Assay Development

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Quantifying Analytes in Paper-Based Microfluidic Devices Without Using External Electronic Readers**

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Point-of-care (POC) and point-of-use assays are critical for identifying and measuring the quantity of analytes in a variety of environments that lack access to laboratory infrastructure. In quantitative versions of these assays, both the duration of the assay and the output signal must be measured. Measurements of time most often are performed using a timer that is external to the platform of the assay.[1,2] Such measurements are relatively simple and inexpensive, and in some cases, can be integrated into the device itself.[2] In contrast, measurements of signal typically are accomplished using hand-held electrochemical,[3,4] absorbance,[5] reflectance,[6] transmittance,[7] or fluorescence readers,[8] and as such, these measurements can be complicated, time-consuming, and expensive, particularly in the context of extremely resource-limited environments such as remote villages in the developing world. The World Health Organization has identified the use of external readers as a challenge that must be overcome when creating ideal POC diagnostic assays for use in the developing world. In fact, they have listed "equipment-free" as one of seven necessary attributes for diagnostic tests in these regions.[9]

Herein, we describe two complimentary assay strategies that address this issue. By using paper-based microfluidic devices, we show that the level of an analyte can be quantified by simply measuring time: no external electronic reader is required for the quantitative measurement (Figure 1). The methods involve either 1) tracking the time required for a sample to react with and ultimately pass through a hydrophobic detection reagent in a single conduit within a three-dimensional (3D) paper-based microfluidic device (Figure 1a) (we call this a digital assay), or 2) counting the number of bars that become colored after a fixed assay period in a related paper-based microfluidic device (Figure 1b; we refer to this as an analog assay). The methods described herein require only a timer, the ability to see color, and/or the ability to count in order to measure the quantity of a specific analyte.

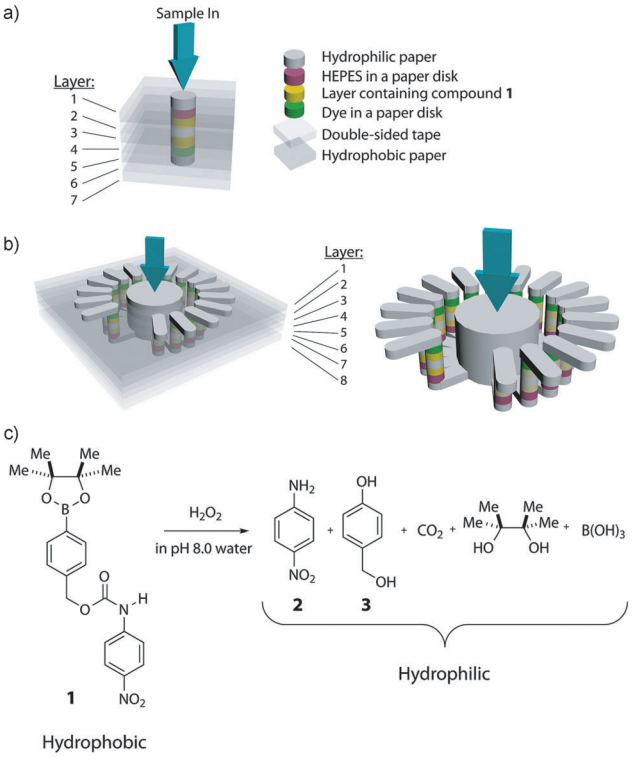

Figure 1. Two 3D paper-based microfluidic devices that are configured to quantify the level of hydrogen peroxide in a sample by simply measuring time. a) Digital assays that use a single conduit device to report the concentration of hydrogen peroxide by measuring the time required for the sample to flow through the device in the *z* direction. b) Analog assays that use a radial paper-based microfluidic device for quantifying the level of hydrogen peroxide by using a fixed assay time and by counting the number of bars that become colored as a function of the concentration of hydrogen peroxide in the sample. c) Both devices contain compound **1**, which modulates the wetting properties of defined hydrophilic regions in the devices in a dose-dependent manner when exposed to hydrogen peroxide. Oxidative cleavage of **1** (which is hydrophobic) by hydrogen peroxide yields hydrophilic products and changes the wetting properties of layers 3 and 5 (a, b).

