A Mobile Phone–Based Approach to Detection of Hemolysis

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Abstract

Preeclampsia and HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome are pregnancy-related complications with high rates of morbidity and mortality. HELLP syndrome, in particular, can be difficult to diagnose. Recent work suggests that elevated levels of free cell hemoglobin in blood plasma can, as early as the first trimester, potentially serve as a diagnostic biomarker for impending complications. We therefore developed a point-of-care mobile phone–based platform that can quickly characterize a patient's level of hemolysis by measuring the color of of blood plasma. The custom hardware and software are designed to be easy to use. A sample of the whole blood (~10 μL or less) is first collected into a clear capillary tube or microtube, which is then inserted into a low-cost 3D-printed sample holder attached to the phone. A 5–10 min period of quiescence allows for gravitational sedimentation of the red blood cells, leaving a layer of yellowish plasma at the top of the tube. The phone camera then photographs the capillary tube and analyzes the color components of the cell-free plasma layer. The software converts these color values to a concentration of free hemoglobin, based on a built-in calibration curve, and reports the patient's hemolysis level: non-hemolyzed, slightly hemolyzed, mildly hemolyzed, frankly hemolyzed, or grossly hemolyzed. The accuracy of the method is ~1 mg dL^{-1}. This phone-based point-of-care system provides the potentially life-saving advantage of a turnaround time of about

10 minutes (versus 4+ hours for conventional laboratory analytical methods) and a cost of approximately one dollar USD (assuming you have the phone and the software are already available).

1. Introduction

Pregnancy-related complications are the fourth-leading cause of death in developing countries (World Health Organization, 2005), where expecting mothers are 36 times more likely to be affected by complications than are mothers in developed countries (World Health Organization, 2015). Major causes of maternal and neonatal deaths include preeclampsia, a hypertensive pregnancy disorder, and HELLP syndrome (*H*emolysis, *E*levated *L*iver enzymes, and *L*ow *P*latelet count), an associated complication. Hemolysis is the rupturing of red blood cells, which release hemoglobin into the blood plasma. According to the Preeclampsia Foundation (2013), the mortality rate of HELLP syndrome has been reported to be as high as 25%. Overall perinatal mortality from HELLP syndrome (stillbirth plus neonatal death) ranges from 8 to 60% (Turgut et al., 2010).

HELLP syndrome is often difficult to diagnose because its symptoms (headache, nausea, blurry vision) are non-specific and can be mistaken for gastritis, flu, acute hepatitis, gall bladder disease, or other conditions. According to the American Congress of Obstetricians and Gynecologists (ACOG), preeclampsia is diagnosed mainly by new-onset hypertension (blood pressure higher than 140/90) in the second half of pregnancy in combination with new-onset proteinuria (excretion of more than 300 mg protein over a 24-hour period) (ACOG, 2013). HELLP syndrome can progress very quickly, potentially resulting in multiple-organ failure, coma, or death in as little as three hours. Thus, rapid and reliable disease diagnosis is critical for patient survival.

Recent findings show that blood plasma samples from preeclamptic women contain elevated levels of cell-free plasma hemoglobin (Hgb) that could serve as a diagnostic biomarker from even the first trimester. The critical sequence of events begins with fetal hemoglobin leaking over to the maternal circulation system. The resulting protein increase causes oxidative damage to the placental barrier (Hansson et al., 2014) and reduces the availability of free NO, which in turn results in vasoconstriction. Examination of the placenta and maternal endothelium indicates that oxidative stress induced by the fetal hemoglobin contributes majorly to the pathology of preeclampsia (Hansson et al., 2014). In preeclampsia, HELLP, and eclampsia, and the coagulation system are both activated by the damaged endothelium, which may in turn cause subacute/acute disseminated intravascular coagulation (Thachil and Toh, 2009). In this condition, a dysregulation of coagulation and fibrinolysis results in lower platelet counts and fibrinogen levels and greater consumption of antithrombin. Fibrin gradually forms, especially in the kidneys and the liver (Hansson, 2014). In this way, acutely high levels of free Hgb contribute to kidney damage (Hansson et al., 2014).

