High-yield paper-based quantitative blood separation system

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ARTICLE

High-yield paper-based quantitative blood separation system

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Interest in developing paper-based devices for point-of-care diagnostics in resource-limited settings has risen remarkably in recent decades. In this paper, we demonstrate what we refer to as "High Yield Passive Erythrocyte Removal" (HYPER) technology, which utilizes capillary forces in a unique cross-flow filtration for the separation of whole blood with performance comparable to centrifuges. As we will demonstrate, state-of-the-art passive blood separation methods implemented in paper-based systems exhibit rapid blood cell clogging on the filtration media or serum outlet and yield only about 10%-30% of the total serum present in the sample. Our innovation results from the inclusion of a differentiation pad, which exploits hydrodynamic effects to reduce the formation of a fouling layer on the blood filtration membrane resulting in more than 60% serum yield with undiluted whole blood as direct input. To demonstrate the effectiveness of the HYPER technology we implement it in a lateral flow system and demonstrate the accurate quantification of vitamin A and iron levels in whole blood samples in 15 minutes.

Introduction

Paper-based diagnostic devices are especially suitable for point-of-care diagnostics because they are low-cost and relatively easy to manufacture, store and operate. Interest in developing paper-based microfluidic analytical devices for these applications has risen remarkably in recent years [1, 2]. Due to the relative inefficiencies in existing integrated red blood cell removal methods however, many of these devices rely on external blood separation to achieve high accuracy[3-5]. Recent trends in blood-serum separation show a rapid shift toward microscale processes. Among these devices, passive blood separation technologies, which are achieved without an external force field are favoured because they can be made easily and can be integrated with other biomedical devices[6].

In this paper, we have developed a new approach which we refer to as the High Yield Passive Erythrocyte Removal (HYPER) platform. The HYPER platform can be integrated with lateral flow and other paper-based systems and produced in large quantities at relatively low cost (<$0.10 each). The technique is based around a unique arrangement of papers and a differentiation pad, which adjusts the concentration of serum in the flow and prevents the formation of the fouling layer on the filtration membrane. Because of this the separation yield of HYPER reaches as much as 80%, significantly improved from state-of-the-art passive separation methods and is comparable with what is obtained from conventional centrifugation. Furthermore, HYPER also includes a unique calibration pad system which enables accurate output volume control, reducing the need for pipetting to obtain consistent yields. To demonstrate efficacy, we integrated the HYPER platform with our previously developed multiplexed test for two of the most prevalent forms of malnutrition and proved that the platform can accurately determine iron and vitamin A status within ~15 minutes, at a cost lower than $1 with whole blood as direct sample input.

Results

Schematics and filtration dynamics

Performance of low-cost paper-based blood separation systems that rely on filtration membranes is commonly limited by blood clogging. Filtration membranes separate blood with pores (typically < 9 µm) smaller than size of blood cells, as shown in Fig. 1a. Initially serum passes the filtration membrane efficiently. But as the blood cells aggregate on the filtration membrane, a fouling layer is rapidly formed which prevents further blood separation, and limits the overall yield to the amounts shown in Table 1.

Driven by capillary flow, the HYPER platform utilizes both hydrodynamics and membrane filtration, and the separation yield reaches 86%, with an average of ~66%. As shown in Fig.1b, the HYPER pad consists of three blood filtration layers: a horizontal blood cell differentiation pad in the top that prevents