Both of the assays are based on selective changes in the wetting properties of paper,[1,2] since hydrogen peroxide (a model analyte) oxidatively cleaves compound **1** (the detection reagent; Figure 1c), which is hydrophobic and is deposited into defined regions of the microfluidic conduits prior to assembling the devices (Figure 1a, b). Reaction of hydrogen peroxide with **1** initiates an elimination reaction that converts **1** into hydrophilic byproducts such as **2** and **3**

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(Figure 1c). This change from hydrophobic to hydrophilic allows the sample to wick through the device and wet a detection region, where the time required to wet the detection region depends on the concentration of the analyte.

The first design (Figure 1a) uses a 3D paper-based microfluidic device (10 mm wide × 10 mm long × 0.9 mm thick) that is formed by stacking alternating layers of wax-patterned paper[10] and patterned double-sided adhesive tape[11] to create a device that contains seven layers (four paper layers and three tape layers) and a single hydrophilic conduit that extends in the *z* direction from one end of the device to the other.

As little as 2 μL of sample is added to the top of the hydrophilic conduit (layer 1) and a colored readout appears on the bottom of the device (layer 7) when the sample has flowed through the entire device (Figure 2). Layer 2 is

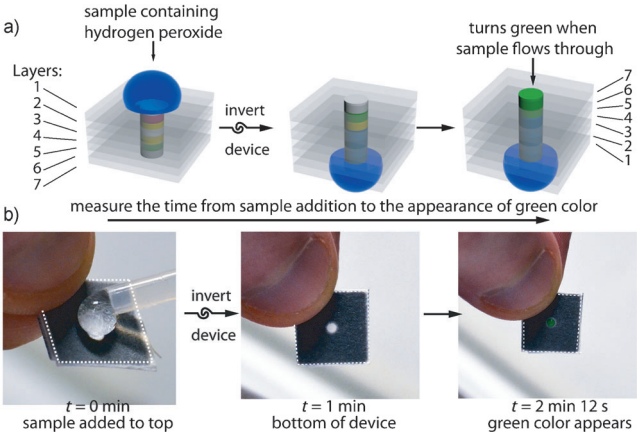

Figure 2. Diagram of the assay procedure for the "digital" device in Figure 1a. Sample is added initially to the top of the device, the device is then inverted to allow the detection layer to become visible. The end of the assay occurs when layer 7 turns bright green. a) 3D representation of the flow-through assay. b) Photographs over time for a device exposed to hydrogen peroxide (100 mM). The dotted white lines mark the edges of each device.

patterned tape that contains a 2.5 mm diameter hole that is filled with a 180 μm thick disk of Whatman Chromatography Paper No. 1. This disk contains 4-(2-hydroxyethyl)-1-piperazineethanesufonic acid buffer (HEPES) that was predeposited (0.25 μL from a 45 mM, pH 8.0 solution) and dried on the paper disk prior to assembling the device. The buffer salts are included in the device to ensure that the pH value of the sample is approximately 8.0, which facilitates rapid oxidative cleavage of **1** by hydrogen peroxide. Layer 4 contains a disk of Whatman Chromatography Paper No. 1 in a hole in patterned tape, whereas layers 3 and 5 are patterned Boise Aspen 30 printer paper that each contain compound **1** in a 2.5 mm diameter hydrophilic region (**1** is predeposited from ethyl acetate solutions ranging in concentration from 7.5 to 130 mM). We included two layers containing **1**, because initial studies showed that two layers provided more reproducible assay results than a single layer of paper containing **1**. Layer 6 is patterned tape, but in this case the 2.5 mm diameter, 180 μm thick disk of Whatman Chromatography paper No. 1 contains predeposited (0.25 μL) green food coloring. Layer 7 is the readout region and contains patterned Boise Aspen 30 printer paper with a 2.5 mm diameter white hydrophilic region that becomes bright green after the sample redissolves the dye in layer 6 and distributes it to layer 7.

