



Correcting the Effect of Hematocrit in Blood Coagulation Analysis on Paper-Based Lateral Flow Device

Journal:	<i>Analytical Methods</i>
Manuscript ID	AY-ART-01-2018-000192.R2
Article Type:	Paper
Date Submitted by the Author:	03-May-2018
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Analytical Methods

COMMUNICATION

Correcting the Effect of Hematocrit in Whole Blood Coagulation Analysis on Paper-Based Lateral Flow Device

Received 00th January 20xx,
Accepted 00th January 20xx

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DOI: 10.1039/x0xx00000x

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A simple method to correct the effect of hematocrit (Hct) on a paper-based lateral flow assay device that is designed to assess whole blood coagulation is reported. By quantifying the effect of Hct on red blood cell (RBC) transport, a calibration procedure is obtained that compensates for the Hct effect, while maintaining the dependence of blood coagulation on RBC transport.

Introduction

Whole blood tests are considered the “gold standard” in clinical diagnostics. These tests (e.g. complete blood count, metabolic panel, lipoprotein panel, blood clotting test, blood enzyme test, etc.) are particularly common tests that aid in a physician’s decision during disease diagnosis and/or therapy by providing critical information on internal organ functions, cardiovascular risk factors, blood sugar levels, immune system wellness and hormone balance¹.

Paper-based point-of-care (POC) diagnostics have gained increasing popularity²⁻¹¹ due to their capability to perform low-cost and rapid diagnostic tests in a patient-friendly non-laboratory setting, which include physician’s office, ambulance, at home and a remote site¹². Lateral flow immunoassay (LFIA) technology has been the driving force behind developing simple and low-cost tests with fast detection and high portability¹³⁻¹⁵. Major applications of LFIA-based test are qualitative or quantitative detection of antigens, antibodies, biomarkers, cells, toxins, pathogens, pesticides, heavy metals, drugs and products of gene amplification¹³⁻¹⁵. Conventional LFIA consists of cellulose sample pad for sample collection, fiber glass conjugate pad for conjugate release, nitrocellulose membrane for bioaffinity reaction, and wicking pad for flow assurance.

However, whole blood has not been the preferred test sample for POC diagnostic devices with colorimetric or optical detection due to its intense red color produced by the

hemoglobin within red blood cells (RBCs) which masks the signal of target analyte¹⁶. As a consequence, RBCs are usually removed from whole blood by centrifugation or filtration, and plasma or serum is typically utilized during the test. The centrifugation step lengthens the sample preparation time and increases the test complexity. The popular on-disk centrifugation method^{17, 18} allows immediate testing after the centrifugation in a continuous spin but requires complicated microfluidic channels inside of the disk and an external centrifugal platform (centrifuge, CD/DVD player, etc.). Additionally, the filtration method^{19, 20} requires either RBC agglutinating agent or plasma separation membrane as an extra device component, which increases device complexity. Therefore, identifying a rapid process that eliminates the effect of RBCs, or the effect of patient-specific RBC variabilities in blood (namely hematocrit) without centrifugation or filtration is an important step toward simple and low-cost whole blood analysis.

We have previously introduced the *no-reaction* lateral flow assay (nrLFA) device for low-cost blood coagulation monitoring, illustrated in Fig. 1. Based on conventional LFIA technology, the nrLFA device consists of fiber glass sample pad, nitrocellulose analytical membrane, cellulose wicking pad and plastic housing. However, no conjugate pad or pre-stored reagents are used. More details on device fabrication can be found in Materials and Methods for nrLFA Device Fabrication section in Supplementary Material. The major characteristic that distinguishes nrLFA from LFIA is the fact that no bioaffinity reaction between antibody and antigen is required for the assessment of a desired blood parameter. Instead, the nrLFA device utilizes the porous nature of nitrocellulose membrane to transport fluids with different viscosity at different rates through capillary action. The reason behind adopting the nrLFA device for blood coagulation monitoring is the fact that the viscosity of whole blood changes during the coagulation process²¹. After the coagulation cascade is activated by intrinsic and/or extrinsic pathways, whole blood viscosity gradually increases and eventually forms cross-linked

