Electronic Supplementary Material (ESI) for Analytical Methods.

Electronic Supplementary Information

Determination of sample stability for whole blood parameters using formal experimental design

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Materials

We purchased Ahlstrom chromatography paper grade 55 (pore size 15 μm) from Laboratory Sales & Services LLC (Branchburg, NH). We purchased ethylenediaminetetraacetic acid (EDTA) from Sigma-Aldrich. We purchased sodium chloride and SafeCrit plastic microcapillary tubes from Fisher Scientific. We purchased Drabkin's reagent and Brij 35 (30% w/w) from Ricca Chemical. We purchased lyophilized hemoglobin standard from Pointe Scientific (Canton, MI). We purchased Critoseal vinyl plastic putty and glass cover slips (No. 1.5) from VWR. We purchased Flexmount Select DF051521 (permeant adhesive double-faced liner) from FLEXcon (Spencer, MA). We purchased Fellowes laminate sheets from Amazon.

Methods

Live Subject Statement

We obtained samples of whole blood from Research Blood Components (Brighton, MA). The vendor follows American Association of Blood Banks guidelines for all donors, which includes IRB approved consent to the use of collected blood for research purposes.[1] All research was approved by the Tufts University Institutional Biosafety Committee.

Development of Experimental Design

We established the fractional factorial design using JMP Pro Statistical Analysis Software (SAS). We selected a 32-run fractional factorial from the design of experiments (DOE) menu in JMP and the values for the levels of each variable (**Table 1**) were entered manually. To ensure that the main effects were clean (i.e., not confounded with each other) we created an alias table in JMP and analyzed the interactions, which showed no aliasing between the main effects. We then generated a randomized design, which created a table (**Table S1**) that indicated which level of variable to select for each of the 32 runs.

Reference Measurement of the Hematocrit

We measured the initial hematocrit of the whole blood sample upon arrival. We added 20 µL of whole blood to a SafeCrit plastic microcapillary tube and sealed the tube at one end with Critoseal. The microcapillary tubes were centrifuged at an RCF of 800 g for 3 minutes. We obtained images of the microcapillary tubes using an 8-bit EPSON Perfection V600 PHOTO scanner with a resolution of 800 dpi. The hematocrit of the sample was calculated by measuring the ratio of the length that RBCs occupied in the tube to the total sample length with ImageJ software.[2] The hematocrit was measured in duplicate for each sample.

Quantification of Percent Hemolysis

We determined the extent of hemolysis in each sample by quantifying hemoglobin using Drabkin's reagent. The Drabkin's reagent was first prepared by adding 25 µL of Brij 35 (30% w/w) to 50 mL of Drabkin's reagent. A calibration curve was prepared with the hemoglobin standard, which was diluted with 18 MΩ deionized water over a concentration range of 3–20 g/dL. The initial hemoglobin concentrations of the whole blood sample and of the isolated plasma were measured immediately upon delivery of the blood sample. We added 4 µL of each sample to 1 mL of the prepared Drabkin's reagent. Each sample incubated with Drabkin's reagent at room temperature (21 ˚C) for 15 minutes before absorbance was measured at 540 nm using a Varioskan LUX microplate reader. Calibration curves were prepared in the same way for each timepoint in this study. Plasma samples from each run were obtained from the microcentrifuge tube used in the reference hematocrit method. We removed 4 µL of plasma from the open end of the tube with a pipette and mixed it with 1 mL of Drabkin's reagent. Each sample incubated with Drabkin's reagent at room temperature (21 ˚C) for 15 minutes before 200 µL of each sample was added to a 96-well plate. Absorbance of each sample was measured at 540 nm using a Varioskan LUX microplate reader. Percent of hemolysis was determined as a ratio of the initial hemoglobin concentration of whole blood to the hemoglobin concentration of the collected plasma sample for each run.

The limit of detection (LOD) of the quantification of hemoglobin method used in this study was determined by measuring the hemoglobin concentration of isolated plasma from fresh, whole blood (n=20 replicates).

