

Electronic Supplementary Information

Measurement of the Hematocrit Using Paper-Based Microfluidic Devices

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Materials

We purchased Ahlstrom chromatography paper grades 6130 (pore size 6 μm), 54 (pore size 10 μm), and 55 (pore size 15 μm) from Laboratory Sales & Service LLC (Branchburg, NJ). We purchased Whatman grade 4 chromatography paper (pore size 25 μm), citric acid monohydrate, D-(+)-Glucose (45% w/v), dimethyl sulfoxide and 0.5 M ethylenediaminetetraacetic acid (EDTA) from Sigma-Aldrich. We purchased sodium chloride, SafeCrit plastic microhematocrit tubes, and glass microscope slides from Fisher Scientific. We received trisodium citric acid dihydrate and Phenol Red free acid from Amresco. We purchased DiIC₁₈(3) general cell membrane stain from Biotium. We obtained Flexmount Select DF051521 (permanent adhesive-double faced liner) from FLEXcon (Spencer, MA). We received Jurkat D1.1 cells (CRL-10915) from ATCC. We purchased RPMI-1640 media from EMD Millipore and fetal bovine serum (FBS) from Biowest. We purchased Fellowes laminate sheets from Amazon. We obtained samples of whole blood samples from Research Blood Components (Brighton, MA). We purchased Critoseal vinyl plastic putty from VWR.

Methods

Fabrication of the paper-based microfluidic device

We designed the hydrophobic barriers that defined the fluidic network in Adobe Illustrator and printed the layers of paper using a Xerox ColorQube 8580 printer.¹ We placed the printed layers into a 150 °C oven for 30 seconds to melt the wax through the full thickness of the paper. We assembled two-layered devices using a sheet of double-sided adhesive that was patterned using a Graphtec Cutting Plotter (CE6000-40). The exposed area of the second layer was sealed with Fellowes laminate sheets to prevent evaporation of the sample and to protect the

user from biological contamination. We assembled the completed device with an Apache AL13P laminator. This process was repeated for all types of paper used.

Preparation of samples of whole blood at desired hematocrits

Samples of whole blood were used the same day that they were received from the vendor. Initial hematocrits were determined using standard centrifugation techniques. Briefly, 20 μL of sample was pipetted into a SafeCrit microhematocrit tube, sealed with Critoseal, and centrifuged at 800 g for 3 minutes. The microhematocrit tube was then scanned using an 8-bit EPSON Perfection V500 PHOTO scanner. The hematocrit of the sample was calculated by measuring the ratio of the length that RBCs occupied the tube to the total sample length in ImageJ.² From each sample of blood, we prepared seven hematocrits that ranged from 30%–60% by adding or removing plasma. To specify, lower hematocrits were prepared by diluting whole blood using plasma obtained from the original sample. To obtain plasma, we placed 10-mL of whole blood into a centrifuge (800 g for 10 minutes) to separate the components of whole blood based on density. After centrifugation, we removed the plasma for immediate use as a diluent. Alternatively, plasma can be aliquoted and stored at $-20\text{ }^{\circ}\text{C}$ until needed. Higher hematocrits were obtained by removing an appropriate amount of volume of plasma determined by Eq. 1, where I is the initial hematocrit fraction, V is the volume of sample, x is the amount of volume of plasma to remove, and F is the desired hematocrit fraction.

$$F = \frac{I}{V - x} \quad \text{Eq. 1}$$

We used standard centrifugation techniques to confirm hematocrits of prepared samples. Prior to adding samples (whole blood or plasma only) to the device, they were incubated in a water bath at $37\text{ }^{\circ}\text{C}$ (i.e., body temperature) for 30 minutes to mimic conditions at the point-of-care where blood would be evaluated immediately after it was drawn. A trace amount of phenol red was

added to samples containing only plasma prior to incubation at 37 °C to help visualize the distance-based readout in the lateral channel.