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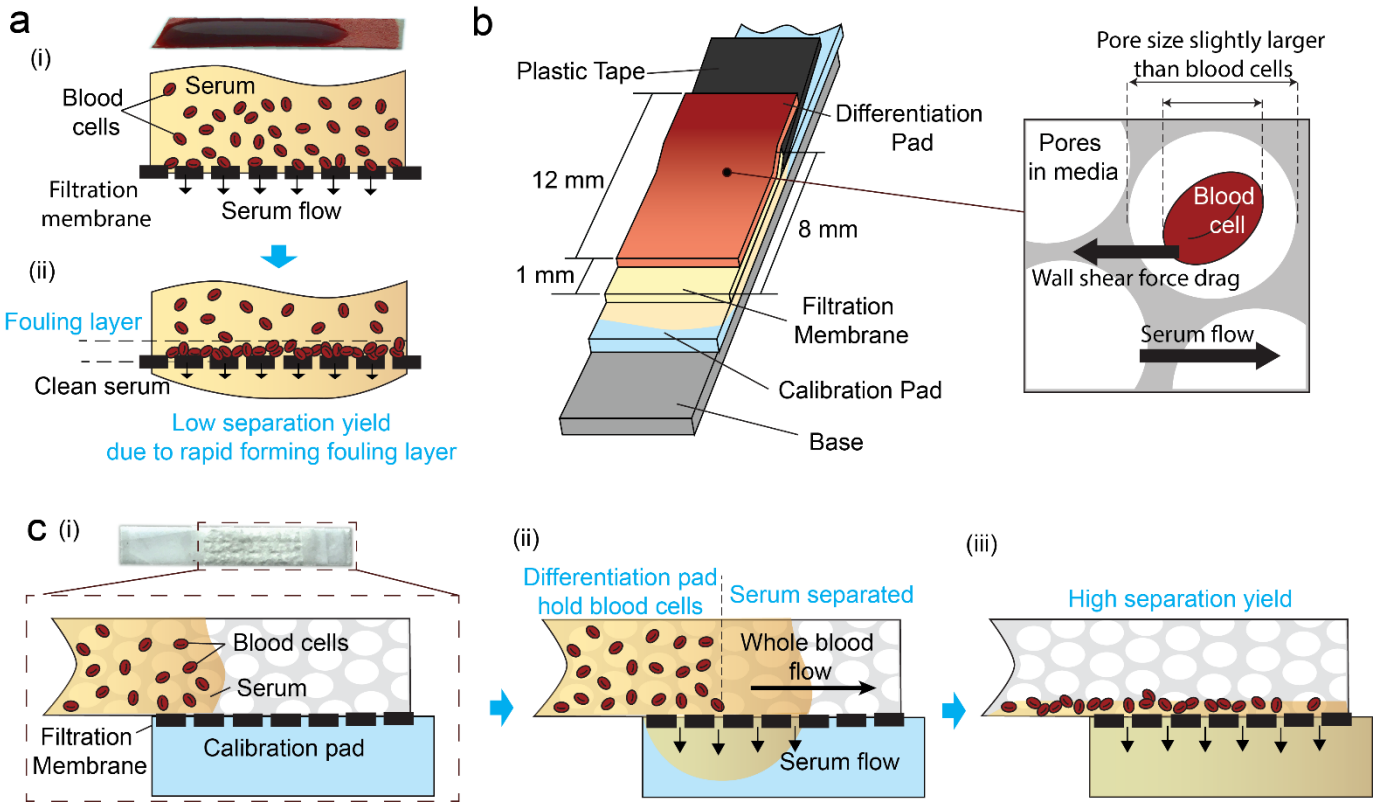

Figure 1. Schematics and filtration dynamic of the HYPER platform (a) Schematic of traditional filtration membrane blood filtration. The separation yield is limited as the fouling layer forms rapidly on the membrane. (b) Schematic of the HYPER platform, the differentiation pad utilizes hydrodynamic effects to slow down blood cell movement in serum flow. (c) Illustration of the flow dynamics of the HYPER platform. The platform slows down fouling layer aggregation by holding blood cells upstream, and thus the separation process becomes faster and more efficient.

fouling layer formation, a filtration membrane in the middle, and a calibration pad to store permeated serum sample. The differentiation pad has an average pore size of 40 µm, marginally larger than the red blood cells. Due to the viscous force between the blood cells and channel walls, the blood cells move slower than serum flow. The filtration membrane is an Asymmetric Polysulfone membrane with a descending pore size from the top side to the bottom side. The estimated mean pore size at the underside of the filtration membrane is 1.9 ± 0.4 µm, reduced from an estimated 50 µm at the topside. It functions as the major filtration medium because only serum can pass through the filtration membrane. To ensure the effectiveness of the separation efficiency, a pore size less than 2.5 µm is usually required for the filtration medium[7]. Outflow serum permeates into the calibration pad, which is fabricated to have a constant liquid holding capacity at 40 µL/cm^2 and stores only the desired amount of serum. Plastic tape is used to ensure contact between layers, and detail in fabrication method is provide in Supplementary Information.

Fig. 1c illustrates the dynamics behind whole blood separation with cross-flow filtration in the HYPER pad. When a whole blood sample is applied to the differentiation pad the blood cells move forward along with serum, driven by capillary force. In the differentiation pad, the blood cells aggregate at the upstream end and serum is differentiated at the flow front. As a result, serum reaches the filtration membrane before the red blood

Table 1. State-of-the-art passive blood separation tools, with their design principle, hematocrit rate and separation yield.

Research group	Design Principle	Hematocrit (Hct %)	Yield %
Yang and Zahn[8]	Bifurcation law T filtration device	45 (Sheep blood)	14-25
Blattert et al[9]	Centrifugal effect and bifurcation law	45	5-10
Kerhoas et al[10]	Combination of constriction-expansion, bifurcation law	45	5
Prabhakar et al[11]	Combination of biophysical effects and geometrical effects	45	3
Li et al[12]	Microbead plug	45 (Sheep blood)	1.8
Chung et al[13]	Membrane filter	45.5	30
Shim et al [14]	Microbeads	43	7
Crowley et al[15]	Porous micro filters	40	1

cells and the clogging on the filtration membrane is reduced. Meanwhile, the blood sample flows horizontally in the differentiation pad, preventing red blood cells from staying at the filtration membrane surface. These two effects significantly slow down the fouling layer formation and raise the yield of blood separation. The calibration pad is a nitrocellulose membrane with properties similar to the differentiation pad. It not only controls volume of outflow serum, but also holds extra blood cells that have accidentally passed the filtration membrane, increasing the overall separation efficiency.