Fabrication of the Paper-Based Hematocrit 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.[3] We heated the printed layers with a Promo Heat press (PRESS-CS-15) at 280 ˚F for 35 seconds to melt the wax through the full thickness of the paper. The paper channel layer was pre-treated with 8 µL of 50 mM NaCl followed by 8 µL of 4.5 mM EDTA. The treated layer was dried in a 65 ˚C oven for 5 minutes prior to assembly. We assembled two-layered devices using a sheet of double-sided adhesive[4] that was patterned using a Graphtex Cutting Plotter (CE6000-40). The exposed area of the second layer of paper was sealed with Fellowes laminate sheets (top and bottom) to prevent evaporation of the sample and to protect the user from biological contamination. We assembled the completed device with a TruLam laminator.

Analysis of Hematocrit Assays Performed in Paper-Based Microfluidic Devices

We initiated our assays by applying 10 µL of whole blood to the top layer of each paper device. Samples were either heated at 37 ˚C with a Grant-bio thermo-shaker for 30 minutes or remained at room temperature before they were applied to the device according to **Table S1**. All runs were performed with n=3 replicates. After 15 minutes, we covered the sample layer with transparent tape to minimize the exposure of biological fluid. The paper devices were then scanned with an 8-bit EPSON Perfection V600 PHOTO scanner with a resolution of 800 dpi. 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 channel layer. We converted the resolution from pixels to inches for our measurements in ImageJ. We then converted the inches to millimeters in Microsoft Excel. We subtracted the length of the circular zone and tapered channel, 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-to-low hematocrits do).

Analysis of Experimental Design Results

We created three response variables in the established fractional factorial design in JMP: reference hematocrit measurement, degree of hemolysis, and transport distance in the paper-based hematocrit device. We set the initial measured values (i.e., the values obtained upon delivery of the whole blood sample, t=0 hours) of the response variables as the target values, as the desired output for each response was to match the target values. We manually entered the averaged measured results from each run for each of the three response variables (**Table S3**). We analyzed the results for each response variable from the experiment using JMP's design of experiments analysis software, where we selected the option to "fit a linear model". This feature fits a mathematical model to the selected response data. We fit separate models for each of the three responses. We then used the JMP platform to perform a complete model reduction by sequentially eliminating the variables with the largest p-values until the only remaining variables had significant p-values (<0.05), displayed in **Table 2**. The p-values adjust as the model reduction is performed, therefore the initial p-values in **Table S4–6** differ slightly from the final p-values in **Table 2**. From this, we determined which variables had a statistically significant impact on the selected measured response.

Additionally, to test for correlation between the response variables, we performed a Spearman rank multivariate correlation (i.e., a nonparametric multivariate method which computes correlation based on the rank of the variables rather than the raw values themselves) with JMP's statistical analysis tools. We used the Mahalanobis distance to identify any outliers and remove them from our data set.

Red Blood Cell Morphology Analysis

We acquired a second sample of whole blood to perform morphology analysis of the RBCs. Upon delivery of the sample, we made 500 µL aliquots. We used a separate aliquot for

each time point (0, 4, 48, 96, and 168 hours). At each of these time points, we performed a 1:100 dilution of the whole blood sample in native plasma to ensure that the cells were at an adequate concentration to facilitate imaging and counting. We added 10 µL of the diluted sample to a glass coverslip and immediately covered the droplet with a secondary glass coverslip. We imaged the RBCs at 63X magnification using the Leica DMi8 with Andor Revolution DSD2 confocal imaging system in brightfield mode. We imaged at least 500 RBCs at each time point to be representative of the entire cell population. We then counted the number of healthy discocytes and the total number of echinocytes at each time point, and subsequently determined the ratio of echinocytes to total number of RBCs. To obtain the percentage of echinocytes in the total population (**Figure S2**), we multiplied the ratio of echinocytes to total RBCs by 100.

Determination of the Gauge Error for the reference hematocrit measurement

To assess the variation associated with the reference hematocrit measurement, we performed a gauge error study. We acquired a separate sample of whole blood, and simulated a normal distribution of hematocrit samples, centered around 45% hematocrit. To create the distribution, we artificially adjusted the hematocrits to the values in **Table S2**. We measured the initial hematocrit with standard centrifugation techniques, described in detail above. To adjust the sample to a lower hematocrit, we diluted whole blood using the native plasma of the original sample. We obtained higher hematocrits by removing the proper amount of plasma (*x)*, as determined by Eq. 1, where *I* is the initial hematocrit fraction, *V* is the volume of the sample and *F* is the desired hematocrit fraction.