The device functions as follows: After the sample is added to layer 1 (Figure 2, left images), it wicks through layers 1 and 2, buffering the sample solution with the redissolved HEPES. Once the sample reaches layer 3, hydrogen peroxide in the sample reacts with **1** to yield hydrophilic products (Figure 2, middle images and Figure 1c).[12] This reaction is repeated in layer 5. The reaction with hydrogen peroxide changes the wetting properties of the paper to allow the sample to continue wicking through the device to layer 6, where it redissolves the green food coloring from the paper disk. This food coloring is then wicked into layer 7 where the colored solution becomes visible (Figure 2, right images). A movie showing an animation of this flow-through process is available in the Supporting Information as Movie S1.

The addition of pure water to this type of "digital" device demonstrates the effect that compound **1** has on the wetting properties of paper (Figure 3). Separate experiments were

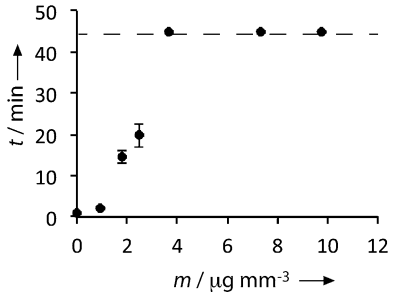

Figure 3. Effect of the quantity of **1** per volume of paper (*m*) on the flow-through time (*t*) of water through the device shown in Figure 1a. The average flow-through time for each experiment was determined by using at least five replicate measurements at 20 °C and 25 % relative humidity. Flow-through times were not measured for assays extending beyond 45 min (dotted line). The error bars reflect the standard deviations from the average values.

conducted using different amounts of compound **1**; from these experiments we found that the presence of only 3.6 $\mu g\,mm^{-3}$ of **1** in layers 3 and 5 is needed to prevent water from flowing through the vertical hydrophilic conduit in Figure 1a within the allotted assay time of 45 min.

When hydrogen peroxide is included in the sample, however, the flow-through time is correlated with the concentration of hydrogen peroxide in the sample (Figure 4, closed circles and in Movie S2 in the Supporting Information). The data obtained for this figure used devices that contained 1.7 $\mu g\,mm^{-3}$ of **1** in layers 3 and 5. The measured flow-through times were recorded when the entire hydrophilic region of layer 7 turned green, which required between less than 1 s and 10 s from the initial appearance of green. (Low concentrations of hydrogen peroxide had the slowest flow-through times and the greatest delay in filling layer 7 with the green solution.)

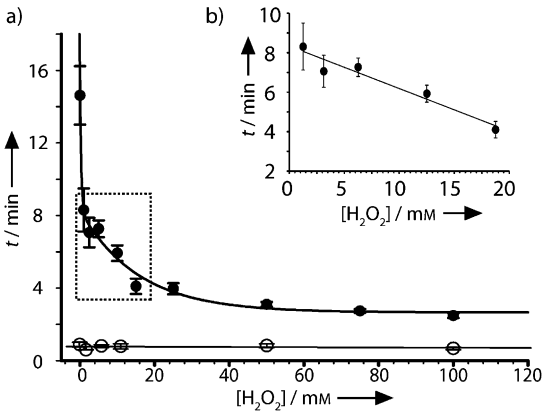

Figure 4. Effect of hydrogen peroxide concentration on flow-through time (t). a) Graph of flow-through time versus hydrogen peroxide concentration (closed circles). Devices were assembled as shown in Figure 1a; layers 3 and 5 each contained 1.7 μg mm^{-3} of **1**. The open circles represent flow-through times when 0 μg mm^{-3} of **1** is included in the device. The average flow-through time for each concentration of hydrogen peroxide was determined using ten replicate measurements at 20 °C and 25 % relative humidity. b) Expanded view of the dotted region in (a). This graph was used to determine the limit of detection for the assay. The error bars reflect the standard deviations from the average values.