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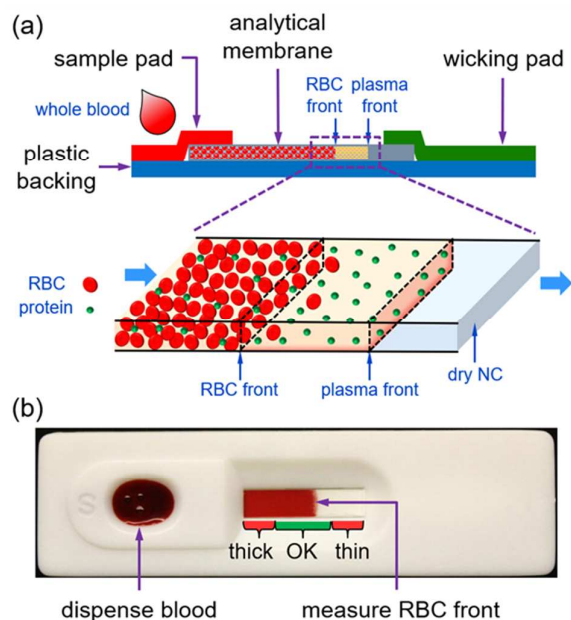


Fig. 1 No-reaction lateral flow assay (nrLFA) schematic.

fibrin clots, during which the physical properties of blood changes from a viscoelastic fluid to a viscoelastic solid²².

For coagulation testing using the nrLFA, whole blood sample is directly applied to the device sample pad allowing rapid transfer to the porous nitrocellulose membrane where RBCs are separate from blood plasma (Fig. 1a). The RBC travel distance on the nrLFA at a given time is related to the coagulation state of blood since whole blood viscosity changes with its coagulation ability²¹. As shown in Fig. 1b, short travel distance indicates the patient's blood coagulates too fast ("thick"), medium travel distance indicates the patient's blood coagulates at desired speed ("OK"), and long travel distance indicates the patient's blood coagulates too slow ("thin"). The RBC front is easily visible to the naked eye and, thus, serves as endpoint marker for the coagulation process on the nrLFA. During the test, the movement of the RBC front is observed through a window opening on the plastic housing. For quantitative analysis, the travel distance is documented in the form of high-resolution digital images using a camera and a timer, and the travel distance is extracted using ImageJ and analyzed using Excel²³⁻²⁵.

The Problem

During the development of a paper-based nrLFA device for blood coagulation monitoring, we reported: (a) the nrLFA's comparable performance to a clinical instrument CoaData 2000 Fibrintimer® (American LAbor, Durham, NC) in clotting time measurements²³; (b) excellent reproducibility of the nrLFA as well as various related membrane materials in device fabrication²⁴; (c) clinical utility of the nrLFA to identify high-risk patients on warfarin therapy²⁵. We also observed that, in addition to the dependence on coagulation ability, RBC

distance on the nrLFA is significantly influenced by the hematocrit of the patient sample – with higher hematocrit resulting in shorter RBC distances, and vice versa²⁴. Hematocrit (Hct) is the packed volume percentage of RBCs in whole blood. It has been reported that the non-Newtonian behavior of blood is mainly caused by the presence of RBCs, and the relationship between whole blood viscosity and Hct is nearly linear to Hct values up to 40-50%²⁶. When the Hct is greater than 50%, whole blood viscosity increases exponentially²⁶. In fact, even a hematocrit test strip was reported²⁷ based on the phenomenon that the elevated viscosity of whole blood due to higher Hct results in reduced travel distance in the porous media. As a consequence, test interpretation may be difficult since RBC travel distance on the nrLFA can be affected by either coagulation or Hct, or both. In order to obtain reliable test results, the objective of this study was to develop a calibration method that allows compensation for the effect of Hct on RBC travel distance on the nrLFA while preserving the clinically relevant diagnostic capabilities of the device for the monitoring of anticoagulation therapy.