Analysis of hematocrit assays performed in paper-based microfluidic devices

We initiated our assays by applying 50- μ L of a sample—whole blood or suspensions of cells, depending on the assay—to the top layer of our devices. For assays performed using only plasma, the volume added to the device corresponded to the theoretical volume of plasma expected based on the target hematocrit. For example, there will be 30 μ L of plasma in the 50- μ L sample of whole blood at a 40% hematocrit. After thirty minutes, we covered the sample layer with transparent tape to minimize the exposure of biological fluid and scanned the hematocrit devices using the 8-bit EPSON Perfection V500 PHOTO scanner with a resolution of 800 dpi. We used the resolution to convert pixels to inches for our measurements in ImageJ. We measured the distance from the top of the circular zone in the second layer to the farthest edge that the RBCs traveled in the layer. We converted inches to millimeters and analyzed the distances in Microsoft Excel. We subtracted the length of the circular zone and wide channel (15 mm), such that the distances presented were in reference to the thin channel allowing for the results to be resolved qualitatively (i.e., high hematocrits do not enter the thin channel, but normal-low hematocrits do enter the channel).

Experimental Details

Determining the ideal geometry for the lateral channel

We investigated several lateral channel geometries to determine the design that: (i) allowed for high concentrations of RBCs to be transported laterally in the second layer and (ii) provided the largest resolved distances between the seven different hematocrit percentages. We

compared devices with the same area (80 mm^2) for the lateral channel but with different dimensions (**Figure S1A**). In the experiments shown, we fabricated devices using $15 \text{ }\mu\text{m}$ paper. We observed that wide lateral channels (4 mm) permitted the transport of high hematocrits into the channel, minimizing a bottleneck effect at the top of the channel. However, lower hematocrits were transported smaller distances in these devices because the fluid in sample wicked radially. There was thus less volume of plasma to carry RBCs longer distances. In contrast, RBCs traveled longer distances in thin channels (2 mm), but in samples of whole blood at higher hematocrits, RBCs often never entered the channel and volume of sample would remain on the top layer (**Figure S1B**). Ultimately, we chose a design for the second layer that combined wide and thin lateral channels.

Transport of white blood cells in paper-based microfluidic devices

Although the concentrations of white blood cells (WBCs) in blood are approximately 1000-fold less than that of RBCs, their presence in a sample of whole blood can have profound negative effects on the performance of the hematocrit assay because larger and stiffer WBCs could inhibit wicking of plasma by occluding pores. Therefore, we investigated the localization of WBCs in devices fabricated from the four types of paper in order to determine the grade(s) of paper that excluded WBCs from entering the lateral channel while permitting the transport of RBCs. We used the Jurkat D1.1 cell line (human T cells) as a representative cell type for white blood cells in paper-based hematocrit assays. D1.1 cells were cultured in RPMI-1640 media supplemented with 10% FBS and incubated at $37 \text{ }^\circ\text{C}$ in a 5% CO_2 atmosphere.

We used fluorescence microscopy to detect where WBCs travel in a paper-based microfluidic device. We stained the D1.1 cells with a general membrane stain, DiIC₁₈(3), by adding $15 \text{ }\mu\text{L}$ of 1 mg/mL DiIC₁₈(3) in DMSO to a 1 mL solution. We incubated the cells for 30

minutes at 37 °C in 5% CO₂ atmosphere. The D1.1 cells were then sedimented at 1100 g for 5 minutes and washed with fresh RPMI-1640/10% FBS medium. The wash step was repeated twice. Finally, samples of D1.1 cells were prepared in Alsever's solution: 42 mg/100 mL NaCl, 80 mg/100 mL trisodium citric acid dihydrate, 5.5 mg/100 mL citric acid monohydrate, and 205 mg/100 mL D-(+)-glucose in 18 MΩ DI H₂O. The final concentration of the D1.1 cells was 6000 cells/μL, which we confirmed using a Countess II Automated Cell Counter (Thermo Fisher). We chose this concentration of cells because it is within expected, normal range of WBCs in whole blood.

We added 50 μL of the sample of stained WBCs to untreated paper-based microfluidic devices to mimic the conditions used in experiments performed with whole blood. After the assays were completed (30 minutes), we disassembled the device and mounted the two separated layers onto FisherBrand glass slides for imaging by fluorescence microscopy. We imaged three regions of the paper-based microfluidic device to determine locations of WBCs within devices (**Figure S5**). Images were obtained using a 20X objective on a Leica DMI8 inverted microscope with an Andor DSD2 differential spinning disk confocal system in widefield mode and Zyla 4.2 CMOS camera. We observed that, in all four grades of paper, the majority of WBCs remained on the top layer and were not able to enter the lateral channel. As a result, the ideal grade of paper could be determined directly by assessing the transport of RBCs in whole blood.

