested CA paper in cartridge under the UV light and electric field conditions. The whole system can process, analyze, and display the results to the patient for about 8 minutes. Compared with related HPLC results, using Gazelle multispectral system with UV imaging coupled with machine learning algorithm can successfully detect different hemoglobin types including Hb C/E/A2, Hb S, Hb F, and Hb A and their fraction in Newborn blood samples. Under the UV light, the separation between different Hb bands can be visible clearly, making the identification and quantification of low concentration Hb variants in newborns possible. In addition, based on the system quantitative analysis results, the Hb identification and quantification of the Gazelle-Multispectral imaging system show a high correlation with HPLC. The system can identify different types of Hb disorders including homozygous Hb SS (SCD), heterozygous Hb AS (SCT), and Hb SC (hemoglobin SC disease) in newborns. The limits of detection of white field (8%) and UV (4%) light for types Hb C/A2, Hb S were further validated through two representative clinical newborn samples (**Figure 6**), indicating

that UV light has high sensitivity and specificity to diagnose them accurately.

Since this platform is designed to analyze individual newborn samples rapidly, patients can receive their results when the test is finished. In addition, this affordable, portable, easy-to-use, accurate, microsystems-based point-of-care (POC) system can remove sample transfer to central laboratories and improve patient access.

4.4 Device Improvements

In this clinical study conducted among 321 newborns in Korle Bu, Ghana, Gazelle-Multispectral demonstrated 100% sensitivity and specificity for identifying SCD and Hb C Disease vs. normal; and SCD and Hb C Disease vs. SCD Trait and Hb C Trait; Additionally, Gazelle-Multispectral demonstrated 98.2% sensitivity and 97.1% specificity for identifying SCD trait and Hb C Trait vs. normal. The common procedure in Hb tests is to confirm all positive test results with a secondary method prior to final diagnostic decision and treatment initiation [26]. Hence, all positive disease tests would likely result in a secondary confirmatory test to eliminate false positives.

Hb electrophoresis techniques overall share a common limitation in discerning certain Hb variants due to their similar electrophoretic mobilities at given condition. For example, it is challenging to discern Hb C and Hb A2, even using the laboratory platform of capillary zone electrophoresis because these Hb S demonstrate partially overlapped peaks within the same detection window [54, 55]. This peak overlapping is also a challenge for the reference standard HPLC as well as its alternatives [54, 56, 57]. Notably, Hb C and Hb E are known to co-migrate in paper-based hemoglobin electrophoresis. Therefore, Gazelle-Multispectral reports Hb C/E instead of reporting Hb C or E individually. However, these Hb variants display distinct geographical prevalence and distribution. For example, Hb C is highly prevalent in West Africa, which is related to this study [58], while Hb E is the most prevalent in the Mediterranean region, Southeast Asia,

and the Indian subcontinent [59]. Test location, the ethnicity of the subject, and clinical history can be used to facilitate differentiate between these co-migrating Hb types. None of the test subjects was identified with Hb E by either Gazelle-Multispectral or HPLC in this study. For example, all Hb variants recognized as Hb C/E were identified as Hb C in this study. Besides, Hemoglobin D is another Hb variants which is also the most frequent abnormal hemoglobin variant in Middle East and South Asia. People who have hemoglobin D disease can reduce the number and size of red blood cells in their body, causing mild anemia. Therefore, expanding our Gazelle-Multispectral system to detect Hb D is a direction for further work.

Secondly, in terms of dilute experiments for limit of detection, considering use fetal patient samples instead of using AA healthy samples will be the next steps to analyze the LoD of Hb S. There is large amount of Hb F inside newborns, which may affect the quantification of Hb S. So using fetal samples can help simulate this situation where the conclusion of LoD will be more reliable.

Preliminary studies of data (**Figure 5** and **Figure 6**) indicate that the LoD should be around 3%, where the required LoD is 4% under UV illumination. Additional image analysis techniques such as using the better fitting curve model or variations in the UV intensity or illuminating wavelength can enhance sensitivity if needed. It is worth noting that in newborns, the total Hb level is not expected to confound the Hb variant percent limit of detection (Hb% LoD) due to the total Hb level being consistently high (non-anemia range) immediately after birth [60].

4.5 Newborn Sickle Cell Screening and Low Hemoglobin Variant Detection

About 50 to 95% of the total hemoglobin in newborns are fetal hemoglobin, which will decline over the course of 6 months. As the levels of hemoglobin F gradually decrease and reach adult levels (less than 1% of total hemoglobin) usually within the first year, as adult forms of hemoglobin begin to be produced [17, 61] Even though, such high expression of fetal Hb will

influence the SCD phenotype in babies which become symptomatic after 6 months [62]. This phenomenon has become an issue for accurately quantifying the other hemoglobin and the mobility-based diagnostic test, resulting in reduced sensitivity and specificity [63].

