



# Draw your assay: Fabrication of low-cost paper-based diagnostic and multi-well test zones by drawing on a paper



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## ABSTRACT

Interest in low-cost diagnostic devices has recently gained attention, in part due to the rising cost of healthcare and the need to serve populations in resource-limited settings. A major challenge in the development of such devices is the need for hydrophobic barriers to contain polar bio-fluid analytes. Key approaches in lowering the cost in diagnostics have centered on (i) development of low-cost fabrication techniques/processes, (ii) use of affordable materials, or, (iii) minimizing the need for high-tech tools. This communication describes a simple, low-cost, adaptable, and portable method for patterning paper and subsequent use of the patterned paper in diagnostic tests. Our approach generates hydrophobic regions using a ball-point pen filled with a hydrophobizing molecule suspended in a solvent carrier. An empty ball-point pen was filled with a solution of trichloro perfluoroalkyl silane in hexanes (or hexadecane), and the pen used to draw lines on Whatman<sup>®</sup> chromatography 1 paper. The drawn regions defined the test zones since the trichloro silane reacts with the paper to give a hydrophobic barrier. The formation of the hydrophobic barriers is reaction kinetic and diffusion-limited, ensuring well defined narrow barriers. We performed colorimetric glucose assays and enzyme-linked immuno-sorbent assay (ELISA) using the created test zones. To demonstrate the versatility of this approach, we fabricated multiple devices on a single piece of paper and demonstrated the reproducibility of assays on these devices. The overall cost of devices fabricated by drawing are relatively lower (< US \$0.001 per device) than those derived from wax-printing (US\$0.05–0.003) or other approaches.

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## 1. Introduction

Low-cost diagnostic devices for point-of-care applications require cheap, readily available and modifiable substrates to support the components needed for diagnosis. Paper is a low-cost, readily available material that has been used as a substrate for chemicals for millennia. [1] In low-cost bioanalysis and diagnostics, paper has been used for years in applications like test for acidity (litmus paper) or mixture separation (chromatography) [2]. One limitation of these early paper-based analytical tools is that only one type of

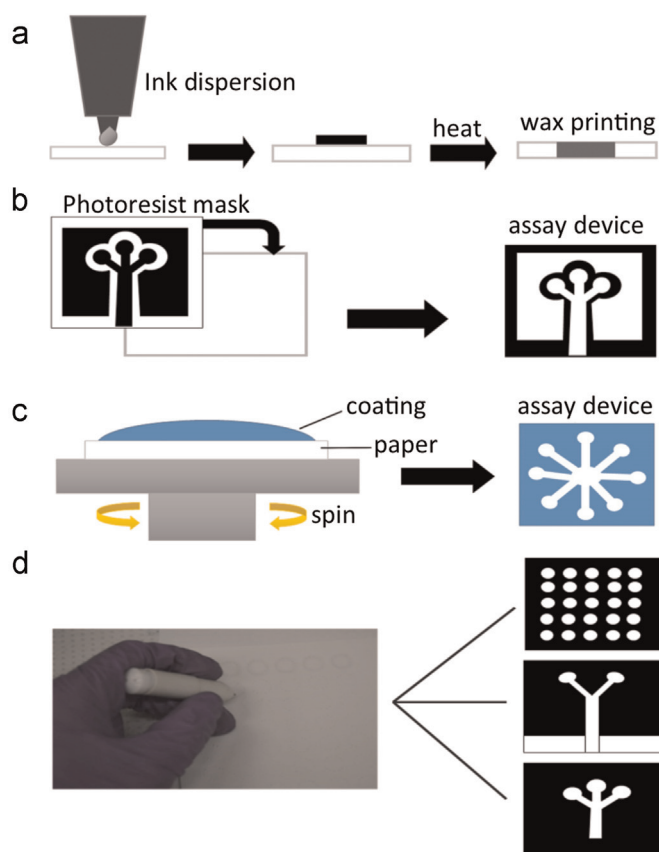
analyte could be detected at a time. Multiplexing on paper requires isolated test zones that can be established through creation of chemical and/or mechanical barriers to limit mixing, through wicking, of the different analytes. Such barriers have been generated by printing, stamping, cutting, embossing, origami, and/or chemical modification of the paper [2–15]. Fig. 1 gives examples of commonly used methods of creating hydrophobic barriers on paper, with Fig. 1d illustrating the hand-drawn test-zones fabrication, the subject of this paper, for comparison. Each of these techniques has its advantages and disadvantages with regard to cost, flexibility in design, reliability, and, technology needs. While most barriers can be easily generated in a clean environment with tools that often require electricity, they are often not adoptable or achievable in resource-limited settings.

