SUPPORTING INFORMATION

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Paper Microzone Plates

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EXPERIMENTAL PROCEDURES

Fabrication of Multi-Zone Paper Plates. We designed photomasks using the program CleWin® (PhoeniX Software, The Netherlands), and printed them onto transparencies using a regular inkjet printer (HP Deskjet D2430, Hewlett-Packard Company, Palo Alto, CA). Four identical masks were printed, stacked and aligned in pairs, and clued together with acrylate based glue (SuperGlue®). This registration is straightforward at the large features sizes involved, and is important to ensure that the black (printed) portions of the masks blocked all the light from reaching the zones.[1,2] We used two masks to sandwich the paper embedded with photoresist between them. Two glass plates pressed the masks against the paper to ensure uniform contact between the masks and the paper facilitating the process of exposure to UV light; see next.

The paper selected to demonstrate the principles of this method was Whatman Chr 1 chromatography paper or Whatman #1 filter paper (Whatman International Ltd., Florham Park, NJ), cut in the size of a microplate–8.5 cm × 13 cm. We spread the commercial photoresist over the paper using a spoon, the flat side of a knife, or the side of a test tube. Pressing the paper with a wood or glass rolling pin squeezed the excess photoresist from the paper; after removing the excess of resist, the plates dried inside the fume hood—vacuum chamber also worked well for this purpose.

We used a UV flood lamp (model Intelliray 600, UVitron International, Inc., West Springfield, MA) for photopatterning the dry paper. The UV lamp has 600 W of power and delivers about 100 mW cm^{-2} at 365 nm. The exposure time was 30 s for each side of the paper; exposure of the paper-photoresist composite to UV light from both sides was necessary to ensure that the pattern extended throughout the entire thickness of the paper. Sandwiching the paper

(1) The printed portions of the mask can present pinholes that allow the light to go through the mask. Since such defects in printing are random, stacking two layers or printing twice eliminates them.
(2) Coltro, W. C. T.; Piccin, E.; da Silva, J. A. F.; do Lago, C.; Carrilho, E. *Lab Chip* **2007**, *7*, 931-934.

between the two masks eliminated the need for re-alignment of the mask on the back of the paper. A 5-min soak in xylenes followed by another 5-min soak in methanol removed the un-polymerized photoresist from the paper. The paper was washed with methanol after these two soaking steps and allowed to dry inside the fume hood.

Fabrication of paper plates with diluted solutions of SC resist followed exactly the same protocol with the exception being the way we applied the photoresist. Solutions of SC resist were readily handled with a disposable pipette, dispensing 2.5 ml directly over the paper for immediate spreading. Flipping the plate a couple of times inside a fume hood evaporated the solvent in two minutes or less.

Patterning Paper with SU-8-like Photoresist. We followed the protocol described by Martinez *et al*.[14] and is similar to that illustrated in Figure 1 with the following exceptions: 1) There is a pre-bake at 95 °C for 10 min before exposing to UV light. 2) The exposure time under UV flood lamp was only 10 s and only in one side. 3) The post-bake was at 135 °C but for only 5 min. 3) Immersion of the paper in acetone (1 × 5 mL/1 min) developed the image and removed the unpolymerized SU-8 from the paper, and 4) a final rinse with 5 mL of propan-2-ol prepared the plate for dryness.

Plasma oxidation. In some particular experiments the paper plates, using air plasma oxidation to completely recovered the hydrophilicity of the paper that had been in intimate contact with hydrophobic photoresists. Due to the size of the plates, we needed to use a plasma cleaner (SPI Plasma-Prep II, Structure Probe, Inc) with a large chamber to accommodate the plate. Typically, the oxidation was carried out for 5 to 10 s at 600 millitorr.

Measuring Fluorescence from Paper Plates Using a Microplate Reader. We prepared solutions of fluorescein-labeled bovine serum albumin (FITC-BSA) ranging in concentrations from 10 μM to 25 nM, and transferred 250 μL of each of these solutions into the wells of a black, plastic 96-well plate designed for measurements of fluorescence (BD Falcon® Microtest® 96-Well Assay Plates, BD Biosciences, San Jose, CA, unit price $3.02). The total number of moles of FITC-BSA in the plastic wells ranged from 2.5 nmol to 6.25 pmol, respectively.

We measured the fluorescence of the plastic 96-well plate using a SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA) microplate reader set at 25 °C in triplicate (three plates on three different days). Each concentration was repeated seven times per plate (total of 21 measurements for each solution). For measurements of fluorescence from paper plates, we transferred 5 μL from each well of the plastic 96-well plate to the corresponding zone in a paper plate, dried the plate in air at 25 °C for 10 min, and then measured the fluorescence of three paper plates using the same microplate reader and settings (Figure 4). The amounts of FITC-labeled BSA transferred to each zone in the paper plate ranged from 50 pmol to 125 fmol.