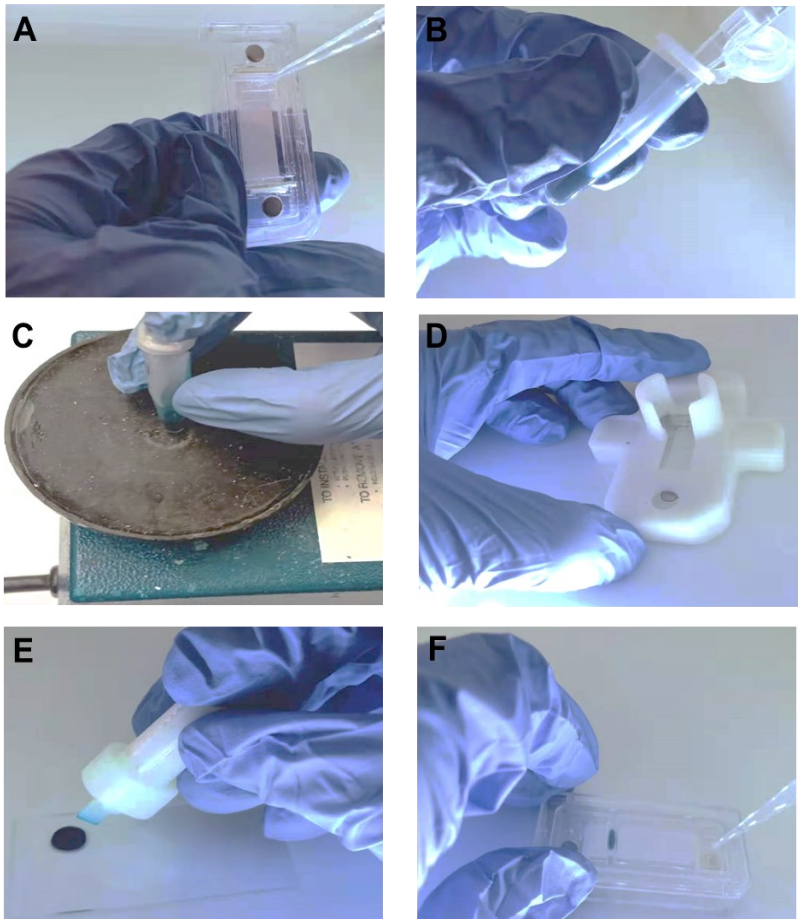
Newborn screening may be integrated to already established immunization programs in Africa [42-44], where babies are vaccinated around 2 months of age. Around this time, Hb F levels in a baby would decrease significantly as they would start replacing Hb F with adult hemoglobin. Besides the normal SCD, Non-beta chain Hb disorder including alpha thalassemia, are also major global health problems in children particularly in Asia region. Our Gazelle-Multispectral system can be extended to this fetal population once the diagnosis of beta-chain abnormalities, such as Hb SS and Hb SC by the system, have been built up. This platform will likely be adaptable to the need for detecting additional variant Hb, such as Hemoglobin Bart's (4 fetal beta-type gamma chains) or Hb H (4 adult beta-type chains), which are elevated in alpha thalassemia.

5. Conclusion

In summary, Gazelle-Multispectral enables affordable and rapid identification of common Hb variants in newborns at the point-of-need. The Gazelle-Multispectral reader provides animated on-screen instructions of step-by-step guidance for test operation procedures to minimize user errors. The internally integrated data analysis algorithm automatically reports Hb variant identification and quantification results in an objective and easily understandable manner. Gazelle-Multispectral system is a versatile, mass-producible, multispectral detection-based electrophoresis platform technology for affordable, rapid, and accurate diagnostic testing and newborn screening programs for SCD at the POC in low resource countries where SCD is at high prevalence. This Gazelle-Multispectral system can further help to reduce the risk of death in baby by initiating early diagnosis and treatment of affected newborns.

Appendix

Appendix 1. Gazelle Cartridge Testing Procedures



(A) CA paper wetting with TBE buffer. **(B, C)** Sample preparation by mixing whole blood with saponin calibrator marker fluid then vortex for complete mixing. **(D, E)** Sample loading using customized manifold and applicator stamper. **(F)** Reservoir filling with background electrolyte (TBE buffer) to complete the electric circuit.