Drawing or writing on paper is an old, well-established art

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**Fig. 1.** Representation of common techniques for creating hydrophobic barrier patterns on paper. (a) Wax printing, (b) photolithography using a washable resist, and, (c) spin-coating of a hydrophobic resist on a pre-treated paper. These techniques require energy input and/or equipment not necessarily amenable to the field. A novel way of creating paper-based assay devices without the need of energy is by drawing on paper as illustrated (d). In this paper, hand-drawing is adopted as a technique to fabricate assay test-zones or create capillary-driven microfluidic devices on paper.

form, which has become ubiquitous to human existence. Drawing on paper is flexible and low-cost, requiring only a paper and ink. The ink (or paint) contains two main components, viz; (i) a chemical—usually a pigment—of which one wishes to deposit on a surface, and, (ii) a carrier, which may or may not be removed after drawing. We adopted this well-established art to create hydrophobic barriers on paper, and therefore, create test-zones that would allow for multiplexing on a single sheet of paper. In this fabrication method, the ink is a hydrophobic alkylsilane, with  $-OH$  reactive moieties, dissolved in an organic solvent as carrier. Trichloro alkylsilanes have been used to modify substrates e.g., metal dioxides, glass, and, metals [16–18] creating reactive and/or hydrophobic surfaces. Silanes have also been used to modify the hydroxyl groups on cellulose introducing different functional groups and altering wettability [2,6,11,12,14,19–21].

In this paper, we combined low-cost materials, paper, a pen filled with an ink of a hydrophobic silane in a hydrophobic solvent, and drawing by hand (or using affordable tools like the craft-cutter) to generate low-cost diagnostic devices. We demonstrated the versatility of these hand-drawn low-cost diagnostic devices by applying them in two types of colorimetric assays viz; (i) detecting glucose concentration in artificial urine, and, (ii) enzyme-linked immuno-sorbent assay (ELISA) for rabbit IgG. Our data compares well with literature and shows that the drawn hydrophobic barriers do not interfere with the assay [4,8,22,23].

## 2. Materials and methods

### 2.1. Materials

Whatman® Chromatography paper #1 was chosen for its lightweight and affordability (~\$0.006 per sheet [11]). Food color kit (Shaw's® supermarket, Tone's Food Color), n-hexane (Sigma Aldrich, ACS reagent grade), n-hexadecane (VWR, MP Biomedical) and trichloro(1H,1H,2H,2H, perfluorooctyl) silane (Sigma Aldrich, 97%) were used as received.

### 2.2. Preparation of test-zones

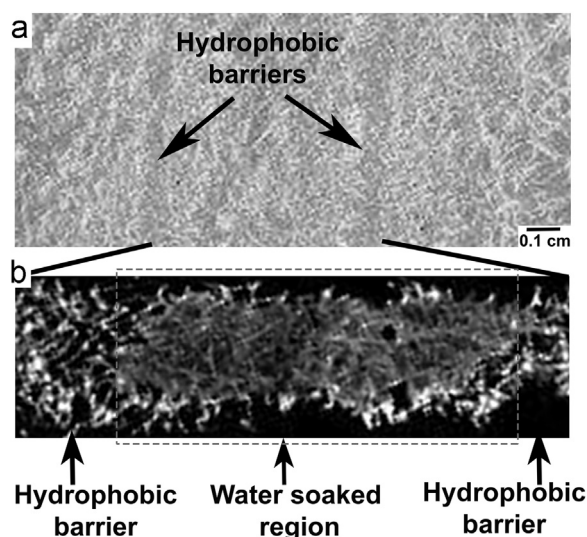
Ink from the cartridge of a silhouette cameo® stencil pen was removed, then the cartridge and the lead of the pen were repeatedly washed with hexanes. After ascertaining that all the ink had been washed off, the pen was rinsed with copious amounts of acetone and allowed to dry at ambient conditions (ca. 12 h). Custom ink was prepared from a 1:50 (v/v) mixture of trichloro perfluorosilane and hexane (or hexadecane where higher viscosity is desired) then placed in the sketch pen. With the custom ink placed on the stencil pen, various patterns were created using either a stencil (e.g. ruler) or by tracing along pre-made markings on a paper (punched holes or pencil traces). After tracing, the paper samples were placed in oven at 95 °C, in vacuo, for ca. 5 min to remove the solvent where hexadecane had been used. When n-hexane was used as the solvent, maintaining the paper under vacuum for ca. 5 min at room temperature removed all the solvents.

