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3D-printed fluidic devices enable quantitative evaluation of blood components in modified storage solutions for use in transfusion medicine
3D-printed fluidic devices enable quantitative evaluation of blood components in modified storage solutions for use in transfusion medicine†

Chengpeng Chen, Yimeng Wang, Sarah Y. Lockwood and Dana M. Spence*

A fluidic device constructed with a 3D-printer can be used to investigate stored blood components with subsequent high-throughput calibration and readout with a standard plate reader.

Introduction

It is estimated that over 16 million units of stored blood are required for transfusion medicine each year in the United States.1 However, complications related to transfusion of blood components still exist and a major effort to determine factors that are considered determinants of those complications is ongoing.2,3 Recently, the levels of in vivo nitric oxide (NO) following transfusion have been investigated and strategies to enhance concentrations of this powerful, gaseous signaling molecule have been reported.4 Motivation for such studies are supported by the fact that patients who receive a transfusion, suffer from insufficient nitric oxide bioavailability (INOBA);2 as a determinant of blood flow, reductions in bioavailable NO would hinder flow of transfused erythrocytes (ERYs), and impair their ability to deliver oxygen to demanding tissues and organs.

There are multiple proposed sources of NO in the bloodstream, including NO release from the ERY itself5,6 or production by other cell types after stimulation by ATP released from the ERYs.7–9 Interestingly, McMahon has shown that the ability of the stored ERYs to release ATP in response to hypoxic conditions is decreased during storage duration.10 In support of these data, using an in vitro model of the bloodstream–endothelium interface, we recently showed that reduced flow-induced ATP release from stored ERYs has a direct effect on endothelium-derived NO production. The inability of the stored ERYs to release normal levels of ATP, which is highly dependent upon the health of the cell, may not be surprising when one considers the various chemical and physical changes of the ERYs during storage.11 Collectively, these changes (often referred to as the red cell storage lesion) are thought to have a significant effect, in vivo, after transfusion has occurred.12–14 The exact cause of the storage lesion is not completely understood, thus promoting various modifications to blood collection and storage solutions, as well as overall blood component storage strategies, to help reduce the occurrence of storage lesion.

The currently approved environment into which ERYs are collected and stored is quite different from in vivo conditions. For example, the bloodstream glucose concentration of a healthy, non-diabetic human is typically in the range of 5–6 mM.14 During the blood storage process, ERYs are collected, and stored in solutions that have initial glucose concentrations >110 mM.15 After the addition of the ERYs to the final storage solution, the final glucose concentration is approximately 40 mM, still much higher than the glucose levels in healthy individuals. While there is minimal clinical evidence suggesting that the high glucose level in storage solutions is the main contributor to the ERY storage lesion, there have been reports showing increased advanced glycation end-products (AGE) on ERYs as the storage duration increased.16 Also, in an in vitro based study, our group recently reported that the collection and storage solutions themselves may adversely affect the ability of the stored ERYs to release ATP, a recognized stimulus of NO production in the endothelium and other cell types. Specifically, it was shown that a modification of the glucose levels in the popular AS-1 storage solution from approximately 110 mM to 5.5 mM resulted in significantly enhanced ATP release from these cells, as well as a significant increase in NO production by an immobilized endothelium. The enhancement in NO production occurred even after 36 days of storage.17 However, each time a measurement was performed during this period, a new microfluidic device (made from poly(dimethylsiloxane), PDMS) was required because the devices were not reusable. Previous devices contained an irreversibly sealed membrane, which results in a device that is essentially single-use. Furthermore, while the PDMS-based devices have a very high experimental success rate, they typically leak between seals after multiple hours (e.g., >8) of use. Furthermore, fabrication of a single device takes hours to complete due to the multiple laboratory steps that must be completed to obtain a finished device. To decrease variability in measurements, an ideal situation would be to employ a single device that was easy to construct, yet be rugged and robust to allow multiple determinations on the stored cells over periods of days to weeks.
Recently, three dimensional (3-D) printing has been utilized in research laboratories to construct scaffolds for tissue growth, pneumatics, and fluidics. 3-D printing is achieved using an additive process, where successive layers of materials are laid down to create a shape, and has been commonly used in manufacturing industries to produce design prototypes. Importantly, in contrast to devices prepared by soft-lithographic methods, 3-D printed devices require no wet laboratory steps; the device is software designed and sent to the printer. Therefore, depending on device complexity, obtaining a finished device can be faster than other fabrication methods. Here, a fluidic device fabricated by 3-D printing technology is used to evaluate ERYS stored in AS-1 (a currently approved storage solution) and AS-1N (a version of AS-1 with modified glucose concentrations). Importantly, upon characterization and validation of the device performance, a week-long evaluation of ERYS was performed using a single 3-D printed device. Our results suggest we demonstrate that reduced glucose levels in the storage solution result in a significantly increased amount of ATP released from the stored ERYS. Further more, a single 3-D printed device was used throughout the week-long study, thus reducing variability in measurements caused by use of multiple devices.

