Electronic Supplementary Material (ESI) for Analytical Methods

Supplementary information for:

Paper-Based Standard Addition Assays: Quantifying Analytes via Digital Image Colorimetry under Various Lighting Conditions

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Table S1. Comparison of results from the standard addition assays and the external calibration assays for three different lighting conditions. All results are reported as the mean ± standard deviation for three trials. The external calibration curve generated from the scanner data was used to calculate the external calibration concentrations for all three lighting conditions.

	Standard Addition, mM			External Calibration, mM		
Sample	Scanner	Outdoor Camera	Indoor Camera	Scanner	Outdoor Camera	Indoor Camera
0 mM	-0.02±0.04	0.07±0.03	0.10±0.07	-0.02±0.03	0.03±0.02	0.03±0.02
1.25 mM	1.3±0.3	1.4±0.3	1.33±0.08	1.31±0.09	0.79±0.04	0.43±0.01
2.50 mM	2.4±0.3	2.2±0.2	2.1±0.4	2.5±0.1	1.20±0.05	0.58±0.03
5.00 mM	4.9±0.3	5.4±0.9	5.2±0.8	5.4±0.5	2.01±0.01	0.91±0.03
Art. Urine (2.50 mM)	2.2±0.3	2.5±0.5	2.2±0.3	2.58±0.07	2.0±0.1	0.84±0.04
Unknown (2.50 mM)	2.5±0.3	2.3±0.5	2.1±0.6	2.8±0.2	1.24±0.03	0.63±0.06

EXPERIMENTAL DESIGN

In order to develop the standard addition assay for glucose, we made three improvements over previously published paper-based glucose assays: (i) we optimized the reagents for the assay in order to achieve more uniform distributions of color in the test zones, (ii) we introduced the use of colored wax barriers to minimize signal interference from the background, and (iii) we introduced the use of a rectangular hyperbolic equation with two parameters for improved external calibration curve fitting for colorimetric paper-based assays. Previous work with paper-based colorimetric assays used quadratic fits and logarithmic fits for the external calibration curves of colorimetric assays with moderate succes.[1,2] The rectangular hyperbolic equation provides excellent fits to plots of signal versus concentration for paper-based colorimetric assays and allows for non-linear standard addition assays to be performed.

Device design and fabrication

The device consisted of a central sample zone from which the sample was distributed through eight channels (spokes) into eight test zones arranged uniformly around the sample zone (Figure 1). We chose to pattern the device by wax printing since this method is simple, rapid, and the patterns can be printed in a variety of colors.[3] The length and width of the spokes were optimized to contain 0.7 μL of solution. The size of the test zones was optimized to store the reagents for the assay and provide a sufficiently large area so that the signal of the assay could be imaged and measured easily. The device was designed to run four replicates of sample (S_0) and four replicates of sample spiked with a known concentration of glucose (S_1). For the spiked replicates, a known concentration of glucose was added to the spokes and dried during fabrication of the device.

Choice of glucose assay

Initial experiments were conducted using a reagent mixture containing glucose oxidase, horseradish peroxidase and potassium iodide, but the brown triiodide produced by the assay tended to concentrate around the rim of the test zone and led to large indeterminate errors (Figure S1). By replacing the potassium iodide with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), a much more uniform color distribution in the test zone was obtained.

Figure S1.

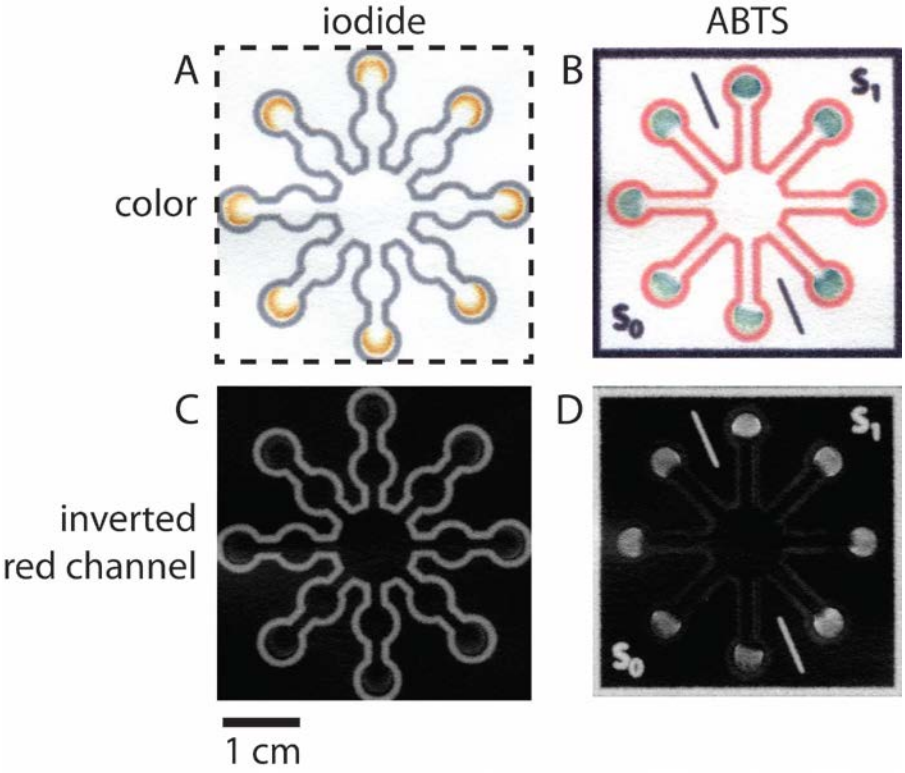

Figure S1. Optimization of the reagents and devices for detection of glucose. (A) Original devices using iodide and black wax barriers. The color generated by the assay accumulated around the edges of the test zones. (B) Inverted red channel of the image shown in (A). The black wax barriers contribute a significant signal, which interferes with the signal of the results. (C) Optimized devices using ABTS and red wax barriers. The distribution of the color generated by the assay is more uniform in the test zones. (D) Inverted red channel of the image shown in (C). The red wax barriers are virtually invisible making it easier to measure the signal from the assay.