Appendix 2. Overall Experimental Protocol

Preparation:

1. Running buffer (1X TBE buffer+0.11mM surfactant)
2. Blue marker fluid (0.0625g Xc-marker in 10ml ultra-pure water with 1% saponin)
3. Coating solution

Experiments:

1. Inverse the microtube which contains coating solution, and coat the applicator tip (dip in the solution, then shake 5 times, and then allow to dry for 5-7 min)
2. Setup all the Gazelle reader and enter the Hb variant test (Username: A, Password: A), Patient ID
3. Mix blood sample and the blue marker fluid at 1:2 v/v ratio (20 μL + 40 μL) in cuvette and vortex for 20 s
4. While slightly angle the cartridge (45 degrees), put 50 μL of running buffer into the hole, mildly tilt and rotate the cartridge to fully wet the paper. Then allow the cartridge to sit vertically for 1 min
5. Put 20 μL of mixture sample on a piece of a glass slide, load the sample onto the applicator (make sure to angle the applicator and load the sample from one side from the middle of the sample droplet on the glass slide)
6. Apply the sample into the cartridge (make sure to use two fingers from one hand instead of two fingers from different hands, press for 5 seconds)
7. Fill the buffer pool on each side of the cartridge with 200 μL of running buffer
8. Place the cartridge in the reader and then start the test.

Appendix 3. Limit of Detection of Hb S Experimental Plan

Patient ID	Dilution Recipe	Calculated % Hb S	White Light % Results	UV % Results
D0 1	Straight RBC	39.3% S	55% A, 45% S	31%S, 69%A
D0 2	Straight RBC	39.3% S	51% A, 49% S	49%S, 51%A
D0 3	Straight RBC	39.3% S	53% A, 47% S	35%S, 65%A
D1 1	462uL RBC + 138uL CL	19.2% S	73% A, 27% S	27%S, 73%A
D1 2	462uL RBC + 138uL CL	19.2% S	67% A, 33% S	19%S, 81%A
D1 3	462uL RBC + 138uL CL	19.2% S	73% A, 27% S	12%S, 88%A
D2 1	306uL RBC + 294uL CL	12.8% S	82% A, 18% S	16%S, 77%A
D2 2	306uL RBC + 294uL CL	12.8% S	80% A, 20% S	8%S, 92%A
D2 3	306uL RBC + 294uL CL	12.8% S	80% A, 20% S	12%S, 81%A
D3 1	229uL RBC + 371uL CL	9.6% S	86% A, 14% S	10%S, 83%A
D3 2	229uL RBC + 371uL CL	9.6% S	85% A, 15% S	16%S, 84%A
D3 3	229uL RBC + 371uL CL	9.6% S	88% A, 12% S	12%S, 95%A
D4 1	153uL RBC + 447uL CL	6.4% S	100% A	8%S, 95%A
D4 2	153uL RBC + 447uL CL	6.4% S	100% A	4%S,98%A
D4 3	153uL RBC + 447uL CL	6.4% S	100% A	6%S, 94%A
D5 1	400uL D4 + 400uL CL	3.2% S	100% A	100% A
D5 2	400uL D4 + 400uL CL	3.2% S	100% A	100% A
D5 3	400uL D4 + 400uL CL	3.2% S	100% A	100% A
D6 1	400uL D5 + 400uL CL	1.6% S	100% A	100% A
D6 2	400uL D5 + 400uL CL	1.6% S	100% A	100%A
D6 3	400uL D5 + 400uL CL	1.6% S	100% A	100%A
D7 1	200uL D6 + 200uL CL	0.8% S	100% A	100%A
D7 2	200uL D6 + 200uL CL	0.8% S	100% A	100%A
D7 3	200uL D6 + 200uL CL	0.8% S	100% A	100%A

Run Limit of detection of Hb S: Start off running straight RBC D0 (AS Cord sample, 59.1% A, 39.3% S, 0.2% F, 1.4% A2 in HPLC), decide on an estimate for S

D1: Dilute RBC in CL blood (AA sample, 96.3% A, 0.4% F, 3.3% A2 in HPLC) to get S to ~19.2%, run 3 times