Results and discussion

Design and fabrication of device

As shown in Fig. 1A, the device is modeled after the dimensions of a standard 96-well plate, making it suitable for direct analysis on a plate reader and use with automated fluidic handling systems. The printed column (1 to 12) and row (A to H) markers make it easy to identify and label wells. This particular device enables parallel analyses as it consists of six channels, each with 3 wells. Membrane inserts, which are removable trans-well inserts often used in cell culture applications, were plugged into the wells (Fig. 1B). 3-D printed threads were designed for both ends of each channel to allow for an amenable connection to external tubing via male luer lock adapters (Fig. 1C). The static wells between channels enable simultaneous calibration and/or internal standards when necessary. In this current application, all 3 wells above each channel were used for dynamic determination of ATP release from ERYS flowing through the channel, while the 4 wells not over a channel were used for calibration by performing static measurements of ATP standards. In this construct, quantitative determinations of ATP could be performed, facilitated by a generated working curve on a single device. Fig. 1D displays the cross section of a single channel, enabling a view of each membrane insert after placement into the device. An evaluation of Fig. 1D also shows how diffusion of the analytes could occur, moving from the channel, across the membrane, and to the area above the insert for eventual measurement. In this study, ATP diffuses from channels to wells, where it is collected into buffer already loaded into the well insert prior to commencement of pumping. Based on diffusion, the amount of ATP accumulated in the well (at some fixed pumping time) is proportional to the concentration in the channel, thereby enabling ATP that is in the channel to be quantitatively determined. After flow of samples in the channels, the device was detached from tubing and directly placed into the plate reader for measurements (Fig. 1E). A more detailed schematic of the entire device design can be seen in Fig. S1 in ESI. Device and well alignment in the plate reader were characterized and validated prior to studies involving ATP by using fluorescein standards ranging from 0 to 20 μM in the membrane inserts, followed by linear regression analysis of the standards as shown Fig. S2 in ESI.
Evaluation of device ruggedness

The trans-well inserts are not fixed into place by any type of glue or epoxy adhesives that lead to permanent combination. There is, however, a wrap of PTFE tape around the side of inserts to help them seal onto the bulk device tightly, yet remain removable if needed. However, to confirm that bulk fluid movement is not occurring from the channel to the area above the membrane insert, or vice versa, 50 μL of doubly deionized water (DDW) was added into membrane inserts in wells B1, E1 and G1 above channel 1. After circulating DDW through the channel for 2 hours at a rate of 50 μL min⁻¹, the remaining volumes of water in the inserts were measured. A control set was performed by adding 50 μL of DDW in inserts placed in static wells A2, D2 and F2, and then measuring the remaining volumes after two hours loading. As shown in Table 1, though a minimal loss of liquid volume in the dynamic inserts (B1, E1 and G1, with flowing underneath) was observed, the lost volume was not likely due to liquid transfer into the channel for a minimal liquid loss was also shown in static inserts (A2, D2 and F2), beneath which, there is no channel. There is no significant difference in remaining volumes between dynamic and static inserts. The results suggest that loaded liquid can be firmly held in dynamic inserts without being drawn into the underlying flow channel for the time duration investigated. The other five channels were examined in the same way, and no bulk liquid was observed. However, it should be noted that changes in flow rate, pore diameter of the membrane, or any flow restrictors at the end of the channels will alter the direction of fluid flow, thus resulting in either bulk fluid movement from channel to insert well or an apparent aspiration of fluid from insert to channel.