Treatment of paper-based microfluidic devices

We screened six concentrations of sodium chloride to evaluate which concentration provided an improvement on the transport of sample, reproducibly (**Figure S7**). We made solutions of NaCl in 18.2 MΩ DI H₂O that ranged from 50 mM to 1 M. To treat the device, we applied 40 μL of a solution of NaCl to the top of the lateral channel, allowed the solution to wet

the entire channel, and then dried the layer for 5 minutes at 65 °C. We prepared a sample of whole blood as previously stated with a hematocrit of 43%. We applied this sample to the paper-based microfluidic devices treated with different concentrations of NaCl and measured the distance the sample traveled into the device. We observed a decrease in transport distances at high concentrations of NaCl solutions (≥ 300 mM) that we attribute to crenated and stiff cells that could no longer travel through the heterogeneous fiber network. Lower concentrations of NaCl (50 and 100 mM) offered improved results over untreated devices and showed comparable results. We chose the lowest concentration (50 mM) to minimize the osmotic stress applied to RBCs during the assay (**Figure S8A**).

For the experiments performed with EDTA-treated devices, we diluted a stock solution of 0.5 M EDTA to a working concentration of 4.5 mM³ using 18.2 M Ω DI H₂O. We added 40 μ L of 4.5 mM EDTA in 18.2 M Ω DI H₂O and allowed the sample to completely wet the channel. After, we dried the layer for 5 minutes at 65 °C. We observed comparable results between hematocrit assays on untreated devices and devices treated with EDTA only (**Figure S8B**). Therefore, we investigated the performance of the hematocrit assay with the combination of additives co-spotted on the lateral channel and observed improved transport of the sample (**Figure S8C**). For devices treated with both NaCl and EDTA, NaCl was applied first in a separate step prior to applying the solution of EDTA.

Reproducibility of hematocrit assays

We evaluated the reproducibility of the of the paper-based microfluidic device by performing: (i) a number of technical replicates with a sample that did not undergo any prior preparation, (ii) comparing multiple donors, (iii) comparing the effects of two common anti-coagulants that treat samples of whole blood. We performed each of these experiments on

devices fabricated with 15 μm paper treated with 50 mM NaCl and 4.5 mM EDTA and prepared the samples of whole blood following the protocol previously described. Outliers found in the multiple replicates (**Figure 5**) were determined using Grubb's test at 95% confidence ($\alpha = 0.05$) in Prism 6.

Cost of paper-based microfluidic devices for hematocrit assays

Paper-based microfluidic devices are typically inexpensive to fabricate, which is a significant advantage of their use as a platform for the development of analytical assays for use in limited-resource settings. The device that we designed to enable the measurement of the hematocrit is simple—they comprise only two layers of paper, one layer of adhesive, two pieces of protective laminate film, and small volumes of salts as reagents. To determine costs associated with manufacturing these paper-based microfluidic devices, we obtained list prices from vendor websites at moderate volumes (i.e., not in bulk)⁴ that are appropriate for the scale of a research laboratory (**Table S1**). From these unit costs, we calculated usage costs associated with each component by scaling for the amount of material or reagent needed to prepare a single device based on the stock concentration. We estimated the cost of wax assuming US\$0.018 per 5% coverage of a standard 8.5 in x 11 in piece of paper⁵ and adjusting for the size of each printed layer based on the design pattern. Since we patterned each layer at 100% coverage, the cost to print wax was US\$0.004/in². Overall, the device has a very small footprint (1.67 in²) and is treated with very small amounts of reagents (ca. μg). As a result, the final cost of the hematocrit assay is US\$0.029 per device. We expect costs to decrease further if materials and reagents are purchased in bulk.

Figure S1. Effect of channel geometry on transport of whole blood. (A) Initial thermometer designs vary in dimensions but have a constant channel area of 80 mm^2 . (B) Selected images of assays performed with hematocrits (Hct) at 40% and 60% using devices fabricated with lateral channels of different dimensions.