With the abovementioned series of events, elevated levels of free-cell hemoglobin can be measured in the first trimester. Preeclamptic women have 53% higher cell-free plasma hemoglobin concentrations than healthy pregnant women (5.51 ± 0.56 mg dL^{-1} versus 3.62 ± 0.37 mg dL^{-1}) (Sandrim et al., 2010). Due to these higher Hgb concentrations, preeclamptic women also consume 63% more nitric oxide (NO). Later in pregnancy, the high Hgb levels correlate with high blood pressure in preeclamptic women (Hansson et al., 2014). The same correlation can be made in other hemolytic diseases such as autoimmune hemolytic anemia, sickle cell anemia, and malaria, which are also known to cause kidney disease (Bunn et al., 1977). High concentrations of hemoglobin in plasma can result in multiple-organ failures and potentially death. For all of these conditions, then,

a cost-effective, compact, point-of-care analyzer for detecting Hgb in blood plasma would be of great benefit (Archibong et al., 2015; Adiga and Yogish, 2016; Šimundić et al., 2010).

Conventional methods for detecting hemolysis rely on spectrophotometric determinations or sometimes visual estimations of a clinical quantity known as the hemolysis index (HI). A patient's blood sample is typically sent to automated laboratory, where the sample is centrifuged and the separated blood plasma is analyzed. The laboratory instruments are bulky (Dolic and Panteghini, 2014; Thomas, 2013) and turnaround times may be long (on the order of hours) relative to the urgency of some medical conditions. Delays in processing also increase the likelihood of cell lysis occurring in the sample during the time between collection and analysis, thus potentially confounding measurements of Hgb concentrations and other medically significant quantities. Recent advances in microfluidics (e.g., Crowley et al., 2005) have been applied to the isolation of blood plasma but large, expensive spectrophotometric instruments are still required to measure analyte concentrations. Clinically, an additional diagnostic challenge is that hemolysis-related events are often inferred from the concentrations of other blood constituents rather than being assessed from direct measurements of Hgb itself.

For some types of chemical analyses, mobile phones provide an ideal analytical platform for a fully automated, point-of-care tool (Breslauer et al., 2009; Zhu et al., 2011). For example, the Ozcan Laboratory has developed a mobile phone–based platform for microscopy and flow cytometry (Zhu and Ozcan, 2013; Zhu, 2011). Advances related to phone-based imaging include colorimetric assays and the development of a super-resolution sample imaging algorithm (Breslauer et al., 2010; Cui et al, 2008; Zheng et al., 2010)). Example medical applications include *E. coli* detection (Zhu et al., 2012) and urinalysis for detection of the prostate cancer marker PCADM-1 (GENTAG Inc and MacroArray Technologies, LLC, 2014). Examples of

environmental applications include analyses of toxic trace elements and pesticides (Wei et al., 2014; Mei at al., 2016).

Mobile phones are also widely available worldwide and are easy to use for mobile health (mHealth) applications (Kahn et al., 2010). An additional benefit is the ability to transmit and receive information in real time. We therefore developed an mHealth platform for the prompt, quantitative, onsite assessment of in vivo hemolysis (i.e., free plasma hemoglobin) in a small, fresh sample of citrated blood. The method requires a high-quality mobile phone camera with a flashlight, a 3D-printed hardware accessory, and a custom image-processing app. The primary user-managed steps in the procedure are to (a) collect the blood sample, (b) wait for gravitational settling of the red blood cells, and (c) photograph the resulting plasma layer. The result is reported to the user as a category of hemolysis: non-hemolyzed, slightly hemolyzed, mildly hemolyzed, frankly hemolyzed, or grossly hemolyzed. The entire procedure requires about 10 minutes.