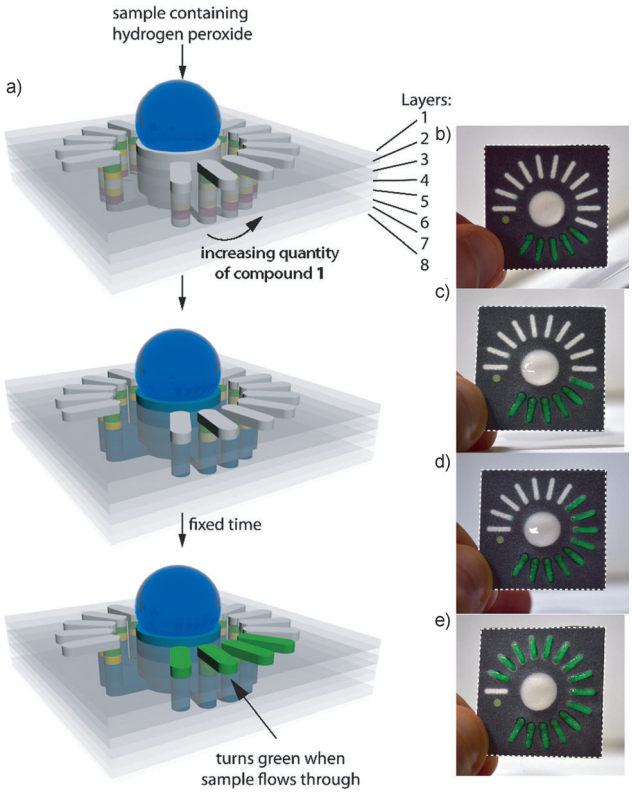

Figure 5. Diagram of the assay procedure for the "analog" device shown in Figure 1b. a) 3D representation of the movement of sample within the device. Sample is initially added to the top of the device, where it wicks down through the device and is distributed to the surrounding conduits. These surrounding conduits have nearly the same configuration as the device in Figure 1a, with the exception that the quantity of **1** increases by 1.1 μg mm^{-3} in each subsequent conduit along the radial configuration. b–e) Photographs after a 10 min assay of devices that were exposed to b) 1 mM, c) 35 mM, d) 75 mM, and e) 100 mM hydrogen peroxide. The edges of the devices shown in (b–e) are marked with white dotted lines.

The exponential relationship between hydrogen peroxide concentration and flow-through time illustrated in Figure 4a indicates that the assay is particularly sensitive to low concentrations of hydrogen peroxide: in this current proof-of-concept assay, the limit of detection[13] is 0.7 mM hydrogen peroxide (Figure 4b) and the dynamic range is 0.7–100 mM.[14]

Figure 4a also reveals that **1** is essential for generating this exponential relationship between hydrogen peroxide concentration and flow-through time: in the absence of **1** (Figure 4a, open circles), the flow-through times are uniform and rapid across the different concentrations of hydrogen peroxide tested.

Since wicking rates in porous media typically are affected by humidity,[2,15] we tested the effect of this variable in our flow-through assay. We found that the flow-through time is independent of humidity over the range of 17–62 % relative humidity (Figure S4 in the Supporting Information), which is a result that we attribute to the exceedingly short path length that the sample must travel in the device (i.e., ca. 900 μm).[16]

Based on these promising results for the "digital" device, we next explored whether a related—but operationally more straightforward—"analog" device could be created. This design (Figure 1b) requires that the user simply counts the number of colored bars that appear after a fixed assay time.

The design uses an 8-layer 3D paper-based microfluidic device (32 mm wide × 32 mm long × 1.0 mm thick) that contains 16 hydrophilic conduits (2.5 mm diameter) arranged in a circle surrounding a central hydrophilic conduit (10 mm diameter). The sample is added to the central conduit in layer 1, which is patterned Whatman Chromatography Paper No. 1, and then wicks through the device in the z direction down to layer 7 (Figure 5a, top image). In layer 7, the central conduit separates the sample evenly into the 16 surrounding conduits. The surrounding conduits direct the sample up (in the z direction), opposite to the direction of flow in the central conduit. This arrangement allows the sample to be added to the top of the device, and the bars for the dial to be visible on the top as well. Layer 8 is a single-sided protective tape that allows the user to place the device on a surface to run the assay. An animation of the distribution process is depicted in Movie S3 in the Supporting Information.