The leakage of fluids from the flowing channel across the membrane and into any pre-loaded reagents on the other side of the membrane have the potential to be detrimental to the quality of analysis using this fluidic device (e.g., dilution can hinder detection limits). To determine if flowing fluids leak across membrane (as opposed to movement of an analyte by diffusion alone), a fluorescein solution was circulated through channel 1, with nothing loaded in the membrane inserts above the channel. After 2 hours of pumping this solution, fluorescence images above the channel were obtained. Fig. 2 was integrated by images observed on separate parts of the channel because of the limited camera view. As shown in Fig. 2, there is some fluorescein at the bottom of each well. This fluorescence was on the apical side of the membrane (the bottom side, touching the channel). Due to the lack of fluorescein entering the well (we would expect a “circle” of fluorescence emission in the well if flowing fluid had moved across the membrane), we concluded that leakage of fluids was not occurring. The other five channels were verified with the same method and none showed any leakage of liquid across membrane. This is important because it will help ensure that only molecules can diffuse or move through the membrane to the other side of inserts.

Optimizing device parameters for the quantitative determination of ATP

There are multiple factors that can affect the overall quantitative determination of ATP release from the stored ERYs flowing through the device channels. One of the first factors to be investigated was the amount of time the ERYs were allowed to flow through the device channel prior to determination of ATP. As the ERYs pass through the channel, they release ATP that diffuses through the porous membrane on the bottom of the trans-well insert. Thus, an increase in the amount of time the ERYs pass through the device channels would increase the collection time and amount of ATP in the well above the channel. To determine the optimal ATP collection time, ATP standards (0 to 800 nM) were circulated for 10 min, 20 min or 30 min through a device channel having a well insert that was loaded with 50 μL of PSS. This circulation time enabled ATP from the ERYs to diffuse through the membrane pores. After the various circulation times of the ERYs (or, the collection time of ERY-derived ATP), an aliquot of a luciferin–luciferase mixture was added to the well insert and the resultant chemiluminescence was recorded using the multi-well plate reader. Calibration curves were prepared to investigate such figures of merit as analytical sensitivity and limits of detection for each collection time. The resultant data, which are summarized in Table 2 and shown graphically in Fig. S3 (ESI†), show that lower limits of detection and higher sensitivity can be achieved as the collection time increased. Although quantifiable results were

![Fig. 2](image-url)
suggest that 10 μL of the luciferin–luciferase mixture yielded the lowest background (y-intercept), and the best linearity, as measured by the coefficient of determination, $R^2$. In fact, larger volumes of the luciferin–luciferase mixture added to the wells lowered analysis quality, likely due to higher background emission. It was also observed that when adding the 20 or 30 μL volumes of the luciferin–luciferase mixture, the signals from 100 nM or 200 nM ATP standards were not statistically different and exhibited reduced linearity and higher detection limits. A calibration curve with 20 min ATP collection time and 10 μL of luciferin–luciferase, the optimal conditions among variations studied here, is shown in Fig. S5 in the ESI.†

Evaluation of analytical features of the device

High-throughput evaluation. With six channels integrated into the 3-D printed device, all of which were amenable to a commercial plate reader, high-throughput applications were explored. Six ATP standards (0 to 0.8 μM) were circulated in the six channels, but in a random order. The membrane inserts in wells B on each channel were loaded with 50 μL of PSS to collect ATP by diffusion. The chemiluminescence intensity detected from each insert as a function of the concentration of ATP flowing in the channels is shown in Fig. S6 in the ESI.† The linearity ($R^2 = 0.99$) and precision of measurement (indicated by error bars representing standard deviation) demonstrated the device can perform 6 quantitative analyses, simultaneously.