Measuring Absorbance from Paper Plates Using a Microplate Reader. We prepared 2.5 mM aqueous solutions of both dyes and filled the first column in a transparent, 96-well plastic plate (BD Biosciences, San Jose, CA, price $2.50/plate) with 200 μL of each solution of dye. Aliquots (100-μL) of each solution of dye was transferred from the previous well to the next one in the plate and diluted two fold with water; this procedure was repeated throughout the 12 columns. The concentrations ranged from 2.5 mM to 1.2 μM.

A microplate reader measured the absorbance of these solutions in the 96-well plastic plate, and then we transferred 5 μL of each solution from the wells to the corresponding zone on a paper plate. The number of moles of analytes deposited in the zones ranged from 12.5 nmol to

6.0 pmol. The paper plate dried in air at 25 °C for 30 min, and then we deposited a thin film of mineral oil to each well using a cotton-swab. The mineral oil soaked into the paper and decreased the scattering of the paper by decreasing the difference between the index of refraction of medium (air *or* mineral oil and cellulose).

A microplate reader measured the absorbance for both plastic 96-well plate and paper 96-zone plates. The background was subtracted from a blank solution in both cases and the baseline was adjusted using the absorbance at 750 nm in the paper data only. Not all data points were used in the graphs. The two highest concentrations saturated the detector for the plastic plates experiments while the three lowest concentrations showed absorbance values that were the same as the lowest point shown in the graph.

On three different days, we prepared a serial dilution of 2^7 fold for both dyes starting at 0.83 mg mL^{-1} (this value corresponds to 1.01 mM for Coomassie blue and 1.38 mM for Amaranth red). We measured the absorbance at the λ_{max} for each dye (620 nm for Coomassie and 530 nm for Amaranth), subtracted the average background from the blank (n = 12), and corrected the absorbance value by subtracting the absorbance of the paper at 750 nm (baseline).

Measuring Reflectance from Paper Plates Using a Desktop Scanner. We used a flatbed scanner (Epson Perfection model 1640SU, Epson, Long Beach, CA) to image all plates—such as those in Figure SI-1—spotted with solutions of Coomassie Brilliant Blue and Amaranth for absorbance measurements, but before the application of mineral oil, *i.e.,* the zones were dry. The resolution of the image was 300 dpi acquired in color mode. For analytical processing, converting the image of the plate from color to gray scale at resolution of 8 bits in the Adobe Photoshop, Creative Suite 3 (Adobe Systems, Inc., San Jose, CA) yielded satisfactory results. As discussed in the text, other color spaces and/or other color channels are also adequate and should

be selected according to the assay of interest. Selecting the entire zone with the marquee tool allowed the program to average the color inside the zone. We used Origin 7.0 (OriginLabs, Northampton, MA) in all plots and regressions.

ADDITIONAL RESULTS AND DISCUSSION

Alternative Papers as Substrates. In this experiment, we analyzed three features of the paper substrate inside the well and how it responded upon application of the samples. The features we evaluated were: i) the capacity of the zones, and ii) the hydrophilicity and the homogeneity of the zones.

To determine the volume of sample—or capacity—that we can add to a paper well, we applied 2, 4, 6, and 8 μL of Amaranth red and Coomassie Brilliant Blue solutions, in six replicates for each volume. It is expected that the absorbance is proportional to the number of absorbing molecules in the optical pathlength, *i.e.,* the thickness of the paper; therefore, the plots of absorbance *vs.* volume added should be linear. Deviations from linearity should indicate effects due to the substrates and how the dyes interact with them. We can see in Figure SI-1, that 2 μL was not enough to fill the zones (the two rows on the bottom of each plate) in thicker papers, but was adequate for thinner papers. Volumes of 4 and 6 μL were adequate to fill the zones in Whatman paper.

To test the hydrophilicity, each one of these plates was split in half. The left-hand side of the plates shown in Figure SI-1 was used without exposure to plasma of oxygen while the right-hand side was submitted to plasma oxidation for 5 s. To test the homogeneity of the paper or the residual hydrophobicity due to exposure of the fibers on the paper to the resist, we observed visually how the sample spread inside the zones and how was the standard deviation of the

optical absorbance values measured in six replicates on a plate reader at the maximum wavelength for each dye.