Choice of wax barrier color

We chose to pattern the hydrophobic barriers in the device using red wax because we found this color interfered the least with signal from the assay (Figure S1). Since the ABTS produced a blue-green color, we found that the best signal to noise ratio could be obtained by splitting the image into its red, green and blue channels in ImageJ, and measuring the intensity of the color in the red channel. The red wax generated much less signal in the red channel compared to black, blue or yellow wax.

Choice of lighting conditions

To demonstrate the capability of the standard addition assay to provide quantitative results that were independent of lighting conditions, the devices were imaged under three different lighting conditions: (i) scanner with an LED light source, (ii) digital camera outdoors in sunlight and (iii) digital camera indoors under fluorescent lighting. The scanner provided uniform and reproducible lighting conditions for imaging. Both the outdoor and indoor lighting conditions varied throughout the day. The devices were imaged on a sunny day around noon, and the devices and camera were arranged in order to minimize the presence of shadows in the image.

Data analysis and calculations

The intensity of the color generated in the test zones was read using ImageJ by inverting the image (to obtain the negative), splitting it into the red, green and blue channels and analyzing the red channel (Figure S1). Our initial experiments focused on preparing external calibration curves by plotting signal versus concentration for a series of known glucose standard solutions and attempting a number of different curve fitting equations. It was only after attending a mock lecture from a biochemistry job candidate

that we realized that the signal versus concentration plot of our colorimetric assay for glucose had a similar shape to the curves observed in enzyme kinetics experiments as described by the Michaelis-Menten equation.[4,5] sA rectangular hyperbolic equation was then used to derive an expression for the non-linear standard addition assay for glucose.

EXPERIMENTAL DETAILS

The devices were designed in CleWin® and the colors of the barriers were adjusted in Adobe Illustrator®. The patterns were printed onto Whatman No. 1 chromatography paper using a Xerox® Phaser 8560 printer. The paper was baked in an MTI Corporation Compact Forced Air Convection Oven for 2 minutes at 195°C and then cooled to room temperature under ambient conditions. All eight test zones were spotted with 0.7 μL of a reaction mixture consisting of 25-mM ABTS, 75-kU·L^{-1} glucose oxidase, 250-kU·L^{-1} horseradish peroxidase and 1-M trehalose dissolved in 1xPBS. The spokes for the test zones labeled S_1 were spotted with 0.7 μL of a 5-mM glucose solution prepared in 1xPBS. All reagents were allowed to dry under ambient conditions for 15 minutes.

The assays were run by adding 35 μL of sample solution to the sample zone while the device was held horizontally. The results were imaged using an EPSON® Perfection V300 scanner and a Nikon® D5100 camera after 30 minutes. The images were opened in ImageJ and split into red, green and blue channels. The red channel was inverted, and the mean intensity of each test zone was measured by selecting the entire circular area of the test zone. The intensity of the sample zone was also measured and subtracted from

the signal of the test zones as background. Outliers were rejected using Grubb's test at a significance level of α=0.05.[6]

An external calibration curve was prepared by running seven devices with glucose concentrations of 0, 0.625, 1.25, 2.50, 5.00, 7.50, and 10.0 mM, all prepared in 1xPBS. No glucose was added to the spokes of the calibration devices. The calibration curve for each lighting condition was fit with Equation 4 using KaleidaGraph® in order to obtain values for S_{max} and K. Four spoke-calibration devices were prepared to determine the magnitude of C_s. A 5-mM glucose solution was added to all eight spokes of the calibration devices, and the devices were run with 0, 0.125, 2.50 and 5.00-mM glucose samples respectively. The determined glucose concentrations from the test zones were plotted versus the known concentrations of the glucose samples, and the data was fit with a linear function. The x-intercept of the resulting equation was taken as the value of C_s.

Standard addition assays were performed in triplicate using a series of known samples with concentrations of 0, 1.25, 2.50 and 5.00-mM glucose. A simulated urine sample containing 2.50-mM glucose and 5-mM Tartrazine, a yellow dye, in 1xPBS was also tested. Finally an unknown sample, which also contained 2.50-mM glucose, was tested via a blind experiment where the assay and data analysis were performed blind to the unknown's true glucose concentration.

All chemicals were purchased from commercial sources and unaltered from factory composition before use: Fisher Scientific 10xPBS Solution, Acros Organics D-(+)-Trehalose Dihydrate (99%), Sigma Aldrich D-(+)-Glucose Anhydrous, Sigma Aldrich Horseradish Peroxidase Type VI, Sigma Aldrich Glucose Oxidase, Alpha Aesar ABTS (98%).

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