The Solution

In our independently developed approach to calibrate RBC distance on the nrLFA device starts by extracting a linear fit from the travel distance of the RBC front on the nrLFA using citrated blood with various Hct values. Citrated rabbit blood was purchased from the vendor (HemoStat Laboratories, Dixon, CA) where fresh rabbit blood was collected in 4 wt.% trisodium citrate solution (anticoagulant) at 4:1 volume ratio to inhibit coagulation so that the effect of Hct alone on RBC distance could be extracted and evaluated. Alterations of the Hct values to 25%, 30%, 35% and 40% were achieved by removing freshly separated plasma from low Hct blood (19-25% for various batches from the vendor) after light centrifugation (Thermo Fisher Scientific accuSpin Micro 17, Osterode am Harz, Germany) for 6 min at 400× g, followed by re-suspending with gentle agitation. All purchased blood was used up to 2 weeks

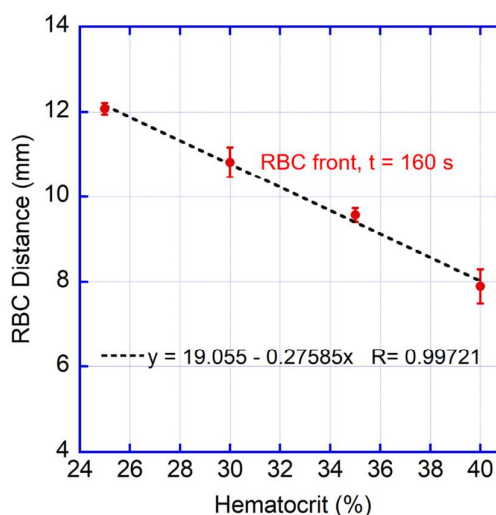


Fig. 2 RBC distance vs. hematocrit when using 30 µL of citrated rabbit blood with 25%, 30%, 35% and 40% Hct

(mean \pm SD, $n = 10$)²⁴.

after receipt and then properly disposed. In the calibration measurements, the moment when the RBC front appears in the observation window is defined as $t = 0$, and the end point was selected to be $t = 160$ s due to the length limit of the observation window. More experimental details can be found in Methods for Extracting Calibration Equation Using Citrated Rabbit Blood section in Supplementary Material. Fig. 2²⁴ shows the mean and standard deviation (SD) of RBC travel distance on the nrLFA when using 30 μ L citrated rabbit blood with 25%, 30%, 35% and 40% Hct ($n = 10$ for each Hct value). As can be seen, the relationship between RBC distance and Hct value exhibits high linearity, with a regression coefficient of 0.99721. Therefore, we adopt the linear fit equation (Eq. 1) as the starting equation for Hct calibration, where x is Hct value and y is RBC distance observed on the nrLFA:

$$y = -0.27585x + 19.055 \quad (\text{Eq. 1})$$

To reduce the number of calibrations required for each coagulation test, we choose Hct = 40% as the center value and decrease or increase RBC travel distance for Hct levels lower or higher than 40%. In other words, after calibration, RBC distance of blood with 40% Hct remains the same, the distance of blood with <40% Hct decreases by a certain calculated value, and the distance of blood with >40% Hct increases by a certain calculated value. We call the calculated value "calibration value", which will be obtained from the final calibration equation and is dependent on the Hct of blood. To obtain the calibration value Δy , we keep the slope of Eq. 1 the same but move the line horizontally to intercept x-axis at (40,0), instead of the original intercepting point of (69.077,0). This gives us an intermediate Eq. 2, which becomes Eq. 3 after simplification.

$$\Delta y = -0.27585(x + 29.077) + 19.055 \quad (\text{Eq. 2})$$

$$\Delta y = -0.27585x + 11.0341 \quad (\text{Eq. 3})$$

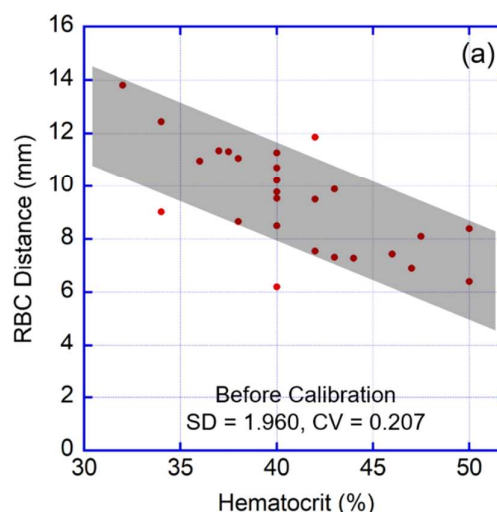
Eq. 3 serves as calibration equation to generate a calibration value (Δy) at various Hct (x). The calibration value is then added to the original RBC distance, which is a negative value for <40% Hct, 0 for 40% Hct, and a positive value for >40% Hct. The final calibrated RBC distance is the original RBC distance plus the calibration value.