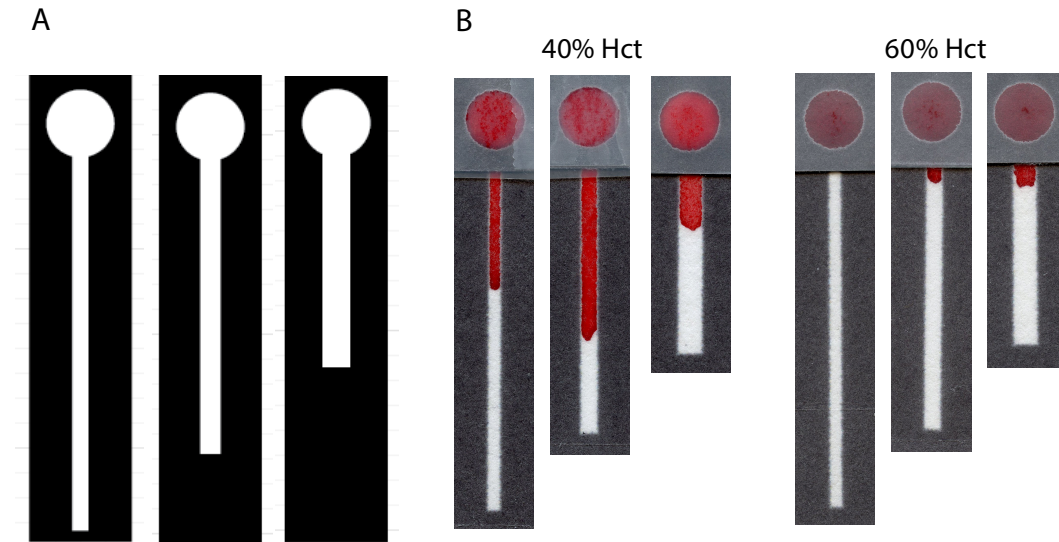


Figure S2. Detailed dimensions of the active layers used in the paper-based microfluidic device for the hematocrit assay.

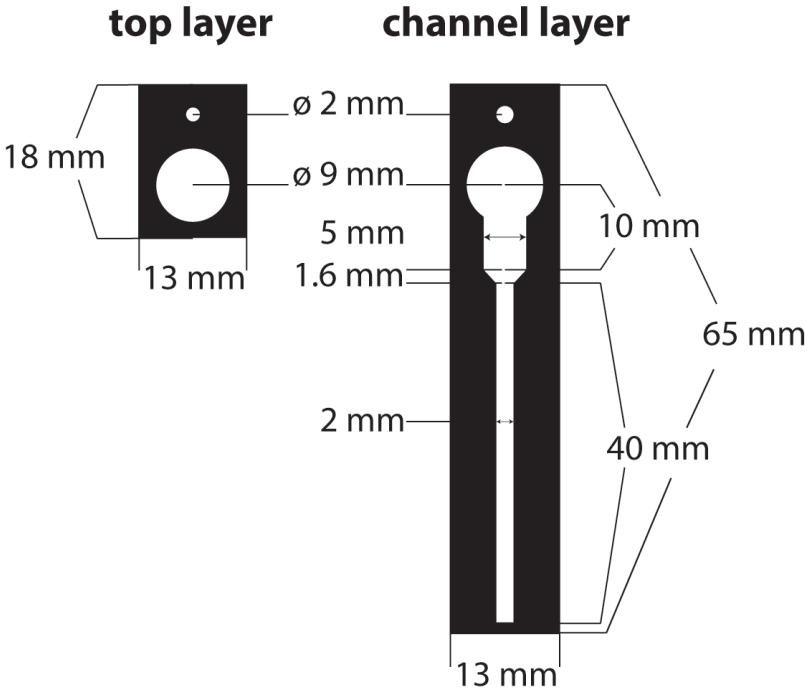


Figure S3. Comparison of the transport of samples of plasma in devices fabricated with types of paper that varied in particle retention size: 6 μ m paper (blue, $R^2 = 0.851$), 10 μ m paper (purple, $R^2=0.963$), 15 μ m paper (red, $R^2 = 0.986$), and 25 μ m paper (black, $R^2 = 0.993$). Each data point is the average of five replicates and the error bars are standard error of the mean. The solid lines are the linear fits of the data series.