2. Design, Materials, and Procedures

2.1. Mobile lab: hardware and software design

The complete point-of-care system for hemolysis detection consists of a mobile phone fitted with an attached sample holder (Figure 1). The prototype platforms used a Samsung Galaxy Nexus 19250 phone and a Nokia Lumia 520 phone. Both use the Android mobile operating system. The Samsung has a five-megapixel camera with a built-in LED flashlight, and the Nokia phone is similar. The primary cost of the platform is the smartphone.

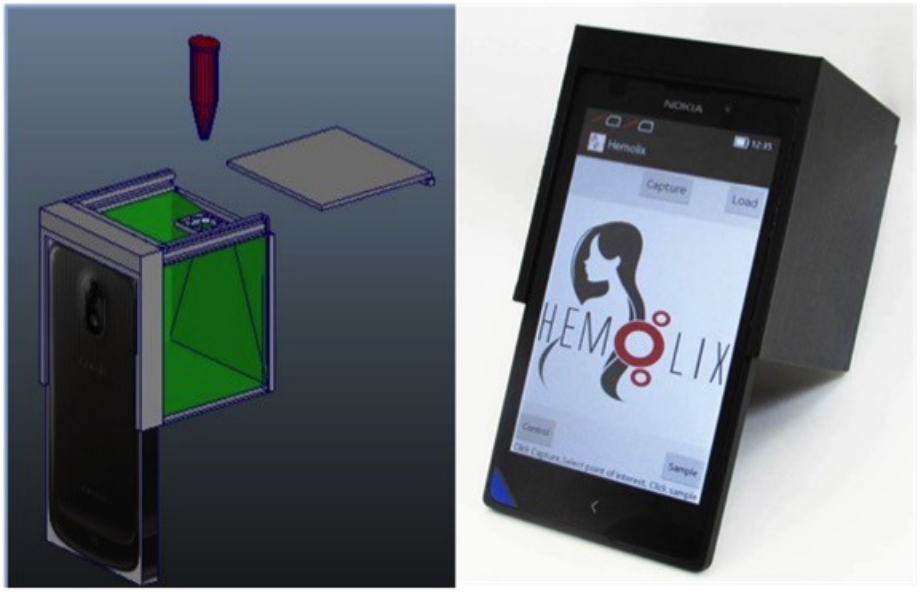

Figure 1: Illustration and photograph of the mobile phone–based hemolysis-measurement platform. Left: The sample holder, with a ring slot and a lid, is connected to the back of the mobile phone. Right: One of the actual prototypes, with the 3D-printed sample holder attached to the phone. The screen on the phone shows the logo of the first software prototype ("Hemolix").

The sample holder includes a slide-on lid and a ring slot (Figure 1). The lid serves to cover the sample compartment during measurements (keeping lighting conditions constant), and the ring slot serves to hold a microtube or capillary tube of blood specimen within the field of view of the smartphone camera. We used a capillary tube of ~1 mm diameter. The holder attachment is designed and built with a 3D printer according to the dimensions of the phone (in this case, 5.33 x 2.67 x 0.35 inches). The design of the holder can be modified to accommodate different phone sizes. Manufacture of the add-on hardware costs 1 USD per unit.

Because the presence of blood cells interferes with optical detection of hemolysis, centrifugation or some other method of plasma separation is required. Our point-of-care system relies on gravitational sedimentation of red blood cells. The sample holder is therefore designed to hold the sample nearly upright during a 5–10 min period of quiescence (Figure 1). During this

time, the red blood cells settle to the bottom of the specimen tube, leaving a layer of cleared plasma (our constituent of interest) at the top of the tube (Figures 2a–b). A layer 1–2 mm thick is required for imaging.