Blood separation protocol and performance

As shown in Fig. 2a, the HYPER platform needs less than 15 seconds of hands-on time to separate serum from whole blood. To begin, around 60 µL of whole blood is applied to the HYPER pad, and cross-flow filtration of the red blood cells starts immediately. Depending on the volume of serum needed, 5-20 minutes are required for the HYPER platform to complete blood separation. After the calibration pad is saturated, the differentiation pad and the filtration membrane are removed to stop the separation process. The running buffer is then applied to initialize the flow and start the test in the integrated paper-based analytical device. As we will demonstrate in the last section of this paper, the serum sample can also be stored for further laboratory-based diagnostic analysis.

To illustrate the performance of the HYPER platform a series of systems with calibration pads of a uniform size (4mm by 25mm) were manufactured. Blood samples with 45% hematocrit were prepared, and 60 µL of whole blood was applied to each HYPER pad. After removal of the differentiation pad and filtration membrane, the volume of serum on HYPER calibration pad is calculated from the measured weight gain. Fig. 2b plots the average volume of serum as a function of time. For each data point, 8 HYPER pads were tested, and the error bar represents the standard deviation. As we will demonstrate later, the hematocrit has significant influence on overall separation efficiency, and we normalized the hematocrit to 45% in this test. The HYPER platform performance results using the whole blood sample collected directly from the participants' vein is provided in Supplementary Information Fig S2. Overall, the HYPER platform reaches a yield of 61.5% within 10 minutes, and a yield of 81.6% within 20 minutes for un-modified whole blood sample.

Separation efficiency and quantitative serum volume control

To demonstrate separation efficiency of the HYPER platform, we show in Fig. 3a a bright field image of the whole blood at the inlet, serum at the outlet, and blood cells trapped on the filtration pad. All the microscopy images were obtained at 400 times magnification. As seen in the images in Fig. 3a, the density of blood cells in the filtration pad is much higher than that in the whole blood sample, demonstrating that the HYPER pad performed effective red blood cell capture. Moreover, because the calibration pad holds extra blood cells that might have accidentally passed through the filtration membrane, few blood cells were found in the final output serum. By checking the number of blood cell in the inflow whole blood and in the outflow serum, we found the HYPER has a separation efficiency of greater than 99%. The number of blood cells was counted with ImageJ software. Details of the method for calculating separation efficiency and blood cell counting can be found in Supplementary Information.