Method Verification

In order to assess validity of the above mentioned calibration method, clinical trial data previously collected from patients on various warfarin dose regimes were utilized. The trial was conducted at the St. Elizabeth Healthcare anticoagulation clinic (Fort Thomas, KY) and included 27 warfarin patients²⁵. For each patient, blood coagulation test was performed on the nrLFA device using 30 μ L capillary blood, and the international normalized ratio (INR) was measured using a commercial POC coagulation analyzer CoaguChek[®] XS (Roche Diagnostics, Mannheim, Germany) for comparison. The INR is the standard

clinical parameters used for routine monitoring of drug efficacy during vitamin K antagonists treatment (e.g. warfarin)²⁵ and is calculated using patient prothrombin time divided by control prothrombin time. Higher INR indicates the patient's blood coagulates slower, and vice versa. Hct of each blood sample was quantified by spinning in a heparin-coated capillary tube for 5 min at 11,000 rpm using a Zipocrit[®] microhematocrit centrifuge (LW Scientific, Lawrenceville, GA) and the value was visually determined using a standard nomogram. Detailed information on the clinical trial can be found in Clinical Trial – Study Setting, Population and Protocol section in Supplementary Material.

Fig. 3 shows the relationship between RBC distance on the nrLFA and blood Hct at $t = 4$ min for all patients before and after distance calibration. A strong dependence of RBC distance on blood Hct before calibration can be observed in Fig. 3a, with the higher Hct resulting in shorter RBC distances. After calibration, as shown in Fig. 3b, higher Hct does not result in shorter RBC distances, and no obvious dependence of RBC distance on blood Hct can be observed. The standard deviation (SD) and coefficient of variation (CV) of scattered data are 1.960 (SD) and



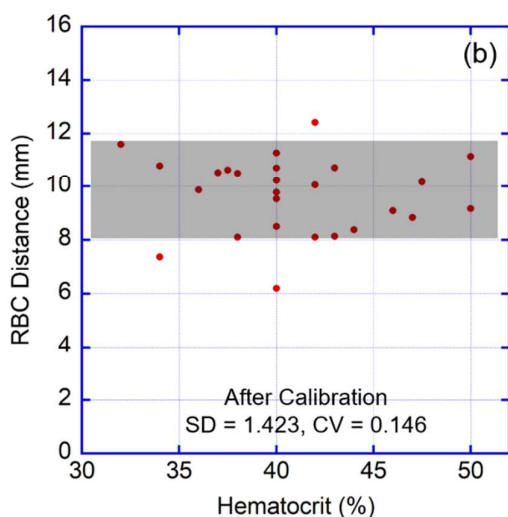


Fig. 3 Data scatter of RBC distance vs. Hct for all patients: (a) before calibration; (b) after calibration ($t = 4$ min). 0.207 (CV) before calibration, and 1.423 (SD) and 0.146 (CV) after calibration. This significant decrease in both SD and CV indicates a reduced scatter in the data with a tighter distribution after the calibration process. It needs to be mentioned that in Fig. 3a and Fig. 3b, 7 data points are present in the range of 41-45% Hct, with two data points having the same Hct and extremely close distance [(43, 10.72095) and (43, 10.72045)].