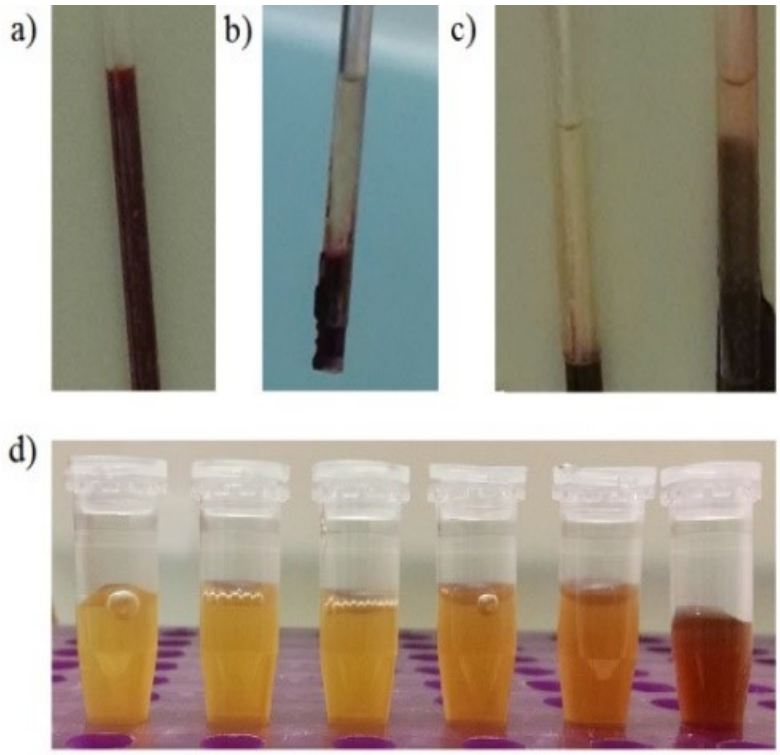

Figure 2: Plasma separation and colorimetric analysis of free hemoglobin concentrations. (a) Human whole blood is first collected into a thin capillary tube (1 mm diameter). (b) Red blood cells are then allowed to settle gravitationally to the bottom of the tube. The top layer (clear color) is isolated plasma. (c) Different levels of hemolysis (i.e., concentrations of hemoglobin) in the plasma are detectable as color differences. The sample on the right is hemolyzed; the sample on the left is not. (d) Solution series showing a range of concentrations of free hemoglobin in plasma in microtubes, from 4 mg dL^{-1} (leftmost sample) to 300 mg dL^{-1} (rightmost sample).

The user then directs the phone software to photograph the blood sample. The custom software app collects 12 sample images and then automatically detects the plasma portion of each image, defining the region of interest (ROI) for further analysis. The ROI is a square of 10 x 10 pixels, which allows for color averaging over the neighborhood of one hundred adjacent pixels.

Red, green, and blue (RGB) values are extracted for each pixel and then converted to CIE*Lab* values, where *L* represents image lightness (brightness), *a* represents the red color component, and *b* represents the yellow color component. These color space components are ideal for characterizing samples that contain hemoglobin (the red component of blood) and bilirubin (the yellow component of plasma).

Color index (CI) is then calculated from the CIE*Lab* parameters:

$$\mathrm{CI} = (\sqrt{(L_i - L_0)^2 + (a_i - a_0)^2 + (b_i - b_0)^2}) \quad \text{Eq. 1}$$

where L_0, a_0, b_0 are the (known) CIELab components of pure plasma and L_i, a_i, and b_i are the (measured) CIELab components of the sample.

Finally, the image-processing algorithm uses a preloaded empirical calibration curve to convert CI to a concentration of free hemoglobin. The result is reported to the user as non-hemolyzed (≤ 5 mg/dl), slightly hemolyzed (5–30 mg/dl), mildly hemolyzed (30– 0 mg/dl), frankly hemolyzed (60–300 mg/dl), or grossly hemolyzed (≥300 mg/dl).