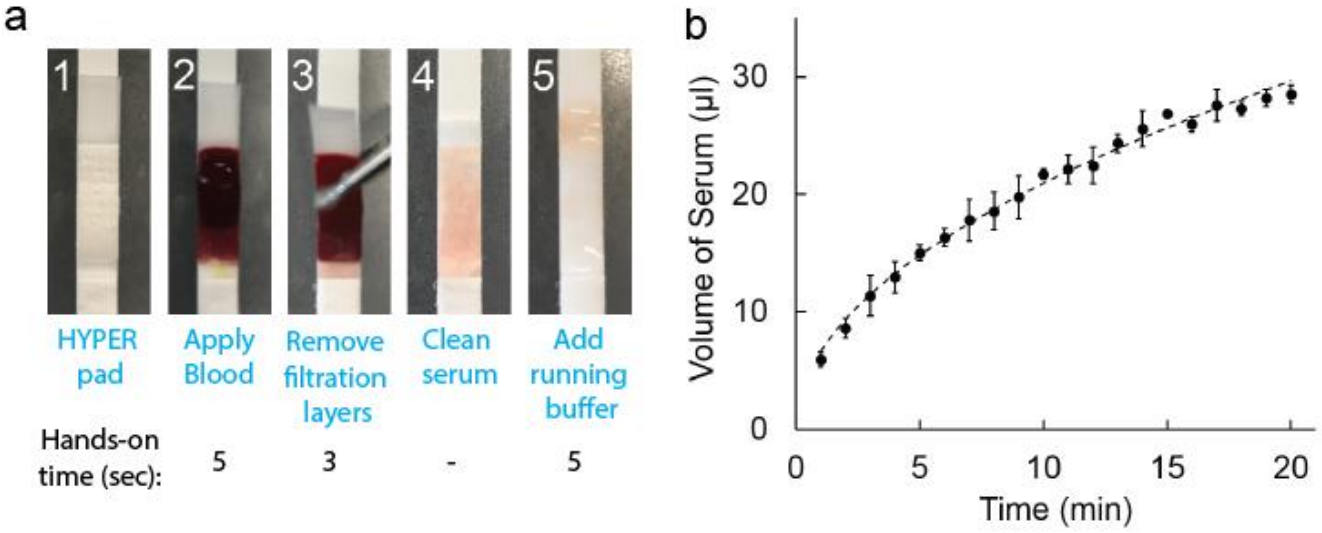

Figure 2. Test protocol and whole blood separation result of the HYPER platform (a) Protocol of operation. First whole blood sample is applied on the differentiation pad. After filtration layers are removed clean serum is obtained. (b) Plot of the volume of serum stored on the calibration pad versus time. In the test 60 µL of whole blood sample with 45% hematocrit rate is used. And the result is fitted to [Volume] = k * t ^ (1/2) according to Washburn's equation. For each data point, 8 HYPER pads were tested.

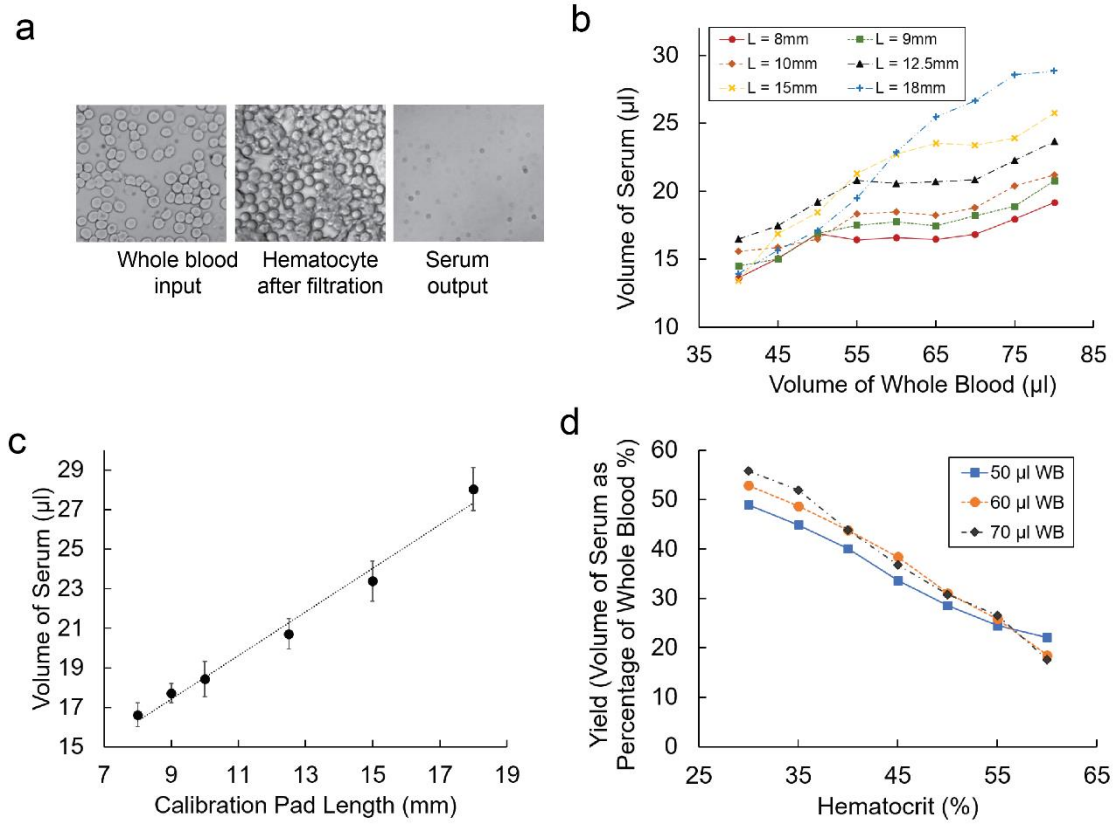

Figure 3. HYPER platform separation efficiency and quantitative outflow serum volume control (a) Optical microscopy of inflow whole blood sample, blood cells remained on the differentiation pad and clean outflow serum. (b) Plot of the volume of serum stored on the calibration pad at various length when amount of whole blood sample changed. (c) Volume of serum stored on calibration pad at specific length and linear fitting of the data point. It indicates that outflow serum volume can be controlled by changing size of calibration pad. 6 HYPER pads were tested for each data point. (d) Yield (serum as volume percentage of whole blood) when whole blood sample with different hematocrit rate.