Detection accuracy, intra-channel reproducibility and repeatability evaluation. Under optimal conditions, ATP standards of known concentrations (150 nM and 250 nM) were evaluated with the device. These two concentrations were determined because a 7% solution of ERYs release ~200 nM ATP. Such measurements were performed on channels 1, 3 and 5. Data shown in Table 4 indicates that the levels of ATP can be detected quantitatively, and the detection results from the three channels did not show any statistically significant difference, which further suggests that quantitation on different channels will yield statistically similar results. Such results are expected due to all channels being printed on the same printer using the same prototype dimensions. To confirm precision between channels, ATP standards were circulated in three channels and resultant calibration curves were compared. As shown in Table 5, these channels were statistically equal in terms of background, sensitivity, linearity, detection limit and accuracy. This intra-channel reproducibility is another outstanding advantage of the 3-D printed device, as it helps reduce variability of devices from different manufactures and fabrication protocols. Another key advantage is the reusability of the 3-D printed device; the

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### Table 2 Optimization of ATP collection time

<table>
<thead>
<tr>
<th>Collection time/min</th>
<th>Y intercept</th>
<th>Slope</th>
<th>$R^2$</th>
<th>Detection limit/nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20.9 ± 5.5</td>
<td>517.5 ± 33.6</td>
<td>0.98 ± 0.005</td>
<td>136.6 ± 24.0</td>
</tr>
<tr>
<td>20</td>
<td>15.8 ± 1.8</td>
<td>565.4 ± 38.6</td>
<td>0.99 ± 0.001</td>
<td>52.4 ± 7.4</td>
</tr>
<tr>
<td>30</td>
<td>19.8 ± 1.0</td>
<td>883.8 ± 69.9</td>
<td>0.99 ± 0.007</td>
<td>33.3 ± 6.8</td>
</tr>
</tbody>
</table>

$a$ n = 3; all errors represent standard deviations.

### Table 3 Optimization of luciferin–luciferase assay volume

<table>
<thead>
<tr>
<th>Assay volume/μL</th>
<th>Y intercept</th>
<th>Slope</th>
<th>$R^2$</th>
<th>Detection limit/nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>15.8 ± 1.8</td>
<td>565.4 ± 38.6</td>
<td>0.99 ± 0.001</td>
<td>52.4 ± 7.4</td>
</tr>
<tr>
<td>20</td>
<td>36.8 ± 3.7</td>
<td>515.9 ± 21.0</td>
<td>0.97 ± 0.02</td>
<td>120.1 ± 13.6</td>
</tr>
<tr>
<td>30</td>
<td>43.8 ± 1.9</td>
<td>549.0 ± 32.0</td>
<td>0.97 ± 0.01</td>
<td>167.7 ± 39.4</td>
</tr>
</tbody>
</table>

$a$ Each assay volume was performed on 5 ATP standards; the resultant calibration curves were evaluated for y-intercept, slope, and linearity which, in turn, were used to determine the detection limit (n = 3; all errors represent standard deviations).

obtained even after 10 minutes of pumping, the 20 minute collection period was utilized for subsequent studies because of its precision and significant reduction in the limit of detection in comparison to the 10 minute pumping period and its suitability for measurements of ERY-derived ATP, which is typically in a range of 90 to 400 nM, depending on the health status of the donor.

A mixture consisting of luciferin–luciferase is required in the chemiluminescent determination of ATP. However, this mixture also contributes to higher background luminescence during the measurement portion of the analysis. Thus, a study was performed to determine which volume of luciferin–luciferase mixture added during the assay provided the best analytical features (detection limit, sensitivity, etc.). To perform this study, ATP standards of identical concentration were circulated in a channel for 20 min and ATP was allowed to diffuse through the membranes of the trans-well inserts that had already been filled with 50 μL of buffer, after which, the device was detached and placed on the sample holder of the plate reader. Aliquots of 10, 20 or 30 μL of the luciferin–luciferase mixture were added to the wells and the chemiluminescence from each trial was acquired. The data from these studies, shown in Table 3 and graphically in Fig. S4 (ESI†),
same device was used for all reported experiments and was cleaned with a simple rinse with DDW. Data in Fig. S3 and S6 in the ESI† were obtained using the same channel on the same device. The error bars, which represent standard deviation, support the reusability of the device, which not only reduces use of materials, but also enables repeated measurements of the same sample on the same device in a reproducible manner.