D2: Dilute D1 in CL blood to get S to ~12.8%, run 3 times

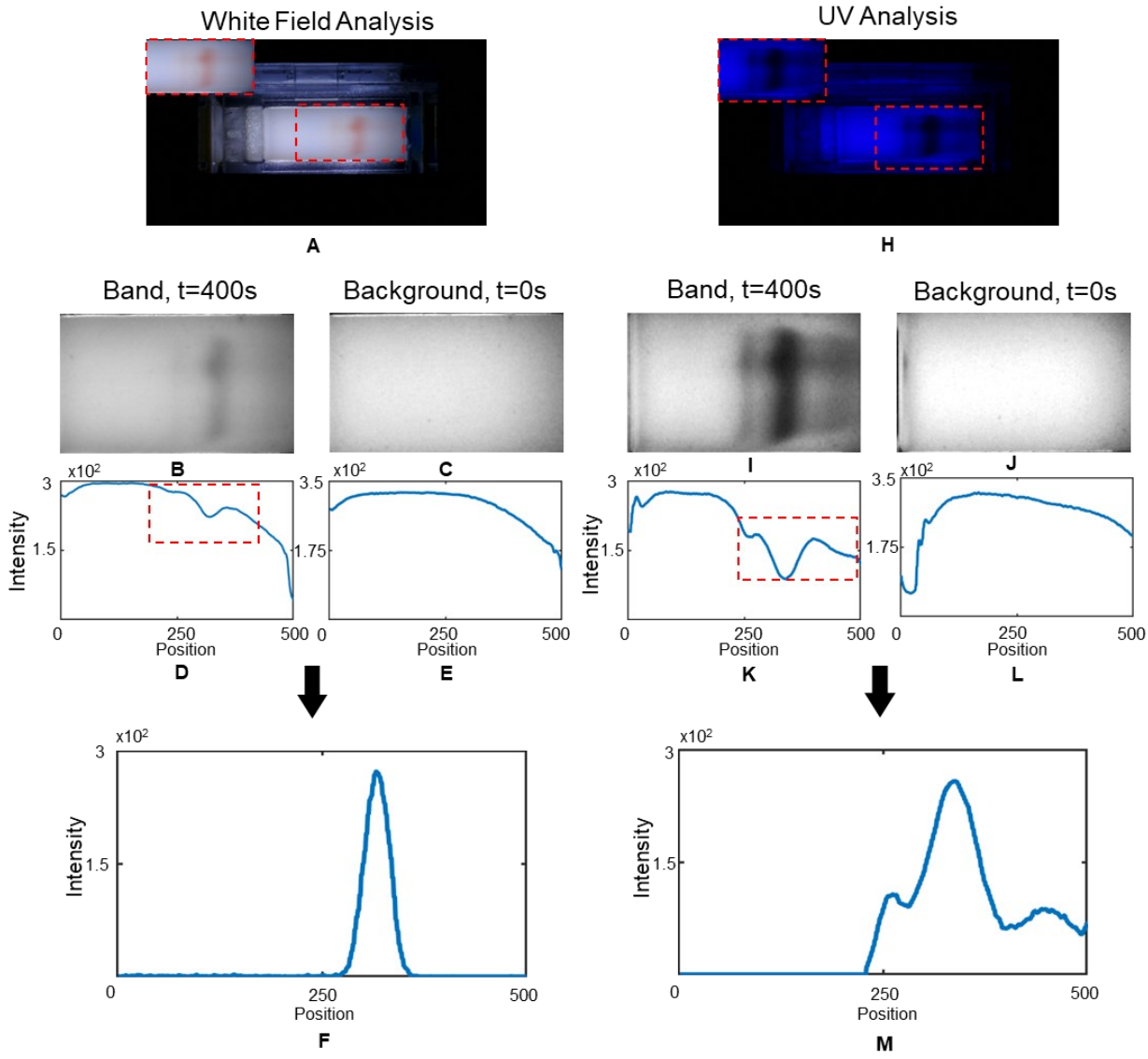
D3: Dilute D2 in CL blood to get S to ~9.6%, run 3 times

D4: Dilute D3 in CL blood to get S to ~6.4%, run 3 times

D5: Dilute D4 in CL blood to get S to ~3.2% run 3 times

Continue to do half dilutions with running 3x until the reader no longer can detect the S band

Appendix 4. The Algorithmic Method for Final Peak Intensity



(**A**) Band separation in white field channel for 400 seconds; (**B-C**) The concentrated areas of white field channel for intensity plot in (**B**) 400 seconds and (**C**) 0 second; (**D-E**) The white field intensity plot for (**D**) 400 seconds and (**E**) 0 second; (**F**) The white field intensity profile when the **plot(E)** subtracts **plot(D)** and taking threshold. (**H**) Band separation in UV channel for 400 seconds; (**I-J**) The concentrated areas of UV channel for intensity plot in (**I**) 400 seconds and (**J**) 0 second; (**K-L**) The UV intensity plot for (**K**) 400 seconds and (**L**) 0 second; (**M**) The UV field intensity profile when the **plot(L)** subtracts **plot (K)** and taking threshold.

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