2.2. Calibration

Our calibration procedure was guided by clinically relevant ranges of free plasma hemoglobin. In healthy humans, the reference range is typically 1–4 mg dL^{-1} (Lippi et al., 2012). Values >10 mg dL^{-1} indicate hemolysis (Lippi et al., 2012). During severe hemolytic episodes, values can exceed 100 mg dL^{-1} (Burka et al., 1966).-Figure 2c shows the color difference between transparent yellowish non-hemolyzed plasma (left tube) versus pink hemolyzed plasma (right tube). Plasma color changes incrementally as the concentration of hemoglobin increases (Figure 2d).

Commercially purchased plasma and hemoglobin (both human and bovine) were used to prepare a series of solutions with hemoglobin concentrations ranging from 0 mg/dL (pure plasma)

to 200 mg/dL (severe hemolysis). A calibration curve was then constructed to relate the solutions' CI color parameters to the known concentrations of plasma hemoglobin.

3. Results and Discussion

3.1. Plasma separation

A thin layer of plasma (1–2 mm) in a capillary tube or microtube is required for the image-based hemolysis measurement. In a test tube, the typical rate of erythrocyte sedimentation rate (ESR) is ~15–20 mm/hour. In cases of pathology, the rate can be as high as 100 mm/hour (Davis et al., 2003). Therefore, testing (imaging) of a plasma layer could potentially begin after 5–10 min of sedimentation.

We measured clearance rates for whole blood samples in a capillary tube (diameter 1 mm) and a microtube (2 μL volume) mounted on a vertical stand. Red blood cells settled gravitationally, leaving a clear plasma layer at the top. In capillary tubes (Figure 3, red bars), the layer was sufficiently thick (>1 mm) for image collection and analysis in less than 5 minutes. For microtubes, the time required for adequate sedimentation and plasma separation was close to 30 min. Therefore, capillary tubes are recommended for high-speed operations.

The observed ESR is sufficient for our needs, and still lower than one expected for pregnant women described in the study (Van den Broek et al., 2001) that examined the effects of gestational age on ESR. For non-anemic women, the sedimentation rate (95% reference range) was 18–48 mm/h in the first half of pregnancy and 30–70 mm/h in the second half. For anemic women, the corresponding ranges were 21–62 mm/h and 40–95 mm/h. This finding suggests that 5 minutes settling time will be adequate to obtain several millimeters of imaginable plasma, especially during the second half of the pregnancy, when HELLP syndrome occurs most frequently.