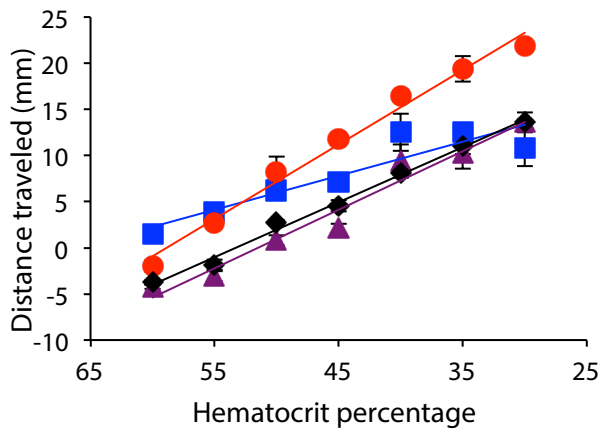


Figure S4. Transport of samples of whole blood on devices fabricated with papers varying in particle retention sizes: 6 μm paper (blue), 10 μm paper (red), 15 μm paper (black), and 25 μm paper (grey). No additives were applied to the devices. Each data point represents an average of three replicates and the error bars represent standard error of the mean for each point. The assays performed on 6 μm , 10 μm , and 15 μm paper were performed on the same day using the same sample of whole blood. The assays performed on the 25 μm paper were performed on a different day using a different sample of whole blood.

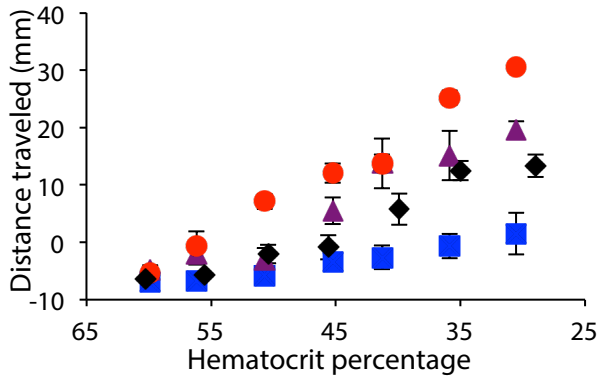


Figure S5. Fluorescence microscopy images of white blood cells in paper-based microfluidic devices. Cells were stained with DiIC₁₈(3) prior to introduction to a device. Images in the top row were acquired in the circular region of the top layer of the device. The second and third rows of images were acquired in the regions specified by the schematic of the lateral channel on the second layer of the device. Scale bars are 500 μm .