Oxidation using air plasma had an unexpected effect—the results for each dye were different. In general, for any given volume of sample applied to the zones, Coomassie presented lower optical absorbance values after oxidation for the same amount of dye deposited in the test zones; Amaranth had a slightly increase (*i.e.,* 5-10%) in the absorbance values. This sensitivity to exposition to air plasma (and thus—we presume—to the hydrophilicity of the paper) probably reflects several factors (since the oxidation can alter the hydrophilicity of the fibers and how the each dye interacts with them).

For the plates not subjected to plasma oxidation the volume of sample applied to the zones, correlated well with the volume—or the total amount of analyte adsorbed—deposited on the fibers of the paper; it was also proportional to the thickness of the paper (thinner papers, *e.g.,* household coffee filter and Kimwipes®, showed some saturation at higher volumes, observed spectrophotometrically. Oxidation of the plates improved the hydrophilicity and the dyes spread homogenously over the zones for all volumes applied. Whatman paper plate made with 10% SC resist presented leakage of the solutions out of the zones indicating that the polymerization of resist for the thick paper was not complete.

Overall Comparison of Photolithographic Methods and Materials for Patterning Multi-Zone Paper Plates. Developing the technology and improving the protocols reduced both the time and the cost to prepare a single paper plate using the FLASH method. Table SI-1 compares the main elements for both methods presented in this work. Using a less expensive photoresist, or diluting a commercial formulation were the main factors that contributed to prepare inexpensive plates ($ 0.45) at the laboratory level, in reasonably short time (~16 min).

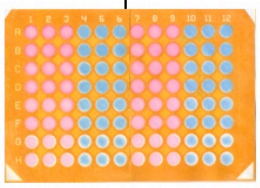
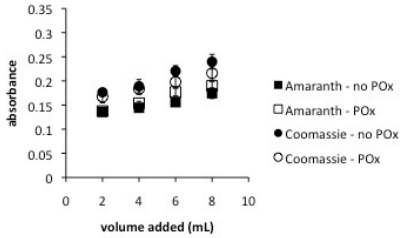
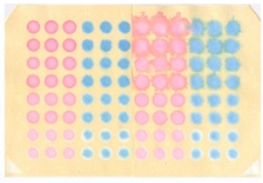
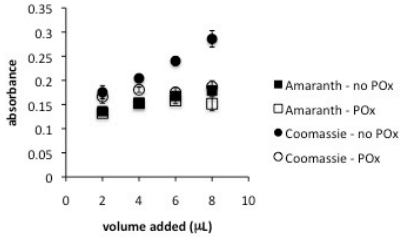
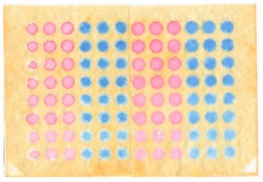
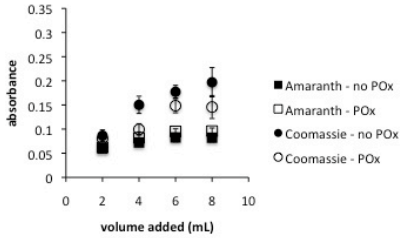
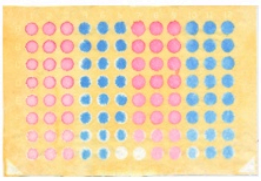
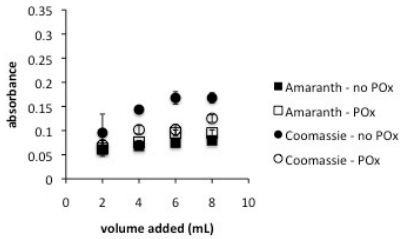
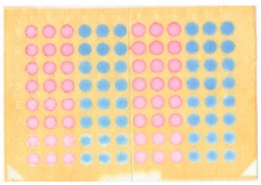
Treatment	No plasma Oxidation / Plasma Oxidation	Thickness of paper (μm)	Absorbance × Volume of Sample
100% SC	 Whatman #1	~180	
10% SC	 Whatman #1	~180	
10% SC	 Coffee filter	~100	
10% SC	 Kimwipes®	~90	
10% SC	 TechniCloth®	~250	Not available because the TechniCloth plate was too flexible to be supported solely by the edges in the plate reader. In a scanner, this is not an issue.

Figure SI-1. Comparison of types of paper, treatment of surface, and capacity of zones as monitored by visual analysis and absorbance detection at the wavelength of maximum absorbance for each dye.

The parallel nature of large-scale production (reel-to-reel) systems can decrease the time per plate substantially. Industrial production processes tens, or even hundreds, of plates at the same time, even if the length of all six steps remains the same (see Table SI-1 for details on times). The continuous batch or reel-to-reel industrial production also decreases the time required in each step, making the process more efficient. The industrial environment reports units as the number of plates per unit of time (for example, 20,000 plates/hour) and not minutes per plate as in an academic study (for example, 25 min/plate). The estimation of the cost of production is less straightforward, but one can imagine that as the production scales goes up, the cost per plate proportionally goes down.