To demonstrate the adaptability of the drawing approach to large scale, roll-to-roll, production of test-zones, the sketch pen was used with a craft-cutter and patterns similar to those drawn by hand were generated in a relatively short time.

## 3. Results and discussion

All devices were fabricated using Whatman® chromatography no. 1 paper with trichloro perfluorooctyl silane suspend in hexane or hexadecane (1:50 v/v) as the treating reagent. Since the silane in hexane is not colored, there were no visible changes on the paper upon drawing the hydrophobic barriers, therefore, small dots were either made with a pencil or by punching into the paper to guide the user as to the locale of the hydrophobic barriers.

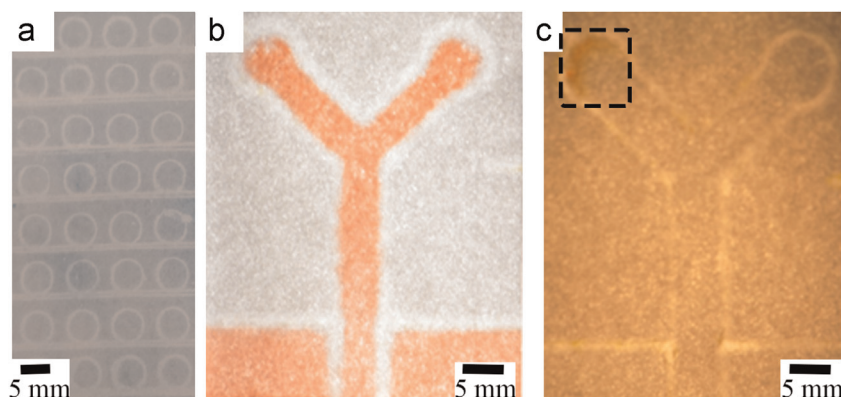
To evaluate whether the barriers, that is the hydrophobizing reagent, permeate the width of the paper, we use x-ray tomography to show that water does not wick through the thickness of the paper in regions where the treatment had been applied. Fig. 2a shows an optical micrograph of a piece of paper after manual treatment with the hydrophobizing ink. The treated regions are highlighted with arrows and can also be identified by the slight indentation of the paper upon drawing a line. Visual inspection of the dry paper does not aid in evaluating the extent of the hydrophobization, and as such, we employed 3D x-ray tomography to evaluate the presence or absence of water across the thickness of the paper (Fig. 2b). Under this technique, regions of the paper that are wet with water appear gray in color while the non-wet regions appear white. Fig. 2b shows that water wicks between two hydrophobic barriers but does not wick at all under the barriers indicating that the treatments do not allow any kind of wetting across the thickness of the paper. This result shows that the barriers are effective at keeping the water from wicking underneath the treatment, but should not be taken as evidence that the chemicals permeate the thickness of the paper as capillary effect could also be a contributing factor. The untreated region of the paper that wicks is highlighted by the dotted box (Fig. 2b)



**Fig. 2.** Analysis of the effectiveness of the drawn barriers to effectively contain water within a pre-defined region. (a) An optical micrograph of Whatman chromatography paper no. 1 with two barriers hand-drawn across the paper surface as highlighted by the arrows. (b) A cross-sectional slice from a 3D x-ray tomography image of the paper after allowing water to wick between the two barriers. Presence of water in the paper is indicated by gray coloration and filling of the pores of the paper. Water is contained within the two barriers in the region highlighted by the dotted box.