Consistent sample volume is an essential parameter for achieving accurate results when working with rapid diagnostic tests. Therefore, we incorporated the calibration pad, which can hold as much as 40 µL/cm2 fluid when saturated, to control the volume of sample input to HYPER. Since the flow in the HYPER pad is primarily driven by capillary force, once the calibration pad is saturated, it no longer provides capillary pressure to the flow. The amount of outflow serum depends on the size of the calibration pad. To determine correlation between calibration pad size and stored sample volume, we manufactured 4 mm wide HYPER pads and cut them to various lengths. As shown in Fig. 3b, we tested the volume of outflow serum obtained with the HYPER platform, 10 minutes after whole blood sample was applied, versus the inflow volume of whole blood. For the HYPER pad at each length, the trend of the curve largely remains the same, indicating that the HYPER platform separates similar amounts of serum even if there is poor control on the input volume of the whole blood sample. Details on quantitative separation mechanics can be found in Supplementary Information.

The length of calibration pad is strongly correlated with the volume of separated serum. In Fig. 3c, we plot the volume of serum separated against the length of the calibration pad. In order to fulfil but not to overflow the calibration pad, 60 µL whole blood was applied on the HYPER pad with the calibration pads shorter than 13 mm, and 80 µL whole blood was used on HYPER pads that were longer than 13 mm. For each data point 6 HYPER pads were tested and the error bar shows the standard deviation. Due to the edge effect of the calibration pad, the fitting result has a non-zero intercept on the y-axis. The linear fitting result has a correlation coefficient R^2 = 0.98 when the calibration pad is 4mm in width and between 8 mm and 18 mm in length, indicating that the calibration pad is capable of metering outflow serum volume between 16 - 28 µL with high accuracy.

The HYPER can also adapt to whole blood samples with varying levels of hematocrit. To demonstrate this we used whole blood samples with hematocrit rate ranging from 30% to 60%. For each test, 50 ~ 70 µL of whole blood was applied to 4 mm HYPER platforms and the volume of serum was measured after 10 minutes. The yield was obtained by calculating the volume of

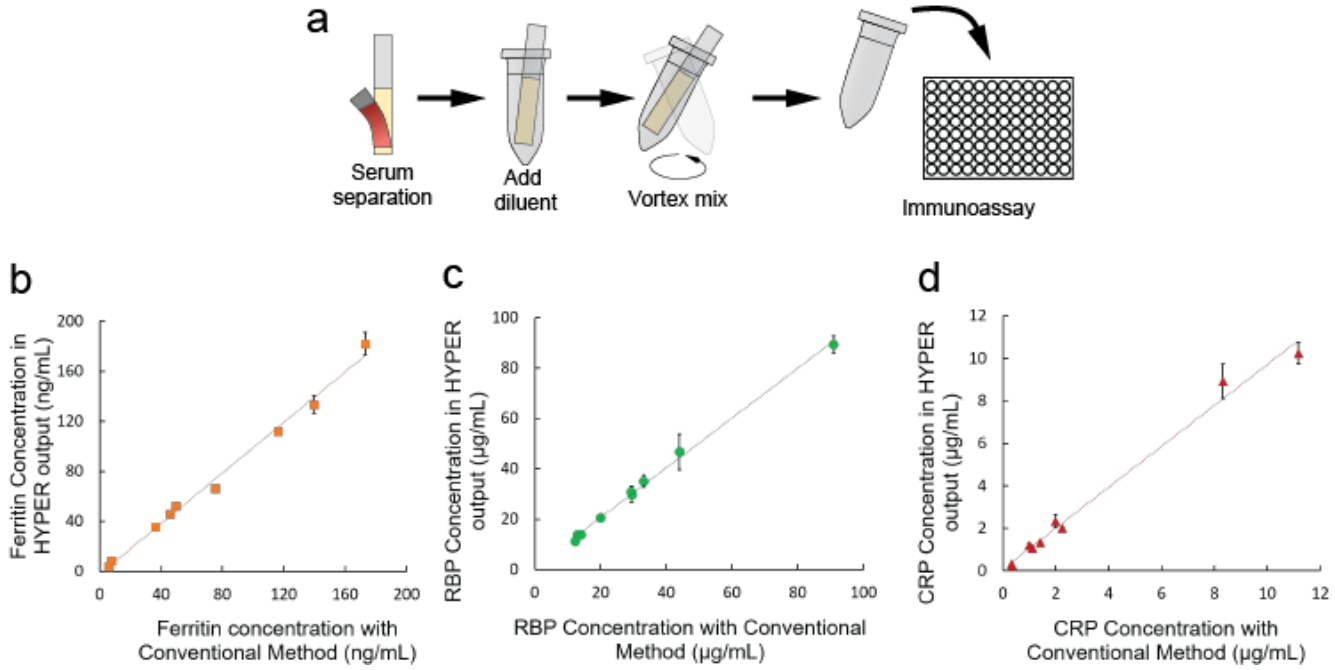

Figure 4. Integration of the HYPER platform and standard ELISA kits (a) Test protocol to use HYPER pad in standard ELISA kits. 20 µL of serum was separated with HYPER platform and diluted to desired rate. (b-d) Plot of the biomarker characterization result. 6 tests were performed at each data point. Concentration of biomarkers in HYPER separated serum shows very high correlation to serum from centrifuge and regression coefficients are close to 1, proving that HYPER can be used as blood separation method for general purpose.