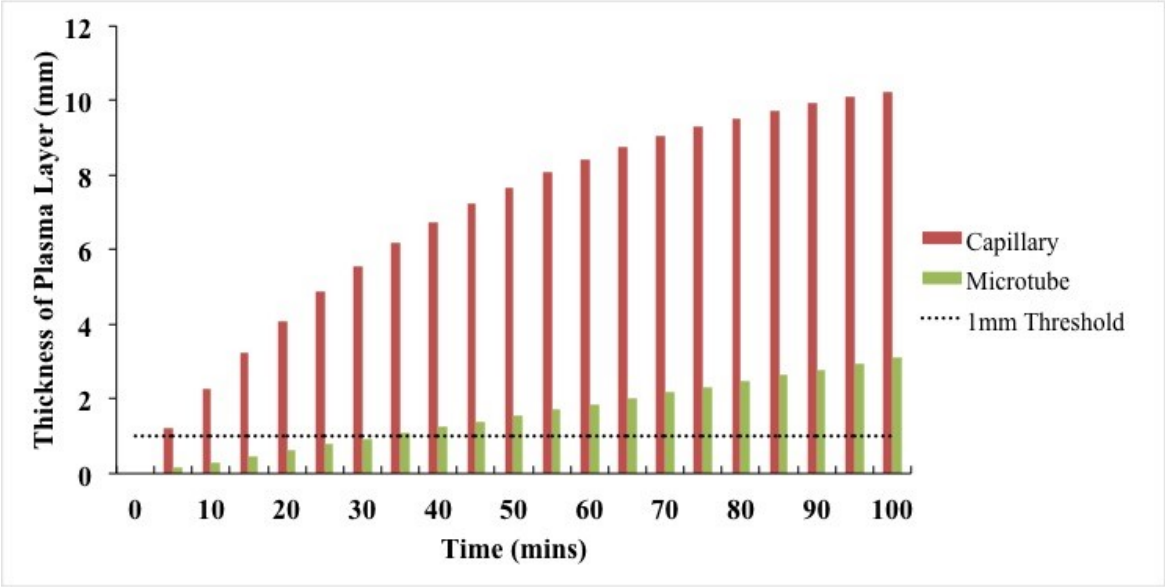

Figure 3: Erythrocyte (red blood cell) sedimentation of Human donor blood in a microtube (green) and a capillary tube (red). The dashed horizontal line shows the minimum thickness (1 mm) required for imaging.

3.2. Plasma color space

The responses of the *L*, *a*, and *b* values for different hemoglobin concentrations were extracted. The intensity of the red value (*a*) is directly proportional to hemoglobin concentrations over the range 0–50 mg/dL, but reaches saturation at concentrations >50 mg/dL. For the channel corresponding to the concentration of proteins in the plasma sample (yellow coloration, *b* values), the intensity is inversely proportional to hemoglobin concentration. Hence, the normalized ratio of all samples to the output values of the blank sample (plasma) increases as the hemoglobin concentration increases. The intensity of parameter *L* (lightness) is also generally inversely proportional to the concentration of free hemoglobin.

3.3. Calibration

An experimentally determined calibration curve is incorporated into the image-processing software for the purpose of converting color index (Equation 1) to a hemoglobin concentration.

These curves vary depending on the phone model. To compare the mobile device with a standard laboratory instrument, a Thermofisher Multiskan Photometer was used to measure absorbance at a wavelength of 405 nm, for a series of reconstituted bovine samples. In order to obtain linear function, we calculated logarithmic values of the exponentially changing dataset. Both instruments perform very similarly and were able to identify unknown samples with agreement within 5-10%.