**Application of the device to quantify ATP released from stored ERYs**

The device was next applied to quantify ATP released from ERYs stored in AS-1, and FDA-approved solution and our modified solution (AS-1N). ATP release from stored ERYs has recently been shown to be reduced during the storage period. ATP plays a key role in blood flow as a recognized stimulus of NO production from endothelial cells, which regulates vessel dilation. A storage period of one week was examined and the ATP release data is consistent with that in Fig. 3, thus confirming that the device has the capability to perform simultaneous calibration and sample quantification in a high-throughput manner.

**Experimental**

**Design and fabrication of device**

The fluidic device was printed on an Objet Connex 350 printer capable of an XY resolution of 600 DPI and Z resolution of 16 μm, (Stratasys Ltd, Eden Prairie, MN) in the department of Electrical and Computer Engineering at Michigan State University. The 3-D design, (Fig. S1 in ESI†) was created using the CAD based engineering software™ package Autodesk Inventor Professional (Autodesk, Inc., San Francisco, CA). The device was printed using Objet VeroClear material (Stratasys Ltd, Eden Prairie, MN) whose exact composition is proprietary, but approximately contains isobornyl acrylate (15–30%), acrylic monomer (15–30%), urethane acrylate (10–30%), acrylic monomer (5–10; 10–15%), epoxy acrylate (5–10; 10–15%), acrylate oligomer (5–10; 10–15%), and a photoinitiator (0.1–1; 1–2%). Once printed, the device is translucent and rigid, but becomes optically transparent and ready to use after a simple polish and clean with sand paper and water. Commercially available membrane inserts (6.5 mm diameter; Corning, Inc., Horseheads, NY) with polyester membranes (0.4 μm pore diameter) were inserted into the dynamic wells above the channels. The inserts function as a semi-permeable barrier between flowing cells or reagents in the channel and reagents that were loaded in the insert (on the opposite side of the membrane). This configuration enables molecular transport through the pores by diffusion. The side of each membrane insert was wrapped by a layer of PTFE seal tape (PL Sourcing, Inc., Newport News, VA) to enhance the seal between the inserts and the wells.

A section of grafted tubing was prepared by connecting two, 20 cm pieces of 1/8” Tygon tubing (Saint-Gobain PPL Corp, Jackson, MI) to the ends of a piece of 15 cm Ismatec tubing (Cole-Parmer Instrument Company, Vernou Hills, IL). The two ends of the tubing were connected to male luer lock adapters (IDEX Health & Science LLC, Oak Harbor, WA), which can be integrated with the two threaded ends of a channel in the printed device, thus forming a loop. The threads were printed in AS-1 than AS-1N (p < 0.03). These results further suggest that the currently approved storage solution (AS-1) may have adverse effects on stored ERYs due to its hyperglycemic character.

The data in Fig. 3 was acquired in two steps, measuring five ATP standards in five channels first, then flowing ERY samples in two random channels for determination of released ATP. In terms of analytical impact, to further examine if the device can perform calibration and sample measurements in only one step, four ATP standards and ERY samples stored in AS-1 and AS-1N were flowed simultaneously on the device. Panel A in Fig. S7 in the ESI† shows the setup of such a measurement on the 3-D printed device, while panel B shows an example of an acquired calibration curve. Panel C contains the quantified ATP release from AS-1 and AS-1N stored ERYs over a seven day storage period. The ATP release data is consistent with that in Fig. 3, thus confirming that the device has the capability to perform simultaneous calibration and sample quantification in a high-throughput manner.

**Table 5**

<table>
<thead>
<tr>
<th>Channel</th>
<th>Y intercept (nM)</th>
<th>Slope (nM)</th>
<th>R²</th>
<th>Detection limit/nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel 1</td>
<td>15.8 ± 1.8</td>
<td>565.4 ± 38.6</td>
<td>0.99 ± 0.001</td>
<td>52.4 ± 7.3</td>
</tr>
<tr>
<td>Channel 3</td>
<td>18.7 ± 0.6</td>
<td>562.2 ± 12.1</td>
<td>0.99 ± 0.006</td>
<td>47.2 ± 7.4</td>
</tr>
<tr>
<td>Channel 5</td>
<td>15.3 ± 2.8</td>
<td>561.1 ± 18.3</td>
<td>0.98 ± 0.005</td>
<td>52.9 ± 5.2</td>
</tr>
</tbody>
</table>

† n = 3; all errors represent standard deviations.
directly from the device design (as opposed to being tapped post-printing) as 10–32 type threads, thus enabling the use of the aforementioned male luer lock adaptors. Fluids or samples were driven through the loop by a 12-roller peristaltic pump (IDEX Health & Science LLC, Oak Harbor, WA).