### 3.1. Evaluating the hydrophobicity of the test-zones

First, the stability and hydrophobicity of the drawn regions was tested by immersing the devices in water. Fig. 3a shows well-plates created by the drawing method after immersion in water containing a blue dye. All non-treated regions became wet (blue) while the hydrophobic regions remain white. These well-plates could be left in water for extended periods without failure (in our case we left them for a week). To demonstrate that the barriers traversed the thickness of the paper, we created capillary-wicking based microfluidic channels on a paper and showed that water, dyed-red, only wicks into regions defined by the drawn barriers (Fig. 3b). Fig. 3c shows a microfluidic device imaged with the light and camera opposite each other (that is, light source and camera are orthogonal to the paper but on opposite sides of the paper) to show the otherwise invisible hydrophobic barriers. Besides using water, we repeated the hydrophobicity and stability tests with artificial urine and artificial plasma and obtained the same results.



**Fig. 3.** By drawing with a silane-hexane ink, we fabricated well plates (a) and “Y” shaped microfluidics (b) – imaged with the light and the camera opposite each other to capture the otherwise invisible drawn barriers. (a) Fabricated devices were tested for wettability with water by soaking them with water containing a blue dye. The non-wetting (hydrophobic) regions still appear white and were stable to water or biological fluids. (b) Drawn lines prevent infiltration by water, thus can be used to guide fluids, by capillary-wicking, into test zones. Water containing a red dye filled the microfluidic channel and, irrespective of the position of the barrier, does not break past the treated regions. (c) Use of the treated zones for glucose assay. The insert highlights the positive test that is absent in the control.

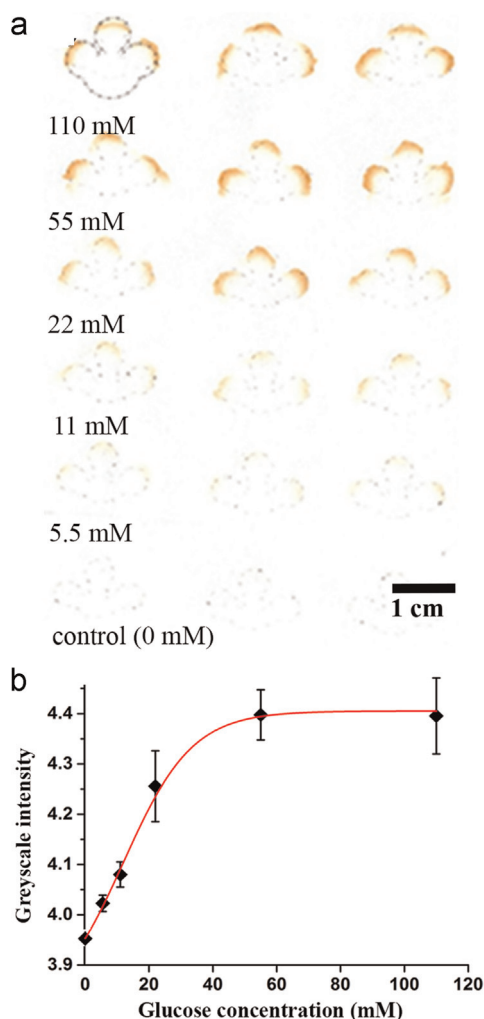
### 3.2. Application of test-zones in colorimetric assays

After ascertaining that the drawn hydrophobic barriers were stable under water or biological fluids (artificial urine and plasma), we evaluated their use in assays. It is well known that the behavior of fluids at interfaces depends on the total composition of the two liquids, as such, we wish to establish whether presence of bio-markers and other assay reagents would lead induce a breach of our hydrophobic barriers. First, we used microfluidic devices for glucose assay using artificial urine (Fig. 3c). We observed that the assay was contained within the test-zone as highlighted (dotted box) in Fig. 3c. One advantage of drawing the test-zone is the promise to rapidly create many small test-zones on a single piece of paper. To further demonstrate the utility of this technique, we applied the ‘draw-your-assay’ approach to create test-zones similar to those described by Whitesides and co-workers [9].