serum as a percentage of the whole blood. The result is plotted in Fig. 3d. The HYPER platform has a yield as high as ~20% even for samples with a very high hematocrit (60%). Though the yield is dependent to hematocrit rate of the sample, the HYPER is able to separate whole blood samples with very large variation in hematocrit rate and volume. This makes the HYPER platform especially suitable for use with finger-pick blood sample, facilitating point-of-care applications.

Characterization of biomarker in outflow serum

In addition to incorporation with paper-based diagnostic tests, the HYPER platform can also be used as a blood separation platform for conventional laboratory tests. We designed a protocol to compare the performance of serum obtained using the HYPER platform to serum obtained via centrifugation by testing with commercially available ELISA kits. As shown in Fig. 4a, to prepare serum samples for ELISA tests with the HYPER platform, HYPER pads that separate 20 µL serum from whole blood sample were manufactured. Once the sample was applied at the inlet and after the differentiation pad and filtration membrane were peeled off, serum stored in the calibration pad was then immersed in 980 µL of diluent buffer to achieve 50 times dilution. To extract the serum sample, the HYPER pad along with the diluent buffer was vortex mixed for 30 seconds until the solution was uniform. Finally, the diluted sample was used as direct input to ELISA kits.

Performance of the HYPER protocol was evaluated by comparing the concentration of the biomarkers in serum obtained from the HYPER platform, and concentration of biomarkers in serum separated with a commercial centrifuge. The serum sample from the centrifuge was also diluted 50 times as well. We utilized ferritin, retinol-binding protein (RBP) and C-reactive protein (CRP) as the target biomarkers for comparison. In the testing, serum ferritin concentration was measured to determine iron status[15]. Since vitamin A binds to RBP in 1:1 ratio, we used serum RBP as the biomarker for vitamin A status[17,18]. Both ferritin and RBP are acute phase proteins whose concentration can be shifted by infections, so we also included CRP to indicate inflammation status[19]. Details about the cut-offs of biomarkers are included in Supplementary Information Table S1.

As shown in Fig. 4b-d, linear regression was applied for each biomarker, and the fitting result was plotted. Concentration of biomarkers in the HYPER outflow serum had a high correlation with results using serum obtained via centrifugation. The three biomarkers we tested show a regression coefficient close to 1 with minimal standard error. Briefly, the ferritin assay has RC at +1.01 ($P<0.0001$), with root mean squared error (RMSE) at 4.9 ng/ml and R^2 at 0.996. The RBP assay has RC at +0.99 ($P<0.0001$), with root mean squared error (RMSE) at 1.3 µg /ml and R^2 at 0.998. The CRP assay has RC at +0.96($P<0.0001$), with root mean squared error (RMSE) at 0.38 µg/ml and R^2 at 0.994. Overall, the HYPER platform, when integrated with laboratory