It is important to point out that all the cost/plate detailed in Table SI-1 were not optimized and only reflected the cost of materials to prepare a single paper plate and disposal of excess paper and solvents used. In an optimized production environment, the waste of paper is minimal and the solvents—purchased by the drum—are recycled. For example, xylenes (production grade) costs \$10.24/L when purchased as 4-L bottle, \$6.76 when purchased as 5-gallon container, and \$5.39 when purchased in a drum (55 gallons).[3] The same xylenes mixture is worth less than \$1/L when negotiated in a market of commodities. Table SI-2 lists the potential cost per plate based for the types of paper tested in this work, as well as other types testes in similar approach from the literature.[14]

[3] Prices obtained on-line from VWR International on 01/08/2009 for xylenes of production grade from BDH brand, www.vwrsp.com.

Table SI-1. Breakdown of Estimates for Time and Cost to Prepare a Single Paper Plate Using FLASH Method with Two Different Photoresists at the Laboratory Scale

fabrication step	SU-8[a]		SC[b]	
	time (min)	cost /plate	time (min)	cost /plate
paper Chr 1		$0.074[c]		$0.083[d]
soak (2.5 mL)[e]	2	$0.240	3	$0.050
pre bake[f]	10	$0.003	0	$0.000
expose (UV)[g]	0.2	$0.003	1	$0.017
post bake[f]	5	$0.002	0	$0.000
develop[h]	1	$0.240	5	$0.210
rinse[i]	1	$0.430	5	$0.176
dry[j]	5	$0.000	2	$0.000
total	24.2	$0.92	16	$0.45

[a] based on reference 14; [b] this work; [c;d] the cost of the paper yielded different values because in each method, it was accounted from a different commercial source of paper (cost per m^2 of chromatography paper reported in the published SU-8 method, and cost per m^2 of chromatography paper used in this work; [e] time to apply and the cost of 2.5 mL of photoresist; [f] cost of total time of use of a simple hot plate ($300) accounted from a five-year depreciation; [g] cost of total time of use of a UV flood lamp ($2000) accounted for 2000 hours of lifetime; [h] time and cost of 20 mL of solvent used for developing the image (acetone for SU-8 and xylenes for SC); [i] time and cost of 20 mL of solvent used to rinse the plate (2-propanol for SU-8 and methanol for SC); [j] time to dry the rinse solvent from the paper on the fume hood.

Table SI-2. Cost per Plate for a Few Selected Types of Paper

Other Papers	Cost/Plate	Base Price
TechniCloth[a]	\$0.013	m^{-2}
towel paper[b]	\$0.002	m^{-2}
Kimwipes[a]	\$0.017	one plate/tissue
coffee filter[a]	\$0.015	one plate/filter

[a] this work; [b] based on reference 14.

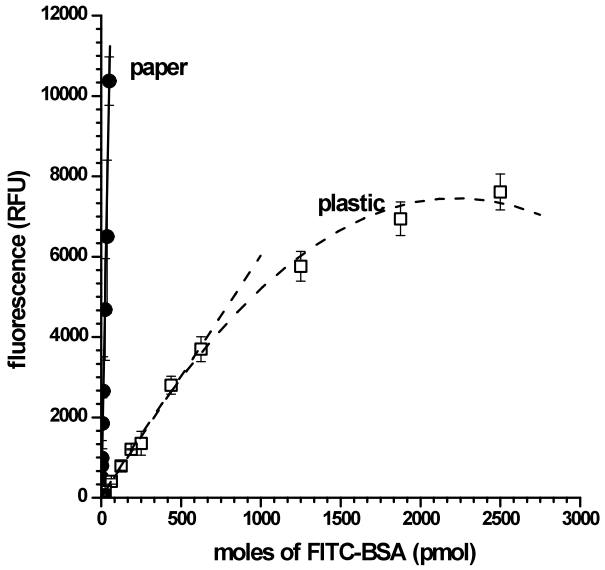


Figure SI-2. Fluorescence data from Figure 4 plotted in linear scales. The equation from the linear regression for the paper plate (●) was $y = 41.7 + 203.6*x$, $r = 0.995$, and for the plastic plate (□) was $y = 0.61 + 6.71*x - 0.002*x^2$, $r^2 = 0.999$ for quadratic regression of the full range or $y = 1.37 + 6.42*x$, $r = 0.999$ for the first ten data points.