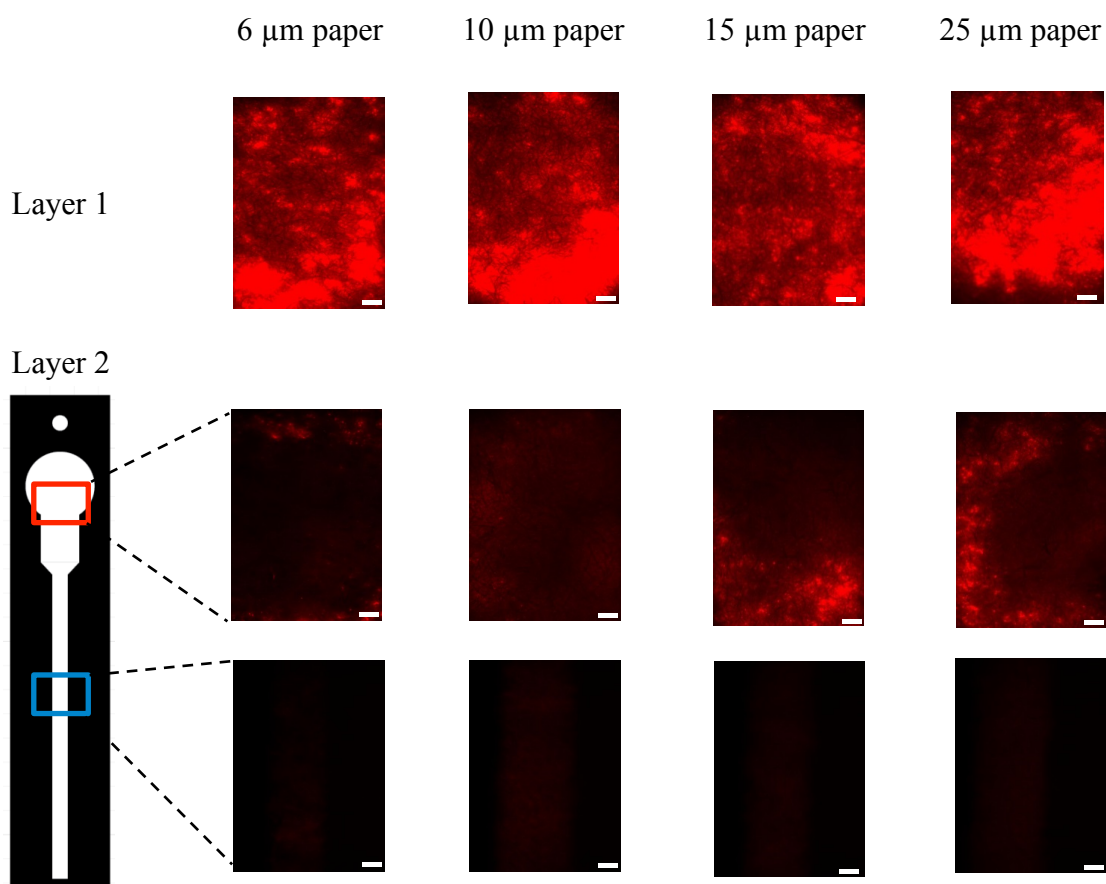


Figure S6. Comparison of the transport of samples of plasma (black) and whole blood (red) in untreated paper-based microfluidic devices. Each data point represents the mean of five replicates for both the samples of plasma and whole blood. The error bars for both data sets represent the standard error of mean.

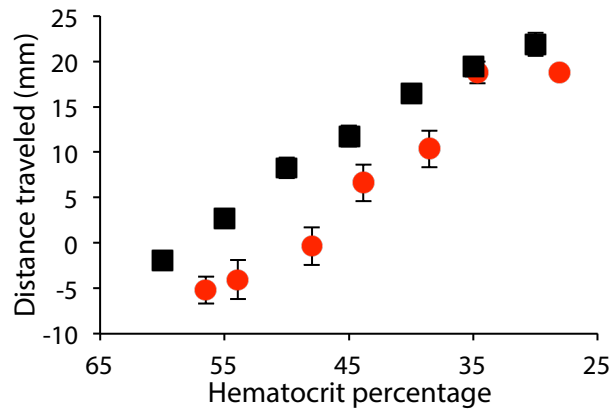


Figure S7. Effect of sodium chloride on transport of whole blood in paper-based microfluidic devices. Each data point is the average of three replicates and the error bars represent the standard error of the mean. The red arrow denotes the results of the 50 mM NaCl solution chosen for the final device treatments.

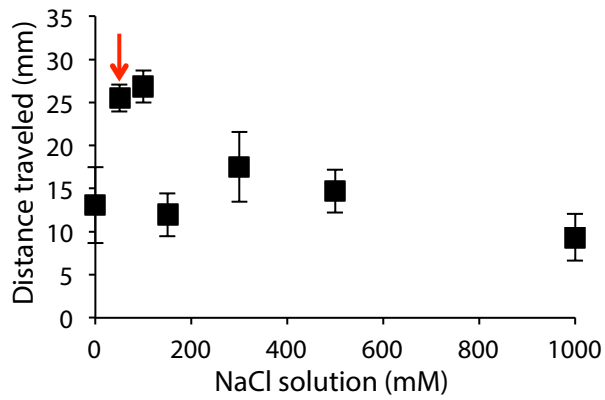


Figure S8. Graphical representation of the performance of hematocrit assays performed on devices treated with additives to improve the transport of samples of whole blood. Comparison of the performance of the hematocrit assay on devices that were untreated (black circles) and those that were: (A) treated with 50 mM NaCl (blue squares), (B) treated with 4.5 mM EDTA (blue squares), and (C) co-spotted with 50 mM NaCl and 4.5 mM EDTA (red squares). Each data point is the average of five replicates and the error bars represent standard error of the mean.