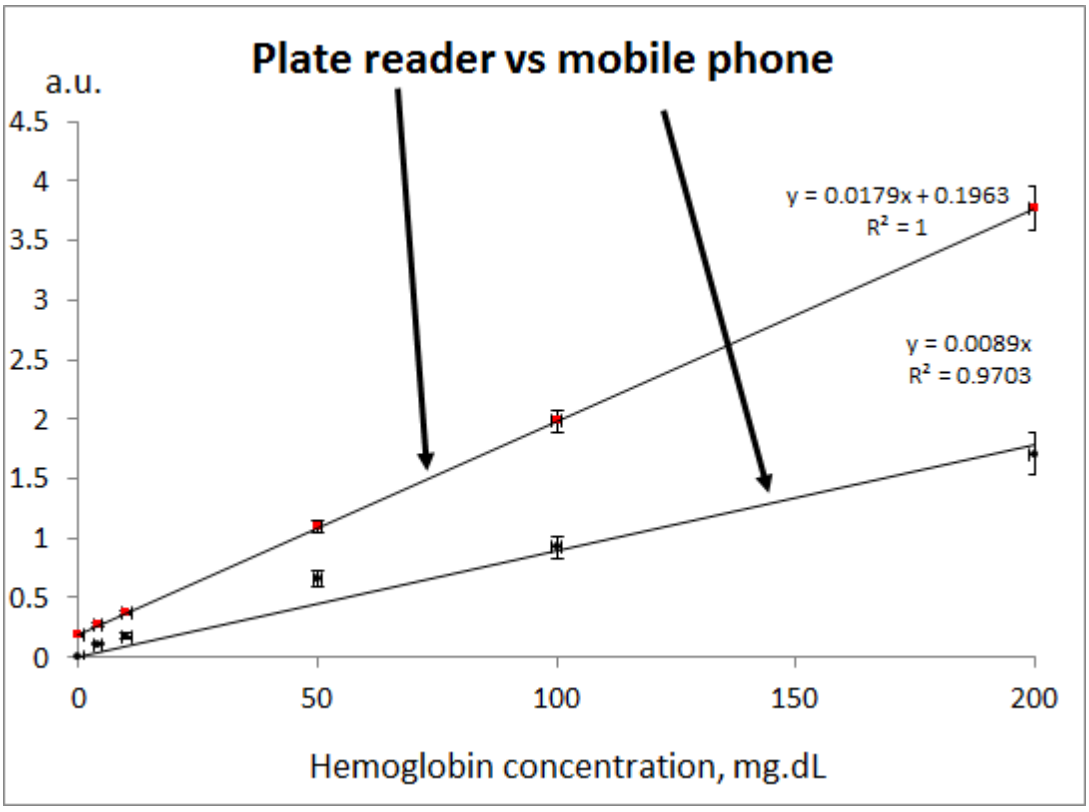

Figure 4: The transfer functions for the plate reader and the mobile phone relating free hemoglobin concentration in a human plasma sample to the plasma color index, CI, as measured on the mobile phone and absorbance value measured by plate reader.

3.4. Assessment and comparison to existing methods

By this method, hemoglobin concentrations can be measured with an accuracy of ~1 mg/dL at lower hemoglobin values. This level of accuracy is sufficient to reliably differentiate the qualitative concentration categories that can be easily interpreted by the user: non-hemolyzed,

slightly hemolyzed, mildly hemolyzed, frankly hemolyzed, and grossly hemolyzed. This approach additionally provides quantitative information about the level of free plasma hemoglobin: ≤5 mg/dL for non-hemolyzed, 5–30 mg/dL for slightly hemolyzed, 30–60 mg/dL for mildly hemolyzed, 60–300 mg/dL for rankly hemolyzed, and ≥300 mg/dL for grossly hemolyzed). Examples of accuracies reported for other systems are 13 mg/dL and 10 mg/dL (Roche Cobas c501 and Siemens Dimension Vista 1500, respectively) (Lippi et al., 2014).

3.5. System operation: the user experience

When the user first opens the Hemolix application, the Welcome screen (Figure 5, left panel) prompts the user to start a new test ("Capture") or load previously collected data ("Load"). A recalibration can be conducted by using the "Control" button, to resets the internal settings of the software. If the user opts to "Capture" (start a new sample analysis), the camera interface prompts the user to manually capture an image. Alternatively, the software can be set by the user to continuously capture images over a 65 sec time period at 5 sec intervals.

The ROI in the plasma layers of the multiple images are automatically determined by the software, and average values for the RGB color components and CIELab values are displayed (Figure 5, middle panel). Finally, the software uses the built-in calibration curve to determine the amount of free hemoglobin, based on the measured color values. The user receives a report about the hemolysis level: normal, slightly hemolyzed, mildly hemolyzed, frankly hemolyzed, or grossly hemolyzed (Figure 5, right panel). At this point, the user can opt to store all data on the phone or send it directly to a care provider via text or email.