Evaluation of device ruggedness
To confirm the absence of bulk fluid movement through the membrane pores into a channel, a 50 µL aliquot of DDW was added to dynamic inserts in wells B1, E1 and G1, while DDW was delivered through the closed loop system at a flow rate of 50 µL min⁻¹ for 2 hours. The amount of water in each insert after pumping was determined by mass measurement. As a control group, the same amount of DDW was loaded into each insert in static wells A2, D2 and F2 (no channel beneath and thus no possible water delivery) for 2 hours. There is no channel underneath the static inserts, thus representing a control group used to account for any change in volumes in the inserts due to evaporation. All other channels were examined in the same way, using static inserts in the next right column as a control set.

To investigate the extent of any leakage of fluids from a channel to the area above membrane inserts, a 30 µM fluorescein solution in DDW was circulated at a rate of 50 µL min⁻¹ in the loop for 2 hours. The device was then detached from the pumping system and placed under the objective lens of a fluorescence microscope (Olympus, Japan). Leakage was investigated for all six channels on the device; any detectable quantity of fluorescein on the outside of the trans-well inserts was considered to be indicative of leaking.

Optimizing device parameters for the quantitative determination of ATP

ATP collection time optimization. The well-established luciferin–luciferase chemiluminescence assay was used for ATP assays.²⁸ Reagents were prepared by dissolving 2.0 mg of d-luciferin (Sigma Aldrich, St. Louis, MO) in 5 mL of DDW, and adding the resultant solution into a single 100 mg vial of DDW, and salt solution (PSS, contains 4.7 mM KCl, 2.0 mM CaCl₂, 1.2 mM MgSO₄, 140.5 mM NaCl, 21.0 mM Tris hydroxymethyl amino-methane, 5.5 mM glucose, and 5% bovine serum albumin at pH = 7.4; all reagents were from Sigma Aldrich). A 50 µL aliquot of PSS was loaded in the insert located in well B. The PSS served as a solution to collect ATP diffusing from the channel through the membrane of the insert. After 10 min, 20 min or 30 min of pumping the various ATP standards, the device was detached and placed in the sample holder of the plate reader (Molecular Devices LLC, Sunnyvale, CA). A 50 µL aliquot of 20 nM ATP standard solution was pipetted into insert in static well 2A as a calibrator, thereby minimizing possible indeterminate error. A 10 µL of luciferin–lucerase was then added into inserts in wells 2A and 1B, simultaneously. After 15 s, the chemiluminescence intensity from both inserts was detected simultaneously, followed by the evaluation of the detection background, detection limit, sensitivity and linearity for the different pumping times under investigation.

Luciferin–luciferase assay amount optimization. To determine the optimal volume of luciferin–luciferase mixture for each assay, ATP standards were prepared and circulated in a channel, as described above, with 50 µL of PSS loaded in the insert in well 1B. ATP standards circulated for 20 min at a rate 50 µL min⁻¹. Next, 10 µL to 30 µL aliquots of the luciferin–luciferase mixture were added to the membrane inserts in dynamic well 1B, as well as static well 2A (containing 50 µL of 20 nM ATP standard as a calibrator) simultaneously. The device was detached and placed in the plate reader sample holder and the optimal assay amount was determined by an evaluation of detection limit, linearity and sensitivity.

Evaluation of analytical features of the device

High-throughput evaluation. ATP solutions of 0, 0.2, 0.3, 0.4, 0.5, 0.8 µM were prepared in PSS and circulated randomly through 6 channels, simultaneously, at a rate 50 µL min⁻¹ for 20 min after 50 µL of PSS were loaded in the membrane inserts in wells B above each channel. The device was then detached from tubing and placed in the plate reader for chemiluminescence detection with the optimal ATP quantitation parameters determined above, on all wells in the row labeled as “B”. A curve of chemiluminescence intensity versus ATP concentration was obtained, and the resulting regression statistics (slope, y-intercept, and coefficient of determination) were calculated.