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

After adjusting to the desired hematocrits, we prepared 4 microcapillary tubes for each of the hematocrits listed in **Table S6.** We measured the hematocrits according to method described above. To calculate the gauge error, we performed ANOVA analysis for each sample (n=4).

Additional Statistical Analyses for Storage Time Points (Equivalence Testing and Kruskal-Wallis Analysis)

To confirm our observation that reference hematocrit measurements change with increased storage time, we obtained a second sample of blood from a different donor for statistical analysis. Upon delivery of the sample, we made 500 µL aliquots. We used a separate aliquot for each time point (0, 4, 48, 96, and 168 hours). At each time point, we prepared 8 microcapillary tubes with the appropriate sample, centrifuged for 3 minutes at an RCF of 800 g, and scanned the tubes with an 8-bit EPSON Perfection V600 PHOTO scanner with a resolution of 800 dpi. The hematocrit of the sample was calculated by measuring the ratio of the length that RBCs occupied in the tube to the total sample length with ImageJ software. We repeated this for each time point.

We compared the measured hematocrits from blood stored at 4, 48, 96, and 168 hours to the initial hematocrit of the blood sample at 0 hours using Kruskal-Wallis analysis (i.e., a non-parametric test for multiple comparisons) at a 95% confidence interval with GraphPad Prism 7.04. We also performed equivalence testing at each time point following the two-one-sided t-test (TOST) procedure as outlined by the ASTM guidelines.[5] We determined our equivalence bounds to coincide with the cutoffs for the normal range of hematocrit, with the lower equivalence bound corresponding to 36% and the upper equivalence bound corresponding to 54%. As the hematocrit at t=0 hours measured 45%, we were able to center and normalize the equivalence bounds around our initial measurement, with a final upper bound of 9% and a lower bound of -9%. Next, we followed the TOST procedure with a 90% confidence interval to determine the mean difference between the hematocrit at each storage time (4, 48, 96, 168 hours) and the initial hematocrit reading (t=0 hours). The results were presented in a mean difference plot (**Figure 4**).

Table S1. Fractional factorial design.

	variable							
test	1	2	3	4	5	6	7	8
1	+	-	+	+	+	-	+	+
2	+	-	-	+	-	+	+	-
3	+	+	-	-	-	-	+	+
4	-	+	+	-	+	+	+	-
5	-	-	-	-	+	-	-	+
6	+	+	-	+	+	-	-	-
7	-	+	+	+	+	+	-	+
8	+	-	-	-	+	+	-	+
9	-	-	-	+	+	+	+	-
10	-	+	-	+	-	-	-	-
11	+	+	+	+	-	+	-	+
12	+	-	+	-	-	+	-	-
13	-	-	+	+	-	-	+	+
14	-	-	+	-	-	-	-	-
15	+	+	+	-	+	-	+	-
16	-	+	-	-	-	+	+	+
17	+	-	-	+++	+	-	+	+
18	+	-	+	+++	+	+	-	-
19	+	+	-	++	+	-	-	+
20	-	-	-	++	+	+	+	-
21	-	-	-	+++	+	+	-	-
22	-	+	-	+++	-	-	+	+
23	+	+	-	+++	-	+	-	-
24	+	+	+	+++	-	+	+	+
25	+	-	-	++	-	-	-	-
26	-	+	+	+++	+	-	-	+
27	-	+	+	+++	-	-	-	-
28	-	+	-	++	-	+	+	+
29	+	-	+	++	+	+	-	+
30	-	-	+	++	-	+	-	+
31	+	+	+	++	-	-	+	-
32	-	-	+	++	+	-	+	-

Table S2. Quantity of tubes prepared for each desired hematocrit for the gauge error experiment.

desired hematocrit (%)	number of tubes prepared
38	1
41	1
43	2
46	3
49	2
51	1
54	1

Table S3. Raw values measured for each response.