Fig. 4a and Fig. 4b show the same data as Fig. 3a and Fig. 3b but stratified in four groups based on Hct values: 31-35%, 36-40%, 41-45%, and 46-50%. The mean value and upper half of SD are plotted for each group. The number within each column indicates the number of patients in that group. Before calibration (Fig. 4a) the dependence of RBC distance on blood Hct is readily apparent, with a clear trend of decreasing RBC distance with increasing Hct values. However, after calibration (Fig. 4b) there is little or no dependence between Hct and RBC distance. We conducted the Independent Samples t Test between the 31-35% group and each of the other groups, and

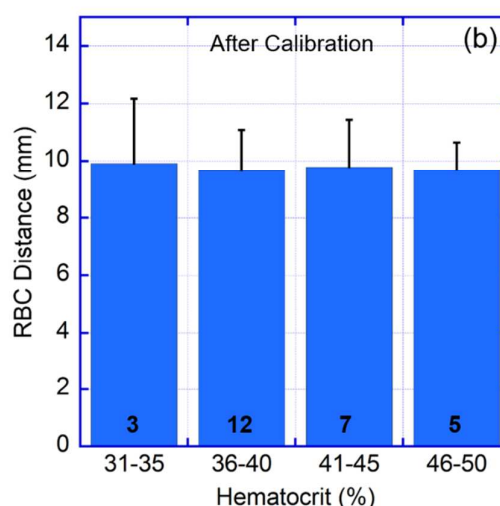
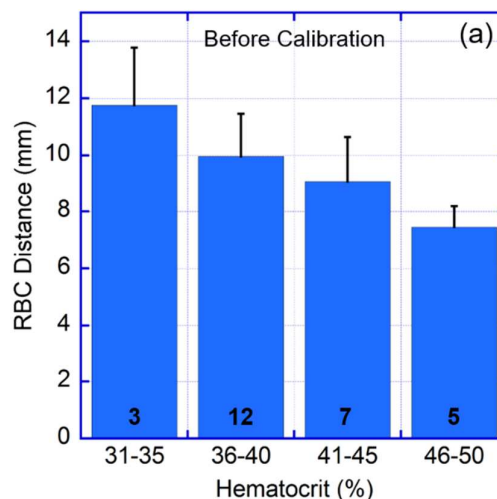


Fig. 4 Stratified data of RBC distance vs. Hct for all patients: (a) before calibration, (b) after calibration ($t = 4$ min). no significant variation was found. This demonstrates that our Hct calibration method is capable of correcting the effect of Hct on RBC travel distance on the nrLFA during coagulation.

To complete the validation of our method, we have investigated the effect of Hct calibration on the dependence of RBC distance on blood coagulation ability, which is represented by the INR value. 25 healthy volunteers are also included in the study to evaluate the nrLFA's capability to distinguish different coagulation ability between healthy volunteers and patients on warfarin medication. Healthy individuals that do not take any anticoagulation drugs (e.g. warfarin), have INR values between 0.9 and 1.1. For patients on warfarin therapy, their INR should be maintained between 2.0 and 3.0 for effective and safe treatment. INR lower than 2.0 indicates the patient's blood is not anticoagulated enough to prevent future thrombotic events, and INR higher than 3.0 indicates the patient's blood is over anticoagulated and severe bleeding may occur. The INR of patients participated in the study has a range of 1.6-3.8 with a

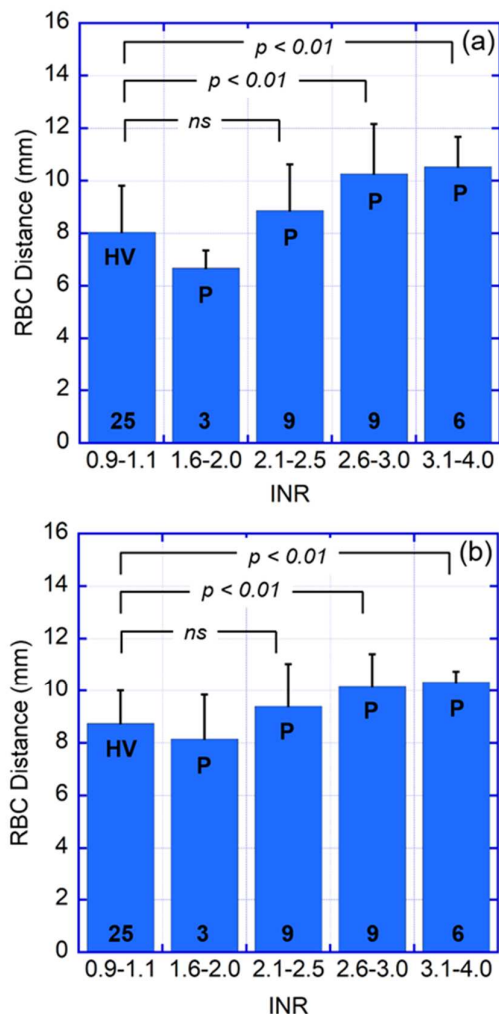


Fig. 5 Stratified data of RBC distance vs. INR for patients (P) and healthy volunteers (HV): (a) before calibration, (b) after calibration ($t = 4$ min).