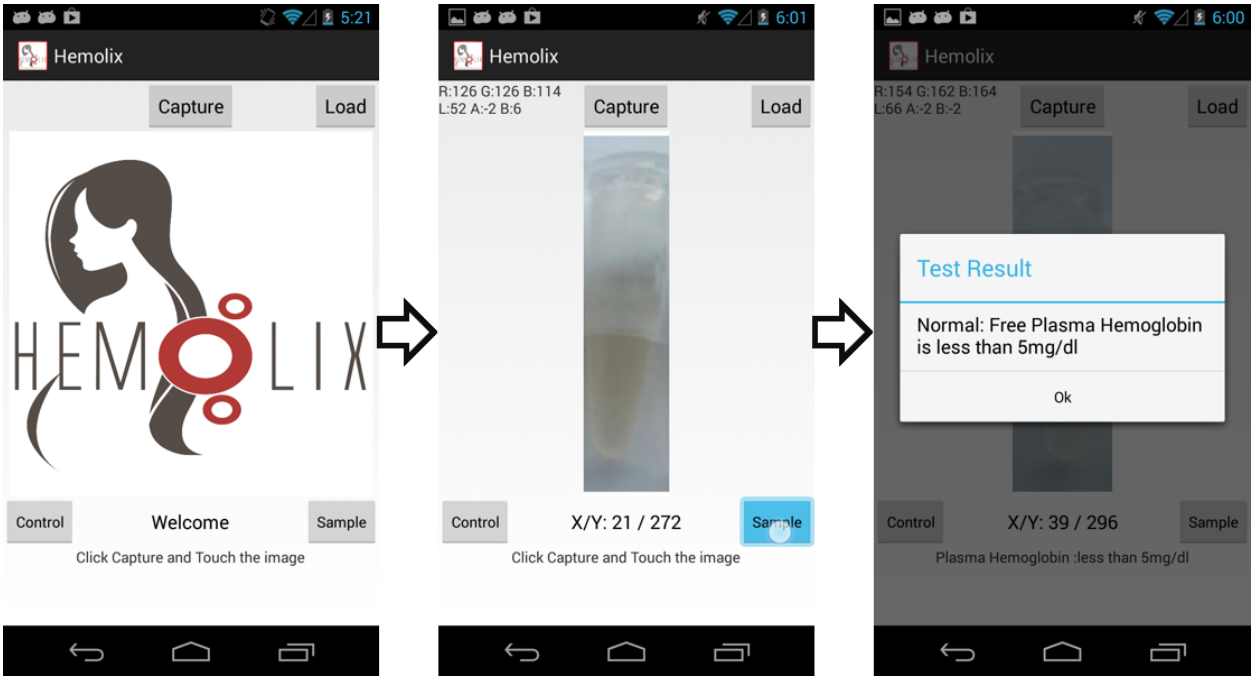

Figure 5: Screenshots of the mobile software interface. Left panel: The Welcome screen. Middle panel: After image capture and plasma-layer color analysis, the screen displays an image of the plasma sample and shows the calculated RGB and CIELab color values (upper left corner). Left panel: Screenshot of the final hemolysis report. In this case, the color analysis determined that the level of free hemoglobin in the blood plasma was Normal.

The rapid turnaround time associated with this POC method of plasma separation is clinically significant. Analysis of blood samples should be conducted with fresh erythrocyte material (fresh blood) only, as delays resulting in aged samples can compromise the results. Standard laboratory analyses can take 4 hours to 4 days for clinicians to receive a patient's results for diagnosis. Our 101-minute POC method can help to significantly speed medical diagnosis and treatment, thus potentially preventing morbidity or mortality. An additional benefit of using capillary tubes is the much smaller volume of blood required.

4. Conclusions

We developed a mHealth platform for low-cost, point-of-care detection of hemolysis. This approach does not require access to specialized laboratory equipment but instead relies on a slightly modified mobile phone and a simple user interface that can be operated by non-

professionals. Blood plasma is separated from the whole blood using gravitational sedimentation and thus can be done promptly without centrifugation. By employing *Lab* color space, which can differentiate varying concentrations of hemoglobin in the blood plasma while also identifying samples with interfering levels of bilirubin we were able to develop an Android application for ultra-fast digital image processing. The LOD of hemoglobin concentration with the mobile device was found to be 1.39 mg/dl, which is within the reference limit of cell-free hemoglobin within plasma. This approach can significantly reduce errors associated with subjective visual methods and can shorten the analysis time relative to that required by standard automated techniques. All of these characteristics could help contribute to improved outcomes for a variety of hemolytic disorders and conditions, especially in developing countries or other settings where resources are limited and prompt detection of hemolysis is critical.

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