First we created a clove shaped (triplex) glucose assay platform and used rows of these devices for serial dilution and the columns for replication to test for reproducibility. Performance and analysis of the assay are as previously reported. Due to the small size of these devices hundreds could be readily created on a single sheet of A4 sized chromatography paper (Fig. 4a). The glucose assays were performed using artificial urine (Sigma-Aldrich) with different concentrations of glucose and in  $3 \times 3$  matrix factorial assays design (Fig. 4a). We observed that, as expected, the assays gave a linear response that eventually asymptotes at higher concentration. There is, however, a significant background from the control probably due to coloration of the paper by the artificial urine (Fig. 4b). Due to significant background coloration, we could not reliably distinguish, by eye, lower concentrations from the blank, hence all images were scanned and color pixels quantified using image J as previously reported. A strong linear correlation was observed ( $R=0.99$ ) for lower concentration (0–55 mM), but the color intensity plateaus at higher concentrations. The linear region compares well with previously paper-based assays [9,24] indicating that the barriers had no effect on the test besides confining the reagents and analytes to the define areas.

Glucose assays are simple and relatively uncomplicated. We therefore desired to test these test zones using an immunoassay that is more elaborate since it requires multiple washings and more biochemical reagents. Immunoassays are a good test for the stability of any barrier material on paper. We used hand-drawn paper-based well-plates (similar to those shown in Fig. 3a) to perform the ELISA in multiples (5 replicates per concentration). We first spotted 20  $\mu$ L of rabbit IgG antigen (at different concentrations) onto the reservoir, and waited for 20 min. We then





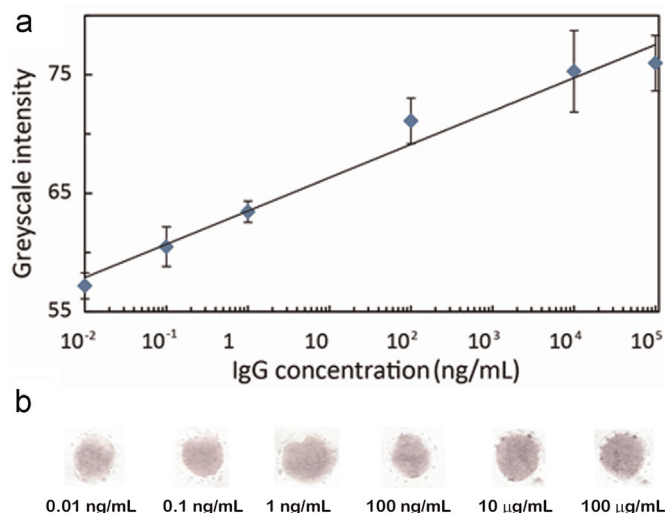
**Fig. 4.** Glucose detection by the colorimetric assays. (a) Glucose assays on clove-shaped drawn test zones. Different columns represent replicates of the same test, while different rows show different concentrations with the concentration decreasing down the column. A serial dilution was done starting with 110 mM solution with the concentration being reduced by half each time. (b) The calibration curve used for quantification of differences in coloration with respect to glucose concentration (mmolar) shows a large linear regime.

added 40  $\mu$ L of blocking buffer followed by an incubation period of 30 min. Next, we applied 18  $\mu$ L of anti-rabbit IgG to each paper well, and waited for 4 min. We washed each well with 60  $\mu$ L PBS for 3 times. After waiting for 20 min, we applied 16  $\mu$ L of BCIP/NBT substrate and waited for 20 min. Finally, we scanned the reaction wells using a desktop scanner and analyzed the grayscale intensity of each well in ImageJ. As expected, the measured grayscale intensity correlates well with the concentration of the rabbit IgG (Fig. 5).

#### 4. Conclusions

We demonstrated that a simple technique, like drawing, can be used to rapidly and efficiently create well defined test-zone through targeted deposition of a hydrophobic reagent on paper. This technique pushes the already low-cost paper devices to new limits since it does not necessarily require any equipment, is easy to use, and can be adopted to any analyte (through surface tension of the ink) or environment.

By defining the test zones using a ball-point pen, we minimize the amount of reagents used and reduce wastage by only creating



**Fig. 5.** Application of circular well-plates in paper-based colorimetric immunoassay, in this case, rabbit IgG detection by direct ELISA. (A) The calibration curve shows a linear dependency of the gray scale intensity with increase in IgG concentration. (B) Images of observed coloration with increase in antibody concentration.

thin ( $\sim 0.1$  mm thick) barriers. Unlike other methods [2–5,7–11,13,15] used to define hydrophobic barriers, targeted deposition of the hydrophobizing material mitigates wastage, and with the low power requirements, this approach to fabrication of low-cost devices qualifies as green engineering.

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