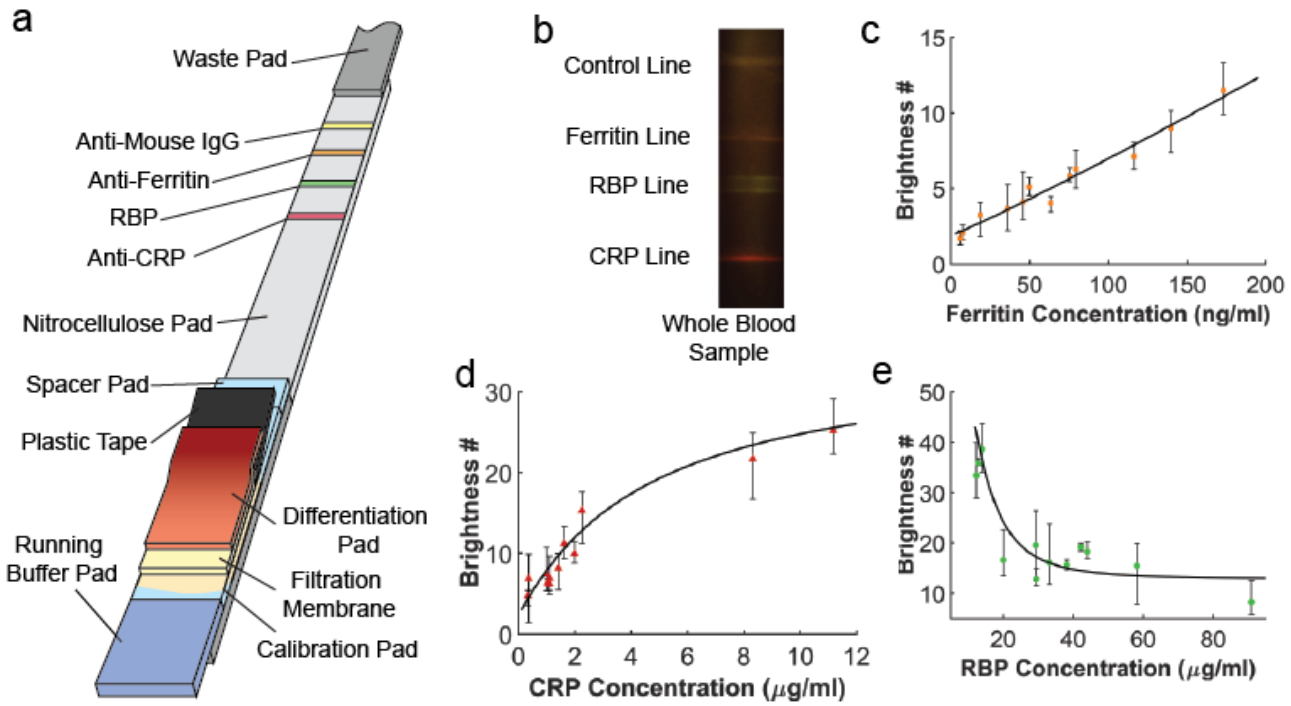

Figure 5. Diagnostic test schematic and calibration result (a) Schematic of the multiplexed test strip. The test strip utilized the HYPER platform as sample pad. The sandwich lateral flow assay used for ferritin and CRP results in higher fluorescence signal intensity with increasing concentration, while higher RBP concentrations result in lower signal intensity due to the use of a competitive assay. (b) Fluorescence image obtained by the TIDBIT platform. (c-e) Data points showing the average intensity of the fluorescence signal for each marker at different concentration and calibration of each marker: [Brightness #] = d + (a - d) / (1 + ([marker] / c) ^ b). For each data point, 3 test strips were used and the error bar shows the *range* of the fluorescence intensity.

standard characterization or diagnostic methods, serves as a suitable alternative to commercially available blood-serum separation methods.

Whole blood multiplexed malnutrition diagnosis

We also integrated the HYPER platform into our previously developed platform[3] for point-of-care vitamin A and iron deficiency diagnosis. Fig. 5a shows a brief schematic of the HYPER pad along with this lateral flow assay. The HYPER pad in this application, had an 8 mm long calibration pad to measure a 16 µL clean serum sample. The HYPER pads were attached to the TIDBIT lateral flow immunoassay test strips as the sample pad. The platform measures ferritin and CRP concentrations with sandwich type immunoassays, and RBP concentration with a competitive immunoassay (further details are available in the supplementary information). 12 whole blood samples from US adults were directly used as the input in this study.

After each test was done, the fluorescence signal intensity on the test strips was compared to the concentration of biomarkers characterized with laboratory standard ELISA methods, and fit to four-parameter logistic curves such that [marker] = f[brightness#], as shown in Fig. 5c-e. Each data point represents 3 test strips using whole blood sample from the same participant, and error bar shows the range of fluorescence intensity. The four-parameter curve fitting results show R^2 = 0.96 ($P < 0.0001$) with standard error SE = 16.6 ng/mL for ferritin, R^2 = 0.81 ($P < 0.0001$) with SE = 3.75 µg/mL for RBP and R^2 = 0.98 ($P < 0.0001$) with SE = 1.37 µg/mL for CRP. Details on parameters of the calibration functions are listed in Supplementary Information Table S2. The test result shows comparable accuracy to our previous work[3] using the centrifuge serum separation, which had the SE at 14.4 ng/mL for ferritin, the SE at 4.34 µg/mL for RBP and the SE at 0.65 µg/mL for CRP, proving that the additional deviation introduced by the HYPER platform is minimal.

Because of the differences in the production process, antibody effectiveness and storage condition of the lateral flow test strips, they are subject to strip-to-strip variation. For single test strip, the HYPER platform has a mean deviation at 24.3%, 20.3% and 26.4% for ferritin, CRP and RBP respectively. As we stated previously, the HYPER platform shows similar SE to the measurement results using centrifugation separation. Therefore the diagnostic conclusion made by the HYPER platform is as accurate if it is based on the average fluorescence intensity of 3 test strips.