	responses		
test	% hematocrit	% hemolysis	transport distance (mm)
initial	45.4	0.0	9.7
1	48.6	3.6	6.3
2	47.0	0.1	8.7
3	46.0	0.8	8.1
4	43.5	0.3	10.6
5	56.7	0.0	6.5
6	50.2	2.5	7.5
7	48.6	0.0	6.7
8	47.9	0.3	11.1
9	48.0	0.8	11.4
10	46.9	2.1	8.7
11	48.6	0.4	5.3
12	45.1	0.3	7.6
13	49.2	0.7	6.6
14	45.6	0.3	10.6
15	44.6	1.1	10.8
16	44.4	0.2	9.7
17	53.2	2.2	6.9
18	71.0	1.0	9.1
19	46.0	2.1	6.6
20	44.3	1.0	4.7
21	55.9	1.2	0.4
22	48.1	1.4	5.3
23	57.3	2.2	9.1
24	52.5	0.3	6.2
25	49.0	4.4	9.4
26	47.3	1.3	5.2
27	57.7	1.8	4.1
28	49.8	2.1	11.6
29	53.1	5.4	6.2
30	52.7	2.5	4.2
31	50.6	1.3	6.4
32	54.0	1.9	6.8

Table S4. Full list of p-values for the effect of each variable on the reference hematocrit measurement prior to model reduction.

	variable	p-value
4	storage time (hrs)	0.0006
7	mixing method	0.1338
2	wide bore tip	0.1553
3	storage temperature (˚C)	0.4502
1	aliquot volume (µL)	0.5475
8	heated (37˚C)	0.6828
5	centrifugation speed (rcf)	0.7041
6	centrifugation time (mins)	0.7401

Table S5. Full list of p-values for the effect of each variable on the transport distance in the paper-based hematocrit device prior to model reduction.

	variable	p-value
4	storage time (hrs)	0.0056
8	heated (37˚C)	0.2458
7	mixing method	0.2984
1	aliquot volume (μL)	0.3231
3	storage temperature (˚C)	0.3587
2	wide bore tip	0.4523
6	centrifugation time (mins)	0.5000
5	centrifugation speed (rcf)	0.9188

Table S6. Full list of p-values for the effect of each variable on the percent hemolysis prior to model reduction.

	variable	p-value
4	storage time (hrs)	0.0813
6	centrifugation time (mins)	0.1481
1	aliquot volume (µL)	0.1675
2	wide bore tip	0.3135
7	mixing method	0.4409
8	heated (37˚C)	0.7907
5	centrifugation speed (rcf)	0.8178
3	storage temperature (˚C)	0.8349

Figure S1. A complete representation of the workflow for performing the fractional factorial experiments. The run depicted here corresponds to test 3 in **Table S1**. The three response measurements (reference hematocrit, the percent of hemolysis, and the blood transport distance) were performed in parallel. This workflow was followed for each of the 32 runs for the fractional factorial design.