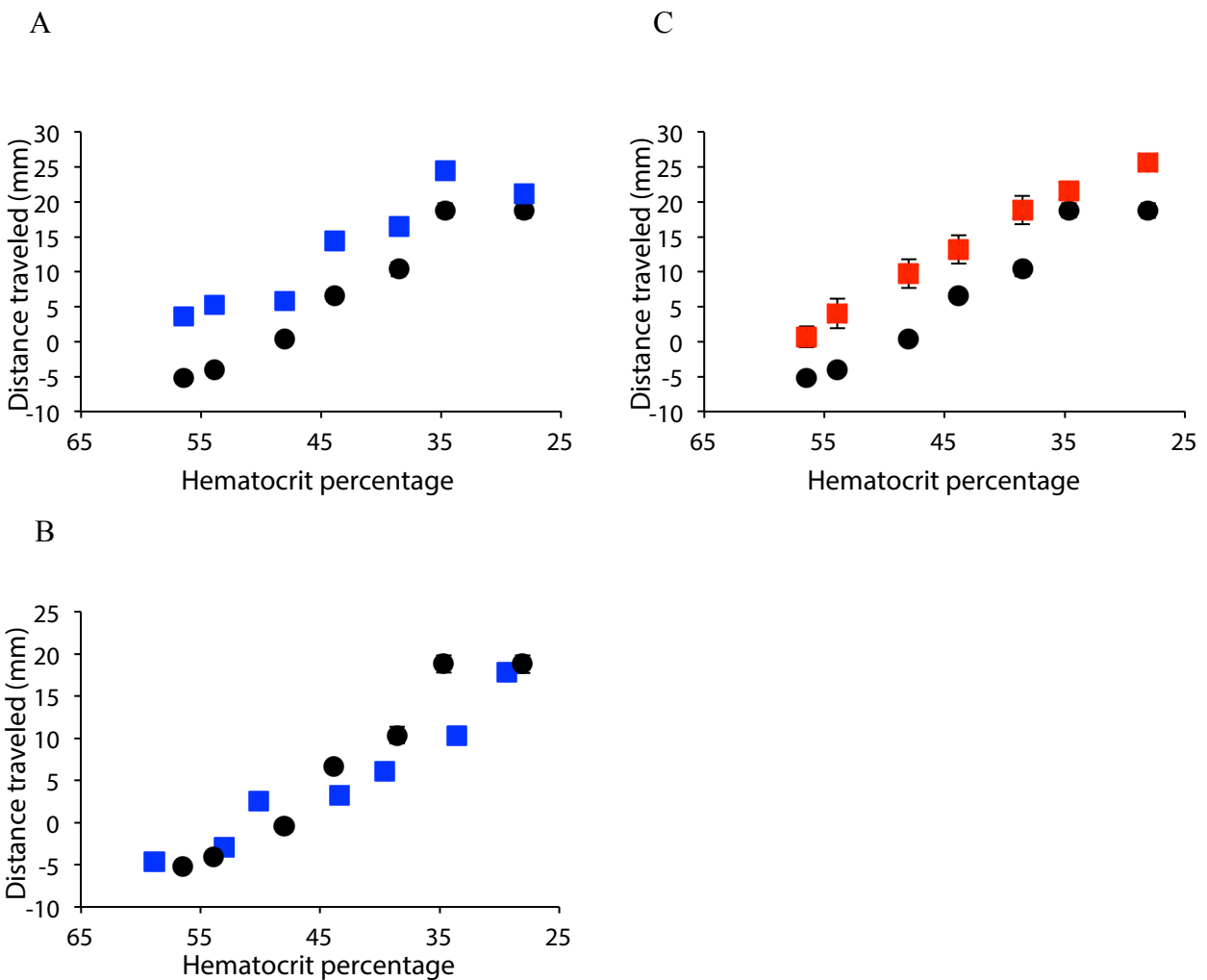


Figure S9. Comparison of the performance of hematocrit assays using whole blood from different donors: Donor 1 (red), Donor 2 (blue), and Donor 3 (black). The samples of whole blood were treated with EDTA as an anti-coagulant. The devices were treated with both NaCl and EDTA. Each data point is the average of five replicates and the error bars represent the standard error of the mean.