Discussion and Conclusion

Current diagnostic methods are heavily dependent on efficient sample purification methods to achieve accurate and reliable results[4-6]. Particularly, molecular diagnostic tests that use blood for analysis require the separation of the red blood cells from the serum present in the sample. While centrifuges are typically used for plasma or serum extraction from whole blood in conventional laboratory methods, they are bulky, expensive and resource dependent. Alternatives proposed to the centrifuge require hands-on operation for extraction[9-10], which adds complexity to test protocols.

The HYPER platform consists of two layers of blood cell filtration membranes with two types of pore geometries, to allow clog-free cross-flow filtration of blood cells on membrane. The channel geometry in the HYPER platform demonstrates a filtration method which achieves high separation yield. Compared to state-of-the-art passive blood separation with less than 30% yield[11-14], the HYPER pad is able to separate approximately 60% of pure serum out of whole blood in less than 10 minutes, making it comparable to the performance achieved by commercial centrifuges. We demonstrate that the HYPER pad is compatible with paper-based diagnostic tests as well as commercial-grade laboratory tests such as ELISA, and the results are comparable to test using centrifuge-based blood separation. In our future work, the HYPER will be integrated into other diagnostics tools, such as microfluidic devices[20-23], that use whole blood as input.

Materials and Methods

HYPER pad manufacture

The HYPER pad has three layers: a differentiation pad, a filtration membrane and a calibration pad. The differentiation pad was made from FR-01 (0.35 mm) horizontal blood filtration pad (Advance Microdevice Pvt. Ltd.). The filtration membrane was an Asymmetric Polysulfone based Vivid Plasma GF Separation Membrane (PALL, Inc.), which serves as the primary blood cell filter. The calibration pad was a Fusion 5 membrane (GE Health) which have 40 µL/cm2 wicking capacity. To manufacture HYPER pad, first FR-01 pad, GF membrane were cut to 12 mm and 8 mm wide strips respectively, and then the two membranes were attached to a piece of plastic tape with a 7 mm overlap. The Fusion 5 membrane was cut to the desired width according to the correlation between the length of calibration pad and the outflow serum volume, and then attached underneath the GF membrane with a 7 mm overlap. For HYPER pads that were integrated into lateral flow assay, they were used as sample pad on the test strips. Otherwise the HYPER pad was then cut into 4 mm wide pieces for blood separation and serum collection. More detailed fabrication method is included in the supplementary information.

Blood sample preparation and lateral flow assay protocols

All human whole blood samples were obtained from commercial provider (Research Blood Components, LLC). The whole blood samples were collected at the commercial provider so no human participants were involved during the research. Whole blood samples for HYPER pad performance evaluation were adjusted to 30% ~ 60% hematocrit rate. To obtain whole blood samples with the specific hematocrit rate, first blood cells were spun down with centrifuge at 2000g for 10 minutes, and then mixed and suspended in serum from the same participant to achieve the desired hematocrit rate. For vitamin A deficiency and ferritin deficiency test, the whole blood samples were applied to the test strips directly, with no adjustment in the hematocrit rates.

In order to run a vitamin A deficiency and ferritin deficiency test, first 60 µL of whole blood was applied to the HYPER pad. After 5 minutes, the differentiation pad and filtration membrane were removed to stop the separation. Then, 40 µL running buffer was added to initialize the flow and the test strip was inserted into the TIDBIT reader immediately. After 10 minutes, the TIDBIT reader automatically took the fluorescence image of the test strip and determined the nutrition status based on the intensity of the test lines on the lateral flow assay.

Conflicts of interest

There are no conflicts to declare.

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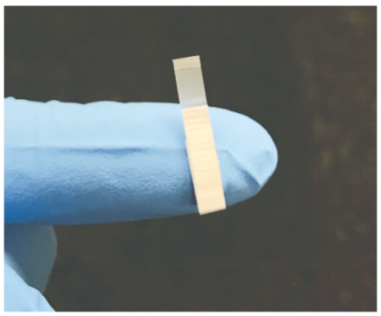

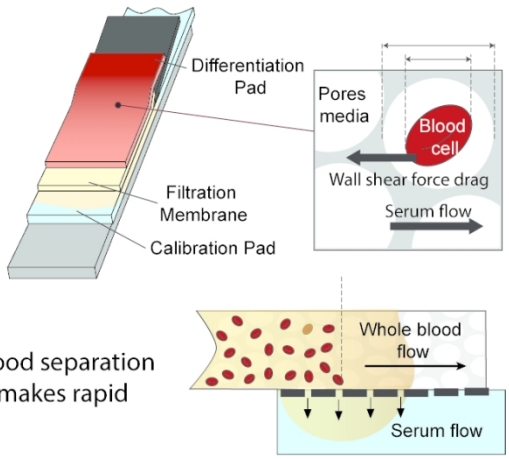

HYPER Platform: Affordable whole blood separation with unique cross-flow filtration that makes rapid diagnostics at point-of-care available