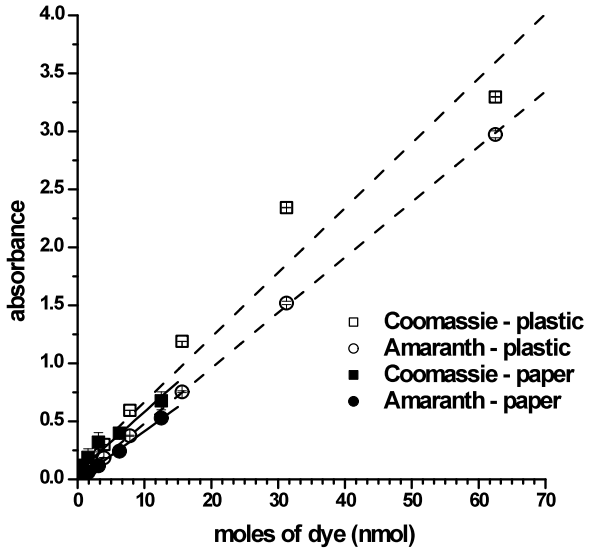
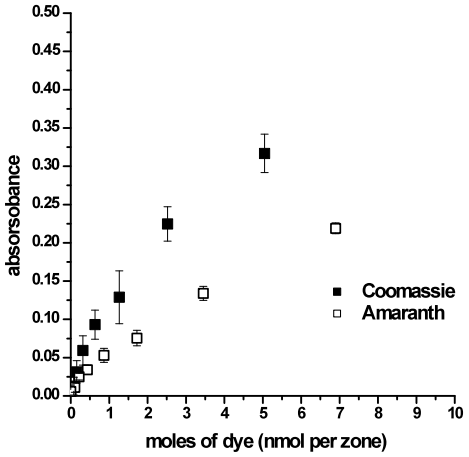
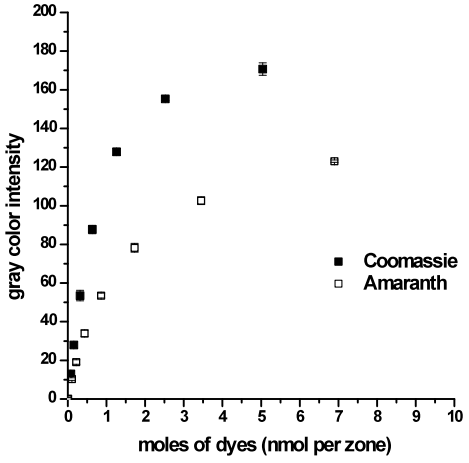


Figure SI-3. Absorbance data from Figure 5 plotted in linear scales. The equations for linear regressions (solid lines) from paper plates are as follow: (■) Coomassie blue: $y = 0.07 + 0.05*x$, $r = 0.977$ and (●) Amaranth red: $y = -0.004 + 0.04*x$, $r = 0.999$. Linear regression equations (broken lines) for plastic plates are: (□) Coomassie blue: $y = 0.16 + 0.06*x$, $r = 0.982$ and (○) Amaranth red: $y = 0.004 + 0.05*x$, $r = 0.999$.

a)


absorsobance
moles of dye (nmol per zone)
Coomassie
Amaranth

b)


gray color intensity
moles of dyes (nmol per zone)
Coomassie
Amaranth

c)

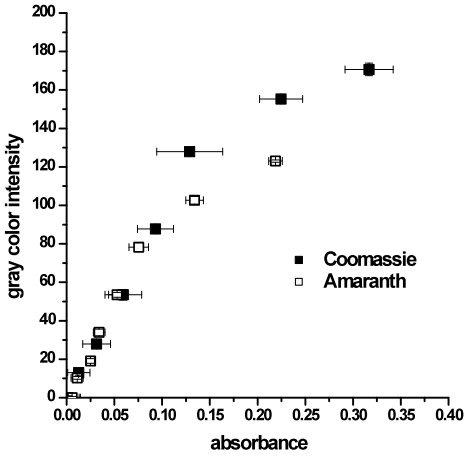

gray color intensity
absorbance
Coomassie
Amaranth

Figure SI-4. Linear scale plots for data from Figure 6 showing the quantitative relationship between amounts of dyes adsorbed on the zones of paper and the signal of the read out mode. (a) Absorbance measured in the microplate reader at the maximum wavelength for each dye after background subtraction. (b) Grey scale intensity measured from the digital image for each dye. (c) Correlation between absorbance and gray scale color intensity for each quantity of dye applied on the wells of paper. Note that the error bars (n = 6) in the gray scale read out are smaller than the size of the symbols.