mean value of 2.6²⁵. Fig. 5 shows the relationship between RBC distance on the nrLFA and INR value for patients and healthy volunteers before and after calibration. The data are stratified in five groups based on INR value. The first group is healthy volunteers (HV) with 0.9-1.1 INR. The remaining four groups are patients (P) with INR groups of 1.6-2.0, 2.1-2.5, 2.6-3.0 and 3.1-4.0. The mean value and upper range of the corresponding SD are plotted for each group. The number within each column denotes the participant number in respective group. As shown in Fig. 5a, RBC distance on the nrLFA increases with increasing INR (except patient group with 1.6-2.0 INR), and a significant difference ($p < 0.01$) in RBC distance can be observed between the healthy volunteer group and the patient group with 2.6-3.0 INR, and between the healthy volunteer group and the patient group with 3.1-4.0 INR. A similar result is obtained from the calibrated data. As shown in Fig. 5b, after calibration, increasing INR also results in increasing RBC distance, and RBC distance of healthy volunteer group is significantly different (p

< 0.01) from patient groups with 2.6-3.0 INR and 3.1-4.0 INR. This demonstrates that our calibration method is able to preserve the dependence of RBC distance on blood coagulation while correcting the dependence of RBC distance on blood Hct.

Discussion and Conclusions

In this manuscript, we reported a mathematical calibration method derived from linear regression to correct the effect of Hct on RBC transport in porous nitrocellulose membrane on the blood coagulation nrLFA device. By utilizing a set of preliminary clinical trial data obtained with patients on warfarin therapy, we demonstrated that our calibration method is capable to correct the effect of Hct on RBC travel in the nrLFA but also retaining the effect of coagulation on RBC travel at the same time. Utilizing the Hct-corrected data set, it is predicted that statistical power analysis will allow more adequate sample size estimation for a future pivotal clinical trial designed to demonstrate clinical validity of the nrLFA device as a clinical screening test for patients on anticoagulant regimens. The intended utilization of the nrLFA device is low-cost blood coagulation monitoring for patients undergoing anticoagulation treatment. The nrLFA device is not designed to substitute existing clinical coagulation analyzers during the treatment, but rather to serve as a screening tool in clinics to decide if a quantitative measurement is necessary for the patient. Despite being a qualitative screening tool, its low cost (<40 cents each²⁵) and simple operation make the nrLFA a promising approach for low-cost monitoring without requiring an expensive coagulation analyzer. Recently, a novel approach of rapid (<1 min) and accurate (to <1%) Hct measurement in whole blood was reported²⁸, utilizing acoustic focusing of RBCs in the center of a microfluidic channel while the plasma component flows along the edges of the channel. The Hct value is determined by optically monitoring the ratio of focused area to channel cross section. By allowing rapid and miniaturized Hct measurement, this new technology makes it possible to integrate Hct and coagulation measurements on the same device, thus giving the nrLFA device the potential to be utilized in patient self-testing at home or in other resource limited settings. The nrLFA device may also be utilized to monitor anticoagulation therapy involving novel oral anticoagulants (NOACs) such as apixaban, edoxaban, rivaroxaban and dabigatran. No clinical monitoring method has been established yet for the patients taking NOACs. Since the RBC travel distance in the nrLFA device is directly related to the overall blood viscosity resulted from coagulation regardless of how the anticoagulant interferes with the coagulation cascade, this simple and low-cost monitoring device can have potential utility in monitoring a larger patient population on anticoagulation therapy.