Detection accuracy evaluation. ATP standards of 150 nM and 250 nM were prepared and circulated in channel 1 at a rate 50 µL min⁻¹ for 20 min, with 50 µL of PSS buffer loaded in the membrane insert in well B (dynamic wells) above the channel to collect ATP by diffusion. A 50 µL aliquot of 20 nM ATP was then added into the membrane insert in wells found in row labeled as “A” (static wells). The device was then detached from tubing and placed in the sample holder of the plate reader. Chemiluminescence was detected 15 s after adding 10 µL of the luciferin–luciferase assay into dynamic and static wells to quantify the ATP, using calibration curves prepared in advance in a similar manner as described above. Two more identical measurements were performed on channel 3 and channel 5.

Evaluation of intra-channel reproducibility and reusability
ATP standards having concentrations ranging from 0 to 800 nM were prepared and simultaneously circulated through channels 1, 3 and 5 at a rate 50 µL min⁻¹ for 20 min, while 50 µL of PSS buffer were loaded in the membrane inserts in well B above each channel. After adding 50 µL of 20 nM ATP to wells 2A, 4A and 6A (as static calibrators for measurements on channels 1, 3, and 5, respectively), the device was detached and placed in the sample holder of the plate reader. An aliquot of 10 µL of the luciferin–luciferase mixture was simultaneously added into inserts above the dynamic and static wells, and chemiluminescence was detected by the plate reader after 15 s. A comparison of sensitivity, detection limit and linearity of results from the 3 channels was performed to determine if the device channels and inserts are statistically equivalent. Precision was
also investigated by performing identical measurements as those described above while changing well inserts in between studies.

Application of the device to quantify ATP released from stored ERYs

Collection and storage of ERYs. Citrate phosphate dextrose solution (CPD) and additive solution 1 (AS-1) were prepared according to standard compositions found in the literature. Specifically, 50 mL of CPD were prepared containing 89.4 mM sodium citrate, 15.6 mM citric acid, 128.8 mM dextrose, and 16.1 mM monobasic sodium phosphate. For AS-1, 200 mL were typically prepared containing 154.0 mM sodium chloride, 41.2 mM mannitol, 1.8 mM adenine, and 111.1 mM dextrose (all chemicals were from Sigma Aldrich). All reagents were prepared from solid forms and used as received without further purification. Normoglycemic versions of CPD and AS-1 (CPD-N and AS-1N, respectively) were prepared in a manner identical to CPD and AS-1, but with the glucose level at 5.5 mM.

All blood was collected by venipuncture under IRB approved protocol. The collection process consisted of preparing 6 non-siliconized and untreated (i.e., no heparin or other anticoagulant) 10 mL glass Vacutainer tubes; 3 of these tubes contained 1 mL of CPD, while the other 3 contained 1 mL of CPD-N. Next, approximately 7 mL of whole blood were collected into each tube, resulting in a total blood volume of ~8 mL. The blood remained in the collection solutions for at least 30 min, but not more than 2 h at room temperature (~20 °C), prior to processing. Whole blood processing consisted of centrifugation at 2000g for 10 min followed by removal of the plasma and buffy coat layers by aspiration. Importantly, an additional top 2 mm layer of the packed ERYs were also removed to minimize white cells remained during subsequent storage in the AS-1 or AS-1N solutions. The purified ERYs from the 3 tubes containing CPD were then combined into a 15 mL tube, followed by the addition of AS-1 such that the ratio of packed ERY volume to AS-1 volume was 2 : 1. The same protocol was followed for ERYs collected in CPD-N and stored in AS-1N. Finally, 2 mL of the ERYs (stored in the AS-1 or AS-1N) were added to PVC bags at 4 °C for up to 7 days. Prior to use, the PVC bags were sterilized under UV light overnight. The PVC bags were prepared in-house using rolled PVC and a heat sealer. All solutions used in collection and storage were autoclaved at 10 bar and 121 °C prior to use. All blood collection and storage processes were performed under sterile conditions. All blood collection procedures from informed and consented donors were approved by the Biomedical and Health Institutional Review Board at Michigan State University.