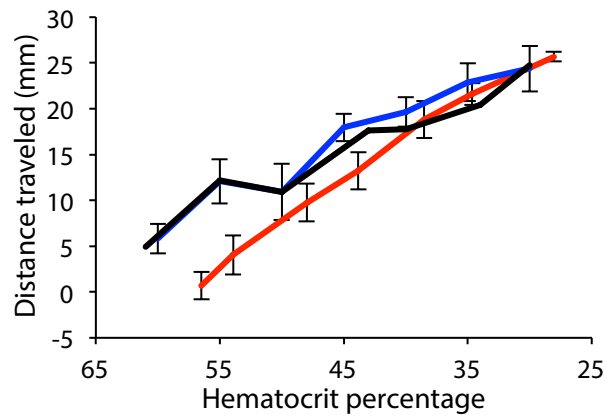


Figure S10. Effect of sodium heparin as an anti-coagulant using whole blood from different donors: Donor 1 (red), Donor 2 (blue), Donor 3 (black). The devices for these experiments were treated with both NaCl and EDTA. Each data point is the average of five replicates and the error bars represent the standard error of the mean.

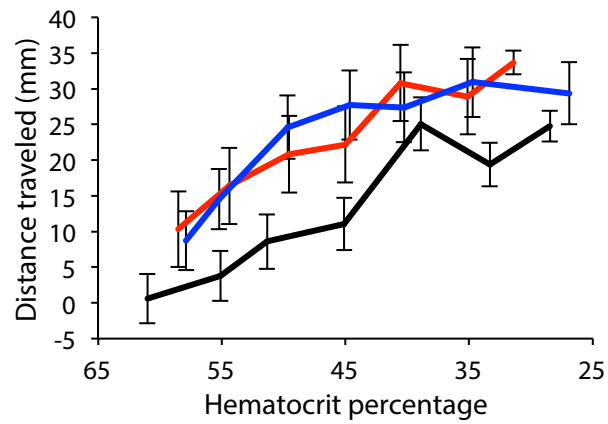


Table S1. Cost analysis to fabricate one paper-based microfluidic device for the hematocrit assay.

Reagent or Material	Vendor	Item #	Item Cost (US\$)	Unit Size	Unit Cost (US\$/unit)	Amount Used Per Device	Usage Cost Per Device
NaCl	Fisher Scientific	S671-500	\$63.87	500 g	\$0.127 / g	117 μ g	\$0.000
EDTA	Sigma-Aldrich	03690-100ML	\$37.60	100 mL at 0.5 M	\$2.573 / g	53 μ g	\$0.000
Ahlstrom Grade 55 paper	Laboratory Sales & Service	N/A	\$74.99	100 sheets (8 in x 10 in)	\$0.009 / in ²	1.672 in ²	\$0.016
Wax [†]	Xerox	N/A	N/A	N/A	\$0.004 / in ²	1.263 in ²	\$0.005
Flexmount adhesive	FLEXcon	DF051521	\$330.54	1 roll (60 in x 150 ft)	\$0.003 / in ²	1.310 in ²	\$0.004
Fellowes laminate	Amazon	B0010K824A	\$12.88	50 sheets (9 in x 12 in)	\$0.002 / in ²	2.255 in ²	\$0.005
TOTAL							\$0.029

[†]Wax costs based on US\$0.018 per 5% coverage of a standard 8.5 in x 11 in piece of paper.

References

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2. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, **9**, 671–675.
3. J. Zhu, L. Chen, Y. Mao, H. Zhou, R. Lui and W. Wang, *Genet. Test Mol. Biomarkers*, 2013, **17**, 10–15.
4. All prices obtained from internet searches performed on July 11, 2016 with the exception of the price of Ahlstrom grade 55 paper, which was obtained directly from the vendor.
5. Xerox Office, URL: <http://www.growtechsolutions.com/Downloads/CostPerPrint.PDF>, (accessed July 2016).