Besides coagulation and hematocrit, a few other factors^{26, 29, 30} also influence the blood viscosity, including the degree of RBC aggregation, the deformability of RBCs, the viscosity of plasma, as well as the temperature. RBC aggregation is mainly dependent on the composition of plasma proteins, the surface properties of RBCs

and the magnitude of shear force²⁹. RBC deformability is mainly dependent on the skeletal proteins of RBC membranes, the RBC's surface area to volume ratio and the cell morphology²⁹. The plasma viscosity is mainly dependent on the plasma protein concentration and the molecular weight and structure of the proteins³¹. We conducted one set of preliminary experiments to study the effect of albumin on blood transport in the nrLFA (see Effect of Albumin on Rabbit Blood Transport in nrLFA section in Supplementary Material) and observed no correlation between increasing albumin concentrations in blood (from 3 to 7 g/dL) and RBC travel distances. A slight decrease in plasma travel distances was observed with increasing albumin concentration, but no effect was observed on RBC travel distance. The effect of RBC aggregation and deformability on the blood transport in nitrocellulose membrane was not investigated in this manuscript.

Compared to experimental data in Fig. 2 (obtained in a university research laboratory), the clinical trial data in Fig. 3, Fig. 4 and Fig. 5 (obtained in an anticoagulation clinic) have relatively large standard deviations. This phenomenon may be due to the variation in sample transportation time from the physician in the examination room where patient's blood was collected and dispensed on the nrLFA device, to the research assistant in the analysis room where the real-time documentation of RBC travel distance was immediately carried out using a digital camera (see Clinical Trial – Study Setting, Population and Protocol section in Supplementary Material). Although all clinical samples were quickly transported from the examination room to the analysis room, the transportation time was not identical for all samples, and thus may have generated larger errors compared to the precisely-controlled laboratory procedures. Additionally, the larger errors of the clinical trial data can also come from patients with different aggregation and deformability of their RBCs. This parameter was not measured during the clinical trial and may have affected the RBC travel distance on the nrLFA device.

Other than whole blood coagulation test, the proposed calibration method can also be employed in other flow-based whole blood tests such as paper-based blood typing^{32, 33} where the eluting pattern of RBCs on paper materials typically serves as an indicator of blood type. Different Hct values may cause variations in the length or the color intensity of RBC eluting pattern which can reduce the test accuracy. This problem can potentially be resolved by calibrating the RBC eluting pattern based on blood Hct value to correct the negative effect of Hct on test results.

Future research on utilizing the nrLFA device for low-cost blood coagulation monitoring includes investigating the effect of RBC aggregation and deformability on RBC travel distance in the nrLFA device, defining the expected lengths of "thick", "OK" and "thin" sections of the observation window for patients on anticoagulation therapy, as well as developing a smartphone app for automatic readout of the test result.

Acknowledgements

This research was supported by the National Science Foundation (PFI:AIR Award #1500236) and by the University

Research Council Interdisciplinary Program from the University of Cincinnati.

This study received IRB approval (study ID: 2016-5324) from the University of Cincinnati and St. Elizabeth Healthcare.

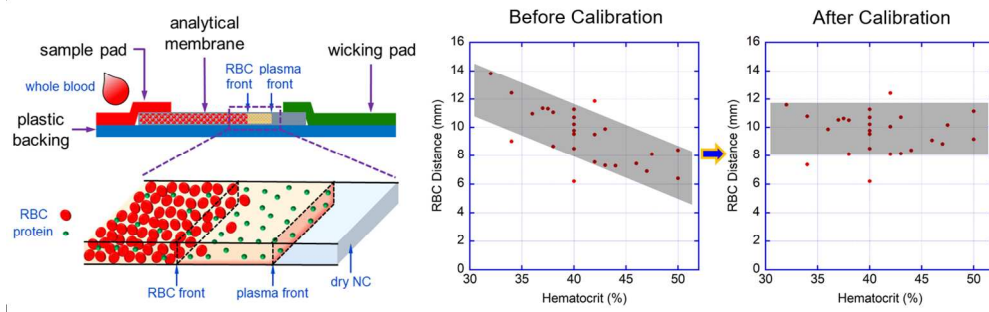
Conflicts of interest

There are no conflicts to declare.

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A simple calibration method corrects for the effect of hematocrit variation in measuring whole blood coagulation in a paper-based lateral flow device.

430x139mm (96 x 96 DPI)