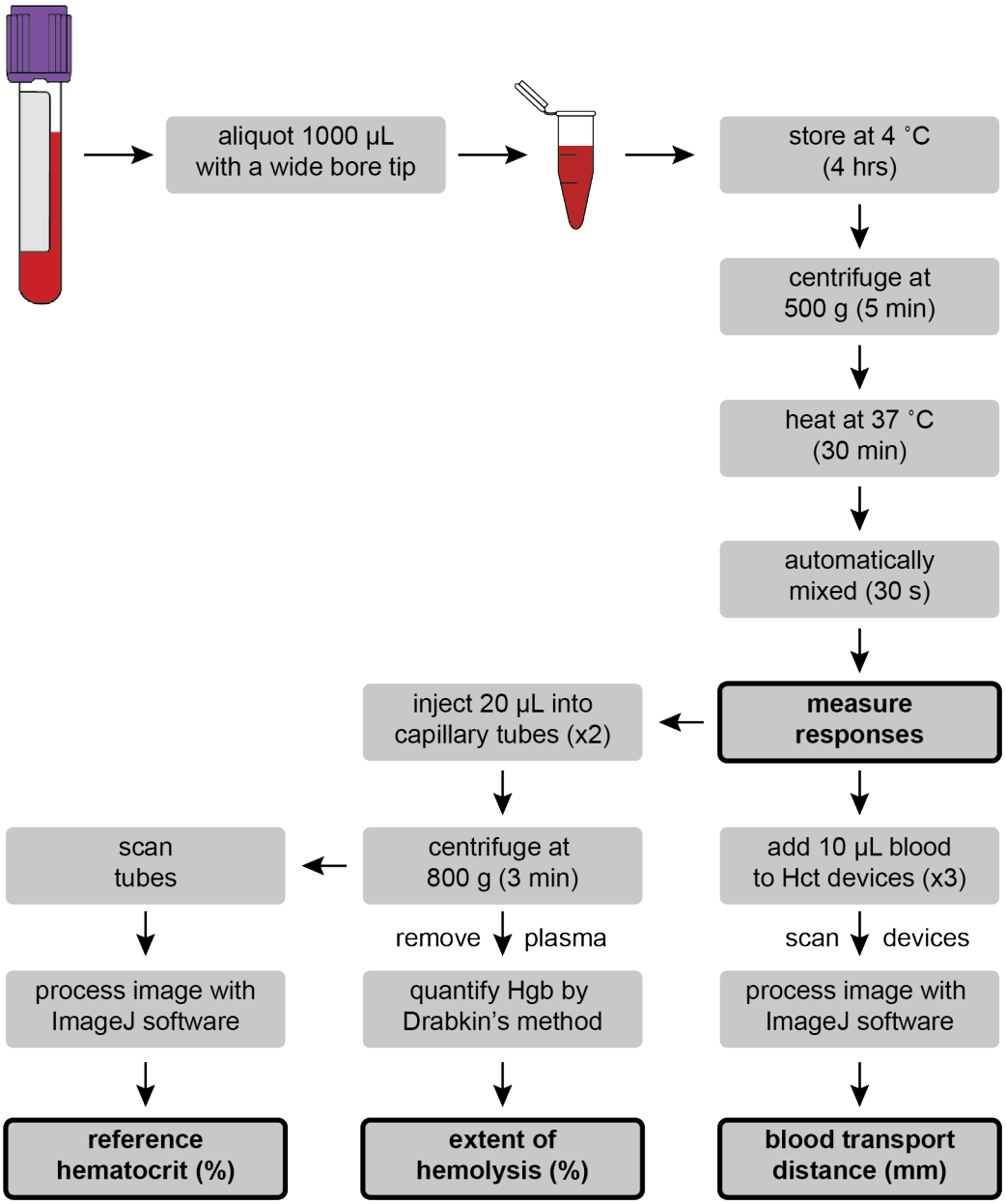
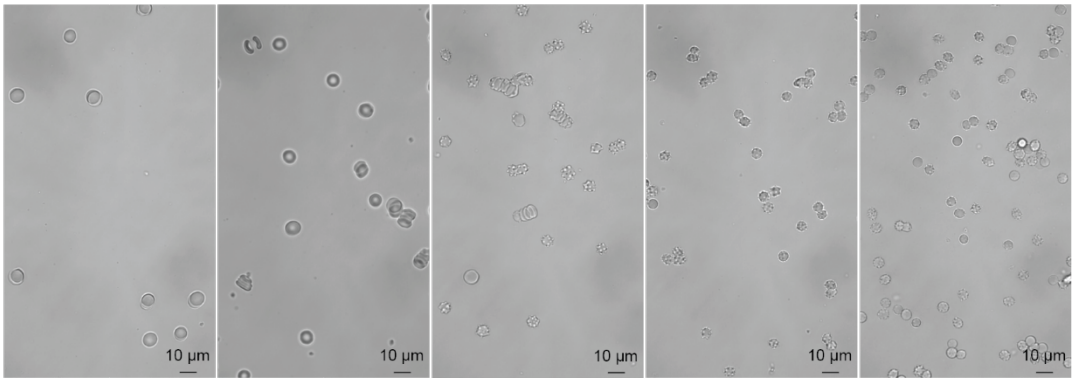


Figure S2. Representative microscopy images with corresponding measured reference hematocrit values as a function of storage time. The percentage of echinocytes was calculated for each time point by taking the ratio of echinocytes to total cells (n>500). The hematocrit appears to decrease at the 168-hour time point. However, we believe this to be due to notable hemolysis in the sample, which we attribute to this specific blood sample.

storage time (hrs)	0	4	48	96	168
Hct (%)	45.9	46.3	49.5	58.1	55.9
echinocytes (%)	0.8	1.7	56.8	90.9	90.0



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