Measurement of ATP released from ERYs stored in AS-1 and AS-1N. A calibration curve was first prepared by flowing ATP standards (0, 0.1, 0.2, 0.4, 0.8 µM) in five channels simultaneously, with 50 µL of AS-1 loaded in wells B above each channel for 20 min to collect diffused ATP, which was then determined by chemiluminescence as described above. After rinsing the device by DDW and AS-1, ERYs stored in AS-1 and AS-1N were diluted with corresponding solution to a final hematocrit of 7%, and circulated on the device in 2 random channels for 20 min at a rate 50 µL min⁻¹. An aliquot of 50 µL of the appropriate additive solution was loaded in the inserts in wells B above corresponding channels to collect ATP by diffusion, after which, the device was detached and placed in the plate reader, 10 µL of the luciferin–luciferase assay mixture were simultaneously added into the well inserts and chemiluminescence intensity was measured after 15 s. In addition, a 50 µL aliquot of 20 nM ATP was added into the insert in well 2A (static well) as a calibrator during both calibration curve preparation and sample measurement. To confirm that the increase in released ATP was not due to cell lysis, an absorbance measurement was performed (to evaluate if free hemoglobin was detected in the supernatant) after flow was concluded. If hemoglobin was detected, that particular sample was discarded due to indication of lysis. In the studies reported here, there were no samples discarded due to lysis.

The ATP released from ERYs was also quantified on the device in a one-step manner. Four ATP standards of 0, 0.1, 0.2 and 0.4 µM in AS-1N were prepared and circulated through channels 1, 3, 5 and 7, while diluted ERY samples stored in AS-1N and AS-1 were flowing in channels 9 and 11. An aliquot of 50 µL of AS-1N was loaded in the inserts in wells B (dynamic well) above all channels to collect ATP by diffusion for 20 min, after which the same procedures as described above were taken to detect chemiluminescence intensity from wells B by the plate reader. Signals from wells 1B, 3B, 5B and 7B, which corresponded to 0, 0.1, 0.2 and 0.4 µM ATP flowing beneath them, respectively, were used for calibration curve, while signals from wells 9B and 11B reflecting the released ATP amount from corresponding ERYs samples.

Statistical analysis of data

For those studies involving blood samples obtained from different donors, the standard error of the mean was used in data evaluation. All other experiments utilized the standard deviation of the mean for evaluation of precision. All statistical comparisons of obtained means were performed using Student’s t-test at 95% confidence, unless otherwise noted.

Conclusion

A 3-D printed fluidic device was successfully employed to facilitate the quantitative determination of ATP release from ERYs stored in different conditions through the use of a plate reader. In terms of preparing the materials necessary to evaluate the stored ERYs, 3-D printing has proven to be a convenient method for device fabrication, is capable of printing devices that can be used in conjunction with popular laboratory instrumentation (i.e., the device reported was modeled after a 96-well plate, which is amenable to a commercial plate reader optical robotics for efficient and high throughput analysis). Compared to conventional PDMS-based fluidic devices, a 3-D printed device is more rugged and robust, did not leak between seals, and was capable of multiple uses over a week-long study. Here, stored ERYs in AS-1 and AS-1N were circulated and tested...
on a single 3-D printed device, thus significantly increasing the reliability of the experimental data by eliminating variability of having to use multiple devices. The rigidity of the material also creates an end-user friendly device. For example, threads can be directly printed at the ends of each channel, allowing the channel to be connected to external tubing by standard maleleur adapters. Six channels integrated onto the device enable high throughput flow analysis and static wells between channels facilitate simultaneous internal standard and/or calibration.

Nonetheless, unlike soft-lithographic methods, where almost any laboratory can design and fabricate fluidic devices, 3-D printers, especially high performance printers, are not widely available. File transfer can help overcome some of the limitation of printer availability; however, as new printers become available, this new method of fabricating fluidic devices is sure to have an impact in the field.

References

3 B. D. Spiess, Transfusion, 2004, 44, 4S–14S.