The outer conduits of this device are composed of the same layers as the conduit in the "digital" device (i.e., a paper disk containing HEPES buffer salts, followed by two layers containing **1**, and then a paper disk containing dried green food coloring). However, in this device, each successive conduit contains increasing amounts of compound **1** compared to the previous conduit (Figure 5a).

Because flow-through time increases as the amount of **1** in the conduit increases (Figure 3), each conduit has a longer flow-through time than the preceding one. By using a fixed assay time (10 min), the number of colored bars (resulting from flow-through) is used to quantify the amount of hydrogen peroxide in a sample. Figure 5b–e and Movie S4 in the Supporting Information show several examples of

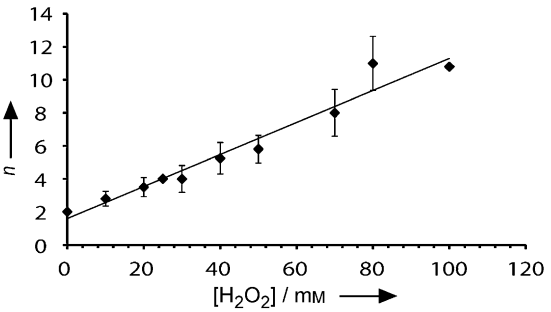

Figure 6. Relationship between the number of bars filled (*n*) in the "analog" device (Figure 1b) and the concentration of hydrogen peroxide (0–100 mM) in a sample. The concentration of **1** increased by 2.3 $\mu g\,mm^{-3}$ increments between conduits in the devices used to obtain this data. The assay is more sensitive if conduits increase in 2.3 $\mu g\,mm^{-3}$ increments rather than 1.1 $\mu g\,mm^{-3}$ (the devices in Figure 5 increased by 1.1 $\mu g\,mm^{-3}$). The average number of bars after a 10 min assay was obtained from five replicate measurements. The error bars reflect the standard deviations from the average values.

devices that were exposed to different concentrations of hydrogen peroxide, and Figure 6 reveals a linear relationship between the number of colored bars and the concentration of hydrogen peroxide. In this initial proof-of-concept analog assay, the dynamic range is 10–100 mM hydrogen peroxide and the limit of detection[17] is 10 mM.

In conclusion, herein we describe two unique quantitative assays that operate by measuring flow-through time or by counting the number of colored bars at a fixed assay time. These assays do not require instruments (other than a timer), thereby making them useful platforms for developing the types of diagnostic assays sought by the World Health Organization for use in the developing world.

While these initial proof-of-concept studies used hydrogen peroxide as a model analyte for demonstrating the idea of analog and digital assays in paper microfluidics, future studies will focus on substantially expanding the scope and sensitivity of the methods. Specifically, we will 1) develop other hydrophobic molecules similar to **1** to enable quantitative detection of a variety of analytes; 2) improve the sensitivity of the assays by increasing the magnitude of change in wetting properties per reaction with the desired analyte; and 3) develop a physical model to explain the effects of compound **1** on flow-through time to guide our efforts to increase the dynamic range and further improve the limits of detection of the assays.

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[12] These byproducts were verified by LC–MS analysis of an analogous solution-phase reaction.
[13] Defined as three times the standard deviation of the lowest non-zero concentration divided by the slope of the linear region of the exponential curve.
[14] While hydrogen peroxide is used only as a model analyte to demonstrate the concepts of digital and analog assays, it should be noted that hydrogen peroxide is a useful analyte in a variety of clinical contexts, including: 1) as a secondary analyte in a variety of assays that use enzyme oxidases (such as glucose assays that operate in the low millimolar range), and 2) as an indicator of inflammation in the lungs.
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